

SIRNA MOLECULE FOR INHIBITING PCSK9 EXPRESSION AND USE THEREOF

FIELD OF THE INVENTION

[0001] The present invention relates to the technical field of gene therapy, and specifically to an siRNA molecule for inhibiting PCSK9 expression, and use thereof.

5 BACKGROUND

- [0002]** Lipoproteins are divided into five categories: chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL). Among them, very low density lipoprotein and low density lipoprotein have a density of less than 1.0063 g/mL, and their main in vivo function is
- 10 to participate in cholesterol metabolism and transport cholesterol from the cells to peripheral blood. Clinically, a high level of VLDL or LDL is an important factor leading to many cardiovascular diseases, especially one of the most important causes of atherosclerosis.
- [0003]** Proprotein convertase subtilisin/kexin type 9 (PCSK9) is expressed in liver, kidney, and small intestine, and mainly in liver. PCSK9 has a molecular weight of 72 kD, and
- 15 consists of three parts: an N-terminal prodomain, a catalytic domain, and a C-terminal domain with unknown function. After the synthesis of PCSK9, the N-terminal prodomain will be autocatalytically cleaved off. However, the cleaved N-terminal prodomain will not leave, but bind to the other two domains, to play a role of molecular chaperone, which partially blocks the binding pocket of the catalytic domain to a substrate.
- 20 **[0004]** PCSK9 was first considered to be related to the liver regeneration and cortical neuron differentiation. However, Abifadel and others found that PCSK9 mutation was related to cholesterol metabolism. Horten and others also found in the study of SREBP that the mRNA level of PCSK9 was related to the cellular cholesterol level. Transfection of mice with Adenovirus to over-express PCSK9 also lead to the increase of LDL level and the decrease of
- 25 the LDL receptor (LDLR), with the mRNA level of LDL unchanged. Then, Lagace TA, et al. found that a similar effect was also observed in the transgenic mice expressing PCSK9, that is, the level of LDL cholesterol increased and the level of the LDLR decreased. After PCSK9 was knocked out, the level of the LDLR increased and the level of LDL cholesterol (LDLC) in plasma decreased greatly. PCSK9 does not directly degrade the LDLR. McNutt MC et al.
- 30 found that inactivated PCSK9 could also lead to the decrease of the LDLR level. After LDL binds to the LDLR, the complex is endocytosed into an endosome. The acidic environment in the endosome causes the structure of the LDLR to change into a hairpin shape. The structural change weakens the binding between the LDLR and LDL to release LDL. The LDLR finally

returns to the surface of the cell, completing the transport of LDL into the cell. However, when PCSK9 binds to the LDLR through the EGF-A domain of the LDLR, the conformational change of the LDLR is inhibited, resulting in the decrease of the LDLR. LDLC in serum enters hepatocytes after binding to the LDLR on the surface of hepatocytes.

- 5 The binding of PCSK9 and the LDL receptor reduces the LDLR, so that the uptake of LDLC by hepatocytes is reduced. Therefore, the uptake of LDLC through LDLR can be enhanced by inhibiting PCSK9 expression.

- [0005] Among the population, 2% of African-Americans have one or two mutations in PCSK9, which cause their LDL cholesterol level to be about 30% lower than that of normal people. The mutation causes about 15% decrease of the LDL cholesterol level in Caucasians. The level of PCSK9 in women is slightly higher than that in men. With the increase of age, the level of PCSK9 in men decreases, while the level of PCSK9 in women increases, which may be related to estrogen. PCSK9 plays an important role in regulating LDL, and the LDL level has a high correlation with cardiovascular diseases, and dyslipidemia. Therefore, PCSK9 has become a very important target for treating cardiovascular diseases and dyslipidemia. In addition, a literature published in Nature in 2020 showed that PCSK9 affects the recognition of tumor cells by CD8⁺ T cells by reducing the expression of MHC I molecules on the surface of tumor cells. PCSK9 knockout or PCSK9 monoclonal antibody can increase the expression of MHC I molecules in tumor cells, thus inhibiting tumor growth.
- 15 PCSK9 has become a very important target for treating cardiovascular diseases and dyslipidemia. In addition, a literature published in Nature in 2020 showed that PCSK9 affects the recognition of tumor cells by CD8⁺ T cells by reducing the expression of MHC I molecules on the surface of tumor cells. PCSK9 knockout or PCSK9 monoclonal antibody can increase the expression of MHC I molecules in tumor cells, thus inhibiting tumor growth.
- 20 This indicates that PCSK9 is also involved in the regulation of tumor immunity.

- [0006] The existing research on drugs inhibiting PCSK9 is mainly focused on polypeptide, antibody, siRNA, and ASO drugs. For polypeptide drugs, a structure similar to the binding site of the LDLR to PCSK9, that is, EGF-A domain, is designed and developed, so that PCSK9 can bind to the polypeptide, to reduce the binding between PCSK9 and the LDLR.
- 25 The monoclonal antibody technology is to block the binding of PCSK9 to the LDLR by developing a class of antibodies that can specifically bind to the proximal catalytic domain of PCSK9. The antibodies such as evolocomab from Amgen, bococizumab from Pfizer and alirocumab from Sanofi and Regeneron have entered clinical research. Except for the discontinuation of bococizumab from Pfizer in November 2016, the other two antibodies are marketed for LDL-lowering treatment. ASO is an antisense nucleotide, which can bind to mRNA and promote mRNA degradation mediated by RNase H, and has a high binding affinity to hinder the translation of mRNA. The anti-sense nucleotide drug targeting PCSK9 developed by Ionis has entered the clinical stage. SiRNAs are a class of small RNAs, a class of naturally occurring small double-stranded nucleic acids, with a length of 19-25 bp. After
- 30 marketed for LDL-lowering treatment. ASO is an antisense nucleotide, which can bind to mRNA and promote mRNA degradation mediated by RNase H, and has a high binding affinity to hinder the translation of mRNA. The anti-sense nucleotide drug targeting PCSK9 developed by Ionis has entered the clinical stage. SiRNAs are a class of small RNAs, a class of naturally occurring small double-stranded nucleic acids, with a length of 19-25 bp. After

binding to a silencing complex, the sense strand is degraded, and the complex bearing the anti-sense strand is complementarily bound to mRNA through the anti-sense strand. The mRNA is degraded by the enzyme in the complex, to block the function of the gene at the mRNA level. Currently, siRNA drugs targeting PCSK9 are marketed, and inclisiran developed by Alnylam has the advantages of long half-life, stability, low toxicity and high potency.

[0007] The action time of the above antibody drugs is short, and they are usually injected once every two weeks to every month. Therefore, the patient compliance is slightly poor. The production and purification cost of the antibody drugs is also high. Because of the limitation by the mode of action, the dosage of ASO drugs is large, the production cost is high, and the toxic side effects are also relatively serious. Additionally, statins, as small-molecule drugs, can effectively reduce the concentration of low-density lipoprotein in blood. However, they need to be taken every day, and drug resistance can be easily developed. Some patients are insensitive to small-molecule drugs like statins. siRNA drugs have the advantages of long time of action, and are usually administered once every six months to every one year. They have the advantages of low dosage, long time interval between administrations, and relatively low production cost. Therefore, the development of an effective and stable siRNA drugs is always a goal pursued by researchers in the art.

SUMMARY OF THE INVENTION

[0008] In the present invention, a modified siRNA molecule is designed by modifying small interfering RNA (siRNA), which can be used for effectively and stably inhibiting the PCSK9 expression, and used in the manufacture of products for treating and/or preventing PCSK9-related diseases.

[0009] The present invention provides an siRNA molecule for inhibiting PCSK9 expression, which includes a sense strand and an anti-sense strand that are complementary to form a double-stranded region, wherein

The sense strand has a sequence of 5'-

mAmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGsmUsB-3', and the anti-sense strand has a sequence of 5'-

30 mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3';

or the sense strand has a sequence of 5'-

BsmAsmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGmU-3', and the anti-sense

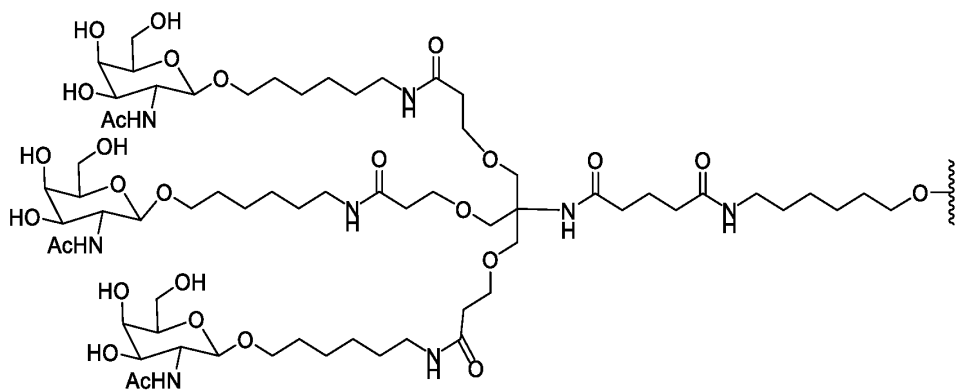
strand has a sequence of 5'-

mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3',

- where A, U, G and C each represents a nucleotide with adenine, uridine, guanine and cytosine as a base, respectively; m means that the nucleotide adjacent to the right side of the letter m is a nucleotide modified with 2'-O-methyl; f means that the nucleotide adjacent to the right side of the letter f is a nucleotide modified with 2'-fluoro; s means that the nucleotides on the left and right sides of the letter s are linked via a phosphorothioate linkage; and B represents an abasic nucleotide.

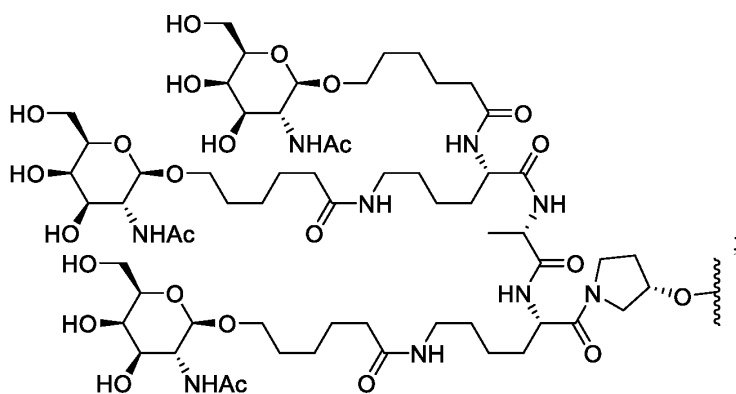
- [0010]** Further included herein is a ligand group for modifying the siRNA molecule with a ligand, wherein the modification with a ligand is a modification of the 3' or 5' end of the sense strand with the ligand group, and the ligand group includes at least one of N-acetylgalactosamine and a N-acetylgalactosamine derivative.

- [0011]** Further, the ligand group includes LICA-1, LICA-2, LICA-3, or L96, wherein LICA-1 has a structural formula of



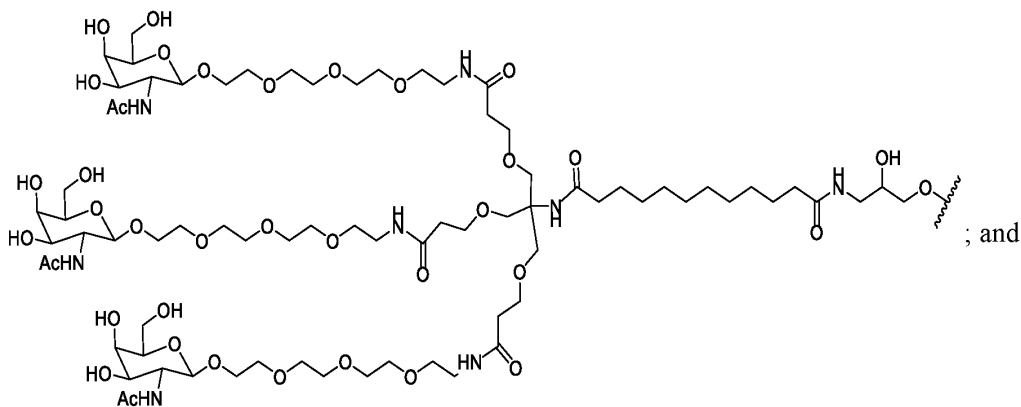
LICA-1(DX₃)

LICA-2 has a structural formula of



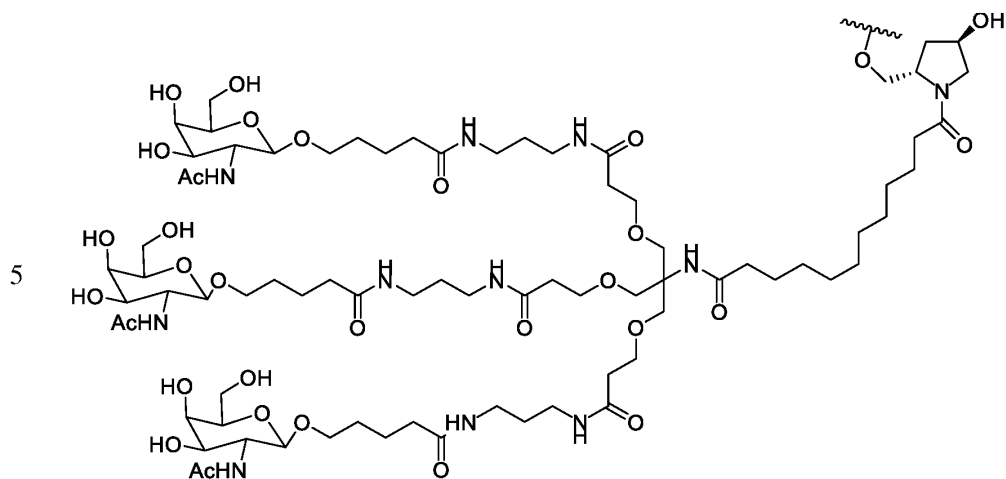
LICA-2

LICA-3 has a structural formula of



LICA-3

L96 has a structural formula of



L96

[0012] Further, the sense strand is linked with the ligand group via a phosphorothioate linkage.

