

IMPROVED SIRNA FOR SILENCING EXPRESSION OF PCSK9 AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Nos. 63/546,217, 63/546,215, and 63/546,216, which were filed on October 29, 2023, the disclosures of each of which are hereby incorporated by reference in their entireties.

SEQUENCE LISTING

[0002] The contents of the electronic sequence listing (129293021501_SL.xml; Size: 739,907 bytes; and Date of Creation: October 28, 2024) is herein incorporated by reference in its entirety.

TECHNICAL FIELD

[0003] Disclosed herein are double-stranded ribonucleic acid molecules targeting Proprotein convertase subtilisin/kexin type 9 (PCSK9) and methods of treating conditions that are modulated by PCSK9 using the same.

BACKGROUND

[0004] The concept of gene silencing by RNA interference (RNAi) was discovered in 1998, as reported, for example, by Hu *et al.* ("Therapeutic siRNA: state of the art," *Signal Transduction and Targeted Therapy* (2020) 5:101). A publication by Tuschl *et al.* (Elbashir, S. M. *et al.* "Duplexes of 21±nucleotide RNAs mediate RNA interference in cultured mammalian cells," *Nature* (2001) 411, 494–498), reports silencing of a specific gene in mammalian cells using a chemically synthesized siRNA. The protein PCSK9 is mainly synthesized in and secreted by the liver. PCSK9 functions in the regulation of Low-Density Lipoprotein (LDL) receptor (LDLR) activity in hepatocytes which bind PCSK9 at the cell membrane surface and/or in the cytoplasm. LDLRs are also primarily responsible for removing Low-Density Lipoprotein-Cholesterol (LDL-C) from the circulation in a competing process, such that a reduction in PCSK9 protein, such as by inhibiting its synthesis, can lead to increased uptake and degradation of LDL-C. On binding of PCSK9 to LDLR on the cell membrane, the complex of PCSK9/LDLR is internalized into endosomes leading to the degradation of both. Conversely, when LDL-C binds to LDLR and the complex is internalized, LDLR dissociates from LDL-C and can be recycled to the surface of the cell,

whereas LDL-C is degraded (Chen *et al.* “A Review of PCSK9 Inhibitors and their Effects on Cardiovascular Diseases,” *Current Topics in Medicinal Chemistry*, 2019, 19(20): 1790-1817). Consequently, the main object of the current invention is to silence the production of PCSK9 protein in hepatocytes by interfering with translation of PCSK9 mRNA, thereby increasing the uptake of serum LDL-C in these same cells more efficiently than previous technology allows.

[0005] Inclisiran, currently marketed as Leqvio® in the United States and Europe, is a first-generation synthetic siRNA targeted to hepatocytes by virtue of a conjugated ligand, triantennary *N*-acetylglucosamine (GalNAc)₃, conjugated to the 3' end of the sense strand (SS) of the siRNA. GalNAc binds to asialoglycoprotein receptors (ASGPR) primarily found on the surface of hepatocytes of the liver, whereupon the conjugate is internalized. Inclisiran is typically administered by subcutaneous injection, whereupon it enters the blood stream and is circulated through the body and taken in by liver cells. Once internalized to the cytoplasm of hepatocytes or other cells, Inclisiran acts by virtue of a natural cell pathway to reduce the expression of PCSK9 by binding to and cleaving PCSK9 messenger RNA (mRNA). Since the binding of the antisense strand (AS) of the siRNA is specific to the sequence of the target mRNA, in theory only the PCSK9 mRNA is affected, thereby reducing (*i.e.*, silencing) the expression of that particular encoding region and protein product.

[0006] The invention of Inclisiran was a major therapeutic breakthrough for patients with hypercholesterolemia, particularly for those who cannot tolerate statins, previously the treatment of choice, or for whom statins are not sufficiently effective. While Inclisiran now provides a relatively new and major step forward in the treatment of such patients, there is still an existing need for significantly more effective forms of PCSK9 directed siRNA.

SUMMARY

[0007] Disclosed herein are a double-stranded ribonucleic acid molecules comprising a sense strand comprising the nucleic acid sequence of SEQ ID NO: 1 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 2, a sense strand comprising the nucleic acid sequence of SEQ ID NO: 3 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 4, a sense strand comprising the nucleic acid sequence of SEQ ID NO: 5 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 6, or a sense strand comprising the nucleic acid sequence of SEQ ID NO: 7 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 8.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] The summary, as well as the following detailed description, is further understood when read in conjunction with the appended drawings. For the purpose of illustrating the disclosed double-stranded ribonucleic acid molecules and methods of using the same, the drawings show exemplary embodiments of the compositions and methods; however, the compositions and methods are not limited to the specific embodiments disclosed. In the drawings:

[0009] **FIG. 1** illustrates the decorated sequence of original Inclisiran.

[0010] **FIG. 2** illustrates a dose-response curve for XD-44731.

[0011] **FIG. 3** illustrates a dose-response curve for XD-44725.

[0012] **FIG. 4** illustrates a dose-response curve for XD-44701.

[0013] **FIG. 5** illustrates a dose-response curve for XD-44695.

[0014] **FIG. 6** illustrates a dose-response curve for XD-44719.

[0015] **FIG. 7** illustrates a dose-response curve for XD-42182.

[0016] **FIG. 8** illustrates a dose-response curve for XD-44689.

[0017] **FIG. 9** illustrates a dose-response curve for XD-44682.

[0018] **FIG. 10** illustrates a dose-response curve for XD-44722 (2).

[0019] **FIG. 11** illustrates a dose-response curve for XD-44728.

[0020] **FIG. 12** illustrates a dose-response curve for XD-44698.

[0021] **FIG. 13** illustrates a dose-response curve for XD-44716.

[0022] **FIG. 14** illustrates a dose-response curve for XD-44692.

[0023] **FIG. 15** illustrates a dose-response curve for XD-44704.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0024] The disclosed double-stranded ribonucleic acid molecules and methods of using the same may be understood more readily by reference to the following detailed description taken in connection with the accompanying figures, which form a part of this disclosure. It is to be understood that the disclosed double-stranded ribonucleic acid molecules and methods of using the same are not limited to the specific compositions and methods described and/or shown herein, and that the terminology used herein is for the purpose of describing particular embodiments by way of example only and is not intended to be limiting of the claimed double-stranded ribonucleic acid molecules and methods of using the same.

[0025] Unless specifically stated otherwise, any description as to a possible mechanism or mode of action or reason for improvement is meant to be illustrative only, and the disclosed double-stranded ribonucleic acid molecules and methods of using the same are not to be constrained by the correctness or incorrectness of any such suggested mechanism or mode of action or reason for improvement.

[0026] Throughout this text, the descriptions refer to double-stranded ribonucleic acid molecules and methods of using said double-stranded ribonucleic acid molecules. Where the disclosure describes or claims a feature or embodiment associated with double-stranded ribonucleic acid molecules, such a feature or embodiment is equally applicable to the methods of using said double-stranded ribonucleic acid molecules. Likewise, where the disclosure describes or claims a feature or embodiment associated with a method of using double-stranded ribonucleic acid molecules, such a feature or embodiment is equally applicable to the double-stranded ribonucleic acid molecules themselves.

[0027] As used herein, the singular forms “a,” “an,” and “the” include the plural.

[0028] When a list is presented, unless stated otherwise, it is to be understood that each individual element of that list, and every combination of that list, is a separate embodiment. For example, a list of embodiments presented as “A, B, or C” is to be interpreted as including the embodiments: “A,” “B,” “C,” “A or B,” “A or C,” “B or C,” or “A, B, or C.”

[0029] Where a range of numerical values is recited or established herein, the range includes the endpoints thereof and all the individual integers and fractions within the range, and also includes each of the narrower ranges therein formed by all the various possible combinations of those endpoints and internal integers and fractions to form subgroups of the larger group of values within the stated range to the same extent as if each of those narrower ranges was explicitly recited. Where a range of numerical values is stated herein as being greater than a stated value, the range is nevertheless finite and is bounded on its upper end by a value that is operable within the context of the herein disclosure. Where a range of numerical values is stated herein as being less than a stated value, the range is nevertheless bounded on its lower end by a non-zero value. It is not intended that the scope of the compositions and methods be limited to the specific values recited when defining a range. All ranges are inclusive and combinable.

[0030] When values are expressed as approximations, by use of the antecedent “about,” it will be understood that the particular value forms another embodiment. Reference

to a particular numerical value includes at least that particular value, unless the context clearly dictates otherwise.

[0031] Various terms relating to aspects of the description are used throughout the specification and claims. Such terms are to be given their ordinary meaning in the art unless otherwise indicated. Other specifically defined terms are to be construed in a manner consistent with the definitions provided herein.

[0032] The term “about” when used in reference to numerical ranges, cutoffs, or specific values is used to indicate that the recited values may vary by up to as much as 10% from the listed value. As many of the numerical values used herein are experimentally determined, it should be understood by those skilled in the art that such determinations can, and often times will, vary among different experiments. The values used herein should not be considered unduly limiting by virtue of this inherent variation. Thus, the term “about” is used to encompass variations of $\pm 10\%$ or less, variations of $\pm 5\%$ or less, variations of $\pm 1\%$ or less, variations of $\pm 0.5\%$ or less, or variations of $\pm 0.1\%$ or less from the specified value.

[0033] “Treat,” “treatment,” and like terms refer to both therapeutic treatment and prophylactic or preventative measures, and includes reducing the severity and/or frequency of symptoms, eliminating symptoms and/or the underlying cause of the symptoms, reducing the frequency or likelihood of symptoms and/or their underlying cause, and improving or remediating damage caused, directly or indirectly, by the double-stranded ribonucleic acid molecules. Treatment also includes prolonging survival as compared to the expected survival of a subject not receiving treatment. Subjects to be treated include those that have the condition or disorder as well as those prone to have the condition or disorder or those in which the condition or disorder is to be prevented.

[0034] As used herein, “administering to said patient” and similar terms indicate a procedure by which double-stranded ribonucleic acid molecules are injected into a patient such that target cells, tissues, or segments of the body of the subject are contacted with the double-stranded ribonucleic acid molecules.

[0035] As used herein, “pharmaceutically acceptable carrier” or “pharmaceutical acceptable excipient” includes any material which, when combined with an active ingredient, allows the ingredient to retain biological activity and is non-reactive with the subject's immune system. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Preferred diluents for aerosol or parenteral

administration are phosphate buffered saline or normal (0.9%) saline. Compositions comprising such carriers are formulated by well known conventional methods (see, for example, Remington's Pharmaceutical Sciences, 18th edition, A. Gennaro, ed., Mack Publishing Co., Easton, Pa., 1990; and Remington, The Science and Practice of Pharmacy 20th Ed. Mack Publishing, 2000).

[0036] The term “subject” as used herein is intended to mean any animal, in particular, mammals. Although treatment of mice and nonhuman primates with double-stranded ribonucleic acid molecules is exemplified herein, any type of mammal can be treated using the disclosed methods. Thus, the methods are applicable to human and nonhuman animals, although preferably used with mice, nonhuman primates, and humans, and most preferably with humans. “Subject” and “patient” are used interchangeably herein.

[0037] The term “comprising” is intended to include examples encompassed by the terms “consisting essentially of” and “consisting of”; similarly, the term “consisting essentially of” is intended to include examples encompassed by the term “consisting of.”

[0038] RNA interference (RNAi) is a naturally occurring biological phenomenon, wherein siRNA functions by interacting with endogenous cellular proteins to form a structure known as RNA-induced silencing complex (RISC). The siRNA comprises a sense strand (SS) and at least partially base-paired antisense strand (AS) which may or may not be covalently linked together. In some embodiments, the siRNA feeds into RISC which separates the two strands and uses the antisense strand (complementary to the targeted mRNA) to guide the complex to the target mRNA transcribed from the source gene and then cleaves it. As a result, the fragmented target mRNA cannot be translated into its encoded protein, thus “silencing” its expression.

[0039] Each protein produced by a cell has a unique sequence translated from the unique gene which encodes it. Therefore, at least theoretically, translation of any gene into its encoded protein can be silenced.

[0040] The intracellular mechanism of siRNA in effecting gene silencing has been recently reviewed (e.g., Hu *et al.* “Therapeutic siRNA: state of the art,” *Signal Transduction and Targeted Therapy* (2020) 5:101). Synthetic siRNA comprises double-stranded RNA (dsRNA), comprising SS and AS wherein each, or nearly each, ribonucleic acid is modified at the 2'-position, for example, by either F or O-methyl group, or another modification. siRNA is introduced into cells by one or more suitable delivery methods which bring the dsRNA in contact with the cells such that the dsRNA enters the cell cytoplasm. The

translocation of the dsRNA across the cell membrane can be either active or passive. As will be understood by those skilled in the art, targeting of the dsRNA comprising the siRNA to a specific cell type and/or body tissue may be desired for treating a particular pathology, reducing or eliminating undesirable side effects such as immune response, and, among other practical considerations, minimizing the effective dose of the siRNA drug needed to effectively treat a subject.

[0041] As noted by Hu *et al.* and others, synthetic and synthetically modified siRNAs were introduced due to issues of poor stability of unmodified siRNA resulting from degradation by RNases and phosphatases, among others. United States Patent No. 10,851,377 (Table B, in particular, incorporated by reference herein) includes numerous examples of synthetically produced, modified nucleotides now known in the art.

[0042] Disclosed herein are siRNAs having modified ribonucleic acids each with 2'-F, or 2'-O-methyl group, among other modifications, with one or more phosphorothioate (PS) bonds present in the backbone, and additionally includes a biostable phosphate mimic, such as a vinyl-phosphonate cap, GalNAc, and other modifications. The latter are included in the design of these synthetic siRNAs as they have been shown to confer some resistance to cleavage of the PS bonds by endogenous nucleases. In some embodiments, GalNAc and/or vinyl- phosphonate cap are optional.

[0043] The decorated sequence of the original Inclisiran is shown in FIG. 1 and Table 1.

Table 1

	Original Inclisiran Chemistry
SS	csusagacCfuGfudTuugcuuuugu(L96)
AS	asCfsaAfAfAfgCfaAfaAfcAfgGfuCfuagsasa

* For letter code modifications, see Table 3.

[0044] The disclosure provides one or more chemically engineered macromolecules, similar to but distinct from Inclisiran, both in sequence and modification, mimicking and further improving the activity of Inclisiran small interfering RNAs (siRNAs). Various sequences of the herein disclosed improved siRNAs without modifications are shown in Table 2.