[0013] Further, the siRNA molecule has a structure of any one of (1)-(4):

(1) a sense strand having a sequence of 5'-LICA-1-

5 smAmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGsmUsB-3', and

an anti-sense strand having a sequence of 5'-

mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3';

(2) a sense strand having a sequence of 5'-LICA-2-

smAmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGsmUsB-3', and

10 an anti-sense strand having a sequence of 5'-

mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3';

(3) a sense strand having a sequence of 5'-

BsmAsmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGmUs-LICA-3-3', and

an anti-sense strand having a sequence of 5'-

15 mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3'; and

(4) a sense strand having a sequence of 5'-

BsmAsmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGmUs-L96-3', and

an anti-sense strand having a sequence of 5'-

mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3'.

20 [0014] The present invention also provides a pharmaceutical composition, which includes any siRNA molecule as described above and a pharmaceutically acceptable carrier.

[0015] The present invention also provides use of any siRNA molecule or any pharmaceutical composition as described above in the manufacture of products for inhibiting PCSK9 protein expression.

25 [0016] The present invention also provides use of any siRNA molecule or any pharmaceutical composition as described above in the manufacture of products for reducing the concentration of low-density lipoprotein or low-density lipoprotein cholesterol in serum.

[0017] The present invention also provides use of any siRNA molecule or any pharmaceutical composition as described above in the manufacture of products for relieving
30 symptoms of PCSK9 gene-mediated diseases.

[0018] Further, the PCSK9 gene-mediated diseases include cardiovascular diseases, dyslipidemia, and neoplastic diseases. The cardiovascular diseases include hypercholesterolemia, and hyperlipidemia; the dyslipidemia includes lipid metabolism

disorders; and the neoplastic diseases include PCSK9 associated melanoma and metastatic liver cancer.

[0019] The present invention has the following advantages.

[0020] Through the design of siRNA sequences of the present invention, siRNA sequences
5 having high activity in degrading mRNA are screened from the ground sequence level, and
then RNA modification (including phosphorothioate modification between sequences, and
addition of an abasic nucleotide at the end) is added, to effectively improve the activity and
stability and extend the in vivo time of action of the drug. In addition, by linking a ligand
10 group that is a targeting ligand directing the target organ liver to the end of siRNA, the
enrichment of the molecule in the liver is increased. Finally, an effective, low-toxic, stable
and long-acting siRNA molecule for silencing PCSK9 is developed, which is used to inhibit
PCSK9 expression, can knock down PCSK9 efficiently, is highly stable, and is useful in the
manufacture of products for treating/preventing PCSK9-related diseases such as
cardiovascular diseases, dyslipidemia, and neoplastic diseases.

15 BRIEF DESCRIPTION OF DRAWINGS

[0021] The accompanying drawings consisting a part of the present invention are intended to
provide further understanding of the present invention and the schematic embodiments and
description thereof in the present invention are provided for explaining the present invention,
and do not constitute a restriction on the present invention. In the accompanying drawings,

20 [0022] Fig. 1 shows the activity (IC_{50}) of PCSK9 inhibition in Huh7 cells, where IC_{50} of
RRG002-51 B1 (the first-batch synthetic sample) and RRG002-52-D B1 (the first-batch
synthetic sample) are 6.0 pM and 4.0 pM, respectively; and IC_{50} of RRG002-51 B2 (the
second-batch synthetic sample, replication) and RRG002-52-D B2 (the second-batch
synthetic sample, replication) are 5.6 and 2.9 pM, respectively, which are all lower than the
25 30.2 pM of the positive control.

[0023] Fig. 2 shows the cytotoxicity (CCK8 assay) in Huh7 cells, where in the range from
0.1 pM to 500 nM, none of the three siRNAs have obvious effects on the cell viability of
Huh7 cells.

30 [0024] Fig. 3 shows the cytotoxicity (CCK8 assay) in HepG2, where in the range from 0.1
pM to 500 nM, none of the three siRNAs have obvious effects on the cell viability of HepG2
cells.

[0025] Fig. 4A-D show RNA-sequencing of primary cells, where A is the negative control in
which only a transfection reagent is added, B is the positive control, C is RRG002-51, and D

is RRG002-52-D. The RNA sequencing after the transfection of primary cells shows that the off-target risk of RRG002-51 and RRG002-52-D is similar to that of the positive control, and there is no off-target risk.

5 [0026] Fig. 5 shows the effect of RRG002-50-D, RRG002-51, RRG002-56-D, and RRG002-57-D on the expression level of PCSK9 protein in transgenic mice.

[0027] Fig. 6 shows the effect of RRG002-50-D, RRG002-51, and RRG002-53 on the expression level of PCSK9 protein in transgenic mice.

[0028] Fig. 7 shows the effect of RRG002-51, RRG002-54, and RRG002-55 on the expression level of PCSK9 protein in transgenic mice.

10 [0029] Fig. 8 shows the effects of RRG002-50-D and RRG002-55 on the expression level of PCSK9 protein in transgenic mice.

[0030] DETAILED DESCRIPTION The technical solutions according to embodiments of the present invention will be clearly and completely described in connection with embodiments of the present invention. Apparently, the embodiments described are merely some of
15 embodiments, but not all of the embodiments of the present invention. The embodiments and the features in the embodiments in the present invention can be combined with each other without conflict.

[0031] In an aspect, an embodiment of the present invention provides an siRNA molecule for inhibiting PCSK9 expression, which includes a sense strand and an anti-sense strand that are
20 complementary to form a double-stranded region.

The sense strand has a sequence of 5'-
mAmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGsmUsB-3', and the anti-sense
strand has a sequence of 5'-

mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3';

25 or the sense strand has a sequence of 5'-

BsmAsmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGmU-3', and the anti-sense
strand has a sequence of 5'-

mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3',

30 where A, U, G and C each represents a nucleotide with adenine, uridine, guanine and cytosine as a base, respectively; m means that the nucleotide adjacent to the right side of the letter m is a nucleotide modified with 2'-O-methyl; f means that the nucleotide adjacent to the right side of the letter f is a nucleotide modified with 2'-fluoro; s means that the nucleotides on the left and right sides of the letter s are linked via a phosphorothioate linkage; and B represents an abasic nucleotide.

[0032] In an embodiment of the present invention, the ability of siRNA to degrade mRNA is improved from the ground sequence level through RNA sequence design, and then RNA modification (including phosphorothioate modification between sequences, and addition of an abasic nucleotide at the end) is added, to improve the activity and stability and extend the time of action of the drug in vivo. The siRNA molecule is used to inhibit PCSK9 expression, can knock down PCSK9 efficiently, and is highly stable. That is, the efficiency of silencing PCSK9 gene is higher and the effect is more persistent.

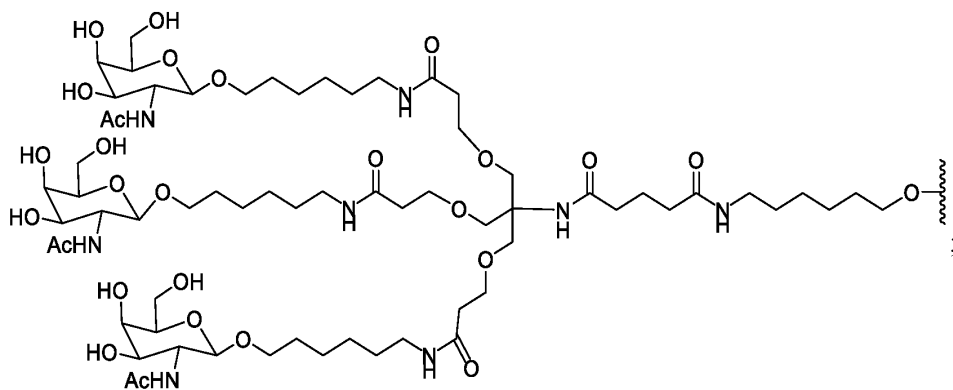
[0033] The main inventive improvements made in an embodiment of the present invention include modifications of the siRNA, including phosphorothioate modification between sequences and specific modification such as addition of an abasic nucleotide at the end. Such inventive improvements enable the modified siRNA molecule, after being modified with a ligand group directing the target organ liver, can deliver active molecules to the liver cells and stably exert their high activity.

[0034] Specifically, the siRNA molecule for inhibiting PCSK9 expression further includes a ligand group for modifying the siRNA molecule with a ligand, where the modification with the ligand is specifically modification of the 3' or 5' end of the sense strand with the ligand group. In an embodiment of the present invention, the siRNA molecule modified with a ligand group is one obtained by linking a ligand group that is a targeting ligand directing the target organ liver to the end of RNA, by which the enrichment of the molecule in the liver is increased. Therefore, an effective, low-toxic, stable and long-acting siRNA molecule targeting liver for silencing PCSK9 is obtained. Meanwhile, RNA sequencing after the transfection of primary cells shows that the RNAi inhibitor of the present invention has a low off-target risk and a good application prospect.

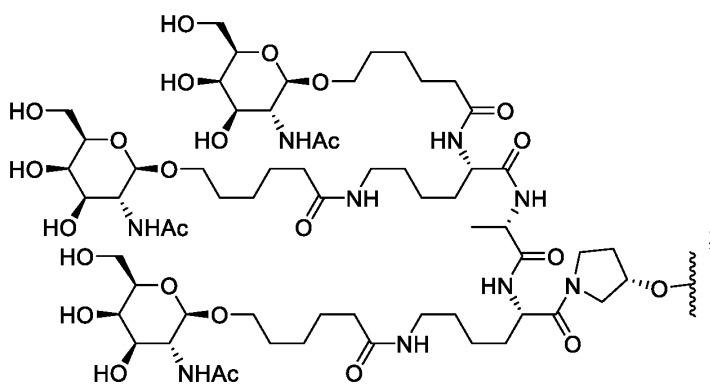
[0035] In a preferred embodiment of the present invention, the ligand group includes at least one of N-acetylgalactosamine and an N-acetylgalactosamine derivative. For example, the ligand group may include one or more N-acetylgalactosamine (GalNAc), or the ligand group may include one or more N-acetylgalactosamine derivatives. In an embodiment of the present invention, because PCSK9 is mainly expressed in the liver, the ligand group containing N-acetylgalactosamine is used as the targeting molecule directing the target organ liver, to directionally deliver the siRNA molecule to hepatocytes. Therefore, the enrichment of the molecule in the liver is increased, to effectively exert the effect of inhibiting PCSK9 expression.