Table 2: Sequence Level Improved siRNAs

	Sense (5'-3')	Antisense (5'-3')
3512-A	C-U-U-U-U-U-A-C-A-G-C-C-A-A- C-U-U-U-U-C-A SEQ ID NO: 1	U-G-A-A-A-A-G-U-U-G-G-C-U-G-U-A- A-A-A-A-G-G-C SEQ ID NO: 2
3512-B	C-U-U-U-U-U-A-C-A-G-C-C-A-A- C-U-U-U-U-C-U SEQ ID NO: 3	A-G-A-A-A-A-G-U-U-G-G-C-U-G-U-A- A-A-A-A-G-G-C SEQ ID NO: 4
3545	C-U-U-U-U-G-U-A-A-C-U-U-G-A- A-G-A-U-A-U-A SEQ ID NO: 5	U-A-U-A-U-C-U-U-C-A-A-G-U-U-A-C- A-A-A-A-G-C-A SEQ ID NO: 6
44687	C-U-A-G-A-C-C-U-G-U-T-U-U-G- C-U-U-U-U-G-A SEQ ID NO: 7	U-C-A-A-A-A-G-C-A-A-A-A-C-A-G-G- U-C-U-A-G-A-A SEQ ID NO: 8

wherein:

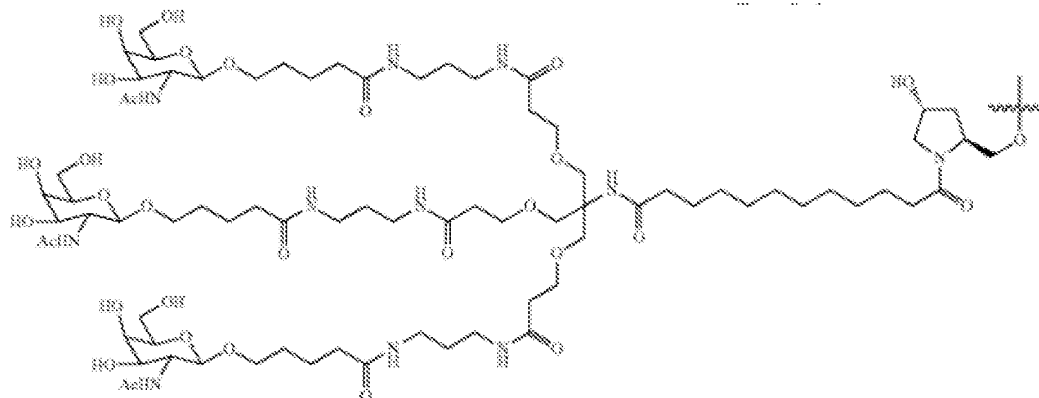
- A' is an adenosine-containing nucleoside,
- C' is a cytosine-containing nucleoside,
- G' is a guanine-containing nucleoside,
- T' is a thymine-containing nucleoside, and
- U' is a uracil-containing nucleoside.

Table 3: Key to Structure of Synthetic, Modified siRNAs

All nucleosides are connected via phosphodiester-linkage unless otherwise stated.

- A Adenine
- a 2'-O-methyladenine
- Ab Adenine 2'-4'-Locked Nucleic Acid
- dA deoxyadenosine

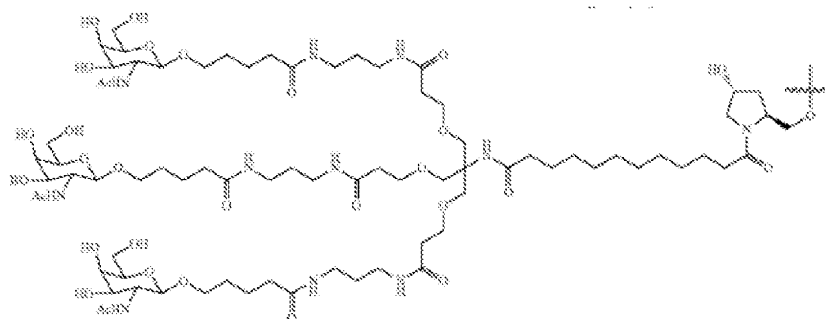
Af 2'-fluoroadenine
 as 2'-O-methyladenine-3'-phosphorothioate
 Afs 2'-fluoroadenine-3'-phosphorothioate
 C Cytosine
 c 2'-O-methylcytosine
 Cb Cytosine 2'-4'-Locked Nucleic Acid
 Cf 2'-fluorocytosine
 cs 2'-O-methylcytosine-3'-phosphorothioate
 Cfs 2'-fluorocytosine-3'-phosphorothioate
 dC deoxycytosine
 G Guanine
 g 2'-O-methylguanine
 Gb Guanine 2'-4'-Locked Nucleic Acid
 Gf 2'-fluoroguanine
 gs 2'-O-methylguanine-3'-phosphorothioate
 Gfs 2'-fluoroguanine-3'-phosphorothioate
 dT deoxythymine
 Tb thymine 2'-4'-Locked Nucleic Acid
 U Uracil
 u 2'-O-methyluracil
 Uf 2'-fluorouracil
 us 2'-O-methyluracil-3'-phosphorothioate
 Ufs 2'-fluorouracil-3'-phosphorothioate
 (vinu)s -> 5'-(E)-vinylphosphonate 2'-O-methyluracil-3'-phosphorothioate
 = phosphorothioate bond -> small "s" after the nucleotide-designating letter preceding the bond (for sequence 5'-3')
 b following a capital letter indicates a nucleotide represents 2'-4'-Locked Nucleic Acid
 (abasic) indicates 1'-H (no base)
 L96 is



[0045] As used herein, bases such as adenine, cytosine, guanine, uracil, and thymine, including their modified and deoxy forms, can refer to the heterocyclic bases themselves or nucleotides or nucleosides having the base.

[0046] In some embodiments of the invention, the double-stranded siRNA conjugates comprise a sense strand 21 nucleotides in length wherein 2'-O-methyladenine conjugated to a triantennary *N*-acetylglucosamine (GalNAc)₃ ligand is in position 21 from the 5' end. The antisense strand of the dsRNA conjugate comprises 23 modified nucleotides. In some embodiments of the invention, nucleotides comprising the sense strand and antisense strand are modified compared to naturally occurring RNAs. In various embodiments of the invention, phosphorothioate bonds connect nucleotides 1=2 and 2=3 of the sense strand and nucleotides 1=2, 2=3, 21=22, and 22=23 of the antisense strand (Formula III), wherein nucleotides 22 and 23 of the antisense strand are generally not base-paired. (The nucleotide number here is indicated starting from the 5' end of each strand).

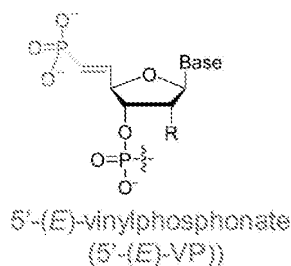
[0047] The triantennary *N*-acetylglucosamine (GalNAc)₃ ligand, also known as L96, is conjugated to the 3' end of the sense strand. The L96 ligand structure is shown, for example, in US Patent Nos. 8,273,866 and 8,828,956 and as Formula I. (GalNAc)₁, mono-, di-, triantennary, etc. is a well-defined liver-targeted moiety benefiting from its high affinity with asialoglycoprotein receptor (ASGPR). By conjugating it directly to the oligonucleotides or decorating it to certain delivery system as a targeting moiety, GalNAc has achieved compelling successes in the development of nucleic acid therapeutics in recent years. Several oligonucleotide modalities are undergoing pivotal clinical studies.



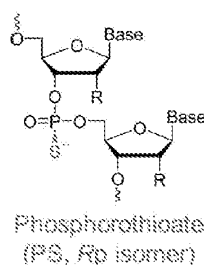
(Formula I)

[0048] In some embodiments, nucleotide 1 of the antisense strand is 2'-O-methyluracil which has a 5'-(*E*)-vinylphosphonate (vinyl-phosphonate cap) as illustrated in Formula II. For double-stranded siRNA, the vinyl-phosphonate cap in this position has been shown to protect the 5' end of the AS from degradation by phosphatases and 5'-3' exonucleases (see, *e.g.*, Haraszti et al., "5'-Vinylphosphonate improves tissue accumulation and efficacy of conjugated siRNAs *in vivo*," *Nucleic Acids Research*, 7 June 2017 45(13):

7581-7592). This protective effect can result in improved stability of siRNA conjugates *in vivo* with the potential for improved clinical efficacy in silencing the expression of the targeted gene, here PCSK9.

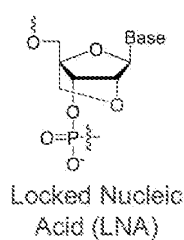


(Formula II)



(Formula III)

[0049] In some embodiments of the invention, modifications to the nucleotides may include abasic ribose rings (1'-H), Locked Nucleic Acids (LNA; see Formula IV), or deoxyribonucleic acids (DNA), with or without further modifications. In some embodiments of the invention, the identity of the individual nucleotides, modified or not, may be any natural or synthetic nucleotide, deoxyribonucleic nucleotides, or the like, the only desirable limitation being that the opposing regions of the sense and antisense strands are mostly complementary such that opposing nucleotides can form base pairs between the sense and antisense strands. Any individual phosphorothioate (PS) bond within either strand of a dsRNA conjugate may be *R_p* or *S_p*, as the chirality of the bond(s) does not seem affect the activity of the complex, moreover, it is difficult to separate during synthesis.



(Formula IV)

[0050] As shown, the SS of Inclisiran comprises 21 modified ribonucleic nucleotides. The AS, on the other hand, comprises 23 modified ribonucleic nucleotides, wherein the nucleotide at the 3' end of the sense strand (or position 21 from the 5' end) is complementary to the nucleotide in position 1 at the 5' end of the sense strand, SS position 20

from the 5' end is complementary to position 2 from the 5' end of the AS, and so forth. Thus, the two nucleotides at positions 22 and 23 of the antisense strand are unpaired when the two strands are otherwise paired.

[0051] As will be recognized by those skilled in the art, the specific sequence of each complementary siRNA strand (SS and AS) which must pair with the other strand of the dsRNA at least for a length of each strand, and any modifications therein, may affect the activity of the siRNA in its intended and unintended function(s) in ways which cannot be predicted. When making modifications to the siRNA strands it can be useful to determine experimentally under certain conditions whether the designed siRNA interacts with the endogenous cellular machinery required for silencing or reducing the expression of a target gene's mRNA, and further, whether and how effectively the siRNA (antisense strand) recognizes the target mRNA based on its specific composition. Another consideration is the rate of degradation of the therapeutic siRNA, both en route to the target cell and within the cell targeted. Recognition of mRNA other than from the target gene has the potential for causing a multitude of off-target side effects. The modified siRNAs also have the potential to trigger unwanted systemic effects, such as an immune response, which must be experimentally detected *in vivo*. Following the identification of a particular gene for targeting, challenges to therapeutic gene silencing include delivery, stability, and specificity.

[0052] Disclosed herein are a double-stranded ribonucleic acid molecules comprising a sense strand comprising the nucleic acid sequence of SEQ ID NO: 1 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 2, a sense strand comprising the nucleic acid sequence of SEQ ID NO: 3 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 4, a sense strand comprising the nucleic acid sequence of SEQ ID NO: 5 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 6, or a sense strand comprising the nucleic acid sequence of SEQ ID NO: 7 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 8.

[0053] In some embodiments, the sense strand comprises the nucleic acid sequence of SEQ ID NO: 1 and the antisense strand comprises the nucleic acid sequence of SEQ ID NO: 2. In some embodiments, the sense strand comprises the nucleic acid sequence of SEQ ID NO: 3 and the antisense strand comprises the nucleic acid sequence of SEQ ID NO: 4. In some embodiments, the sense strand comprises the nucleic acid sequence of SEQ ID NO: 5 and the antisense strand comprises the nucleic acid sequence of SEQ ID NO: 6. In some

embodiments, the sense strand comprises the nucleic acid sequence of SEQ ID NO: 7 and the antisense strand comprises the nucleic acid sequence of SEQ ID NO: 8.

[0054] The sense strand of any of the herein disclosed double stranded nucleic acid molecules can comprise L96. The L96 can be conjugated to the 5' end of the sense strand. The L96 can be conjugated to the 3' end of the sense strand.

[0055] The antisense strand of any of the herein disclosed double stranded nucleic acid molecules can comprise a biostable phosphate mimic, such as a vinyl-phosphonate cap. In some embodiments, the vinyl-phosphonate cap is (vinu). The (vinu) can be conjugated to the 5' end of the antisense strand.

[0056] The herein disclosed double-stranded ribonucleic acid molecules can comprise a sense strand and/or an antisense strand comprising one or more modified nucleotides. In some embodiments, the sense strand comprises one or more modified nucleotides. In some embodiments, the antisense strand comprises one or more modified nucleotides. In some embodiments, the sense strand and the antisense strand comprise one or more modified nucleotides.

[0057] The one or more modified nucleotides include but are not limited to the modifications described in Table 3. For example, the double-stranded ribonucleic acid molecule can comprise one or more modified nucleotides are selected from 2'-O-methyladenosine, 2'-fluoroadenosine, 2'-O-methylcytidine, 2'-fluorocytidine, 2'-O-methylguanosine, 2'-fluoroguanosine, 2'-O-methyluridine, and 2'-fluorouridine, or any combination thereof. The double-stranded ribonucleic acid molecule can comprise 2'-O-methyladenosine. The double-stranded ribonucleic acid molecule can comprise 2'-fluoroadenosine. The double-stranded ribonucleic acid molecule can comprise 2'-O-methylcytidine. The double-stranded ribonucleic acid molecule can comprise 2'-fluorocytidine. The double-stranded ribonucleic acid molecule can comprise 2'-O-methylguanosine. The double-stranded ribonucleic acid molecule can comprise 2'-fluoroguanosine. The double-stranded ribonucleic acid molecule can comprise 2'-O-methyluridine. The double-stranded ribonucleic acid molecule can comprise 2'-fluorouridine.

[0058] The herein disclosed double-stranded ribonucleic acid molecules can comprise one or more 2'-deoxynucleotides selected from 2'-deoxyadenosine, 2'-deoxycytidine, 2'-deoxythymidine, and 2'-deoxyguanosine, or any combination thereof. The double-stranded ribonucleic acid molecule can comprise 2'-deoxyadenosine. The double-stranded ribonucleic acid molecule can comprise 2'-deoxycytidine. The double-stranded

ribonucleic acid molecule can comprise 2'-deoxythymidine. The double-stranded ribonucleic acid molecule can comprise 2'-deoxyguanosine.

[0059] The herein disclosed double-stranded ribonucleic acid molecules can comprise nucleotides that are connected by one or more phosphodiester bonds, one or more phosphorothioate bonds, or any combination of phosphodiester bonds and phosphorothioate bonds. The double-stranded ribonucleic acid molecule can comprise one or more phosphodiester bonds. The double-stranded ribonucleic acid molecule can comprise one or more phosphorothioate bonds. The double-stranded ribonucleic acid molecule can comprise one or more phosphodiester bonds and one or more phosphorothioate bonds.

[0060] The herein disclosed double-stranded ribonucleic acid molecules can comprise one or more glycol nucleic acid (GNA) nucleotides.

[0061] The herein disclosed double-stranded ribonucleic acid molecules can comprise one or more 2'-4'-Locked Nucleic Acid (LNA) nucleotides. The double-stranded ribonucleic acid molecules can comprise adenine 2'-4'-LNA nucleotides, cytosine 2'-4'-LNA nucleotides, guanine, 2'-4'-LNA nucleotides, thymine 2'-4'-LNA nucleotides, or any combination thereof. In some embodiments, the double-stranded ribonucleic acid molecules comprise one or more adenine 2'-4'-LNA nucleotides. In some embodiments, the double-stranded ribonucleic acid molecules comprise one or more cytosine 2'-4'-LNA nucleotides. In some embodiments, the double-stranded ribonucleic acid molecules comprise one or more guanine 2'-4'-LNA nucleotides. In some embodiments, the double-stranded ribonucleic acid molecules comprise one or more thymine 2'-4'-LNA nucleotides.