[0036] In a preferred embodiment of the present invention, the ligand group includes LICA-1, LICA-2, LICA-3 or L96, wherein

LICA-1 has a structural formula of

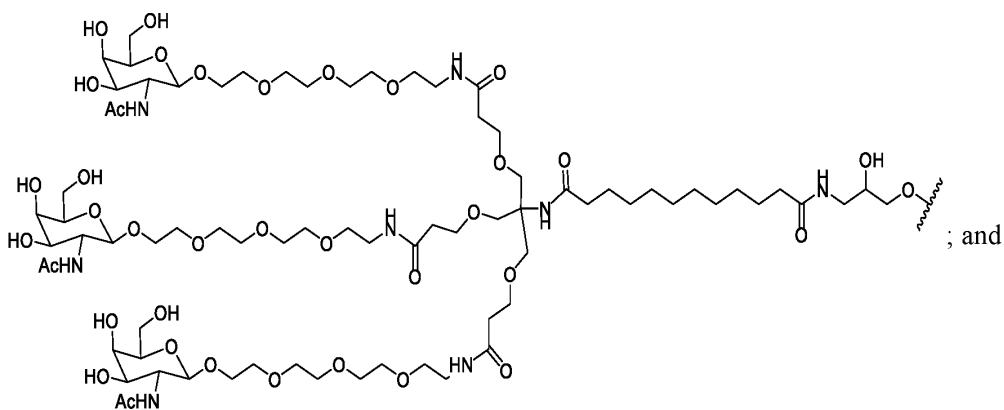
LICA-1(DX₃)

LICA-2 has a structural formula of



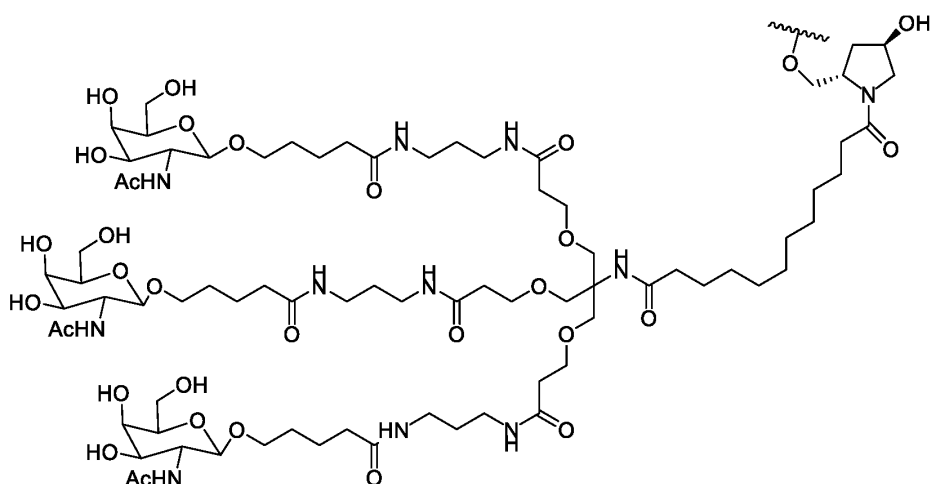
LICA-2

5 LICA-3 has a structural formula of



LICA-3

L96 has a structural formula of



L96

[0037] It is to be noted that the ligand group LICA-1, LICA-2, LICA-3 or L96 are GalNAc delivery vectors well known in the art.

[0038] In an embodiment of the present invention, the sense strand is linked with the ligand group via a phosphorothioate linkage. Specifically, the sense strand is linked with the ligand group LICA-1, LICA-2, LICA-3 or L96 via a phosphorothioate linkage.

[0039] In a preferred embodiment of the present invention, the siRNA molecule has a structure, wherein the sense strand has a sequence of 5'-LICA-1-smAmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGsmUsB-3', and
the anti-sense strand has a sequence of 5'-

mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3'.

[0040] In a preferred embodiment of the present invention, the siRNA molecule has a structure, wherein the sense strand has a sequence of 5'-LICA-2-smAmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGsmUsB-3', and
the anti-sense strand has a sequence of 5'-

mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3'.

[0041] In a preferred embodiment of the present invention, the siRNA molecule has a structure, wherein the sense strand has a sequence of 5'-BsmAsmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGmUs-LICA-3-3', and
the anti-sense strand has a sequence of 5'-

mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3'.

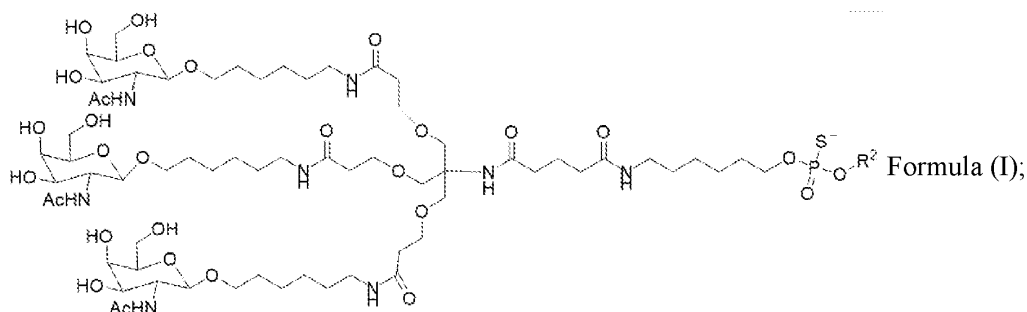
[0042] In a preferred embodiment of the present invention, the siRNA molecule has a structure, wherein the sense strand has a sequence of 5'-

BsmAsmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGmUs-L96-3', and

the anti-sense strand has a sequence of 5'-

5 mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3'.

[0043] Specifically, after the ligand group LICA-1 is conjugated to the nucleotide at the 5' end of the sense strand of siRNA via a phosphorothioate linkage, the obtained siRNA molecule has a structure of Formula (I):



10 where in Formula (I), R^2 is siRNA, which includes a sense strand and an anti-sense strand that are complementary to form a double-stranded region, wherein

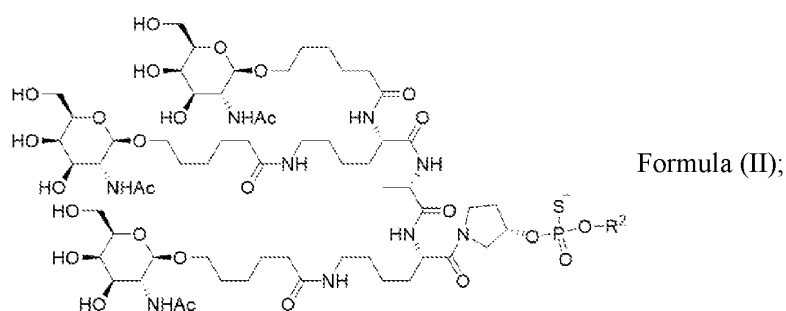
the sense strand has a sequence of 5'-

mAmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGsmUsB-3', and

the anti-sense strand has a sequence of 5'-

15 mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3'.

Specifically, after the ligand group LICA-2 is conjugated to the nucleotide at the 5' end of the sense strand of siRNA via a phosphorothioate linkage, the obtained siRNA molecule has a structure of Formula (II):



20 where in Formula (II), R^2 is siRNA, which includes a sense strand and an anti-sense strand that are complementary to form a double-stranded region, wherein

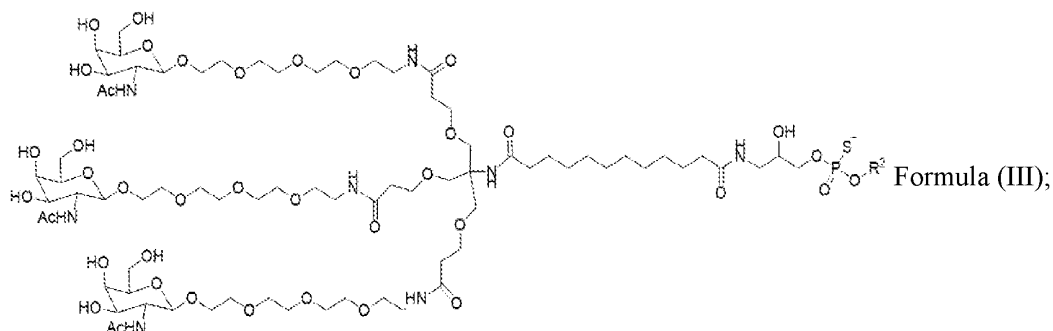
the sense strand has a sequence of 5'-

mAmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmGsmUsB-3', and

the anti-sense strand has a sequence of 5'-

mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3'.

- 5 **[0044]** Specifically, after LICA-3 is conjugated to the nucleotide at the 3' end of the sense strand of siRNA via a phosphorothioate linkage, the obtained siRNA molecule has a structure of Formula (III):



where in Formula (III), R² is siRNA, which includes a sense strand and an

- 10 anti-sense strand that are complementary to form a double-stranded region, wherein

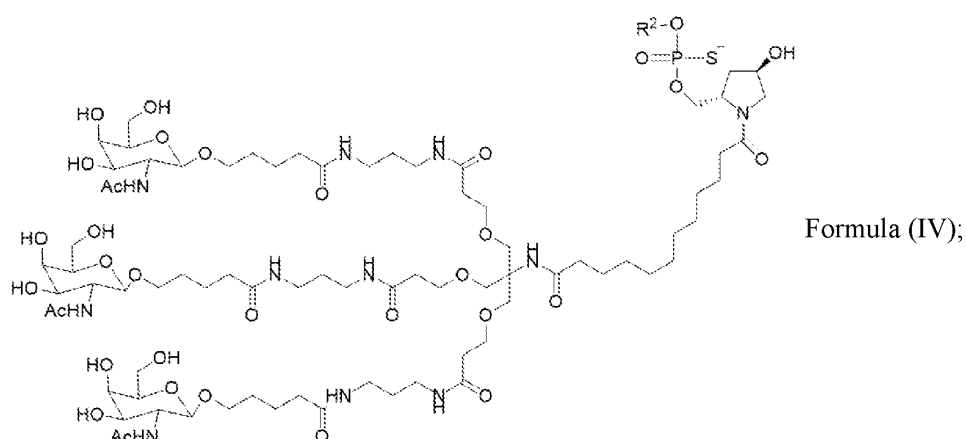
the sense strand has a sequence of 5'-

BsmAsmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGmU-3', and

the anti-sense strand has a sequence of 5'-

mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3'.

- 15 **[0045]** Specifically, after L96 is conjugated to the nucleotide at the 3' end of the sense strand of siRNA via a phosphorothioate linkage, the obtained siRNA molecule has a structure of Formula (IV):



where in Formula (IV), R^2 is siRNA, which includes a sense strand and an anti-sense strand that are complementary to form a double-stranded region, wherein

the sense strand has a sequence of 5'-

BsmAsmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGmU-3', and

5 the anti-sense strand has a sequence of 5'-

mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3'.

[0046] Moreover, an embodiment of the present invention also provides a pharmaceutical composition, which includes any siRNA molecule as described above and a pharmaceutically acceptable carrier. Specifically, the pharmaceutically acceptable carrier includes magnetic
10 nanoparticles, carbon nanotubes, chitosan and the like.

[0047] Preferably, the pharmaceutical composition may also include an additional pharmaceutically acceptable component. The additional pharmaceutically acceptable component includes saline, a pH buffer, and a diluent and the like.

In another aspect, an embodiment of the present invention also provides use of any siRNA
15 molecule or any pharmaceutical composition as described above in the manufacture of products for inhibiting PCSK9 protein expression. In an embodiment of the present invention, the product can inhibit the PCSK9 expression in human, monkeys, rats or mice.

[0048] The siRNA molecule provided in an embodiment of the present invention can effectively knock down PCSK9 when used for inhibiting PCSK9 expression, and has strong
20 stability. The PCSK9 inhibition activity measuring result of the siRNA molecule obtained in the present invention in Huh7 cells is as follows: IC₅₀ of RRG002-51 B1 (the first-batch synthetic sample) and RRG002-51 B2 (the second-batch synthetic sample) are 6.0 pM and 5.6 pM, respectively, which are both lower than the value of the positive control of 30.2 pM. The siRNA molecule obtained in the present invention reaches the peak efficacy about 20
25 days after being administered in transgenic mice (PCSK9 humanized mice), and the knock-down efficiency of RRG002-51 and RRG002-53 reaches 86%. 56 days after administration, the PCSK9 content returns to the baseline in the positive control; and the knock-down activity in the modified and optimized two groups (RRG002-51 and RRG002-53) remains at 61%. In the test in transgenic mice with another batch, on day 56 after administration, the
30 knock-down efficiency of RRG002-51 is 73%, and the knock-down efficiency of modified sequences (RRG002-54 and RRG002-55) with a substituted ligand is about 70-80%.

Meanwhile, RNA sequencing after the transfection of primary cells shows that the RNAi inhibitor of the present invention has a low off-target risk and a good application prospect.

[0049] In another aspect, an embodiment of the present invention also provides use of any siRNA molecule or any pharmaceutical composition as described above in the manufacture of products for reducing the concentration of low-density lipoprotein (LDL) or low-density lipoprotein cholesterol (LDLC) in serum.

- 5 [0050] In another aspect, an embodiment of the present invention also provides use of any siRNA molecule or any pharmaceutical composition as described above in the manufacture of products for relieving symptoms of PCSK9 gene-mediated diseases.

[0051] Specifically, the PCSK9 gene-mediated diseases include, but are not limited to, cardiovascular diseases, dyslipidemia, and neoplastic diseases. The cardiovascular diseases
10 include hypercholesterolemia, and hyperlipidemia. The dyslipidemia includes lipid metabolism disorders. The neoplastic diseases include PCSK9 associated melanoma and metastatic liver cancer.

[0052] The present invention will be described in detail by way of examples with reference to the accompanying drawings.

- 15 [0053] Example 1: Design of RNA sequence

The coding region (CD) sequence of human PCSK9 gene transcription product was obtained from NCBI website. The CD region of human PCSK9 mRNA was input into an online siRNA design platform <http://biodev.extra.cea.fr/DSIR/DSIR.html>, and the first 100 pairs were selected from the sequences generated with default parameters. Then the generated siRNAs
20 were sorted according to off-target analysis obtained by comparing with the human transcriptome and scoring. In the alignment process, the 2nd to 9th nucleotides from the 5' end of a single strand are the most important, followed by the 10th and 11th nucleotides; and the 1st or 21st nucleotide at the end has little influence on off-target. After alignment, 50 pairs of siRNAs as shown in Table 1 below were obtained by sorting according to the weight of
25 importance.