[0062] Disclosed herein are double-stranded ribonucleic acid molecules comprising a sense strand and an antisense strand comprising: SEQ ID NOs: 9 and 10, respectively; SEQ ID NOs: 11 and 12, respectively; SEQ ID NOs: 13 and 14, respectively; SEQ ID NOs: 15 and 16, respectively; SEQ ID NOs: 17 and 18, respectively; SEQ ID NOs: 19 and 20, respectively; SEQ ID NOs: 21 and 22, respectively; SEQ ID NOs: 23 and 24, respectively; SEQ ID NOs: 25 and 26, respectively; SEQ ID NOs: 27 and 28, respectively; SEQ ID NOs: 29 and 30, respectively; SEQ ID NOs: 31 and 32, respectively; SEQ ID NOs: 33 and 34, respectively; SEQ ID NOs: 35 and 36, respectively; SEQ ID NOs: 37 and 38, respectively; SEQ ID NOs: 39 and 40, respectively; SEQ ID NOs: 41 and 42, respectively; SEQ ID NOs: 43 and 44, respectively; SEQ ID NOs: 45 and 46, respectively; SEQ ID NOs: 47 and 48, respectively; SEQ ID NOs: 49 and 50, respectively; SEQ ID NOs: 51 and 52, respectively; SEQ ID NOs: 53 and 54, respectively; SEQ ID NOs: 55 and 56, respectively; SEQ ID NOs:

57 and 58, respectively; SEQ ID NOs: 59 and 60, respectively; SEQ ID NOs: 61 and 62, respectively; SEQ ID NOs: 63 and 64, respectively; SEQ ID NOs: 65 and 66, respectively; SEQ ID NOs: 67 and 68, respectively; SEQ ID NOs: 69 and 70, respectively; or SEQ ID NOs: 71 and 72, respectively.

[0063] Disclosed herein are double-stranded ribonucleic acid molecules comprising a sense strand comprising the nucleic acid sequence of SEQ ID NO: 15 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 16.

[0064] Disclosed herein are double-stranded ribonucleic acid molecules comprising a sense strand comprising the nucleic acid sequence of SEQ ID NO: 49 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 50.

[0065] Disclosed herein are double-stranded ribonucleic acid molecules comprising a sense strand comprising the nucleic acid sequence of SEQ ID NO: 21 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 22.

[0066] Disclosed herein are double-stranded ribonucleic acid molecules comprising a sense strand comprising the nucleic acid sequence of SEQ ID NO: 47 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 48.

[0067] Disclosed herein are double-stranded ribonucleic acid molecules comprising a sense strand comprising the nucleic acid sequence of SEQ ID NO: 57 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 58.

[0068] Disclosed herein are double-stranded ribonucleic acid molecules comprising a sense strand comprising the nucleic acid sequence of SEQ ID NO: 53 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 54.

[0069] Disclosed herein are methods of treating a condition that is modulated by PCSK9 in a subject, the method comprising administering to the subject an effective amount of a double-stranded ribonucleic acid molecules of any one of the previous claims. The condition that is modulated by PCSK9 can be hypercholesterolemia or mixed dyslipidemia. In some embodiments, the condition modulated by PCSK9 is hypercholesterolemia. In some embodiments, the condition modulated by PCSK9 is mixed dyslipidemia. The herein disclosed methods of treatment can result in a reduction in PCSK9 protein levels and/or a reduction in low-density lipoprotein (LDL) levels. The herein disclosed methods of treatment can result in a reduction in PCSK9 protein levels. The herein disclosed methods of treatment can result in a reduction in low-density lipoprotein (LDL) levels.

[0070] The herein disclosed methods of treatment can comprise administering to the subject a dose of about 0.25 mg/kg to about 5 mg/kg of any of the disclosed double-stranded ribonucleic acid molecules. In some embodiments, the dose is about 0.25 mg/kg. In some embodiments, the dose is about 0.5 mg/kg. In some embodiments, the dose is about 1.0 mg/kg. In some embodiments, the dose is about 2.0 mg/kg. In some embodiments, the dose is about 5 mg/kg. The dose can be administered as a pharmaceutical composition. The dose can be administered by injection. In some embodiments, the injection is a subcutaneous injection.

[0071] Disclosed herein are pharmaceutical compositions comprising any of the herein disclosed double-stranded ribonucleic acids and a pharmaceutically acceptable carrier.

[0072] The herein disclosed siRNAs alternative to Inclisiran both by sequence and novel and non-obvious chemical modifications, result in lower IC₅₀ as compared to original Inclisiran. Thus, the siRNAs of the invention directed against PCSK9 are wholly different from those of Inclisiran.

EXAMPLES

[0073] The following examples are provided to further describe some of the embodiments disclosed herein. The examples are intended to illustrate, not to limit, the disclosed embodiments.

[0074] The siRNAs of the inventions are related to Inclisiran but have novel and non-obvious chemical modifications, resulting in particular in lower IC₅₀ than original Inclisiran under same experimental conditions (“for apples-to-apples comparison”).

Example 1. Screening Protocol for modified siRNA conjugates

1. siRNA manufacturing

2'-modified oligoribonucleotide synthesis

[0075] The single stranded oligoribonucleotides were synthesized according to the conventional solid-phase oligonucleotide synthesis technology using the standard phosphoramidite-based oligomerization chemistry, whereby the oligonucleotides were assembled on solid support, employing a Mermade 96E synthesizer (LGC Bioautomation) in a 96 well format, controlled by the Poseidon software package.

[0076] Syntheses were performed on a solid support made of controlled pore glass (CPG). Specifically, guide strands were assembled on a 500 Å universal solid support available from Biocomma (#DS0500, porosity of 500 Å). For passenger strands modified with a tri-antennary GalNAc-cluster based ligand on the 3'-end (L96-tri-N-acetylgalactosamine (GalNAc)-cluster (J. Am. Chem. Soc. 2014, 136, 16958)), synthesis was performed on the L96-GalNAc-cluster immobilised CPG solid support sourced from Hongene biotech, China (#ON-469, porosity of 500 Å, 55 µmol/g loading).

[0077] All 2'-modified RNA phosphoramidites as well as majority of the ancillary reagents were purchased from SAFC, Proligo, Sigma Aldrich (now Merck, Hamburg, Germany). Specifically, the following 2'-O-Methyl phosphoramidites were used: (5'-O-dimethoxytrityl-N6-(benzoyl)-2'-O-methyl-adenosine-3'-O-(2-cyanoethyl-N,N-diisopropylamino) phosphoramidite, 5'-Odimethoxytrityl-N4-(acetyl)-2'-O-methyl-cytidine-3'-O-(2-cyanoethyl-N,N-diisopropylamino) phosphoramidite, (5'-O-dimethoxytrityl-N2-(isobutyryl)-2'-O-methyl-guanosine-3'-O-(2-cyanoethyl-N,N-diisopropylamino) phosphoramidite, and 5'-O-dimethoxytrityl-2'-O-methyl-uridine-3'-O-(2-cyanoethyl-N,N-diisopropylamino) phosphoramidite. The 2'-deoxy-2'-fluoro-phosphoramidites carried the same protecting groups as the 2'-O-methyl RNA amidites. In addition to these, the other phosphoramidites were sourced as follows: 5'-(E)-vinyl phosphonate-2'-OMe-U (POM protected) (#PR5-032) phosphoramidite and (S)-glycol nucleic acid phosphoramidites (GNA-A(bz) (#OP-033), GNA-C(ac) (#OP-041), GNA-G(ibu) (#OP-194), GNA-U (#OP-193) and GNA-T (#OP-032)) were purchased from Hongene biotech, Hamburg; 5'-O-dimethoxytrityl-2'-O-deoxy-thymidine-3'-O-(2-cyanoethyl-N,N-diisopropylamino) phosphoramidite was purchased from SAFC, Proligo, Sigma Aldrich (now Merck, Hamburg, Germany). Amidite solutions (50 mM) used were prepared from the respective amidites, as commercially sourced, in anhydrous acetonitrile and molecular sieves (3 Å, 8-12 mesh, Car Roth, #N893.1) were added.

[0078] 5-Ethyl thiotetrazole (ETT, 500 mM in acetonitrile, 99.8%, Biosolve #0022112402BS) was used as activator solution. Coupling times were 6 minutes. In order to introduce phosphorothioate linkages a 100 mM solution of 3-Amino-1,2,4-dithiazole-5-thione (or Xanthane hydride obtained from TCI Chemicals, Germany, (Purity: >96.0%(HPLC) cas: 6846-35-1)) dissolved in ACN-pyridine (2:3 v/v) was employed as sulfurizing agent. The other ancillary reagents used were as follows: Iodine-Oxidizer (50 mM I₂ (in Pyridine-H₂O

(9:1 v/v))), TCA (3% in DCM) for deblocking, Cap A (Acetic anhydride in THF (9.1:90.9 v/v)) and Cap B (THF, N-Methylimidazole and Pyridine (8:1:1 v/v/v)) as capping agents. Oligonucleotides were all synthesized with the removal of the final DMT protecting group ("DMT-Off").

Cleavage and deprotection of support bound oligomer

[0079] After finalization of the solid phase synthesis, oligonucleotides were cleaved from the solid support by addition of AMA (1:1 (v/v) mixture of concentrated aqueous ammonia and 40% aqueous methylamine, both available from Sigma Aldrich) and collected in 96 well plates. To achieve quantitative removal of all the protecting groups, the solutions were incubated with shaking at 33 °C. Samples were then dried under reduced pressure (SpeedVac concentrator (ThermoFisher)) and solid residues were thereafter reconstituted in 250mM Tris(hydroxymethyl)aminomethane (TRIS) (pH 7.0) to yield crude sample solutions for subsequent purification.

Purification of oligoribonucleotides

[0080] Crude preparations were thereafter purified by Anion Exchange Chromatography using a Dionex DNA Pac100 (9 x 250 mm)-column (ThermoFisher, Dreieich, Germany) on an ÄKTA Purifier system (GE Healthcare, Freiburg, Germany) equipped with an auto-sampler (A905) and a fraction collector (Frac-950). Buffer A was 20 mM TRIS pH 7.4 and contained 20% acetonitrile and buffer B contained 500 mM sodium perchlorate in buffer A. A flow rate of 5 mL/min and a gradient starting from 10% to 100% buffer B within 15 column volumes (CV) was employed. UV traces at 260 and 280 nm were recorded to monitor product elution. Appropriate fractions were pooled and precipitated overnight in the freezer using 3M NaOAc, pH=5.2 in ethanol (1:32 v/v). Pellets were collected by centrifugation and reconstituted in purified water. The purified sense and antisense strands were quantified by measuring the UV absorption at 260 nm. Subsequently, the materials were assessed with respect to fulfilment of pre-agreed specifications for MS-identity (+/-0.05% of calculated mass (by ESI-MS)) and purity (single strand purity > 85%, as per integration of the UV signal of the analytical AEX trace).

Annealing of oligoribonucleotides to generate siRNA

[0081] The purified complementary strands were then mixed in an equimolar ratio and dried in a SpeedVac concentrator. Subsequently, they were formulated in annealing

buffer (40 mM NaH₂PO₄·H₂O + Na₂HPO₄ buffer, pH 6.8, 0.2 M NaCl) and annealed (duplex solutions were placed into a water bath at 70 °C, which was subsequently cooled to room temperature within 3 h) to yield desired amount (in nmols or mg) of the siRNAs required for the follow-up experiment. The resultant siRNA-duplexes were characterized by size-exclusion chromatography (SEC) towards fulfillment of pre agreed specification of purity (duplex purity > 90%, as per integration of the UV signal of the analytical SEC trace).

2. Cell culture work

Cell culture and transfection

[0082] HuH-7 were obtained from CLS (via the German distributor Hoelzel Diagnostics, cat.Nr #300156) and cultured in DMEM medium (Biochrom #F0435). Hep3B cells were obtained from ATCC (ATCC in partnership with LGC Standards, Wesel, Germany, cat. #HB-8064) and cultured in EMEM medium (#ATCC #30-2003). Both media were supplemented to contain 10% fetal calf serum (#1248D, Biochrom GmbH, Berlin, Germany), and 100 U/ml Penicillin / 100 µg/ml Streptomycin (#A2213, Biochrom GmbH, Berlin, Germany). Cells were incubated at 37 °C in an atmosphere with 5% CO₂ in a humidified incubator. For transfection with siRNAs, 15,000 cells/well were seeded into 96-well tissue culture plates.

[0083] Transfection of siRNAs was carried out with Lipofectamine 2000 for HuH7 cells (0.5 µl/well), and Lipofectamine RNAiMax for Hep3B cells (0.3 µl/well), both obtained from Invitrogen/Thermo (Karlsruhe, Germany) according to manufacturer's instructions for reverse transfection. Dual dose screen was performed in HuH7 cells, with siRNAs in quadruplicates at 20 nM and 0.3 nM. As controls served siRNAs targeting Ahsa1, Firefly-Luciferase and FVII as unspecific controls and a mock transfection. The Ahsa1 siRNA also served as positive control for transfection efficiency (detecting Ahsa1 mRNA level instead of PCSK9). Dose-response experiments were performed in 10 concentrations transfected in quadruplicates, starting at 30 nM in 5-fold dilutions steps down to ~15 fM. After 24h incubation, medium was removed and cells were lysed in 150 µl Medium-Lysis Mixture (1 volume lysis buffer, 2 volumes cell culture medium) and then incubated at 53 °C for 30 minutes. Lysate were kept frozen until analysis.

Direct uptake in primary hepatocytes

[0084] Cryopreserved primary hepatocytes were purchased from Primacyt (Schwerin, Germany, Lot CHM2221-HE-C for human hepatocytes, Lot# CH141204 for

cynomolgus hepatocytes). Dose-response experiments were performed in 10 concentrations incubated in quadruplicates, starting at 5 μ M in 4-fold dilutions steps down to ~20 pM. After 5 hours, the plating medium containing the siRNAs was changed to maintenance medium (Primacyt cat#HHMM). The medium was changed every 24 hours and after 48 h incubation, medium was removed and cells were lysed in 200 μ l Medium-Lysis Mixture (1 volume lysis mixture, 2 volumes cell culture medium containing 1 μ L/mL Proteinase K) and then incubated at 53°C for 30 minutes. Lysates were kept frozen until further analysis.

mRNA quantification

[0085] A branched DNA assay, in the Quantigene Singleplex version (Thermo, #QS0014) was used to quantify mRNA levels after transfection. The assay was performed according to manufacturer's instructions with probesets to PCSK9 and GAPDH for the respective species, both designed by ThermoFisher Scientific and synthesized by Metabion (Planegg, Germany).

Table 4: Probesets to PCS97 and Human GAPDH

Probe ID	Sequence derivation, location and assignment	5'-3'-sequence (reduced by proprietary sequence parts for CE & LE)
QG2 hsPCSK9 1	NM_174936.1005.1023.LE	TGGCCTGTCTGTGGAAGCG
QG2 hsPCSK9 2	NM_174936.1024.1044.LE	TGCCATGACTGTCACACTTGC
QG2 hsPCSK9 3	NM_174936.1045.1061.CE	CACCCCTGCCAGGTGGG
QG2 hsPCSK9 4	NM_174936.1062.1077.CE	CATCCCGGCCGCTGAC
QG2 hsPCSK9 5	NM_174936.1078.1092.LE	CCTTGGCCACGCCGG
QG2 hsPCSK9 6	NM_174936.1093.1109.LE	GCTGCGCATGCTGGCAC
QG2 hsPCSK9 7	NM_174936.1110.1127.CE	GCAGTTGAGCACGCGCAG
QG2 hsPCSK9 8	NM_174936.1128.1145.LE	AACCGTGCCCTTCCCTTG
QG2 hsPCSK9 9	NM_174936.1146.1163.LE	GCCTATGAGGGTGCCGCT
QG2 hsPCSK9 10	NM_174936.1164.1185.CE	GGCTTTTCCGAATAAACTCCAG
QG2 hsPCSK9 11	NM_174936.1186.1204.LE	CCCACAGGCTGGACCAGCT
QG2 hsPCSK9 12	NM_174936.1205.1223.LE	CAGCAGCACCACCAGTGGC
QG2 hsPCSK9 13	NM_174936.1224.1238.CE	CCCACCCGCCAGGGG
QG2 hsPCSK9 14	NM_174936.1239.1257.LE	CGTTGAGGACGCGGCTGTA
QG2 hsPCSK9 15	NM_174936.1258.1271.LE	GCGCTGGCAGGCGG
QG2 hsPCSK9 16	NM_174936.1272.1287.CE	CCCCAGCCCTCGCCAG
QG2 hsPCSK9 17	NM_174936.1288.1305.LE	CAGCGGTGACCAGCACGA
QG2 hsPCSK9 18	NM_174936.1306.1321.LE	TCCCGGAAGTTGCCGG
QG2 hsPCSK9 19	NM_174936.1322.1341.CE	GGGAGTAGAGGCAGGCATCG
QG2 hsPCSK9 20	NM_174936.1342.1359.LE	CCTCGGGAGCTGAGGCTG
QG2 hsPCSK9 21	NM_174936.1360.1379.LE	GGTGGCCCCAACTGTGATGA
QG2 hsPCSK9 22	NM_174936.1380.1397.CE	CGGCTGGTCTTGGGCATT
QG2 hsPCSK9 23	NM_174936.1398.1416.LE	CCAAAGTCCCCAGGGTCAC

QG2 hsPCSK9 24	NM 174936.1417.1434.LE	AGCGGCCAAAGTTGGTCC
QG2 hsPCSK9 25	NM 174936.1435.1453.LE	GGGGCAAAGAGGTCCACAC
QG2 hsPCSK9 26	NM 174936.1454.1473.LE	CACCAATGATGTCTCCCT
QG2 hsPCSK9 27	NM 174936.1474.1491.LE	TGCTGCAGTCGCTGGAGG
QG2 hsPCSK9 28	NM 174936.1492.1513.LE	CCACTCTGTGACACAAAGCAGG
QG2 hsPCSK9 29	NM 174936.1514.1532.BL	GGCAGCAGCCTGTGATGTC
QG2 hsPCSK9 30	NM 174936.1533.1550.CE	TGCAATGCCAGCCACGTG
QG2 hsPCSK9 31	NM 174936.1551.1569.BL	CGGCAGACAGCATCATGGC
QG2 hsPCSK9 32	NM 174936.1570.1587.CE	CCAGGGTGAGCTCCGGCT
QG2 hsGAPDH 1	NM 002046.881.898.CE	CAGGATGCCCTTGAGGGG
QG2 hsGAPDH 2	NM 002046.899.919.LE	CACCTGGTGCTCAGTGTAGCC
QG2 hsGAPDH 3	NM 002046.920.942.LE	TCGCTGTTGAAGTCAGAGGAGAC
QG2 hsGAPDH 4	NM 002046.943.962.CE	CAAAGGTGGAGGAGTGGGTG
QG2 hsGAPDH 5	NM 002046.963.979.LE	AATGCCAGCCCCAGCGT
QG2 hsGAPDH 6	NM 002046.980.999.LE	ACAAAGTGGTCGTTGAGGGC
QG2 hsGAPDH 7	NM 002046.1000.1022.CE	TGTCATACCAGGAAATGAGCTTG
QG2 hsGAPDH 8	NM 002046.1023.1044.LE	CTGTTGCTGTAGCCAAATTCGT
QG2 hsGAPDH 9	NM 002046.1045.1063.LE	GGCCATGAGGTCCACCACC
QG2 hsGAPDH 10	NM 002046.1064.1084.LE	TTACTCCTTGAGGCCATGTG
QG2 hsGAPDH 11	NM 002046.1085.1102.LE	GCTGGTGGTCCAGGGGTC
QG2 hsGAPDH 12	NM 002046.1103.1122.CE	CCTCTTGTGCTCTTGCTGGG
QG2 hsGAPDH 13	NM 002046.1123.1145.BL	CCAGCAGTGAGGGTCTCTCTCTT
QG2 hsGAPDH 14	NM 002046.1146.1165.BL	CTGAGTGTGGCAGGGACTCC
QG2 hsGAPDH 15	NM 002046.1166.1185.BL	AGATTCAGTGTGGTGGGGGA
QG2 hsGAPDH 16	NM 002046.1186.1204.LE	TGGCAACTGTGAGGAGGGG
QG2 hsGAPDH 17	NM 002046.1205.1225.LE	CCCCTCTTCAAGGGGTCTACA
QG2 hsGAPDH 18	NM 002046.1226.1242.CE	CGGCTCCCTAGGCCCT

[0086] Luminescence was read using 1420 Luminescence Counter (WALLAC VICTOR Light, Perkin Elmer, Rodgau- Jügesheim, Germany) following 30 minutes incubation at RT in the dark. For each well, the PCSK9 mRNA level was normalized to the respective GAPDH mRNA level. The activity of a given PCSK9 siRNA was expressed as percent PCSK9 mRNA concentration (normalized to GAPDH mRNA) in treated cells, relative to the PCSK9 mRNA concentration (normalized to GAPDH mRNA) averaged across control wells.

3. RNA-Seq specificity analysis

In vitro work and library preparation

[0087] *Cell culture work in HepB3 cells:* For the cell line RNA-Seq analysis Hep3B purchased from ATCC (ATCC in partnership with LGC Standards, Wesel, Germany, cat. #HB-8064) were used. For the RNA-Seq experiment, Hep3B cell line was transfected using

Lipofectamine RNAiMAX (4.5 µL/well, Thermo Fisher Scientific, Karlsruhe, Germany) in a 6-well plate in triplicates at 10 nM final siRNA concentration for 24 hours.

[0088] *Library preparation:* Following siRNA treatment, the Hep3B cells were lysed in RNA Tissue Lysis Buffer (Mannheim, Germany, cat#03604721001) and total RNA was isolated using the MagNa Pure 96 Cellular RNA Large Volume Kit (Mannheim, Germany, cat#05467535001) on Roche's MagNa Pure 96 system (Roche, Mannheim, Germany). RNA quality was monitored using RNA ScreenTape Analysis kit (Santa Clara, USA, cat#50675576; 50675578; 50675577) on Agilent's TapeStation system (Santa Clara, USA). Only a RIN^e value > 8 was used for subsequent RNA-Seq library preparation. Concentration of the RNA was quantified using the Qubit® RNA broad range kit (Thermo Fisher Scientific, Karlsruhe, Germany, cat#Q10211) on a Qubit® analyzer (Karlsruhe, Germany). Sequencing libraries were prepared using the TruSeq stranded mRNA kit (Illumina, Berlin, Germany, cat#20020595), with 500 ng RNA input on a fully automated NGS Star (Hamilton, Switzerland) according to manufacturer's instruction. After completion of preparation, the libraries were quantified using the Qubit® dsDNA HS quantification kit (Thermo Fisher Scientific, Karlsruhe, Germany, cat#Q32854) on a Qubit® analyzer and library size distribution were analyzed using DNA ScreenTape Analysis kit (Santa Clara, USA, cat# cat#50675586; 50675583; 50675582) on Agilent's TapeStation system (Santa Clara, USA). After calculation of the final library concentration, libraries were normalized to 10 nM and pooled for subsequent single read sequencing. Pooled libraries were denatured, diluted to 1.7 pM and loaded on the reagent cartridge for sequencing according to manufacturer's instruction. Sequencing was performed on a NextSeq550 sequencer (Illumina, Darmstadt, Germany) with a High Output Kit v2.5 75 cycles (Darmstadt, Germany, cat#20024906). 75 bp single read sequencing was performed with an estimate 20 – 30 million reads for each library. Adapter trimmed raw reads were handed over for bioinformatic analysis.

Data analysis

[0089] *Raw read quality checks:* The raw reads (fastq.gz file format) generated on the Illumina NextSeq550 were used as input for quality checks (PHRED scores, reads per library, read lengths) using the software FastQC (version 0.11.0) (bioinformatics.babraham.ac.uk/projects/fastqc/).

[0090] *Read quality trimming:* Raw reads with a PHRED score < 28 were removed using the software TrimGalore (version 0.6.4)

(bioinformatics.babraham.ac.uk/projects/trim_galore/). Afterwards, FastQC (version 0.11.0) (bioinformatics.babraham.ac.uk/projects/fastqc/) was repeated on the trimmed reads.

[0091] *Read mapping against the human reference genome:* The trimmed reads were mapped against the human genome (ftp.ncbi.nlm.nih.gov/genomes/all/annotation_releases/9606/GCF_000001405.40-RS_2023_03/GRCh38_major_release_seqs_for_alignment_pipelines/GCA_000001405.15_GRCh38_full_analysis_set.fna.gz) and the according reference annotation (ftp.ncbi.nlm.nih.gov/genomes/all/annotation_releases/9606/GCF_000001405.40-RS_2023_03/GRCh38_major_release_seqs_for_alignment_pipelines/GCA_000001405.15_GRCh38_full_analysis_set.refseq_annotation.gtf.gz) from NCBI using the software STAR (version 2.7.3) (STAR: Dobin, Alexander, et al. "STAR: ultrafast universal RNA-seq aligner." *Bioinformatics* 29.1 (2013): 15-21), resulting in BAM files.

[0092] *Read counting and normalization:* The BAM files generated by read mapping were used to count the reads per gene using the software featureCounts (version 2.0.3) (featureCounts (subread): Liao, Yang, Gordon K. Smyth, and Wei Shi. "featureCounts: an efficient general purpose program for assigning sequence reads to genomic features." *Bioinformatics* 30.7 (2014): 923-930), resulting in a read counts matrix. The read counts were then normalized using the DESeq2 method (DESeq2: Love, Michael I., Wolfgang Huber, and Simon Anders. "Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2." *Genome biology* 15 (2014): 1-21).

[0093] *Principal Component Analysis and Differential Expression Analysis:* The normalized read counts were used as input for the software DESeq2 to perform the Principal Component Analysis (PCA) and the Differential Expression Analysis (DEA). For the PCA, the variance-stabilizing transformation (VST) was conducted as suggested in the manual. DEA was conducted following the DESeq2 manual. Here, the log fold change shrinkage was applied to consider lowly expressed and potentially noisy genes (method: ash).

[0094] *Further analyses and data visualizations:* All further analyses and visualizations were generated using R Statistical software.

[0095] The dual dose screen was performed with GalNAc-conjugated PCSK9 siRNAs incubated in quadruplicates at 5 μ M and 50 nM, respectively, with a positive control siRNA targeting PCSK9 as a gold standard (XD-15878 (original Inclisiran) or its derivative XD-36954 shown in Table 5, with equal activity present on each cell culture plate at respective dose. Untreated cells served as negative control.

Table 5

SS	csusasgacCfuGfudTuugcuuuugu(L96)
AS	asCfsasAfAfAfgCfaAfaAfcAfgGfuCfuasgsasa

[0096] Initial dose-response experiments (data not shown) were done with designed PCSK9 siRNA conjugates obtained from first set of initial applications at 5 concentrations (10.0, 1.429, 0.204, 0.029, and 0.004 μM) incubated in quadruplicates. Of note, original Inclisiran was also tested at these concentrations in the same experiment.

[0097] Subsequently, experiments were done with PCSK9 siRNAs in 10 concentrations incubated in quadruplicates, starting at 10 μM sequentially diluted in either 3 or 4-fold dilution steps down to ~ 500 pM or 40 pM.

[0098] In both DRC experiments, Inclisiran was used as positive control on each plate and untreated cells served as negative control.

Sequential Optimization of Modifications of Improved Inclisiran Potency

Example 2. Vinu-modified Inclisiran [XD-42180]

[0099] Nucleic acid sequences for the sense strand (SS 5'-3') and antisense strand (AS 5'-3') of the XD-42180 designed siRNA duplexes are represented in Table 6 according to the notation provided in Table 3.

Table 6

	Original Chemistry vinu
SS	csusagacCfuGfudTuugcuuuugu(L96)
AS	(vinu)sCfsaAfAfAfgCfaAfaAfcAfgGfuCfuagsasa

[00100] The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes at a series of 10 concentrations was determined as described in Example 1. The data are shown in Table 7 and Table 8. The IC_{50} as determined from the Dose Response Curve (DRC) was 0.015 μM , as compared to $\text{IC}_{50} = 0.059$ μM for the original Inclisiran under same experimental conditions (hepatocyte batch), which shows ~ 4 -fold improvement in activity.

Table 7

ID	dose [μ M]	((Target / GapDH) / MV ctrl) * 100				MV quadruplicat es [% residual target mRNA]	SD quadrupli- cates
		Well 1	Well 2	Well 3	Well 4		
XD-42180	10.0	20	22	20	19	21	1
	3.33	20	23	22	21	22	1
	1.11	25	42	26	23	29	9
	0.37	25	34	25	26	27	4
	0.12	31	31	33	30	31	1
	0.041	43	36	42	32	38	5
	0.014	46	61	53	43	51	8
	0.005	60		58	72	63	7
	0.0150	90	88	72	100	88	12
	0.0005	85	76	81	80	81	4
	untreated	101	91	96	112	100	9

Table 8

ID	dose [μ M]	((Target / GapDH) / MV ctrl) * 100				MV quadruplicates [% residual target mRNA]	SD quadruplicates
		Well 1	Well 2	Well 3	Well 4		
XD-15878 (Inclisiran)	10.0	24	25	29	27	26	2
	3.33	26	27	29	26	27	1
	1.11	29	34	36	35	33	3
	0.37	34	43	36	43	39	5
	0.12	43	35	45	42	41	4
	0.04	48	54	51	63	54	6
	0.01	59	65	67	71	66	5
	0.00	68	73	77	75	73	4
	0.00	72	83		91	82	10
	0.00	85	89	102	105	95	10
	untreated	101	88	102	109	100	9

Example 3. DV18-Vinu Modified Inclisiran [XD-44683]

The nucleic acid sequences for the sense strand (SS 5'-3') and antisense strand (AS 5'-3') of the duplexes of the designed siRNA conjugates tested as represented in Table 9 according to the notation provided in Table 3. The IC₅₀ of DV18-vinu modified Inclisiran as determined

from the Dose Response Curve (DRC) was 0.005 μM , as compared to $\text{IC}_{50} = 0.033 \mu\text{M}$ for Inclisiran measured in the same conditions, which shows over 6-fold improvement in activity.