Table 1: siRNA sequence

siRNA No.	Sense strand (5'-3')	Anti-sense strand (5'-3')
RRG002-0	CUAGACCUGUUUUGCUUUUGU (SEQ ID NO.1)	ACAAAAGCAAAACAGGUCUAGAA (SEQ ID NO.2)
RRG002-1	GGUCUGGAAUGCAAAGUCA	UGACUUUGCAUUC CAGACCUG
RRG002-2	GGUGGAGGUGUAUCUCCUA	UAGGAGAUACACCUCCACCAG
RRG002-3	CCUACGUGGUGGUGCUGAA	UUCAGCACCACCACGUAGGUG
RRG002-4	GGACUGUAUGGUCAGCACA	UGUGCUGACCAUACAGUCCUG
RRG002-5	GCACCUGCUUUGUGUCACA	UGUGACACAAAGCAGGUGCUG
RRG002-6	GGGUCAUGGUCACCGACUU	AAGUCGGUGACCAUGACCCUG
RRG002-7	CCAAGCAAGCAGACAUUUA	UAAAUGUCUGCUUGCUUGGGU
RRG002-8	GUCUGGAAUGCAAAGUCA	UUGACUUUGCAUUC CAGACCU
RRG002-9	CAAGCAAGCAGACAUUUAU	AUAAAUGUCUGCUUGCUUGGG

RRG002-10	GGUUAGCGGCACCCUCAUA	UAUGAGGGUGCCGCUAACCGU
RRG002-11	CCUACUUCACAGAGGAAGA	UCUUCCUCUGUGAAGUAGGGG
RRG002-12	CCUGAUUAAUGGAGGCUUA	UAAGCCUCCAUUAAUCAGGGA
RRG002-13	CCAAGAUCUGCAUGUCUU	AAGACAUGCAGGAUCUUGGUG
RRG002-14	GAGUUGAGGCAGAGACUGA	UCAGUCUCUGCCUCAACUCGG
RRG002-15	GGCAGUGUGCAGUGGUGCA	UGCACACUGCACACUGCCGA
RRG002-16	GAAGCACCGAGCACGGAA	UUCGUGCUCGGGUGCUUCGG
RRG002-17	GGGACGAUGCCUGCCUCUA	UAGAGGCAGGCAUCGUCCCGG
RRG002-18	GGUACAGCCGCGUCCUCAA	UUGAGGACGCGGCUGUACCCA
RRG002-19	CAGAGGAAGAAACCUGGAA	UUCAGGUUUCUCCUCUGUG
RRG002-20	CCAAAGAUGUCAUCAUGA	UCAUUGAUGACAUCUUGGCA
RRG002-21	GCAUUUCACCAUUCAAACA	UGUUUGAAUGGUGAAAUGCCC
RRG002-22	GCAGAAUGACUUUUAUUGA	UCAAUAAAAGUCAUUCUGCC
RRG002-23	GGAACUUUUUCCGUUAUCA	UGAUAAACGGAAAAAGUCCAU
RRG002-24	GUAACUUGAAGAUUUUAU	AUAAAUAUCUUAAGUACAA
RRG002-25	GGGUUUUGUAGCAUUUUUA	UAAAAUAGCUACAAAACCCAG
RRG002-26	GUAGCAUUUUUAUUAAUAU	AUAUUAAUAAAAUAGCUACAA
RRG002-27	GGUCACCGACUUCGAGAAU	AUUCUCGAAGUCGGUGACCAU
RRG002-28	GAGGCAGAGACUGAUCCAC	GUGGAUCAGUCUCUGCCUCAA
RRG002-29	GGCAGAGACUGAUCCACUU	AAGUGGAUCAGUCUCUGCCUC
RRG002-30	GGGCAUUUCACCAUUCAAA	UUUGAAUGGUGAAAUGCCCCA
RRG002-31	GUUUCUGAGCCACCUUUA	UAAAGGUGGCUCAGGAAACCA
RRG002-32	GCCACCUUUACUCUGCUCU	AGAGCAGAGUAAAGGUGGCUC
RRG002-33	UGGUCACCGACUUCGAGAA	UUCUCGAAGUCGGUGACCAUG
RRG002-34	GGUCACCGACUUCGAGAAU	AUUCUCGAAGUCGGUGACCAU
RRG002-35	CAGAGUGACCACCGGGAAA	UUUCCCGGUGGUCACUCUGUA
RRG002-36	CGUGGAACCUUGGAGCGGAU	AUCCGCUCCAGGUUCCACGGG
RRG002-37	CCGGGACGUCAGCACUACA	UGUAGUGCUGACGUCCCGGCU
RRG002-38	CAAGCAGACAUUUAUCUUUU	AAAAAGAUAAAUGUCUGCUUGCU
RRG002-39	CAAGCAAGCAGACAUUUAUCU	AGAUAUAAUGUCUGCUUGCUUGGG
RRG002-40	UUCUAGACCUUUUUGCUUUU	AAAAGCAAAACAGGUCUAGAAAA
RRG002-41	UAGACCUUUUUGCUUUUGUA	UACAAAAGCAAAACAGGUCUAGA
RRG002-42	UUUUCUAGACCUUUUUGCUU	AAGCAAAACAGGUCUAGAAAAGU
RRG002-43	AAGCAAGCAGACAUUUAUCUU	AAGAUAAAUGUCUGCUUGCUUGG
RRG002-44	UGUAACUUGAAGAUUUUAUU	AAUAAAUAUCUUAAGUUACAAA
RRG002-45	UUUCUAGACCUUUUUGCUUU	AAAGCAAAACAGGUCUAGAAAAG
RRG002-46	UUUUGUAACUUGAAGAUUUU	AAAUAAUCUUAAGUUACAAAAGC
RRG002-47	CACCCAAGCAAGCAGACAUUU	AAAUUGUCUGCUUGCUUGGGUGG
RRG002-48	CCAAGCAAGCAGACAUUUAUU	AAUAAAUGUCUGCUUGCUUGGGU
RRG002-49	CCCAAGCAAGCAGACAUUUAU	AUAAAUGUCUGCUUGCUUGGGUG

Example 2: Detection methods of PCSK9 Protein content and cell viability

2.1: Method for detecting PCSK9 protein content in cell supernatant by ELISA involved in the present invention

5 2.1.1: Cell culture and transfection

2.1.1.1: Cell inoculation (first day)

HepG2 cells at a density of 1.5×10^5 cells/ml or Huh-7 cells at a density of 0.75×10^5 cells/ml were inoculated into a 24-well plate with a volume of 500 μ L per well, so the cells during transfection can reach 30-50% confluence. The cell plate was incubated in a cell incubator for 18-24 h. Two duplicate wells were set for each siRNA.

5 **[0054]** 2.1.1.2: Transfection (second day)

Before transfection, the culture medium in each well was refreshed with 450 μ L DMEM.

[0055] Preparation of Lipofectamine RNAiMAX dilution: The volume of Lipofectamine prepared was calculated according to the number of wells for transfection. For each group of duplicate wells, 3.6 μ L of Lipofectamine and 56.4 μ L of OPTI-MEM medium were mixed to give a final volume of 60 μ L, and then mixed upside down.

10 **[0056]** siRNA positive controls (RRG002-0 sequence and RRG002-50-D sequence), negative control (with only a transfection reagent) and a dilution of the sample to be tested were prepared. 60 μ L of Lipofectamine dilution was mixed with 60 μ L of siRNA sample dilution, pipetted until uniform, and let stand at room temperature for 15 min. 50 μ L of the mixed solution was added dropwise into the corresponding wells of the 24-well plate, mixed well by gently shaking the 24-well plate, and incubated in a cell incubator.

[0057] 2.1.1.3: Collection of culture supernatant and detection of cell viability (fourth day) 48 h after transfection, 200 μ L of the culture supernatant from each well was collected into a centrifuge tube. The culture supernatant was stored at -80°C if it was not detected

20 immediately.

[0058] 2.1.2: Detection of PCSK9 by ELISA

The PCSK9 ELISA kit was purchased from R&D Systems, article number: DY3888.

[0059] Coating: The plate was coated with 100 μ L per well of Capture Ab having a final concentration of 2 $\mu\text{g/ml}$ and allowed to stand at 4°C overnight.

25 **[0060]** Blocking: Capture Ab was discarded, and Reagent diluent was added in an amount of 300 μ L per well, and allowed to stand at room temperature for 2-3 h.

[0061] Preparation of standard: The standard was reconstituted according to the instruction of the kit, to give a concentration of 32 ng/ml. The solution was then 2-fold diluted to 16 ng/ml, and then 2-fold diluted with Reagent Diluent to give 7 dilutions.

30 **[0062]** The HepG2/Huh-7 supernatant was 10-fold diluted.

[0063] Plate washing: The plate was washed 3 times with a plate washer in an amount of 300 μ L per well, and beaten dry.

[0064] The standard or sample was added in an amount of 100 μ L per well, and allowed to stand at room temperature for 2 h.

- [0065] Plate washing: The plate was washed 5 times with a plate washer in an amount of 300 μ L per well, and beaten dry.
- [0066] 100 μ L of Detection Ab was added, and allowed to stand at room temperature for 2 h.
- [0067] Plate washing: The plate was washed 5 times with a plate washer in an amount of 300 μ L per well, and beaten dry.
- [0068] 100 μ L of Streptavidin-HRP was added, and allowed to stand in the dark at room temperature 20 min.
- [0069] Plate washing: The plate was washed 5 times with a plate washer in an amount of 300 μ L per well, and beaten dry.
- [0070] Development: The TMB solution was drawn in advance and balanced at room temperature. 100 μ L TMB was added and used for development in the dark for 15 min, and 50 μ L of ELISA stop solution was added.
- [0071] The plate was read on a microplate reader at 450 nm and 570 nm. The values of OD₄₅₀-OD₅₇₀ were taken as the measured OD values, and then the sample concentration was calculated from the corresponding standard curve.
- [0072] 2.2: Method for detecting cell viability by CCK8 assay involved in the present invention
- [0073] 40 μ L of CCK-8 solution was added to the cell supernatant in each well of the 24-well plate 48 h after the transfection in step 2.1.1.2. The plate was incubated in an incubator for 1 h, and the absorbance at 450 and 650 nm was measured on a microplate reader. The values of OD₄₅₀-OD₆₅₀ obtained were taken as the measured OD values, and cell viability was presented as the ratio of the OD values of the treatment group and the control group.
- [0074] Example 3: Screening of unmodified sequence of siRNA in vitro
- [0075] 3.1: Effect on expression level of PCSK9 protein
- [0076] To evaluate the in-vitro activity of siRNA knocking down PCSK9 mRNA, the in vitro activity of 50 pairs of siRNAs in Table 1 was evaluated by measuring their effects on the expression level of PCSK9 protein by using the ELISA method in Part 2.1 of Example 2. The results were shown in Table 2.
- [0077] As can be seen from Table 2, after screening the activity of siRNA at two concentrations (0.01 nM, and 0.001 nM), it is found that the sequence of the positive control RRG002-0 has the highest activity.
- Table 2. Expression level of PCSK9 protein in SiRNA group vs negative control group (Huh7 cells)

	0.01 nM (final concentration of siRNA in the system)		0.001 nM (final concentration of siRNA in the system)	
siRNA No.	Average remaining PCSK9 (%)	SD	Average remaining PCSK9 (%)	SD
RRG002-0	40.99	1.15	71.58	14.54
RRG002-1	90.53	2.30	112.93	0.44
RRG002-2	111.14	4.23	106.05	4.79
RRG002-3	94.72	0.82	99.27	0.22
RRG002-4	101.73	1.42	102.39	0.06
RRG002-5	92.64	0.57	107.95	0.78
RRG002-6	87.27	1.70	108.17	1.79
RRG002-7	60.35	0.59	104.61	0.58
RRG002-8	85.23	0.96	102.86	1.37
RRG002-9	59.75	0.29	91.51	0.50
RRG002-10	103.94	1.22	99.89	0.70
RRG002-11	89.96	2.04	102.54	0.33
RRG002-12	85.00	3.02	101.98	1.66
RRG002-13	85.16	1.63	102.61	0.94
RRG002-14	93.39	0.10	103.82	1.46
RRG002-15	60.35	0.37	97.03	3.66
RRG002-16	81.75	2.05	101.98	3.50
RRG002-17	93.67	5.73	104.88	1.95
RRG002-18	92.62	3.66	96.29	2.66
RRG002-19	73.42	3.88	98.57	3.70
RRG002-20	89.00	2.59	95.98	1.13
RRG002-21	51.22	1.40	94.03	3.16
RRG002-22	92.77	0.99	95.86	0.05
RRG002-23	70.34	0.76	99.94	1.75
RRG002-24	60.02	0.72	100.18	1.25
RRG002-25	53.69	1.10	106.80	1.35
RRG002-26	88.63	1.27	113.95	0.05
RRG002-27	97.30	0.37	110.77	1.70
RRG002-28	93.74	0.10	105.32	0.86
RRG002-29	87.68	0.90	103.27	0.00
RRG002-30	60.72	0.77	97.03	0.43

RRG002-31	87.08	0.28	104.44	1.93
RRG002-32	78.54	0.22	108.72	2.74
RRG002-33	89.50	1.63	108.10	2.41
RRG002-34	97.72	0.30	114.79	0.64
RRG002-35	92.10	2.68	115.09	0.38
RRG002-36	86.51	0.75	109.88	0.89
RRG002-37	100.71	0.32	106.86	1.50
RRG002-38	68.74	0.37	104.07	1.20
RRG002-39	54.64	1.07	88.94	0.29
RRG002-40	46.25	0.03	93.36	2.20
RRG002-41	45.06	0.04	91.71	0.46
RRG002-42	40.93	0.53	94.96	2.49
RRG002-43	52.07	0.07	103.50	0.38
RRG002-44	75.05	0.14	103.71	0.77
RRG002-45	37.87	0.08	90.60	2.60
RRG002-46	59.95	0.17	100.47	0.80
RRG002-47	67.93	1.21	103.73	5.02
RRG002-48	50.07	5.16	101.03	4.38
RRG002-49	56.39	0.52	95.53	7.24

[0078] Example 4: Preparation and Screening of modified sequences

[0079] 4.1: Preparation of modified sequences

[0080] Through the screening of unmodified sequences in Example 3, it is found that the sequence with the highest activity is still the positive control RRG002-0. To further improve the activity, on the basis of the positive control sequence, the positive control sequence was engineered by modification, including phosphorothioate modification between sequences, the addition of abasic and 5(E)VP at the end, and modification with a ligand group. The modified siRNA sequences are shown in Table 3.

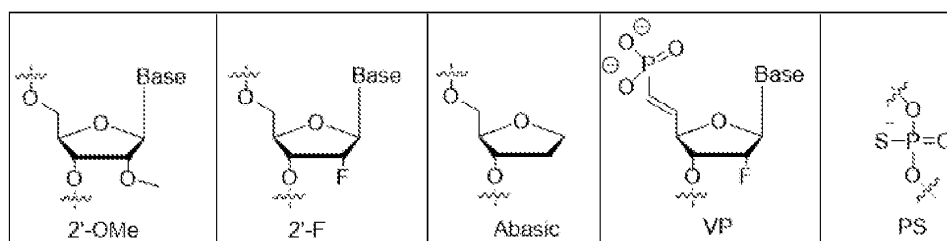
- 10 **[0081]** In Table 3, siRNA-1 and siRNA2 are siRNA molecules of the present invention without modification with a ligand. RRG002-51, RRG002-53, RRG002-54, and RRG002-55 are siRNA molecules of the present invention modified with a ligand group. RRG002-50-D, RRG002-52-D, RRG002-56-D, and RRG002-57-D are reference siRNA molecules for highlighting the performance of the siRNA molecules of the present invention by comparing
- 15 with the siRNA molecules of the present invention.

Table 3. Modified sequence

siRNA No.	Sense strand (5'-3')	Anti-sense strand (5'-3')
siRNA-1	mAmGmAmCmCmUfGmUfUfUfU mGmCmUmUmUmUmGsmUsB	mAsfCsmAmAmAfAmGmCmAmAmA mAmCfAmGfGmUmCmUsmAsmG
siRNA-2	BsmAsmGmAmCmCmUfGmUfUfU fUmGmCmUmUmUmUmGmU	mAsfCsmAmAmAfAmGmCmAmAmA mAmCfAmGfGmUmCmUsmAsmG
RRG002-50-D	mCsmUsmAmGmAmCfCmUfGmUd TmUmUmGmCmUmUmUmUmGm U-L96	mAsfCsmAfAfAfAmGfCmAfAmAfAm CfAmGfGmUfCmUmAmGsmAsmA
RRG002-51	DX3-smAmGmAmCmCmUfGmUfU fUfUmGmCmUmUmUmUmGsmUs B	mAsfCsmAmAmAfAmGmCmAmAmA mAmCfAmGfGmUmCmUsmAsmG
RRG002-52-D	DX3-smAmGmAmCmCmUfGmUfU fUfUmGmCmUmUmUmUmGsmUs B	vAsfCsmAmAmAfAmGmCmAmAmA mAmCfAmGfGmUmCmUsmAsmG
RRG002-53	BsmAsmGmAmCmCmUfGmUfUfU fUmGmCmUmUmUmUmGmUs-L9 6	mAsfCsmAmAmAfAmGmCmAmAmA mAmCfAmGfGmUmCmUsmAsmG
RRG002-54	LICA-2-smAmGmAmCmCmUfGm UfUfUfUmGmCmUmUmUmUmGs mUsB	mAsfCsmAmAmAfAmGmCmAmAmA mAmCfAmGfGmUmCmUsmAsmG
RRG002-55	BsmAsmGmAmCmCmUfGmUfUfU fUmGmCmUmUmUmUmGmUs-LI CA-3	mAsfCsmAmAmAfAmGmCmAmAmA mAmCfAmGfGmUmCmUsmAsmG
RRG002-56-D	DX3-mAmGmAmCmCmUfGmUfUf UfUmGmCmUmUmUmUsmGsmU	mAsfCsmAmAmAfAmGmCmAmAmA mAmCfAmGfGmUmCmUsmAsmG
RRG002-57-D	DX3-mAmGmAmCmCmUfGmUfUf UfUmGmCmUmUmUmUmGsmUsB	mAsfCsmAmAmAfAmGmCmAmAmA mAmCfAmGfGmUmCmUsmAsmG

wherein, m means that the nucleotide adjacent to the right side of the letter m is a nucleotide modified with 2'-O-methyl (2'OMe); f means that the nucleotide adjacent to the right side of the letter f is a nucleotide modified with 2'-fluoro (2'F); s represents PS, that is, the two riboses/groups on the left and right sides of the letter s are linked via a phosphorothioate linkage; and B represents abasic (an abasic nucleotide), v represents 5(E)VP, and the wavy line represents the previous or next nucleotide. The specific mode of modification is shown in Table 4 below.

10 Table 4: Scheme of modification

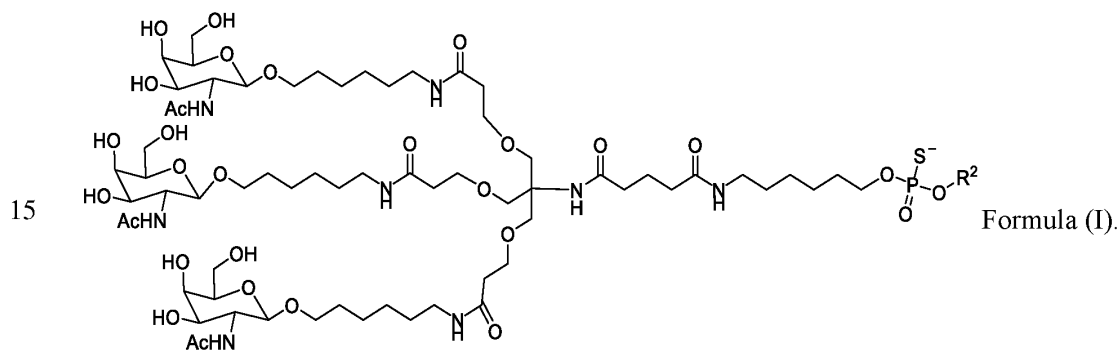


wherein, 2'-OMe means that 2'H of the nucleotide is replaced by methoxy; 2'-F means that 2'H of the nucleotide is replaced by fluoro; Abasic means that the base of the nucleotide at this site is removed; VP means that P-O-C at the 5' end is replaced by P-C=C, PS means that one of the oxygen atom in phosphate is replaced by S, and Base means the base A, U, G or C.

5 **[0082]** Specifically, the siRNA molecule RRG002-51 has a structure represented by Formula (I):

In Formula (I), R² is siRNA-1, which includes a sense strand and an anti-sense strand that are complementary to form a double-stranded region, wherein the sense strand has a sequence of 5'-mAmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGsmUsB-3', the anti-sense

10 strand has a sequence of 5'-
mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3'. DX₃ (i.e.,
LICA-1) is a delivery vector having the ligand GalNAc linked to the sense strand of siRNA,
and DX₃ (that is, LICA-1) is connected to the 5' end of the sense strand of siRNA-1 via a
phosphorothioate linkage.



RRG002-51

[0083] Reference siRNA molecule RRG002-52-D: Compared with the structure of RRG002-51, (E)VP modification is added at the 5' end of the anti-sense strand.

[0084] Reference siRNA molecule RRG002-56-D: Compared with the structure of RRG002-51, the ligand of the sense strand is linked to the siRNA sequence via a phosphate linkage instead of a phosphorothioate linkage, and the side without the connection of a ligand has no Abasic.

[0085] Reference siRNA molecule RRG002-57-D: Compared with the structure of RRG002-51, the ligand of the sense strand is linked to the siRNA sequence via a phosphate linkage instead of a phosphorothioate linkage.

25 **[0086]** Reference siRNA molecule RRG002-50-D: Compared with the structure of RRG002-
51, the sense strand and the anti-sense strand are both 2 nt longer, the ligand of the sense

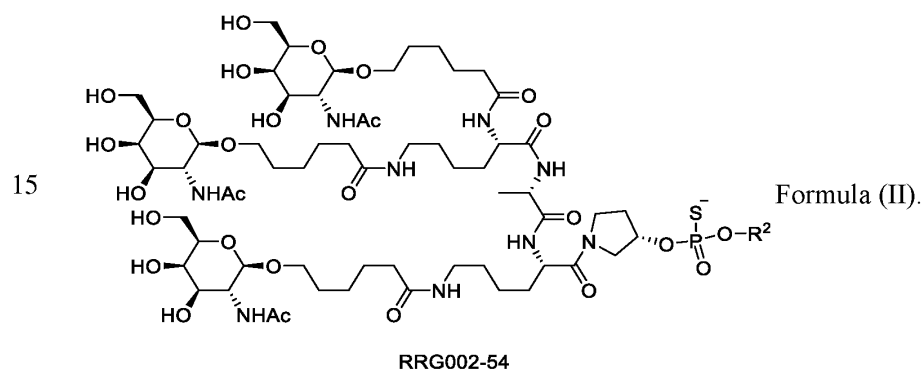
strand is linked to the siRNA sequence via a phosphate linkage instead of a phosphorothioate linkage, and the side without the connection of a ligand has no Abasic. The positions in the sense strand substituted with fluoro are positions 7 and 9, and the positions in the anti-sense strand substituted with fluoro are positions 2, 4, 5, 6, 8, 10, 12, 14, 16, and 18. Position 11 in

the sense strand is dT.

[0087] The siRNA molecule RRG002-54 has a structure represented by Formula (II):

In Formula (II), R^2 is siRNA-1, which includes a sense strand and an anti-sense strand that are complementary to form a double-stranded region, wherein the sense strand has a sequence of 5'-mAmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGsmUsB-3', and the anti-

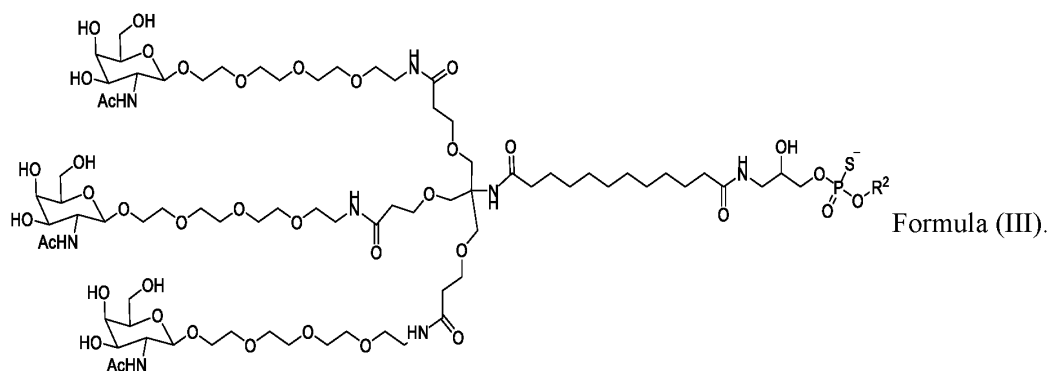
sense strand has a sequence of 5'-mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3'. LICA-2 is a delivery vector having the ligand GalNAc connected to the sense strand of siRNA, and LICA-2 is connected to the 5' end of the sense strand of siRNA-1 via a phosphorothioate linkage.