Table 9

	DV18 vinu
SS	csusagacCfuGfUfUfuugcuuuuga(L96)
AS	(vinu)sCfsaaaAfgCfAfaaacAfgGfucuagsasa

Table 10: DV18-Vinu Modified Inclisiran

ID	dose [μ M]	((Target / GapDH) / MV ctrl) * 100				MV quadruplicates [% residual Rluc activity]	SD quadruplicates
		Well 1	Well 2	Well 3	Well 4		
XD-15878 (Inclisiran)	10	36	35	35	31	34	2
	1.429	41	33	33	27	34	6
	0.204	45	43	35	30	38	7
	0.029	57	50	53	49	52	4
	0.004	78	72	68	58	69	9
	untreated	112	92	88	87	95	12

Table 11

ID	dose [μ M]	((Target / GapDH) / MV ctrl) * 100				MV quadruplicates [% residual Rluc activity]	SD quadruplicates
		Well 1	Well 2	Well 3	Well 4		
XD-44683	10	25		22	20	22	2
	1.429	28	27	26	24	26	2
	0.204	33	26	26	27	28	3
	0.029	44	35	30	31	35	6
	0.004	62	53	48	47	52	7
	untreated	112	92	88	87	95	12

Example 4. Exemplary Modified Inclisiran DV18_vinu_4F_SS11DNA [XD-44687]

[00101] The nucleic acid sequences for the sense strand (SS 5'-3') and antisense strand (AS 5'-3') of the duplexes of the designed siRNA conjugates tested in the second stage are represented in Table 12 according to the notation provided in Table 3.

Table 12

	4F SS11DNA
SS	csusagacCfuGfUfdTuugcuuuuga(L96)
AS	(vinu)sCfsaaaAfgcaaaacAfgGfucuagsasa

Table 13: DV18 + 4F_SS11DNA Modified Inclisiran

ID	dose [μM]	MV ctrls	((Target / GapDH) / MV ctrl) * 100				MV quadruplicates [% residual target mRNA]	SD quadruplicates
			Well 1	Well 2	Well 3	Well 4		
	Untreated	0.04112	83	85	79	78	82	3
XD-44687	10.0		38	34	33	33	35	2
	2.50		37	24	36	30	32	6
	0.63		43	35	36	34	37	4
	0.16		54	48	41	41	46	6
	0.04		63	50	53	52	55	6
	0.01		74	58	62	54	62	9
	0.002		82	77	68	65	73	8
	0.001		93	78	71	77	80	9
	0.0002		97	85	77	80	85	9
	0.00004		225	200	199	231	214	17
	Untreated		110		90	100	100	10

Table 14

ID	dose [μM]	((Target / GapDH) / MV ctrl) * 100				MV quadruplicates [% residual target mRNA]	SD quadruplicates
		Well 1	Well 2	Well 3	Well 4		
XD-15878 (Inclisiran)	10.0	52	48	42	42	46	5
	2.50	53	40	40	42	44	6
	0.63	61	48	41	42	48	10
	0.16	61	60	49	48	54	7
	0.04	74	60	55	54	61	9
	0.01		73	64	66	68	5
	0.002		88	73	82	81	8
	0.001		91	82	90	87	5
	0.0002		94	92	95	93	1
	0.00004	94	98	81	103	94	10
	Untreated	110	104	87		100	12

*Empty cells represent outliers removed from quadruplicate.

[00102] The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes at a series of 10 concentrations was determined as described in Example 1. The data are shown in Table 13 and Table 14. The

IC₅₀ as determined from the Dose Response Curve (DRC) was 0.03 μM, as compared to IC₅₀ = 0.37 μM for original Inclisiran measured in the same conditions, which shows greater than 12-fold improvement in activity.

Example 5. Unmodified Inclisiran

[00103] The nucleic acid sequences for the sense strand (SS 5'-3') and antisense strand (AS 5'-3') of the duplexes of the designed siRNA conjugates additionally tested are represented in Table 1. The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes was determined as described in Example 1. The data are shown in Table 14. The IC₅₀ as determined from the Dose Response Curve (DRC) was IC₅₀ = 0.37 μM.

[00104] The nucleic acid sequences for the sense strand (SS 5'-3') and antisense strand (AS 5'-3') of the duplexes of the designed siRNA conjugates tested in the second stage are represented in Table 15 according to the notation provided in Table 3. Where a sequence comprises one or more Locked nucleic acids (LNA), the strand (SS or AS) which comprises this modification is identified in the name (Short form seq mod) followed by the number(s) of the modified nucleotide from the 5' end and "LNA." For example, "3512_4F_AS6OMe_16LNA" comprises an LNA in the AS at position 16. Other modifications comprising the respective sequence information are indicated by the sequence notation itself.

Table 15: 3512-based sequences

Short-form abbreviation	XD	siRNA Duplex	SS 5'-3' AS-5'-3'	IC50 (μ M)
3512_4F_AS6OMe	XD-44719	csusuuuuAfcAfGfCfcaacuuuua(L96)\ (vinu)sGfsaaaaguuggcGfuAfaaaggsgc		IC50=0.02 μ M
3512_4F_AS6DNA	XD-44725	csusuuuuAfcAfGfCfcaacuuuua(L96)\ (vinu)sGfsaaadAguugggcGfuAfaaaggsgc		IC50=0.05 μ M
3512_4F_SS7+10LNA	XD-44701	csusuuuuAbcAfGbCfcaacuuuua(L96)\ (vinu)sGfsaaaAfguugggcGfuAfaaaggsgc		IC=0.08 μ M
3512_4F_SS11ab	XD-44695	csusuuuuAfcAfGf(abasic)caacuuuua(L96)\ (vinu)sGfsaaaAfguugggcGfuAfaaaggsgc		IC=0.10 μ M
3512_DV18_vinu	XD-42182	csusuuuuAfcAfGfCfcaacuuuua(L96)\ (vinu)sGfsaaaAfgUfUfggcGfuAfaaaggsgc		IC50=0.07 μ M
3512_4F_AS6OMe_16LNA	XD-44731	csusuuuuAfcAfGfCfcaacuuuua(L96)\ (vinu)sGfsaaaaguuggcGfuAbaaaggsgc		IC50=0.11 μ M
3512_4F_SS11DNA	XD-44689	csusuuuuAfcAfGfdCcaacuuuua(L96)\ (vinu)sGfsaaaAfguugggcGfuAfaaaggsgc		IC50 =0.14 μ M
3512_4F_AS6OMe_16+14LNA	XD-44737	csusuuuuAfcAfGfCfcaacuuuua(L96)\ (vinu)sGfsaaaaguuggcGbuAbaaaggsgc		Data not shown
3512_DV18	XD-38572	csusuuuuAfcAfGfCfcaacuuuua(L96)\ usGfsaaaAfgUfUfggcGfuAfaaaggsgc		Data not shown
3512_4F_SS10DNA_9+11LNA	XD-44713	csusuuuuAfcAbdGCbcaacuuuua(L96)\ (vinu)sGfsaaaAfguugggcGfuAfaaaggsgc		N/A
3512_4F_SS7+10LNA_9+11DNA	XD-44707	csusuuuuAbcdAGbdCcaacuuuua(L96)\ (vinu)sGfsaaaAfguugggcGfuAfaaaggsgc		N/A

[00105] The nucleic acid sequences for the sense strand (SS 5'-3') and antisense strand (AS 5'-3') of the duplexes of the designed siRNA conjugates tested in the second stage are represented in Table 16 according to the notation provided in Table 3. Where a sequence comprises one or more Locked nucleic acids (LNA), the strand (SS or AS) which comprises this modification is identified in the name (Short form seq mod) followed by the number(s) of the modified nucleotide from the 5' end and "LNA.". For example, "3545_4F_AS6OMe_16LNA" comprises an LNA in the AS at position 16. Other modifications comprising the respective sequence information are indicated by the sequence notation itself.

Table 16. 3545-based sequences

Short-form abbreviation	XD	siRNA Duplex SS 5'-3' AS 5'-3'	Ex. No. IC50 (μ M)
3545 4F AS6OMe 16LNA	XD-44728	csusuugUfaAfCfUfugaagauua(L96)\ (vinu)sAfsuaucuucaaguUfaCbaaaagscsa	0.02
3545 4F AS6DNA	XD-44722	csusuugUfaAfCfUfugaagauua(L96)\ (vinu)sAfsuauCuucaaguUfaCfaaaagscsa	0.01
3545 DV18 vinu	XD-44682	csusuugUfaAfCfUfugaagauua(L96)\ (vinu)sAfsuauCfuUfCfaaguUfaCfaaaagscsa	0.03
3545 4F AS6OMe	XD-44716	csusuugUfaAfCfUfugaagauua(L96)\ (vinu)sAfsuaucuucaaguUfaCfaaaagscsa	0.02
3545 4F SS11ab	XD-44692	csusuugUfaAfCf(abasic)ugaagauua(L96)\ (vinu)sAfsuauCfuucaaguUfaCfaaaagscsa	0.08
3545 4F SS7+10LNA	XD-44698	csusuugTbaAfCbUfugaagauua(L96)\ (vinu)sAfsuauCfuucaaguUfaCfaaaagscsa	0.07
3545 4F SS7+10LNA 9+11DNA	XD-44704	csusuugTbadACbdTugaagauua(L96)\ (vinu)sAfsuauCfuucaaguUfaCfaaaagscsa	0.13
3545 DV18	XD-38578	csusuugUfaAfCfUfugaagauua(L96)\ usAfsuauCfuUfCfaaguUfaCfaaaagscsa	Data Not Shown
3545 4F SS10DNA 9+11LNA	XD-44710	csusuugUfaAbdCTbugaagauua(L96)\ (vinu)sAfsuauCfuucaaguUfaCfaaaagscsa	Data Not Shown
3545 4F AS6OMe 16+14LNA	XD-44734	csusuugUfaAfCfUfugaagauua(L96)\ (vinu)sAfsuaucuucaaguTbaCbaaaagscsa	Data Not Shown
3545 4F SS11DNA	XD-44686	csusuugUfaAfCfdTugaagauua(L96) (vinu)sAfsuauCfuucaaguUfaCfaaaagscsa	Data Not Shown

Example 6. 3512_4F_AS6OMe_16LNA [XD-44731]

[00106] The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes at a series of 10 concentrations was determined as described in Example 1. The data are shown in FIG. 2 and Table 17. The IC₅₀ as determined from the Dose Response Curve (DRC) was 0.11 μ M, compared to IC₅₀ = 0.37 μ M for original Inclisiran measured in the same experiment, which shows greater than a 3-fold improvement in activity.

Table 17

ID	Conc (μ M)	MV Controls	((Target / GapDH) / MV ctrl) * 100				Data*	Data**
			Well 1	Well 2	Well 3	Well 4		
XD-44731	Mock	0.03954	114	110	109	105	109	4
	10.0		37	43	37	36	38	3
	2.50		38	44	39	33	38	4
	0.63		41	44	39	37	40	3
	0.16		54	53	44	50	50	5
	0.04		61	N/A	70	50	60	10
	0.01		72	65	60	54	63	8
	0.002		90	94	87	83	88	4
	0.001		103	95	85	82	91	10
	0.0002		102	100	86	86	93	9
	0.00004		103	109	94	86	98	10

Data*: MV quadruplicates [% residual target mRNA]

Data**: SD quadruplicates

Example 7. 3512_4F_AS6DNA [XD-44725]

[00107] The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes at a series of 10 concentrations was determined as described in Example 1. The data are shown in FIG. 3 and Table 18. The IC₅₀ as determined from the Dose Response Curve (DRC) was 0.05 μ M, compared to IC₅₀ = 0.37 μ M for original Inclisiran measured in the same experiment, which shows greater than a 7-fold improvement in the designed siRNA activity in vitro.

Table 18

ID	dose [μ M]	((Target / GapDH) / MV ctrl) * 100				MV quadruplicates [% residual target mRNA]	SD quadruplicates
		Well 1	Well 2	Well 3	Well 4		
XD-44725	10.0	38	32	32	29	33	4
	2.50	35	35	31	30	33	2
	0.63	40	45	37	35	39	4
	0.16	49	48	43	44	46	3
	0.04	50	49	48	47	48	1
	0.01	58	54	57	58	56	2
	0.002	91		80	92	88	7
	0.001	96	89	85	86	89	5
	0.0002		87	87	89	88	1
	0.00004	95	93	90	88	92	3
	Mock	102	103	91	105	100	6

Data*: MV (mean value) quadruplicates [% residual target mRNA]

Data**: SD (standard deviation) quadruplicates

Example 8. 3512_4F_SS7+10LNA [XD-44701]

[00108] The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes at a series of 10 concentrations was determined as described in Example 1. The data are shown in FIG. 4 and Table 19. The IC_{50} determined from the Dose Response Curve (DRC) was 0.08 μ M, compared to $IC_{50} = 0.37$ μ M for original Inclisiran measured in the same experiment, which shows greater than a 4-fold improvement in activity.

Table 19

ID	Conc (μ M)	MV Controls	((Target / GapDH) / MV ctrl) * 100				Data*	Data**
			Well 1	Well 2	Well 3	Well 4		
	Inclisiran, XD-15878K5	0.06508	72	64	56	54	62	8
XD-44701	10.0		37	36	33	29	34	4
	2.50		43	36	39	38	39	3
	0.63		44	40	43	39	42	2
	0.16		49	46	47	43	46	3
	0.04		55	58	58	54	56	2
	0.01		72	68	76	66	71	4
	0.002		78	82	86	75	80	5
	0.001		94	89	97	110	97	9
	0.0002		101	92	98	102	98	4
	0.00004		106	99	101	105	103	3
	Mock		96	102	103	99	100	3

Data*: MV quadruplicates [% residual target mRNA]

Data**: SD quadruplicates

Example 9. 3512_4F_SS11ab [XD-44695]

[00109] The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes at a series of 10 concentrations was determined as described in Example 1. The data are shown in FIG. 5 and Table 20. The IC₅₀ as determined from the Dose Response Curve (DRC) was 0.10 μ M, compared to IC₅₀ = 0.37 μ M for original Inclisiran measured in the same experiment, which shows greater than a 3-fold improvement in activity.

Table 20

ID	Conc [μ M]	MV Controls	((Target / GapDH) / MV ctrl) * 100				Data*	Data**
			Well 1	Well 1 2	Well 1 3	Well 4		
Inclisiran, XD-15878K5	0.04 μ M (IC ₅₀)	0.07516	71	66	49	54	60	10
XD-44695	10.0		38	39	33	32	35	3
	2.50		38	36	37	N/A	37	1
	0.63		43	40	39	38	40	2
	0.16		53	46	42	40	45	6
	0.04		60	54	54	53	55	3
	0.01		70	69	67	65	67	2

	0.002		83	81	75	74	78	5
	0.001		91	81	90	87	87	4
	0.0002		95	95	90	88	92	3
	0.00004		87	85	86	85	86	1

Data*: MV quadruplicates [% residual target mRNA]

Data**: SD quadruplicates

Example 10. 3512_4F_AS6OMe [XD-44719]

[00110] The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes at a series of 10 concentrations was determined as described in Example 1. The data are shown in FIG. 6 and Table 21. The IC₅₀ determined from the Dose Response Curve (DRC) was 0.02 μ M, compared to IC₅₀ = 0.37 μ M for original Inclisiran measured in the same experiment, which shows greater than an 18-fold improvement in activity.