[0088] The siRNA molecule RRG002-55 has a structure represented by Formula (III):

[0089] In Formula (III), R^2 is siRNA-2, which includes a sense strand and an anti-sense strand that are complementary to form a double-stranded region, where the sense strand has a sequence of 5'-BsmAsmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGmU-3', and

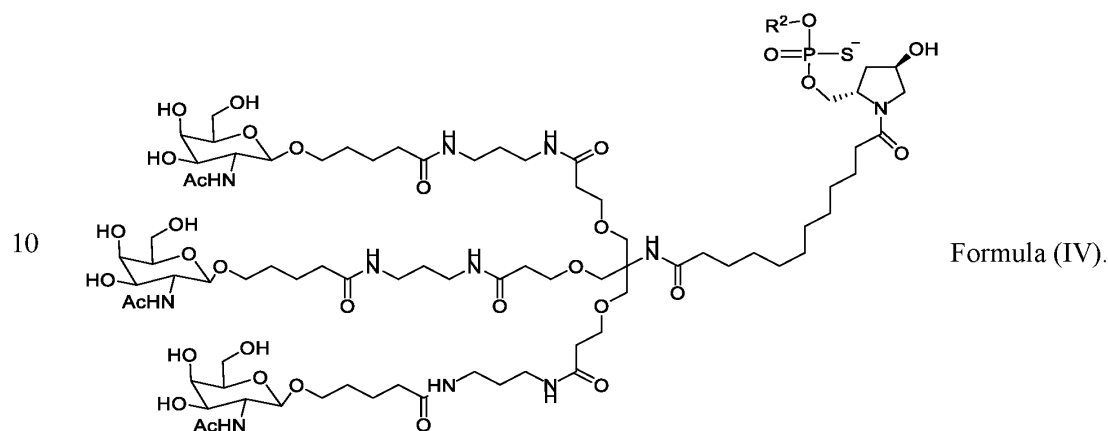
the anti-sense strand has a sequence of 5'-mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3'. LICA-3 is a delivery vector having the ligand GalNAc connected to the sense strand of siRNA, and LICA-3 is connected to the 3' end of the sense strand of siRNA-2 via a phosphorothioate linkage.



RRG002-55

[0090] The siRNA molecule RRG002-53 has a structure represented by Formula (IV):

In Formula (IV), R² is siRNA-2, which includes a sense strand and an anti-sense strand that are complementary to form a double-stranded region, where the sense strand has a sequence of 5'-BsmAsmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGmU-3', and the anti-sense strand has a sequence of 5'-mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3'. L96 is a delivery vector having the ligand GalNAc connected to the sense strand of siRNA, and L96 is connected to the 3' end of the sense strand of siRNA-2 via a phosphorothioate linkage.



RRG002-53

[0091] The siRNA molecule was prepared through a method including the following steps.

[0092] (1) Solid-phase synthesis of RNA

[0093] The siRNAs in Table 1 and Table 3 could be prepared by an entrusted synthesis company by solid-phase synthesis, or synthesized by the following method.

[0094] The sense strand and anti-sense strand of siRNA were prepared by solid-phase phosphoramidite synthesis of nucleic acid well known in the art. A general solid-phase carrier

(Unylinker, Loading: 50 $\mu\text{mol/g}$) or a ligand containing solid-phase carrier was used to start the cycle. The nucleoside monomers were connected one by one in the sequence of 3'-5'. The connection of each nucleoside monomer had four steps: deprotection, coupling, capping, and oxidation (or thiolation).

5 [0095] 1) The synthesis conditions were as follows:

[0096] The nucleoside monomer was provided as a 0.05M solution in acetonitrile.

[0097] ① Deprotection: The conditions for the deprotection reaction in each step were the same. That is, the temperature was 10-25°C, the reaction time was 60 sec, the deprotection reagent was a solution (3% v/v) of trichloroacetic acid in toluene, and the molar ratio of
10 trichloroacetic acid to 4, 4'-dimethoxytrityl protecting group on the solid-phase carrier was 5:1.

[0098] ② Coupling: The conditions for the coupling reaction in each step were the same. That is, the temperature was 10-25°C, the coupling reagent was a 0.25 M solution of 5-ethylthio-1H-tetrazole (ETT) in acetonitrile, the molar ratio of nucleic acid sequence
15 connected on the solid-phase carrier to the nucleoside monomer was 1:5, the molar ratio of the nucleic acid sequence connected on the solid-phase carrier to the coupling reagent was 1:3.3, and the reaction time was 360 seconds.

[0099] ③ Capping: The capping conditions in each step were the same. That is, the temperature was 10-25°C, and the reaction time was 90 seconds. The capping reagent
20 solution was a mixed solution of Cap1 and Cap2 at a molar ratio of 1:1, and the molar ratio of the capping reagent to the nucleic acid sequence connected on the solid-phase carrier was acetic anhydride: N-methylimidazole: nucleic acid sequence connected on the solid-phase carrier = 1: 1: 1.

[0100] ④ Oxidation: The conditions for the oxidation reaction in each step were the same.
25 That is, the temperature was 10-25°C, and the reaction time was 90 sec, and the oxidation reagent was iodine water with a concentration of 0.05 M. The molar ratio of iodine to the nucleic acid sequence connected on the solid-phase carrier in the coupling step was 30:1, and the reaction was carried out in a mixed solvent of tetrahydrofuran: water: pyridine = 3: 1: 1.

[0101] ⑤ Thiolation: The conditions for the thiolation reaction in each step were the same.
30 That is, the temperature was 10-25°C, the reaction time was 90 seconds, the thiolation reagent was phenylacetyl disulfide (PADS) with a concentration of 0.25 M, and the molar ratio of PADS to the nucleic acid sequence connected on the solid-phase carrier in the thiolation step

was 40:1. Finally, in this step, the solid-phase carrier was conjugated to the 3' end of the sense strand or the anti-sense strand of siRNA.

[0102] 2) Cleavage and deprotection

[0103] After the solid-phase synthesis, the RNA was cleaved from the solid-phase carrier and
5 deprotected.

[0104] The cleavage and deprotection steps were as follows. The solid-phase carrier was placed in a pressure-resistant test tube, and 1.0-3.0 mL of 28% aqueous ammonia was added, sealed, and reacted with vibration or stirring at 50°C for 15 h. After the reaction, the filtrate was centrifuged and concentrated to dryness after filtration. Where the sequence had a 2'-
10 TBDMS protecting group, 350 μ L solution of DMSO: triethylamine: triethylamine trihydrofluoride = 1:1:1 was added, and reacted at 70°C for 3 h, to remove the 2'-TBDMS protecting group on ribose.

[0105] 3) Purification and desalting

[0106] The nucleic acid sequence was purified by a preparative reversed-phase
15 chromatographic column (SinoPak BEH AQ-C18), eluting with eluent A: 4 mM triethylamine and 50 mM hexafluoroisopropanol aqueous solution, and eluent B: methanol, over gradient of 10%-35% B. The product-containing eluates were collected and combined, and desalted by an ultrafiltration centrifuge tube (made of regenerated cellulose, having a molecular weight cut-off of 3000).

20 **[0107]** 4) Annealing

[0108] The sense strand and anti-sense strand of siRNA were obtained according to the above method, and annealed to obtain a double-stranded siRNA.

[0109] The annealing operation was as follows. The concentrations of the sense strand and the anti-sense strand were detected by an ultraviolet spectrophotometer. The sense strand and
25 the anti-sense strand at a ratio of 1: 1 were added to the same container, heated to 90°C for 5 min, and then naturally cooled to room temperature, so that a double-stranded structure was formed through hydrogen bonding. siRNA having a double-stranded structure was obtained after annealing.

[0110] (2) Connection of ligand

30 **[0111]** When the ligand was connected to the 3' end of the sense strand (RRG002-50-D, RRG002-53, and RRG002-55), a solid-phase carrier containing the ligand L96 (L96-CPG, Chengdu HitGen Inc., article number: GN-0003) was used as a raw material to start the cycle at a loading of 41.88 μ mol/g, or a solid-phase carrier containing the ligand LICA-3 (LICA-3-CPG, Shanghai Hongene Biotech, article number: ON-433) was used as a raw material to

start the cycle at a loading of 55 $\mu\text{mol/g}$. The nucleoside monomers were connected one by one in the sequence of 3'-5'. The connection of each nucleoside monomer had four steps: deprotection, coupling, capping, and oxidation (or thiolation). The specific preparation process was consistent with the solid-phase synthesis of RNA in Step (1). RRG002-50-D, 5 RRG002-53, and RRG002-55 were obtained respectively.

[0112] When the ligand was connected to the 5' end of the sense strand (RRG002-51, RRG002-52-D, RRG002-54, RRG002-56-D, and RRG002-57-D), a general solid-phase carrier (Unylinker, Loading: 50 $\mu\text{mol/g}$) was used as a raw material to start the cycle. The nucleoside monomers were connected one by one in the sequence of 3'-5'. In the conjugation 10 of the ligand DX₃ to the 5' end, a 0.1 M solution of DX₃-phosphoramidite monomer (Shanghai Hongene Biotech, article number OP-026) in acetonitrile was used as a raw material. In the conjugation of the ligand LICA-2 to the 5' end, a 0.1 M solution of LICA-2-phosphoramidite monomer (Shanghai Refreshgene Therapeutics Co., Ltd., homemade, article number KAK4) in acetonitrile was used as a raw material. The coupling reagent was a 0.25 15 M solution of 5- ethylthio -1H-tetrazole (ETT) in acetonitrile, the molar ratio of the nucleic acid sequence connected on the solid-phase carrier to the phosphoramidite monomer of the ligand was 1:8, the molar ratio of the nucleic acid sequence connected on the solid-phase carrier to the coupling reagent was 1:5, and the reaction time was 600 sec. Other steps were consistent with the solid-phase synthesis of RNA in Step (1). RRG002-51, RRG002-52-D, 20 RRG002-54, RRG002-56-D, and RRG002-57-D were obtained respectively.

[0113] 4.2. Screening of modified sequences: effect on PCSK9 protein expression level

[0114] To evaluate the in-vitro activities of the 3 modified siRNAs, namely RRG002-50-D, RRG002-51, and RRG002-52-D in Table 3 to knock down PCSK9 mRNA, the in-vitro activity of the 3 modified siRNAs in Table 3 was evaluated by evaluating their effects on the 25 expression level of PCSK9 protein using the ELISA method in Part 2.1 of Example 2. The results are shown in Table 5 and Fig. 1.

[0115] Fig. 1 shows the PCSK9 activity (IC₅₀) in Huh7 cells. It can be seen from Fig. 1 that IC₅₀ of RRG002-51 B1 (the first-batch synthetic sample) and RRG002-52-D B1 (the first-batch synthetic sample) are 6.0 pM and 4.0 pM, respectively; and IC₅₀ of RRG002-51 B2 30 (the second-batch synthetic sample, replication) and RRG002-52-D B2 (the second-batch synthetic sample, replication) are 5.6 and 2.9 pM, respectively, which are all lower than 30.2 pM of the positive control RRG002-50-D.

Table 5. Expression level of PCSK9 protein in modified siRNA group vs negative control group (Huh-7 cells)

	RRG002-50-D	RRG002-51 B1	RRG002-52-D B1	RRG002-51 B2	RRG002-52-D B2
Concentration (nM)	Average remaining PCSK9 (%)	Average remaining PCSK9 (%)	Average remaining PCSK9 (%)	Average remaining PCSK9 (%)	Average remaining PCSK9 (%)
500	22.71	23.32	16.32	38.54	24.48
100	17.88	13.47	12.48	17.79	14.83
10	14.76	15.80	12.39	8.12	11.54
4	20.74	16.75	9.98	9.11	9.23
1	21.67	15.54	8.52	13.75	8.52
0.4	21.47	13.44	11.18	13.19	8.44
0.1	30.76	19.14	17.07	24.37	17.09
0.01	87.05	53.12	38.61	40.43	38.42
0.001	103.24	82.10	81.41	92.97	71.84
0.0001	118.19	91.43	89.23	90.84	86.96

[0116] 4.3. Screening of modified sequences: cell viability

[0117] To evaluate the effect of modified siRNAs on cell viability, the effect of siRNAs of RRG002-50-D, 51 and 52 in Table 3 on the cell viability was evaluated by the CCK8 method in Part 2.2 of Example 2. The results are shown in Figs. 2 and 3. The three pairs of siRNAs have no effect on the viability of Huh7 and HepG2 cells within the concentration range of 0.1 pM to 500 nM, and have low toxicity (Inclisiran in Figs. 2 and 3 is RRG002-50-D).

[0118] 4.4. Assessment of off-target risk in primary cells

[0119] To verify that the modified siRNA has no off-target risk in the cells, 10 nM RRG002-50-D, 51, and 52 were respectively transfected in human primary cells, and then RNAseq analysis was performed. The results are shown in Fig. 4. The off-target risks of RRG002-51 and RRG002-52-D are similar to that of the positive control RRG002-50-D, and there is no off-target risk. The specific experiment was shown below.

[0120] siRNA was transfected into human primary hepatocytes (PHHs) as follows.

[0121] 1) Preparation of siRNA dilution and transfection reagent: siRNA was diluted with PBS to a concentration that was 20 times the final concentration (for example, when the test concentration was 10 nM, it was diluted to 200 nM). A mixture of Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen -13778-150): Opti-MEM™ I Reduced Serum Medium (Gibco-31985-070) = 1.5 : 23.5 was prepared, and incubated at room temperature for 15 min.

- [0122] 2) Thawing and counting of human primary hepatocytes (PHHs): During the incubation in the previous step, the frozen human primary hepatocytes (PHHs) (WBU) was removed from liquid nitrogen, and thawed in a water bath at 37°C. The human primary hepatocytes were removed after they were almost completely thawed. Subsequently, the thawed PHHs was transferred to a cell culture medium containing 10% fetal bovine serum and mixed evenly. A small amount of cell suspension was added to and mixed evenly with an AO/PI double staining reagent (Count star-RE010212). Then, the cells were counted by using an automatic cell fluorescence analyzer (Countstar Rigel2) and the cell density was adjusted to 6.7×10^5 cells/mL according to the cell count.
- 5
- 10 [0123] 3) Preparation of siRNA transfection mixture: 100 μ L of diluted siRNA was added into 100 μ L of mixed solution prepared in Step 1), mixed uniformly, and incubated for 15 min.
- [0124] 4) Plating: The above mixture was added into a 24-well plate pre-coated with collagen in a volume of 50 μ L/well. siRNA test conditions: 1 concentration point, and triplicate wells.
- 15 Control group: transfection reagent blank control (PBS in place of test siRNA) and cell well control.
- [0125] 5) Cell culture: PHHs of 6.7×10^5 cells/mL was added to the 24-well plate pre-coated with collagen in Step 4) in a volume of 450 μ L/well. The final concentration of the test siRNA was 10 nM. The cells were cultured in an incubator with 5% CO₂ at 37°C for 24 h.
- 20 [0126] Second generation sequencing procedure of mRNA expression was as follows.
- [0127] 1) Total RNA was purified from cells according to the manufacturer's instructions (RNA extraction kit (RNeasy® Mini Kit, Qiagen-74106), and Qiagen-79254_RNase-Free DNase Set).
- [0128] 2) The concentration of total RNA was detected by using a micro-volume UV/Vis spectrophotometer (Nanodrop One), and the RNA integrity was analyzed according to the manufacturer's guidelines (Agilent 2100 Bioanalyzer, and Agilent RNA 6000 Nano Kit (Agilent-5067-1511)).
- 25 [0129] 3) The library was prepared according to the manufacturer's instructions (VAHTS® Universal V6 RNA-seq Library Prep Kit for Illumina (Vazyme-NR604)).
- 30 [0130] 4) The library concentration was detected according to the manufacturer's guidelines (fluorometer (Qubit 4 fluorometer, Thermo Fisher Scientific), and Qubit 1X dsDNA HS Assay Kit (Thermo Fisher Scientific-Q33231)). The library fragment size was analyzed according to the manufacturer's guidelines (Agilent 2100 Bioanalyzer, and Agilent DNA 1000 Kit (Agilent-5067-1504)).

[0131] 5) The library was sequenced according to the manufacturer's guidelines (sequencer (NextSeq 550, Illumina), and NextSeq 500/550 High Output Kit v2.5 (300 Cycles), (Illumina-20024908)).

[0132] Example 5. In-vivo activity detection of siRNA molecule in transgenic mice

5 [0133] This example was mainly used to test that the in-vivo activity detection effect of the reference siRNA molecules RRG002-50-D, RRG002-56-D, and RRG002-57-D with different modifications in transgenic mice was poor than that of the siRNA molecule RRG002-51 of the present invention.

10 [0134] The in-vivo activity of siRNA was tested in 30 6-8-week-old humanized PCSK9 male mice (Shanghai Model Organisms Center, Inc., C57BL/6J-Pcsk9^{em2(hPCSK9)Smoc}, product number NM-HU-00075). The mice were divided into 5 groups (6 animals in each group), including a PBS control group; a RRG002-50-D (3 mg/kg) positive control group; a RRG002-51 (3 mg/kg) treatment group; a RRG002-56-D (3 mg/kg) treatment group; a RRG002-57-D (3 mg/kg) treatment group.

15 [0135] On day -1 (before administration), the plasma samples of mice before administration were obtained (Submandibular Blood). On the first day, the sample for administration was diluted with PBS and subcutaneously injected once at a dose of administration of 3 mg/kg, and a concentration of 0.3 mg/ml (dissolved in PBS). Plasma was collected on the 28th day (Submandibular Blood).

20 [0136] The level of PCSK9 protein in plasma was detected by using a PCSK9 ELISA reagent (R&D systems). The relative proportion was calculated based on the PCSK9 protein level at baseline (before administration). The results are shown in Fig. 5.

[0137] It can be seen from Fig. 5 that the average PCSK9 content in the plasma of the PBS group is 27.82 ng/ml, the average PCSK9 content in the plasma of the positive control group is 10.50 ng/ml, the average PCSK9 content in the plasma of the RRG002-56-D group is 14.49 ng/ml, the average PCSK9 content in the plasma of the RRG002-57-D group is 11.29 ng/ml, and the average PCSK9 content in the plasma of the RRG002-51 group is 4.74 ng/ml.

25 [0138] It can be seen that the difference between RRG002-57-D and RRG002-56-D is that the knock-down effect in vivo is improved by adding Abasic nucleotide on the opposite side of siRNA connected with a ligand. The difference between RRG002-51 and RRG002-57-D is that by adding a modification with a phosphorothioate linkage at the connection between the ligand and the siRNA, the knock-down effect of RRG002-51 in vivo is greatly improved, and the effect of the drug in mice is better than that in positive control at the same dose. This example shows that by using a modification with a phosphorothioate linkage at the

connection between the ligand and the siRNA and using an Abasic modification on the side opposite to the side where the ligand is connected to siRNA, the in vivo activity of the sequence can be improved.

[0139] Example 6. In-vivo activity test of siRNA molecule in transgenic mice

5 **[0140]** (1) First-batch test of in-vivo activity of RRG002-50-D, RRG002-51, and RRG002-53 in transgenic mice

[0141] To evaluate the in-vivo activity of modified siRNA molecules (RRG002-50-D, RRG002-51, and RRG002-53), the in-vivo activity of siRNA was tested in 24 6-8-week-old humanized PCSK9 male mice (Shanghai Model Organisms Center, Inc., C57BL/6J-
10 $Pcsk9^{em2(hPCSK9)Smoc}$, product number NM-HU-00075). The mice were divided into 4 groups (6 animals in each group) including a PBS control group; a RRG002-50-D (3 mg/kg) positive control group; a RRG002-51 (3 mg/kg) treatment group; and a RRG002-53 (3 mg/kg) treatment group.

[0142] On day -1 (before administration), the plasma samples of mice before administration
15 were obtained (Submandibular Blood). On the first day, the sample for administration was diluted with PBS and subcutaneously injected once at a dose of administration of 3 mg/kg, and a concentration of 0.3 mg/ml (dissolved in PBS). Plasma was collected on the 14th, 21th, 28th, 35th, 42th, 49th, and 56th day, respectively (Submandibular Blood).

[0143] The level of PCSK9 protein in plasma was detected by using a PCSK9 ELISA reagent
20 (R&D systems). The relative proportion was calculated based on the PCSK9 protein level at baseline (before administration). The results are shown in FIG. 6.

[0144] It can be seen from Fig. 6 that all the drug treatment groups reach the peak efficacy and the knock-down efficiency reach the highest about 20 days after administration, wherein the knock-down efficiency reaches 77% in the positive control and 86% in the two modified
25 and optimized groups (RRG002-51 and RRG002-53).

[0145] It can be seen that the difference between RRG002-51 and 53 is that different GalNAc (DX₃, L96) ligands are conjugated at different ends of the sense strand. In RRG002-51, DX₃ is conjugated at the 5' end, and in RRG002-53, L96 is conjugated at the 3'end, with the other modifications being basically the same. 56 days after administration, the PCSK9 content
30 returns to the baseline in the positive control; and the knock-down activity in the modified and optimized two groups still remains at 61%. It can be seen that both the efficacy and the time of action are significantly improved compared with the positive control. Moreover, it also shows that the active products provided in the present invention modified by connecting different ligands at different positions achieve better efficacy.

[0146] (2) Second-batch test of in-vivo activity of RRG002-51, RRG002-54, and RRG002-55 in transgenic mice

[0147] To evaluate the in-vivo activity of modified siRNA molecules (RRG002-51, RRG002-54, and RRG002-55), the in-vivo activity of siRNA was tested in 24 6-8-week-old humanized PCSK9 male mice (Shanghai Model Organisms Center, Inc., C57BL/6J-Pcsk9^{em2(hPCSK9)}Smoc, product number NM-HU-00075). The mice were divided into 4 groups (5 animals in each group) including a PBS control group; a RRG002-51 (3 mg/kg) treatment group; a RRG002-54 (3 mg/kg) treatment group; and a RRG002-55 (3 mg/kg) treatment group.

[0148] On day -1 (before administration), the plasma samples of mice before administration were obtained (Submandibular Blood). On the first day, the sample for administration was diluted with PBS and subcutaneously injected once at a dose of administration of 3 mg/kg, and a concentration of 0.3 mg/ml (dissolved in PBS). Plasma was collected on the 7th, 14th, 21st, 28th, 35th, 42nd, 56th, and 70th day, respectively (Submandibular Blood).

[0149] The level of PCSK9 protein in plasma was detected by using a PCSK9 ELISA reagent (R&D systems). The relative proportion was calculated based on the PCSK9 protein level at baseline (before administration). The results were shown in FIG. 7.

[0150] As shown in Fig. 7, all the drug treatment groups reach the peak efficacy about 7-21 days after administration. On day 56 after administration, the knock-down efficiency of RRG002-51 is 73%, the knock-down efficiency of RRG002-54 is 87%, and the knock-down efficiency of RRG002-55 is 76%. On day 70 after administration, the knock-down efficiency of RRG002-51 is 66%, the knock-down efficiency of RRG002-54 is 83%, and the knock-down efficiency of RRG002-55 is 65%.

[0151] It can be seen that in RRG002-51 and RRG002-54, different GalNAc (LICA-1/DX₃, and LICA-2) ligands are conjugated at the 5' end of the sense strand; in RRG002-53 and RRG002-55, different GalNAc (L96, and LICA-3) ligands are conjugated at the 3' end of the sense strand, with the other modifications being basically the same. Therefore, the active products provided in the present invention modified by connecting different ligands at different positions achieve better efficacy.

[0152] Example 7. In vivo activity study of RRG002-55 and positive control RRG002-50-D in transgenic mice

[0153] To evaluate the in vivo activity of modified siRNA molecules (RRG002-55, RRG002-50-D), the in vivo activity of siRNA was measured in 50 6-to-8-week-old PCSK9 humanized mice (Shanghai Model Organisms Center, Inc., C57BL/6J-Pcsk9^{em2(hPCSK9)}Smoc, product number NM-HU-00075), half male and half female, and randomly divided into 5 groups

according to the pre-treatment PCSK9 level, namely, vehicle control group, RRG002-50-D control group (3 mg/kg), RRG002-55 low, medium and high dose groups (1, 3 and 10 mg/kg), 5 mice/(sex·group). Subcutaneous injection, the dosage volume is 10 mL/kg, single dose. The PCSK9 levels were measured on D-8 and D-3 (before administration) and on D8, D15, D22, D29, D36, D50, and D64 (after administration).

[0154] The PCSK9 protein level in plasma was measured using a PCSK9 ELISA reagent (R&D systems). The relative proportion was calculated based on the PCSK9 protein level at baseline (average of D-8 and D-3 before administration). The results were shown in FIG. 8.

[0155] At D64, the knock-down efficiency of plasma PCSK9 protein by 3 mg/kg RRG002-50-D, 1 mg/kg RRG002-55, 3 mg/kg RRG002-55, and 10 mg/kg RRG002-55 were 35.1%, 42.8%, 69.1%, and 92.4%, respectively. The PCSK9 level of animals in each dose group of RRG002-55 decreased in a dose-related manner. Compared with the 3 mg/kg RRG002-50-D (Inclisiran) control group, the PCSK9 level of animals in the same dose group of RRG002-55 decreased to a greater extent. The decrease in PCSK9 levels in the 3 mg/kg RRG002-50-D (Inclisiran) control group was similar to that in the 1 mg/kg RRG002-55 dose group.

[0156] Example 8. In vitro serum stability study of RRG002-55

[0157] To test the in vitro serum stability of RRG002-55, the test product RRG002-55 was incubated in serum of ICR mouse, SD rat, cynomolgus monkey and human at 1 µg/mL and 100 µg/mL concentrations for 0, 2, 4, 8 and 24 h at 37 (±1) °C. Each sample was repeated 3 times in parallel. The LC-MS/MS method was used to semi-quantitatively analyze the sense and antisense chains of RRG002-55.

[0158] After 24 h of co-incubation, when the incubation concentration was 1 µg/mL, the remaining percentages of the RRG002-55 sense strand in the serum of various species were 106.77% (mouse), 105.12% (rat), 95.03% (cynomolgus monkey), and 100.00% (human), and the remaining percentages of the RRG002-55 anti-sense strand in the serum of various species were 95.15% (mouse), 93.10% (rat), 98.96% (cynomolgus monkey), and 101.37% (human); when the incubation concentration was 100 µg/mL, the remaining percentages of the RRG002-55 sense strand in the serum of various species were 104.31% (mouse), 100.44% (rat), 85.87% (cynomolgus monkey), and 92.05% (human), and the remaining percentages of the RRG002-55 anti-sense strand in the serum of various species were 101.24% (mouse), 86.53% (rat), 83.74% (cynomolgus monkey), and 89.22% (human).