Table 21

ID	Concentration μ M	MV controls	((Target / GapDH) / MV ctrl) * 100				Data *	Data**
			Well 1	Well 2	Well 3	Well 4		
	Inclisiran, XD-15878K5 0.04 μ M (IC ₅₀)		65	66	62	68	65	2
XD-44719	10.0	0.04291	29	32	31	33	31	2
	2.50		27	27	28	34	29	3
	0.63		29	31	33	33	32	2
	0.16		35	38	17	44	34	12
	0.04		42	42	43	50	45	4
	0.01		47	50	49	66	53	9
	0.002		66	82	80	86	78	9
	0.001		65	80	75	89	77	10
	0.0002		71	79	88	100	84	12
	0.00004		74	82	87	N/A	81	7
	Mock		N/A	94	100	106	100	6

Data*: MV quadruplicates [% residual target mRNA]

Data**: SD quadruplicates

Example 11. 3512_DV18_vinu [XD-42182]

[00111] The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes at a series of 10 concentrations was determined as described in Example 1. The data are shown in FIG. 7 and Table 22. The IC₅₀ determined from the Dose Response Curve (DRC) was 0.07 μ M, compared to IC₅₀ = 0.37

μM for original Inclisiran measured in the same experiment, which shows greater than a 5-fold improvement in activity.

Table 22

ID	Concentration [μM]	MV controls	((Target / GapDH) / MV ctrl) * 100				Data*	Data**
			Well 1	Well 2	Well 3	Well 4		
XD-42182	Mock	0.06917	108	113	104	92	104	9
	10.0		41	39	32	34	37	4
	2.50		44	43	38	37	40	4
	0.63		50	41	40	38	42	5
	0.16		50	46	44	41	45	4
	0.04		58	55	50	54	54	3
	0.01		72	63	60	61	64	5
	0.002		82	76	74	71	76	5
	0.001		94	N/A	80	84	86	7
	0.0002		98	90	93	95	94	3
	0.00004		101	96	90	95	96	5

Data*: MV quadruplicates [% residual target mRNA]

Data**: SD quadruplicates

Example 12. 3512_4F_SS11DNA [XD-44689]

[00112] The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes at a series of 10 concentrations was determined as described in Example 1. The data are shown in FIG. 8 and Table 23. The IC_{50} determined from the Dose Response Curve (DRC) was $0.14 \mu\text{M}$, compared to $\text{IC}_{50} = 0.37 \mu\text{M}$ for original Inclisiran measured in the same experiment, which shows greater than a 2-fold improvement in activity.

Table 23

ID	dose [μM]	MV ctrls	((Target / GapDH) / MV ctrl) * 100				MV quadruplicates [% residual target mRNA]	SD quadruplicates
			Well 1	Well 2	Well 3	Well 4		
	Mock	0.07933	113	104	102	93	103	8
XD-44689	10.0		44	42	38	35	40	4
	2.50		42	36	43	44	41	4
	0.63		49		46	40	45	4

	0.16		47	47	44	46	46	1
	0.04		67	54	64	62	62	6
	0.01		62	62	67	68	65	3
	0.002		97	67	71	76	78	13
	0.001		81	79	75	83	79	3
	0.0002		99	88	89	85	91	6
	0.00004		107	94	94	96	98	6

Data*: MV quadruplicates [% residual target mRNA]

Data**: SD quadruplicates

Example 13. 3545_DV18_vinu [XD-44682]

[00113] The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes at a series of 10 concentrations was determined as described in Example 1. The data are shown in FIG. 9 and Table 24. The IC₅₀ determined from the Dose Response Curve (DRC) was 0.03 μ M, compared to IC₅₀ = 0.37 μ M for original Inclisiran measured in the same experiment, which shows greater than a 12-fold improvement in activity.

Table 24

ID	Conc [μ M]	MV Controls	((Target / GapDH) / MV ctrl) * 100				Data*	Data**
			Well 1 1	Well 1 2	Well 1 3	Well 4		
XD-44682	10.0	0.03747	61	70	72	84	72	10
	2.50		28	27	34	31	30	3
	0.63		31	33	31	33	32	1
	0.16		36	38	34	35	36	1
	0.04		45	45	48	44	45	2
	0.01		52	45	43	46	46	4
	0.002		56	54	55	49	53	3
	0.001		N/A	86	85	88	86	1
	0.0002		89	86	92	93	90	3
	0.00004		84	84	93	101	90	8
	Mock		88	88	95	102	93	7
			94	97	110	100	100	7

Data*: MV quadruplicates [% residual target mRNA]

Data**: SD quadruplicates

Example 14. 3545_4F_AS6DNA [XD-44722]

[00114] The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes at a series of 10 concentrations was

determined as described in Example 1. The data are shown in FIG. 10 and Table 25. The IC_{50} determined from the Dose Response Curve (DRC) was 0.01 μ M for both data sets, which is the most improved result. Compared to $IC_{50} = 0.37 \mu$ M for original Inclisiran measured in the same experiment, this variant shows about a 37-fold improvement in activity.

Table 25

ID	Conc, μ M	MV controls	((Target / GapDH) / MV ctrl) * 100				Data*	Data**
			Well 1	Well 2	Well 3	Well 4		
XD-44722 (2)	10.0	0.04496	75	68	74	71	72	3
	2.50		66	61	50	54	58	7
	0.63		25	22	24	26	24	1
	0.16		26	26	28	28	27	1
	0.04		27	28	29	31	29	2
	0.01		32	35	38	40	36	3
	0.002		40	35	40	45	40	4
	0.001		46	47	46	49	47	1
	0.0002		56	55	56	61	57	2
	0.00004		69	68	71	75	71	3
	Mock		93	N/A	76	87	86	9
			85	81	82	93	86	5
			N/A	95	100	105	100	5

Data*: MV quadruplicates [% residual target mRNA]

Data**: SD quadruplicates

Example 15. 3545_4F_AS6OMe_16LNA [XD-44728]

[00115] The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes at a series of 10 concentrations was determined as described in Example 1. The data are shown in FIG. 11 and Table 26. The IC_{50} determined from the Dose Response Curve (DRC) was 0.02 μ M, compared to $IC_{50} = 0.37 \mu$ M for original Inclisiran measured in the same experiment, which shows greater than an 18-fold improvement in the modified siRNA activity.

Table 26

ID	dose [μ M]	MV ctrls	((Target / GapDH) / MV ctrl) * 100				MV quadruplicates [% residual target mRNA]	SD quadruplicates
			Well 1	Well 2	Well 3	Well 4		
XD-44728	10.0	0.04395	29	29	28	32	29	2

	2.50		32	30	30	36	32	3
	0.63		31	34	33	38	34	3
	0.16		40	38	37	41	39	2
	0.04		45	45		44	45	1
	0.01		43	51	49	56	50	5
	0.002		72	76	71	75	73	2
	0.001		76	78	72	80	77	3
	0.0002		97		86	80	88	9
	0.00004		80	82	79	89	83	4
	Mock		107	94	96	103	100	6

Data*: MV quadruplicates [% residual target mRNA]

Data**: SD quadruplicates

Example 16. 3545_4F_SS7+10LNA [XD-44698]

[00116] The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes at a series of 10 concentrations was determined as described in Example 1. The data are shown in FIG. 12 and Table 27. The IC₅₀ was determined from the Dose Response Curve (DRC) was 0.07 μ M, compared to IC₅₀ = 0.37 μ M for original Inclisiran measured in the same experiment, which shows greater than a 5-fold improvement in activity.

Table 27

ID	dose [μ M]	MV ctrls	((Target / GapDH) / MV ctrl) * 100				MV quadruplicates [% residual target mRNA]	SD quadruplicates
			Well 1	Well 2	Well 3	Well 4		
XD-44698	10.0	0.03483	35	35	36	39	36	2
	2.50		40	40	36	38	39	2
	0.63		38	35	34	43	38	4
	0.16		52	47	47	53	50	3
	0.04		55	51	52	59	54	3
	0.01		64	60	66	65	64	2
	0.002		91	82	92	92	89	5
	0.001		91	90	91	95	92	2
	0.0002		92	87	96	98	93	5
	0.00004		96	94	105	105	100	6
	Mock		99	92	105	103	100	6

Data*: MV quadruplicates [% residual target mRNA]

Data**: SD quadruplicate

Example 17. 3545_4F_AS6OMe [XD-44716]

[00117] The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes at a series of 10 concentrations was determined as described in Example 1. The data are shown in FIG. 13 and Table 28. The IC₅₀ was determined from the Dose Response Curve (DRC) was 0.02 μ M, compared to IC₅₀ = 0.37 μ M for original Inclisiran measured in the same experiment, which shows greater than an 18-fold improvement in activity.

Table 28

ID	Conc, μ M	MV controls	((Target / GapDH) / MV ctrl) * 100				Data*	Data**
			Well 1	Well 2	Well 3	Well 4		
XD-44716	Mock	0.05183						
	10.0		41	36	28	26	33	7
	2.50		41	34	30	26	32	6
	0.63		41	33	30	26	33	7
	0.16		53	51	41	32	45	10
	0.04		54	47	36	38	44	9
	0.01		66	62	46	47	55	10
	0.002		83	69	58	54	66	13
	0.001		89	N/A	62	58	69	17
	0.0002		N/A	102	71	65	79	20
	0.00004		105	84	74	70	83	16
	Mock		118	115	85	82	100	19

Data*: MV quadruplicates [% residual target mRNA]

Data**: SD quadruplicates

Example 18. 3545_4F_SS11ab [XD-44692]

[00118] The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes at a series of 10 concentrations was determined as described in Example 1. The data are shown in FIG. 14 and Table 29. The IC₅₀ determined from the Dose Response Curve (DRC) was 0.08 μ M, compared to IC₅₀ = 0.37 μ M for original Inclisiran measured in the same experiment, which shows greater than a 4-fold improvement in activity.

Table 29

ID	Conc, μ M	MV controls	((Target / GapDH) / MV ctrl) * 100				Data*	Data**
			Well 1	Well 2	Well 3	Well 4		
XD-44692	Mock	0.04362	92	97	97	N/A	95	3
	10.0		38	31	33	32	33	3
	2.50		38	33	35	34	35	2
	0.63		40	36	36	33	36	3
	0.16		52	45	42	44	46	4
	0.04		50	52	N/A	71	57	11
	0.01		59	51	59	61	57	5
	0.002		86	77	72	77	78	6
	0.001		96	81	80	84	85	7
	0.0002		149	86	80	169		45
	0.00004		92	85	81	84	86	5
	Mock		96	88	99	117	100	12

Data*: MV quadruplicates [% residual target mRNA]

Data**: SD quadruplicates

Example 19. 3545_4F_SS7+10LNA_9+11DNA [XD-44704]

[00119] The activity of the designed siRNA complex in reducing the expression of PCSK9 mRNA in cultured primary human hepatocytes at a series of 10 concentrations was determined as described in Example 1. The data are shown in FIG. 15 and Table 30. The IC₅₀ was determined from the Dose Response Curve (DRC) was 0.13 μ M, compared to IC₅₀ = 0.37 μ M for original Inclisiran measured in the same experiment, which shows almost a 3-fold improvement in activity.

Table 30

ID	Conc, μ M	MV controls	((Target / GapDH) / MV ctrl) * 100				Data*	Data**
			Well 1	Well 2	Well 3	Well 4		
XD-44704	Mock	0.03611	106	106	96	105	104	5
	10.0		37	37	40	38	38	1
	2.50		45	41	43	37	42	3
	0.63		45	43	40	39	42	3
	0.16		58	54	59	48	55	5
	0.04		61	66	55	54	59	5
	0.01		76	72	73	67	72	4
	0.002		103	89	101	92	96	7

	0.001		101	98	97	99	99	2
	0.0002		107	98	96	87	97	8
	0.00004		104	101	97	91	98	5
	Mock		112	93	103	93	100	9

Data*: MV quadruplicates [% residual target mRNA]

Data**: SD quadruplicates

Example 20. Sequence Optimization

[00120] Seven siRNAs and Inclisiran were analyzed in a single experiment to assure an appropriate head to head comparison.

Table 31: Dose Response Analysis of 7 Selected siRNAs and Inclisiran in Primary Human Hepatocytes and in Huh7 Cells:

ID	prim. human hepatocytes		Hu7 cells	
	IC 50 [nM]	max. KD[%]	IC 50 [pM]	max. KD[%]
Inclisiran	49.2	70	550	82
XD-44687	12.6	71	40	87
XD-44719	19.0	79	50	89
XD-44725	14.7	80	60	89
XD-42182	14.2	76	40	88
XD-44722	10.5	82	30	90
XD-44716	15.4	80	30	91
XD-44682	14.4	74	30	90

[00121] Three siRNAs were selected for further development and experiments as shown in Tables 32-35.

Table 32: Activity Analysis of Selected siRNAs in Primary Cynomolgus Monkey Hepatocytes

ID	IC50 [nM]	max. KD [%]
Inclisiran	9.4	69
XD-44687	6.0	78
XD-44719	6.6	76
XD-44722	3.4	80

Table 33: Synthesis of Two Variants of Each Parental Sequence with GNA Modifications and Dose Response Analysis in of Nine siRNAs and Inclisiran in Huh7 cells

ID	GNA Modification	IC50 [pM]	max. KD [%]
Inclisiran		285	79
XD-44687		25	82
XD-78829	GNA6 (XD-44687)	26	82
XD-78830	GNA7 (XD-44687)	317	62

XD-44719		30	85
XD-78826	GNA6 (XD-44719)	75	80
XD-78825	GNA7 (XD-44719)	365	68
XD-44722		18	85
XD-78828	GNA6 (XD-44722)	88	73
XD-78827	GNA7 (XD-44722)	34	83

Table 34: RNA-Seq Analysis of six siRNAs and Inclisiran

ID	GNA Modification	Number of differentially expressed genes log2-fold change: < 0.5 and > -0.5 significance (padj): >0.05
Inclisiran		8
XD-44687		9
XD-78829	GNA6 (XD-44687)	13
XD-44719		38
XD-78826	GNA6 (XD-44719)	7
XD-44722		201
XD-78827	GNA7 (XD-44722)	17

Example 21. Mouse Experiments

[00122] Mice. Human PCSK9-Exon 12(UTR) knock-in mice in C57BL6/N background were generated by CRISPR-Cas9. The knock-in strategy was designed to replace the entire mouse Exon 12 (1230 bp total, encoding the C-terminal 70 amino acids with 1020 bp 3' untranslated region) with that of human Exon 12 (1484 bp total, encoding the C-terminal 71 amino acids with 1271 bp 3' untranslated region).

[00123] Male homozygous human PCSK9-Exon 12(UTR) knock-in mice were ad lib fed with 2018 Teklad global 18% protein rodent diet (Inotiv, Cat.#2018). Each mouse (4-5 per group) received a single s.c injection of the indicated dose of Inclisiran or COR-1003. Tail-nick blood collection (in EDTA-treated blood collection tubes) was done prior (Day 0) to injection or at Day 14, Day 28 after injection. After centrifugation at 3500 g for 10 min at 4°C, the plasma was transferred to fresh tubes and stored at -80c. Plasma PCSK9 levels were determined by Elisa assay as described previously (PNAS 105:11915-20, 2008. pubmed.ncbi.nlm.nih.gov/18695239/). The plasma PCSK9 levels of mice treated with the control, Inclisiran, or XD-78827 at the indicated doses at the indicated time points are shown in Table 35.

Table 35: PCSK9 Levels (ng/mL) in Mice

Group	Dose	Day 0 (mean±SD)	Day 14 (mean±SD)	Day 28 (mean±SD)
Vehicle	-	119 ± 6	130 ± 29	109 ± 11
Inclisiran	0.25 mg/kg	99 ± 15 (<i>p</i> =0.079)	110 ± 23 (<i>p</i> =0.294)	94 ± 10 (<i>p</i> =0.076)
	0.5 mg/kg	130 ± 5 (<i>p</i> =0.025)	99 ± 3 (<i>p</i> =0.081)	117 ± 29 (<i>p</i> =0.616)
	1.0 mg/kg	97 ± 18 (<i>p</i> =0.081)	82 ± 29 (<i>p</i> =0.048)	75 ± 9 (<i>p</i> =0.005)
	2.0 mg/kg	85 ± 17 (<i>p</i> =0.0078)	42 ± 5 (<i>p</i> =0.0023)	69 ± 5 (<i>p</i> =0.0003)
XD-78827	0.25 mg/kg	113 ± 6 (<i>p</i> =0.189)	110 ± 8 (<i>p</i> =0.215)	98 ± 15 (<i>p</i> =0.281)
	0.5 mg/kg	130 ± 27 (<i>p</i> =0.434)	88 ± 11 (<i>p</i> =0.030)	101 ± 20 (<i>p</i> =0.462)
	1.0 mg/kg	120 ± 8 (<i>p</i> =0.886)	101 ± 20 (<i>p</i> =0.126)	144 ± 16 (<i>p</i> =0.014)
	2.0 mg/kg	94 ± 6 (<i>p</i> =0.0007)	57 ± 10 (<i>p</i> =0.0034)	88 ± 19 (<i>p</i> =0.1215)

Example 22. Non-Human Primate Experiments

[00124] The pharmacodynamic effect of XD-44719, XD-44722, XD-44687, and XD-78827 for the reduction of PCSK9 and LDL cholesterol levels were evaluated in female cynomolgus monkeys at Charles River Laboratories (Study Number NC-PCS-24-001). Following a single subcutaneous injection to four groups of animals (n=3-4/group), circulating plasma PCSK9 protein and serum LDL cholesterol levels were measured. Briefly, PCSK9 protein levels (ng/mL) in plasma were measured using the Human PCSK9 Quantikine ELISA Kit from R&D systems. PCSK9 protein levels were quantified using the Molecular Devices SPECTRAmax® M@ or SPECTRAmax® M5 microplate reader. Serum LDL cholesterol levels (mg/dL) in serum were measured using Cobas® 6000 analyzer from Roche Diagnostics.

[00125] The PCSK9 protein levels of monkeys treated with XD-44719, XD-44722, XD-44687, or XD-78827 at the indicated doses at the indicated time points are shown in Table 36. The LDL levels of monkeys treated with XD-44719, XD-44722, XD-44687, or XD-78827 at the indicated doses at the indicated time points are shown in Table 37.

Table 36: PCSK9 Protein Levels (ng/mL)

Group	Dose	PT1	PT2	PT3	Week 0	Week 1	Week 2	Week 3	Week 4	Week 6	Week 8	Week 10	Week 12
XD-44719	0.5	Mean	274.9	199.6	281.1	193.4	247.1	134.0	138.8	165.9	158.5	140.5	112.0
	mg/kg	SD	37.08	162.5	80.98	159.0	145.2	19.16	25.45	1.32	38.59	34.32	134.6
	2.0	Mean	161.3	230.9	174.3	188.9	109.9	64.67	92.77	78.15	70.19	81.32	144.2
	mg/kg	SD	59.07	72.88	56.32	61.42	12.85	2.32	12.47	26.47	12.43	6.70	56.83
	5.0	Mean	154.3	319.5	182.4	218.7	91.83	51.70	55.16	71.36	61.58	83.15	140.7
	mg/kg	SD	49.48	61.32	24.79	34.05	8.56	17.94	17.81	38.12	22.58	41.51	35.61
XD-44722	0.5	Mean	199.8	201.4	153.9	185.0	87.57	103.3	82.55	113.4	117.3	104.2	166.1
	mg/kg	SD	171.1	98.87	54.33	104.5	26.89	38.71	32.79	41.50	73.04	46.76	99.96
	2.0	Mean	253.2	209.2	203.9	222.1	101.1	87.04	100.8	65.94	76.66	80.67	145.2
	mg/kg	SD	84.94	57.43	80.27	72.99	18.38	20.01	15.92	14.38	6.58	4.55	17.83
	5.0	Mean	251.5	229.9	257.9	246.4	76.42	57.96	47.99	49.20	47.50	55.03	95.18
	mg/kg	SD	29.61	101.0	107.4	58.94	1.99	4.43	3.61	5.19	4.20	8.02	10.11
XD-44687	0.5	Mean	256.6	247.6	218.1	240.7	175.7	101.2	173.2	126.2	133.6	145.6	186.1
	mg/kg	SD	15.68	68.11	24.04	27.67	95.89	18.83	82.22	33.51	48.63	35.93	31.25
	2.0	Mean	185.4	222.4	246.7	218.2	146.5	90.35	123.2	87.52	99.01	111.6	136.5
	mg/kg	SD	71.92	10.51	68.52	45.50	11.84	8.74	53.44	30.38	53.94	55.58	67.14
	5.0	Mean	138.7	89.88	119.5	116.0	44.81	28.88	27.13	37.10	36.98	33.81	46.07
	mg/kg	SD	102.8	72.20	109.3	94.75	28.48	17.99	18.30	-	27.82	22.90	44.12
XD-78827	0.5	Mean	229.4	202.5	261.2	231.1	253.9	182.0	215.8	285.4	154.1		
	mg/kg	SD	133.6	62.93	125.5	104.6	103.5	78.70	99.79	107.6	47.42		
	2.0	Mean	233.5	146.3	194.8	191.6	249.2	176.1	161.0	214.7	159.1		
	mg/kg	SD	121.1	41.41	26.62	49.99	80.95	38.21	27.76	56.06	39.43		
	5.0	Mean	152.3	154.0	183.3	163.2	131.4	87.30	70.32	81.95	72.31		
	mg/kg	SD	65.82	69.66	55.63	58.80	71.02	42.73	37.79	25.21	34.00		

PT=Pretreatment / SD=Standard deviation

Table 37: LDL Levels (mg/dL)

Group	Dose		Day -21	Day -20	Day -15	Day -14	Day -8	Day 7	Day 14	Day 28	Day 56	Day 84	Day 91	Day 98	Day 112	Day 126	Day 140
XD-44719	0.5	Mean		49.3	57.0		47.0	49.7	52.7	46.0	43.3	39.3	42.7				
	mg/kg	SD		6.5	4.4		2.0	9.0	6.7	2.6	4.2	1.5	5.5				
	2.0	Mean		52.0	57.0		52.3	37.7	37.3	34.7	42.0	39.3	43.0				
	mg/kg	SD		10.4	13.9		12.3	6.5	7.0	4.6	5.6	3.5	9.2				
	5.0	Mean		51.7	50.3		45.0	34.3	40.3	34.7	33.7	38.3	37.3				
	mg/kg	SD		3.21	7.63		8.18	2.88	7.02	4.93	5.50	0.57	3.21				
XD-44722	0.5	Mean		62	66		60	63	61	60	52	58	50				
	mg/kg	SD		9.07	6.08		5	7.63	14.1	9.16	7.93	12.8	14.7				
	2.0	Mean		59	65		62	51	52	54	51	58		61	58	55	
	mg/kg	SD		14	13.7		13.2	8.62	4.04	2.08	5.03	6.08		7	4.93	12.1	
	5.0	Mean		65	66		55	47	41	37	38	37		50	45	53	62
	mg/kg	SD		7.02	7.21		8.88	14	8.54	5.85	11	12.4		9.71	8.50	8.54	10.9
XD-44687	0.5	Mean		56	59		59	49	55	51	48	49	50				
	mg/kg	SD		23.5	18.3		20.5	24.0	25.0	24.5	19.0	22.7	23.8				
	2.0	Mean		56	71		62	51	52	52	50	48		60	60	56	60
	mg/kg	SD		11.1	13.2		10.5	5.85	14.5	12.3	16.7	15.0		20.6	15.3	17.8	11.7
	5.0	Mean		41	51		41	27	30	24	24	32		41	31	37	44
	mg/kg	SD		2.51	4.72		2.88	2.51	5.56	6.50	10.5	17.2		12.6	12.1	12.5	11.6
XD-78827	0.5	Mean	85		74		68	67	70	72	63						
	mg/kg	SD	26.5		17.6		20.7	17.3	19.5	22.0	20.1						
	2.0	Mean	52		55	46	47	40	40	42	46						
	mg/kg	SD	10.0		9.19		13	5.56	7.54	7.32	8.28						
	5.0	Mean	79			72	64	50	56	43	54						
	mg/kg	SD	21.0			38.5	25.7	17.7	25.6	20.2	23.9						

[00126] Those skilled in the art will appreciate that numerous changes and modifications can be made to the preferred embodiments disclosed herein and that such changes and modifications can be made without departing from the spirit of the invention. It is, therefore, intended that the appended claims cover all such equivalent variations as fall within the true spirit and scope of the invention.

[00127] The disclosures of each patent, patent application, and publication cited or described in this document are hereby incorporated herein by reference, in its entirety.

Table 38: Sequences

Sequence Name	Sequence	SEQ ID NO
3512-A SS	CUUUUUACAGCCAACUUUUCA	1
3512-A AS	UGAAAAGUUGGCUGUAAAAAGGC	2
3512-B SS	CUUUUUACAGCCAACUUUUCU	3
3512-B AS	AGAAAAGUUGGCUGUAAAAAGGC	4
3545 SS	CUUUUGUAACUUGAAGAUUA	5
3545 AS	UAUAUCUUCAAGUUACAAAAGCA	6
44687 SS	CUAGACCUGUTUUGCUUUUGA	7
44687 AS	UCAAAGCAAAAACAGGUCUAGAA	8
XD-36954 SS	csusagacCfuGfudTuugcuuuugu(L96)	9
XD-36954 AS	asCfsasAfAfAfgCfaAfaAfcAfgGfuCfuasgsasa	10
XD-42180 SS	csusagacCfuGfudTuugcuuuugu(L96)	11
XD-42180 AS	(vinu)sCfsaAfAfAfgCfaAfaAfcAfgGfuCfuagsasa	12
XD-44683 SS	csusagacCfuGfUfUfuugcuuuuga(L96)	13
XD-44683 AS	(vinu)sCfsaaaAfgCfAfaaacAfgGfucuagsasa	14
XD-44687 SS	csusagacCfuGfUfdTuugcuuuuga(L96)	15
XD-44687AS	(vinu)sCfsaaaAfgcaaacAfgGfucuagsasa	16
XD-78829 SS	csusagacCfuGfUfdTuugcuuuuga(L96)	17
XD-78829 AS	(vinu)sCfsaaa(GNA-A)gcaaacAfgGfucuagsasa	18
XD-78830 SS	csusagacCfuGfUfdTuugcuuuuga(L96)	19
XD-78830 AS	(vinu)sCfsaaaAf(GNA-G)caaacAfgGfucuagsasa	20
XD-44719 SS	csusuuuuAfcAfGfCfcaacuuuua(L96)	21
XD-44719 AS	(vinu)sGfsaaaaguuggcuGfuAfaaaagsgsc	22
XD-78826 SS	csusuuuuAfcAfGfCfcaacuuuua(L96)	23
XD-78826 AS	(vinu)sGfsaaa(GNA-A)guuggcuGfuAfaaaagsgsc	24
XD-78825 SS	csusuuuuAfcAfGfCfcaacuuuua(L96)	25
XD-78825 AS	(vinu)sGfsaaaa(GNA-G)uuggcuGfuAfaaaagsgsc	26
XD-44725 SS	csusuuuuAfcAfGfCfcaacuuuua(L96)	27
XD-44725 AS	(vinu)sGfsaaadAguuggcuGfuAfaaaagsgsc	28
XD-44701 SS	csusuuuuAbcAfGbCfcaacuuuua(L96)	29
XD-44701 AS	(vinu)sGfsaaaAfguuggcuGfuAfaaaagsgsc	30
XD-44695 SS	csusuuuuAfcAfGf(abasic)caacuuuua(L96)	31
XD-44695 AS	(vinu)sGfsaaaAfguuggcuGfuAfaaaagsgsc	32
XD-42182 SS	csusuuuuAfcAfGfCfcaacuuuua(L96)	33

XD-42182 AS	(vinu)sGfsaaaAfgUfUfggcuGfuAfaaaagsgsc	34
XD-44731 SS	csusuuuuAfcAfGfCfcaacuuuua(L96)	35
XD-44731 AS	(vinu)sGfsaaaaguuggcuGfuAbaaaagsgsc	36
XD-44689 SS	csusuuuuAfcAfGfdCcaacuuuua(L96)	37
XD-44689 AS	(vinu)sGfsaaaAfguuggcuGfuAfaaaagsgsc	38
XD-44737 SS	csusuuuuAfcAfGfCfcaacuuuua(L96)	39
XD-44737 AS	(vinu)sGfsaaaaguuggcuGbuAbaaaagsgsc	40
XD-38572 SS	csusuuuuAfcAfGfCfcaacuuuua(L96)	41
XD-38572 AS	usGfsaaaAfgUfUfggcuGfuAfaaaagsgsc	42
XD-44713 SS	csusuuuuAfcAbdGCbcaacuuuua(L96)	43
XD-44713 AS	(vinu)sGfsaaaAfguuggcuGfuAfaaaagsgsc	44
XD-44707 SS	csusuuuuAbcdAGbdCcaacuuuua(L96)	45
XD-44707 AS	(vinu)sGfsaaaAfguuggcuGfuAfaaaagsgsc	46
XD-44728 SS	csusuuugUfaAfCfUfugaagauua(L96)	47
XD-44728 AS	(vinu)sAfsuaucaaguUfaCbaaaagscsa	48
XD-44722 SS	csusuuugUfaAfCfUfugaagauua(L96)	49
XD-44722 AS	(vinu)sAfsuauCcucaaguUfaCfaaaagscsa	50
XD-78828 SS	csusuuugUfaAfCfUfugaagauua(L96)	51
XD-78828 AS	(vinu)sAfsuau(GNA-C)uucaguUfaCfaaaagscsa	52
XD-78827 SS	csusuuugUfaAfCfUfugaagauua(L96)	53
XD-78827 AS	(vinu)sAfsuau(GNA-U)uucaguUfaCfaaaagscsa	54
XD-44682 SS	csusuuugUfaAfCfUfugaagauua(L96)	55
XD-44682 AS	(vinu)sAfsuauCfuUfCfaaguUfaCfaaaagscsa	56
XD-44716 SS	csusuuugUfaAfCfUfugaagauua(L96)	57
XD-44716 AS	(vinu)sAfsuaucaaguUfaCfaaaagscsa	58
XD-44692 SS	csusuuugUfaAfCf(abasic)ugaagauua(L96)	59
XD-44692 AS	(vinu)sAfsuauCfucaaguUfaCfaaaagscsa	60
XD-44698 SS	csusuuugTbaAfCbUfugaagauua(L96)	61
XD-44698 AS	(vinu)sAfsuauCfucaaguUfaCfaaaagscsa	62
XD-44704 SS	csusuuugTbadACbdTugaagauua(L96)	63
XD-44704 AS	(vinu)sAfsuauCfucaaguUfaCfaaaagscsa	64
XD-38578 SS	csusuuugUfaAfCfUfugaagauua(L96)	65
XD-38578 AS	usAfsuauCfuUfCfaaguUfaCfaaaagscsa	66
XD-44710 SS	csusuuugUfaAbdCTbugaagauua(L96)	67
XD-44710 AS	(vinu)sAfsuauCfucaaguUfaCfaaaagscsa	68
XD-44734 SS	csusuuugUfaAfCfUfugaagauua(L96)	69
XD-44734 AS	(vinu)sAfsuaucaaguTbaCbaaaagscsa	70
XD-44686 SS	csusuuugUfaAfCfdTugaagauua(L96)	71
XD-44686 AS	(vinu)sAfsuauCfucaaguUfaCfaaaagscsa	72
Inclisiran SS	csusagacCfuGfudTuugcuuuugu(L96)	73
Inclisiran AS	asCfsaAfAfAfgCfaAfaAfcAfgGfuCfuagsasa	74
QG2 hsPCSK9 1	TGGCCTGTCTGTGGAAGCG	75
QG2 hsPCSK9 2	TGCCATGACTGTCACTTGC	76
QG2 hsPCSK9 3	CACCCCTGCCAGGTGGG	77
QG2 hsPCSK9 4	CATCCCGGCCGCTGAC	78
QG2 hsPCSK9 5	CCTTGGCCACGCCG	79
QG2 hsPCSK9 6	GCTGCGCATGCTGGCAC	80
QG2 hsPCSK9 7	GCAGTTGAGCACGCGCAG	81