[0159] According to Leqvio Assessment Report (EMA/696912/2020), after the positive molecule RRG002-50-D was co-incubated with serum of mouse, rat, cynomolgus monkey, and human at 37°C for 24 hours, the remaining percentages of the sense strand in the serum

of each species were 95% (mouse), 91% (rat), 91% (cynomolgus monkey), and 87% (human), and the remaining percentages of the anti-sense strand in the serum of each species were 85% (mouse), 82% (rat), 72% (cynomolgus monkey), and 80% (human).

5 [0160] This shows that the stability of RRG002-55 in the serum of various species is better than that of RRG002-50-D.

[0161] Example 9. In vitro liver S9 metabolic stability study of RRG002-55

[0162] To test the in vitro liver S9 metabolic stability of RRG002-55, the test product RRG002-55 was co-incubated with liver S9 of four species, namely mice, rats, cynomolgus monkeys and humans, at a concentration of 1 μ M. A blank control group, a negative control group (the test product was co-incubated with inactivated mixed-species liver S9), a positive control group (Testosterone was co-incubated with liver S9 of different species), and test groups were set up. The test products were analyzed using LC-MS/MS, and the metabolic stability was evaluated by calculating the residual percentage by measuring the peak area ratio of the analyte to the internal standard.

15 [0163] After the test product RRG002-55 was co-incubated with liver S9 for 24 hours, the remaining percentages of the RRG002-55 sense strand in the liver S9 of different species were 93.65% (mouse), 80.86% (rat), 82.23% (cynomolgus monkey), and 94.52% (human); the remaining percentages of the RRG002-55 anti-sense strand in the liver S9 of different species were 81.29% (mouse), 74.48% (rat), 86.13% (cynomolgus monkey), and 96.33% (human).

[0164] According to Leqvio Assessment Report (EMA/696912/2020), after the positive molecule RRG002-50-D was co-incubated with human liver S9 at 37°C for 24 hours, the remaining percentage of the sense strand was 71%, and the remaining percentage of the anti-sense strand was 41%.

25 [0165] This shows that the stability of RRG002-55 in human liver S9 is better than that of RRG002-50-D.

[0166] Example 10. Study on drug distribution in liver for rats after single RRG002-55 administration

30 [0167] To test the in vivo drug distribution of RRG002-55, 18 male and 18 female SD rats were randomly divided into 6 groups (6 rats in each group, half male and half female). Each group was subcutaneously injected with 5 mg/kg RRG002-55, and the brain, skin (abdomen), fat, muscle, testis (uterus), prostate (ovary), lymph node, heart, lung, thyroid, pancreas, spleen, stomach, duodenum, adrenal gland, kidney, liver and skin (injection site) were collected at 1 h, 4 h, 24 h, 72 h, D11 (240 h) and D29 (672 h) after administration. The

concentration of RRG002-55 anti-sense strand in SD rat tissues was measured by LC-MS/MS method (the lower limit of quantification was 50.00 ng/g).

5 [0168] After subcutaneous administration, RRG002-55 was mainly distributed in the liver and kidney tissues. The concentration of RRG002-55 anti-sense strand in the liver was the highest, reaching $C_{\max}$ 4 hours after administration. The average concentration of RRG002-55 anti-sense strand in the liver was 2043 ng/g 240 hours after administration (D11). RRG002-55 anti-sense strand in the liver was still detectable 672 hours after administration(D29), with an average concentration of 85.0 ng/g and a half-life of 87.5 hours.

10 [0169] According to the Leqvio Assessment Report (EMA/696912/2020), the last time point that the positive molecule RRG002-50-D can be detected in the liver after a single dose (1, 5 or 25 mg/kg) in rats is 336 h (about 14 days).

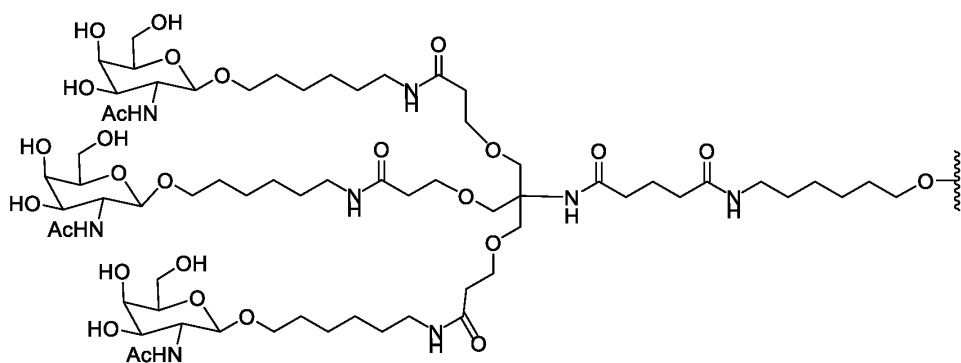
[0170] This shows that the stability of RRG002-55 in rat liver is better than that of RRG002-50-D. The skilled in the art would appreciate that the main tissue where this type of drug acts is liver, and the drug exposure time in liver is positively correlated with the duration of drug efficacy.

15 [0171] It is to be noted that in different batches of tests, due to different batches of animals, different age and state, the effects are slightly different, but the final overall results are consistent.

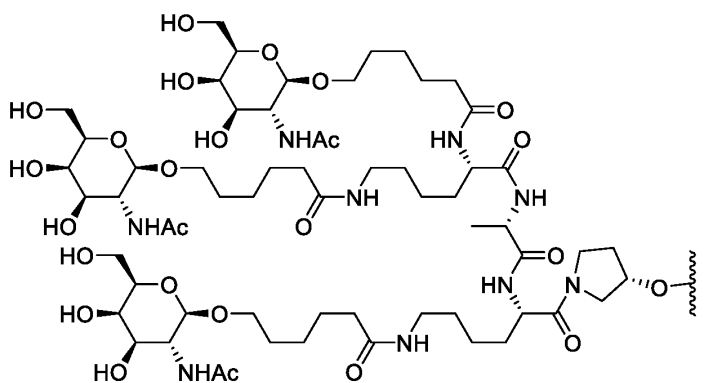
[0172] The above embodiments are only several implementations of the present invention, and are described in detail, without limitation to the scope of the present invention. It is to be understood that for a person of ordinary skill in the art, several variations, combinations, and improvements can be made to the above embodiments by those of ordinary skill in the art without departing from the idea of the present invention, which are all contemplated in the protection scope of the present invention. Therefore, the protection scope of the present invention shall be defined by the claims.

WHAT IS CLAIMED IS:

1. An siRNA molecule for inhibiting PCSK9 expression, comprising a sense strand and an anti-sense strand that are complementary to form a double-stranded region, wherein
5 the sense strand has a sequence of 5'-
mAmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGsmUsB -3', and the anti-sense
strand has a sequence of 5'-
mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3';
or the sense strand has a sequence of 5'-
10 BsmAsmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGmU-3', and the anti-sense
strand has a sequence of 5'-
mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3',
wherein A, U, G and C each represent a nucleotide with adenine, uridine, guanine and
cytosine as a base, respectively; m means that the nucleotide adjacent to the right side of the
15 letter m is a nucleotide modified with 2'-O-methyl; f means that the nucleotide adjacent to the
right side of the letter f is a nucleotide modified with 2'-fluoro; s means that the nucleotides
on the left and right sides of the letter s are linked via a phosphorothioate linkage; and B
represents an abasic nucleotide.
2. The siRNA molecule for inhibiting PCSK9 expression according to claim 1,
20 further comprising a ligand group for modifying the siRNA molecule with a ligand, wherein
the modification with the ligand is a modification of the 3' or 5' end of the sense strand with
the ligand group, and
the ligand group comprises at least one of N-acetylgalactosamine and a N-
acetylgalactosamine derivative.
- 25 3. The siRNA molecule for inhibiting PCSK9 expression according to claim 2, wherein
the ligand group comprises LICA-1, LICA-2, LICA-3, or L96, wherein
LICA-1 has a structural formula of

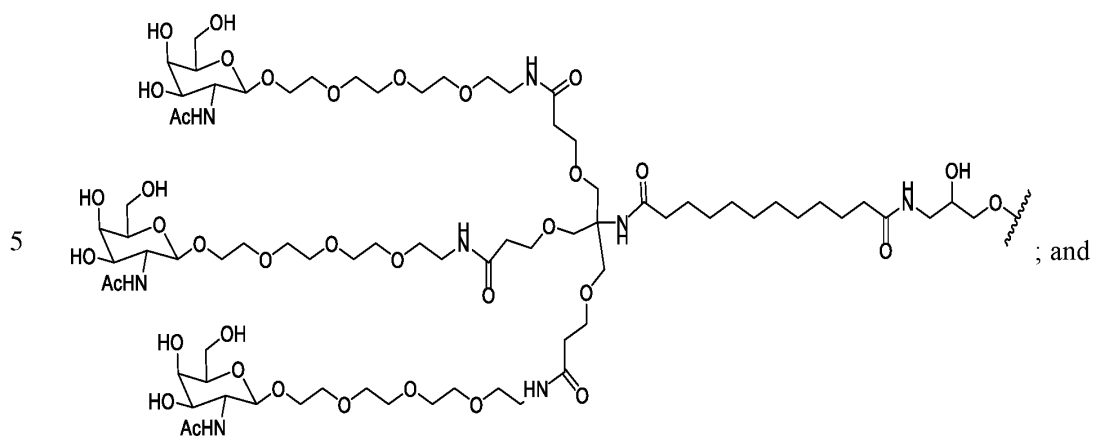
LICA-1(DX₃)

LICA-2 has a structural formula of



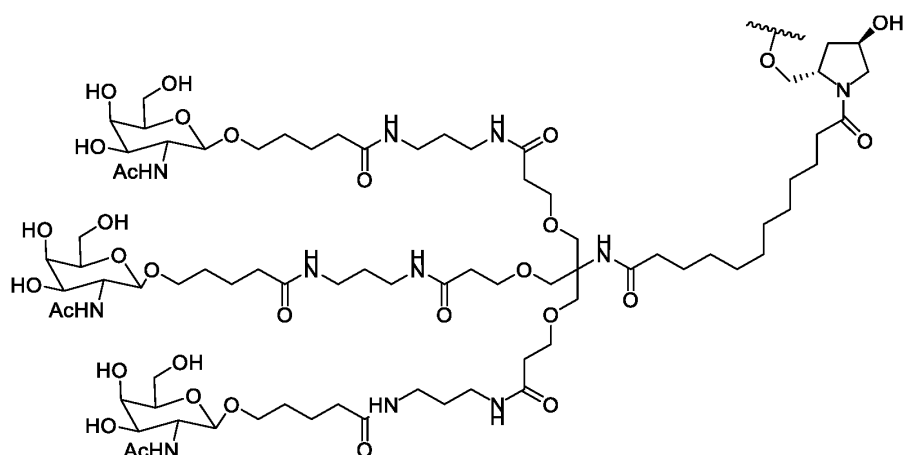
LICA-2

LICA-3 has a structural formula of



LICA-3

L96 has a structural formula of



L96

4. The siRNA molecule for inhibiting PCSK9 expression according to claim 2 or 3, wherein the sense strand is linked with the ligand group via a phosphorothioate linkage.
5. The siRNA molecule for inhibiting PCSK9 expression according to claim 4, having a structure of any one of (1)-(4):
 - (1) a sense strand having a sequence of 5'-LICA-1-smAmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmGsmUsB-3', and an anti-sense strand having a sequence of 5'-mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3';
 - (2) a sense strand having a sequence of 5'-LICA-2-smAmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGsmUsB-3', and an anti-sense strand having a sequence of 5'-mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3';
 - (3) a sense strand having a sequence of 5'-BsmAsmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGmUs-LICA-3-3', and an anti-sense strand having a sequence of 5'-mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3'; and
 - (4) a sense strand having a sequence of 5'-BsmAsmGmAmCmCmUfGmUfUfUfUmGmCmUmUmUmUmGmUs-L96-3', and an anti-sense strand having a sequence of 5'-mAsfCsmAmAmAfAmGmCmAmAmAmAmCfAmGfGmUmCmUsmAsmG-3'.
6. A pharmaceutical composition, comprising the siRNA molecule according to any one of claims 1 to 5 and a pharmaceutically acceptable carrier.

7. Use of the siRNA molecule according to any one of claims 1 to 5 or the pharmaceutical composition according to claim 6 in the manufacture of products for inhibiting PCSK9 protein expression.
8. Use of the siRNA molecule according to any one of claims 1 to 5 or the pharmaceutical composition according to claim 6 in the manufacture of products for reducing the concentration of low-density lipoprotein or low-density lipoprotein cholesterol in serum.
9. Use of the siRNA molecule according to any one of claims 1 to 5 or the pharmaceutical composition according to claim 6 in the manufacture of products for relieving symptoms of PCSK9 gene-mediated diseases.
10. The use according to claim 9, wherein the PCSK9 gene-mediated diseases comprises cardiovascular diseases, dyslipidemia, and neoplastic diseases, wherein the cardiovascular diseases comprise hypercholesterolemia and hyperlipidemia; the dyslipidemia comprises lipid metabolism disorders; and the neoplastic diseases comprise PCSK9 associated melanoma and metastatic liver cancer.