QG2 hsPCSK9 8	AACCGTGCCCTTCCCTTG	82
QG2 hsPCSK9 9	GCCTATGAGGGTGCCGCT	83
QG2 hsPCSK9 10	GGCTTTTCCGAATAAACTCCAG	84
QG2 hsPCSK9 11	CCCACAGGCTGGACCAGCT	85
QG2 hsPCSK9 12	CAGCAGCACCACCAGTGGC	86
QG2 hsPCSK9 13	CCCACCCGCCAGGGG	87
QG2 hsPCSK9 14	CGTTGAGGACGCGGCTGTA	88
QG2 hsPCSK9 15	GCGCTGGCAGGCGG	89
QG2 hsPCSK9 16	CCCCAGCCCTCGCCAG	90
QG2 hsPCSK9 17	CAGCGGTGACCAGCACGA	91
QG2 hsPCSK9 18	TCCCGGAAGTTGCCGG	92
QG2 hsPCSK9 19	GGGAGTAGAGGCAGGCATCG	93
QG2 hsPCSK9 20	CCTCGGGAGCTGAGGCTG	94
QG2 hsPCSK9 21	GGTGGCCCCAACTGTGATGA	95
QG2 hsPCSK9 22	CGGCTGGTCTTGGGCATT	96
QG2 hsPCSK9 23	CCAAAGTCCCCAGGGTCAC	97
QG2 hsPCSK9 24	AGCGGCCAAAGTTGGTCC	98
QG2 hsPCSK9 25	GGGGCAAAGAGGTCCACAC	99
QG2 hsPCSK9 26	CACCAATGATGTCTCCCCCT	100
QG2 hsPCSK9 27	TGCTGCAGTCGCTGGAGG	101
QG2 hsPCSK9 28	CCACTCTGTGACACAAAGCAGG	102
QG2 hsPCSK9 29	GGCAGCAGCCTGTGATGTC	103
QG2 hsPCSK9 30	TGCAATGCCAGCCACGTG	104
QG2 hsPCSK9 31	CGGCAGACAGCATCATGGC	105
QG2 hsPCSK9 32	CCAGGGTGAGCTCCGGCT	106
QG2 hsGAPDH 1	CAGGATGCCCTTGAGGGG	107
QG2 hsGAPDH 2	CACCTGGTGCTCAGTGTAGCC	108
QG2 hsGAPDH 3	TCGCTGTTGAAGTCAGAGGAGAC	109
QG2 hsGAPDH 4	CAAAGGTGGAGGAGTGGGTG	110
QG2 hsGAPDH 5	AATGCCAGCCCCAGCGT	111
QG2 hsGAPDH 6	ACAAAGTGGTCGTTGAGGGC	112
QG2 hsGAPDH 7	TGTCATACCAGGAAATGAGCTTG	113
QG2 hsGAPDH 8	CTGTTGCTGTAGCCAAATTCGT	114
QG2 hsGAPDH 9	GGCCATGAGGTCCACCACC	115
QG2 hsGAPDH 10	TTACTCCTTGAGGCCATGTG	116
QG2 hsGAPDH 11	GCTGGTGGTCCAGGGGTC	117
QG2 hsGAPDH 12	CCTCTTGCTCTTGCTGGG	118
QG2 hsGAPDH 13	CCAGCAGTGAGGGTCTCTCTTT	119
QG2 hsGAPDH 14	CTGAGTGTGGCAGGGACTCC	120
QG2 hsGAPDH 15	AGATTCAAGTGTGGTGGGGGA	121
QG2 hsGAPDH 16	TGGCAACTGTGAGGAGGGG	122
QG2 hsGAPDH 17	CCCCTCTTCAAGGGGTCTACA	123
QG2 hsGAPDH 18	CGGCTCCCTAGGCCCT	124

EMBODIMENTS

The following list of embodiments is intended to complement, rather than displace or supersede, the previous descriptions.

Embodiment 1A. Any of the novel PCSK9 siRNAs, whose IC₅₀ is at least 25%, 50%, 75% or at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 times less than that of the original Inclisiran under the same conditions.

Embodiment 2A. A method of using the PCSK9 siRNA of embodiment 1, comprising administering the siRNA to a subject, e.g., a human, thereby treating disease or condition.

Embodiment 3A. A method of making the PCSK9 siRNA, comprising synthetically producing the siRNA.

Embodiment 1B. A double-stranded ribonucleic acid molecule comprising:
a sense strand comprising the nucleic acid sequence of SEQ ID NO: 1 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 2;
a sense strand comprising the nucleic acid sequence of SEQ ID NO: 3 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 4;
a sense strand comprising the nucleic acid sequence of SEQ ID NO: 5 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 6; or
a sense strand comprising the nucleic acid sequence of SEQ ID NO: 7 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 8.

Embodiment 2B. The double-stranded ribonucleic acid molecule of embodiment 1B, wherein the sense strand comprises the nucleic acid sequence of SEQ ID NO: 1 and the antisense strand comprises the nucleic acid sequence of SEQ ID NO: 2.

Embodiment 3B. The double-stranded ribonucleic acid molecule of embodiment 1B, wherein the sense strand comprises the nucleic acid sequence of SEQ ID NO: 3 and the antisense strand comprises the nucleic acid sequence of SEQ ID NO: 4.

Embodiment 4B. The double-stranded ribonucleic acid molecule of embodiment 1B, wherein the sense strand comprises the nucleic acid sequence of SEQ ID NO: 5 and the antisense strand comprises the nucleic acid sequence of SEQ ID NO: 6.

Embodiment 5B. The double-stranded ribonucleic acid molecule of embodiment 1B, wherein the sense strand comprises the nucleic acid sequence of SEQ ID NO: 7 and the antisense strand comprises the nucleic acid sequence of SEQ ID NO: 8.

Embodiment 6B. The double-stranded ribonucleic acid molecule of any one of the previous embodiments, wherein the sense strand comprises L96.

Embodiment 7B. The double-stranded ribonucleic acid molecule of any one of the previous embodiments, wherein the antisense strand comprises (vinu).

Embodiment 8B. The double-stranded ribonucleic acid molecule of any one of the previous embodiments, wherein the sense strand and/or the antisense strand comprise one or more modified nucleotides.

Embodiment 9B. The double-stranded ribonucleic acid molecule of embodiment 8B, wherein the one or more modified nucleotides are selected from 2'-O-methyladenosine, 2'-fluoroadenosine, 2'-O-methylcytidine, 2'-fluorocytidine, 2'-O-methylguanosine, 2'-fluoroguanosine, 2'-O-methyluridine, and 2'-fluorouridine.

Embodiment 10B. The double-stranded ribonucleic acid molecule of any one of the previous embodiments, comprising one or more deoxynucleotides selected from deoxyadenosine, deoxycytidine, and deoxythymidine.

Embodiment 11B. The double-stranded ribonucleic acid molecule of any one of the previous embodiments, comprising nucleotides that are connected by one or more phosphodiester bonds, one or more phosphorothioate bonds, or any combination of phosphodiester bonds and phosphorothioate bonds.

Embodiment 12B. The double-stranded ribonucleic acid molecule of any one of the previous embodiments, comprising one or more glycol nucleic acid (GNA) nucleotides.

Embodiment 13B. The double-stranded ribonucleic acid molecule of any one of the previous embodiments, comprising one or more 2'-4'-Locked Nucleic Acid (LNA) nucleotides.

Embodiment 14B. The double-stranded ribonucleic acid molecule of embodiment 1B, wherein said sense strand comprises the nucleic acid sequence of SEQ ID NO: 15 and said antisense strand comprises the nucleic acid sequence of SEQ ID NO: 16.

Embodiment 15B. The double-stranded ribonucleic acid molecule of embodiment 1B, wherein said sense strand comprises the nucleic acid sequence of SEQ ID NO: 49 and said antisense strand comprises the nucleic acid sequence of SEQ ID NO: 50.

Embodiment 16B. The double-stranded ribonucleic acid molecule of embodiment 1B, wherein said sense strand comprises the nucleic acid sequence of SEQ ID NO: 21 and said antisense strand comprises the nucleic acid sequence of SEQ ID NO: 22.

Embodiment 17B. The double-stranded ribonucleic acid molecule of embodiment 1B, wherein said sense strand comprises the nucleic acid sequence of SEQ ID NO: 47 and said antisense strand comprises the nucleic acid sequence of SEQ ID NO: 48.

Embodiment 18B. The double-stranded ribonucleic acid molecule of embodiment 1B, wherein said sense strand comprises the nucleic acid sequence of SEQ ID NO: 57 and said antisense strand comprises the nucleic acid sequence of SEQ ID NO: 58.

Embodiment 19B. The double-stranded ribonucleic acid molecule of embodiment 1B, wherein said sense strand comprises the nucleic acid sequence of SEQ ID NO: 53 and said antisense strand comprises the nucleic acid sequence of SEQ ID NO: 54.

Embodiment 20B. A method of treating a condition that is modulated by PCSK9 in a subject, the method comprising administering to the subject an effective amount of a double-stranded ribonucleic acid molecule of any one of the previous embodiments.

Embodiment 21B. The method of embodiment 20B, wherein the condition that is modulated by PCSK9 is hypercholesterolemia or mixed dyslipidemia.

Embodiment 22B. A pharmaceutical composition comprising a double-stranded ribonucleic acid molecule of any one of the previous embodiments and a pharmaceutically acceptable carrier.

What is claimed:

1. A double-stranded ribonucleic acid molecule comprising:
 - a sense strand comprising the nucleic acid sequence of SEQ ID NO: 1 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 2;
 - a sense strand comprising the nucleic acid sequence of SEQ ID NO: 3 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 4;
 - a sense strand comprising the nucleic acid sequence of SEQ ID NO: 5 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 6; or
 - a sense strand comprising the nucleic acid sequence of SEQ ID NO: 7 and an antisense strand comprising the nucleic acid sequence of SEQ ID NO: 8.
2. The double-stranded ribonucleic acid molecule of claim 1, wherein the sense strand comprises the nucleic acid sequence of SEQ ID NO: 1 and the antisense strand comprises the nucleic acid sequence of SEQ ID NO: 2.
3. The double-stranded ribonucleic acid molecule of claim 1, wherein the sense strand comprises the nucleic acid sequence of SEQ ID NO: 3 and the antisense strand comprises the nucleic acid sequence of SEQ ID NO: 4.
4. The double-stranded ribonucleic acid molecule of claim 1, wherein the sense strand comprises the nucleic acid sequence of SEQ ID NO: 5 and the antisense strand comprises the nucleic acid sequence of SEQ ID NO: 6.
5. The double-stranded ribonucleic acid molecule of claim 1, wherein the sense strand comprises the nucleic acid sequence of SEQ ID NO: 7 and the antisense strand comprises the nucleic acid sequence of SEQ ID NO: 8.
6. The double-stranded ribonucleic acid molecule of any one of the previous claims, wherein the sense strand comprises L96.
7. The double-stranded ribonucleic acid molecule of any one of the previous claims, wherein the antisense strand comprises (vinu).

8. The double-stranded ribonucleic acid molecule of any one of the previous claims, wherein the sense strand and/or the antisense strand comprise one or more modified nucleotides.

9. The double-stranded ribonucleic acid molecule of claim 8, wherein the one or more modified nucleotides are selected from 2'-O-methyladenosine, 2'-fluoroadenosine, 2'-O-methylcytidine, 2'-fluorocytidine, 2'-O-methylguanosine, 2'-fluoroguanosine, 2'-O-methyluridine, and 2'-fluorouridine.

10. The double-stranded ribonucleic acid molecule of any one of the previous claims, comprising one or more deoxynucleotides selected from deoxyadenosine, deoxycytidine, and deoxythymidine.

11. The double-stranded ribonucleic acid molecule of any one of the previous claims, comprising nucleotides that are connected by one or more phosphodiester bonds, one or more phosphorothioate bonds, or any combination of phosphodiester bonds and phosphorothioate bonds.

12. The double-stranded ribonucleic acid molecule of any one of the previous claims, comprising one or more glycol nucleic acid (GNA) nucleotides.

13. The double-stranded ribonucleic acid molecule of any one of the previous claims, comprising one or more 2'-4'-Locked Nucleic Acid nucleotides.

14. The double-stranded ribonucleic acid molecule of claim 1, wherein said sense strand comprises the nucleic acid sequence of SEQ ID NO: 15 and said antisense strand comprises the nucleic acid sequence of SEQ ID NO: 16.

15. The double-stranded ribonucleic acid molecule of claim 1, wherein said sense strand comprises the nucleic acid sequence of SEQ ID NO: 49 and said antisense strand comprises the nucleic acid sequence of SEQ ID NO: 50.

16. The double-stranded ribonucleic acid molecule of claim 1, wherein said sense strand comprises the nucleic acid sequence of SEQ ID NO: 21 and said antisense strand comprises the nucleic acid sequence of SEQ ID NO: 22.

17. The double-stranded ribonucleic acid molecule of claim 1, wherein said sense strand comprises the nucleic acid sequence of SEQ ID NO: 47 and said antisense strand comprises the nucleic acid sequence of SEQ ID NO: 48.

18. The double-stranded ribonucleic acid molecule of claim 1, wherein said sense strand comprises the nucleic acid sequence of SEQ ID NO: 57 and said antisense strand comprises the nucleic acid sequence of SEQ ID NO: 58.

19. The double-stranded ribonucleic acid molecule of claim 1, wherein said sense strand comprises the nucleic acid sequence of SEQ ID NO: 53 and said antisense strand comprises the nucleic acid sequence of SEQ ID NO: 54.

20. A method of treating a condition that is modulated by PCSK9 in a subject, the method comprising administering to the subject an effective amount of a double-stranded ribonucleic acid molecule of any one of the previous claims.

21. The method of claim 20, wherein the condition that is modulated by PCSK9 is hypercholesterolemia or mixed dyslipidemia.

22. A pharmaceutical composition comprising a double-stranded ribonucleic acid molecule of any one of the previous claims and a pharmaceutically acceptable carrier.