Modified RNAi Agents

RELATED APPLICATION

This application claims priority to U.S. Provisional Application No. 61/561,710, filed on November 18, 2011, the entire contents of which are hereby incorporated herein by reference.

FIELD OF THE INVENTION

The invention relates to RNAi duplex agents having particular motifs that are advantageous for inhibition of target gene expression, as well as RNAi compositions suitable for therapeutic use. Additionally, the invention provides methods of inhibiting the expression of a target gene by administering these RNAi duplex agents, *e.g.*, for the treatment of various diseases.

BACKGROUND

RNA interference or "RNAi" is a term initially coined by Fire and co-workers to describe the observation that double-stranded RNAi (dsRNA) can block gene expression (Fire *et al.* (1998) *Nature* **391**, 806-811; Elbashir *et al.* (2001) *Genes Dev.* **15**, 188-200). Short dsRNA directs gene-specific, post-transcriptional silencing in many organisms, including vertebrates, and has provided a new tool for studying gene function. RNAi is mediated by RNA-induced silencing complex (RISC), a sequence-specific, multi-component nuclease that destroys messenger RNAs homologous to the silencing trigger. RISC is known to contain short RNAs (approximately 22 nucleotides) derived from the double-stranded RNA trigger, but the protein components of this activity remained unknown.

Double-stranded RNA (dsRNA) molecules with good gene-silencing properties are needed for drug development based on RNA interference (RNAi). An initial step in RNAi is the activation of the RNA induced silencing complex (RISC), which requires degradation of the sense strand of the dsRNA duplex. Sense strand was known to act as the first RISC substrate that is cleaved by Argonaute 2 in the middle of the duplex region. Immediately after the cleaved 5'-end and 3'-end fragments of the sense strand are

removed from the endonuclease Ago2, the RISC becomes activated by the antisense strand (Rand *et al.* (2005) *Cell* 123, 621).

It was believed that when the cleavage of the sense strand is inhibited, the endonucleolytic cleavage of target mRNA is impaired (Leuschner et al. (2006) *EMBO Rep.*, 7, 314; Rand *et al.* (2005) *Cell* 123, 621; Schwarz *et al.* (2004) *Curr. Biol.*14, 787). Leuschner et al. showed that incorporation of a 2'-O-Me ribose to the Ago2 cleavage site in the sense strand inhibits RNAi in HeLa cells (Leuschner et al. (2006) *EMBO Rep.*, 7, 314). A similar effect was observed with phosphorothioate modifications, showing that cleavage of the sense strand was required for efficient RNAi also in mammals.

Morrissey et al. used a siRNA duplex containing 2'-F modified residues, among other sites and modifications, also at the Ago2 cleavage site, and obtained compatible silencing compared to the unmodified siRNAs (Morrissey *et al.* (2005) *Hepatology* 41, 1349). However, Morrissey's modification is not motif specific, e.g., one modification includes 2'-F modifications on all pyrimidines on both sense and antisense strands as long as pyrimidine residue is present, without any selectivity; and hence it is uncertain, based on these teachings, if specific motif modification at the cleavage site of sense strand can have any actual effect on gene silencing acitivity.

Muhonen et al. used a siRNA duplex containing two 2'-F modified residues at the Ago2 cleavage site on the sense or antisense strand and found it was tolerated (Muhonen *et al.* (2007) *Chemistry & Biodiversity* 4, 858-873). However, Muhonen's modification is also sequence specific, e.g., for each particular strand, Muhonen only modifies either all pyrimidines or all purines, without any selectivity.

Choung et al. used a siRNA duplex containing alternative modifications by 2'-OMe or various combinations of 2'-F, 2'-OMe and phosphorothioate modifications to stabilize siRNA in serum to Sur10058 (Choung *et al.* (2006) *Biochemical and Biophysical Research Communications* 342, 919–927). Choung suggested that the residues at the cleavage site of the antisense strand should not be modified with 2'-OMe in order to increase the stability of the siRNA.

There is thus an ongoing need for iRNA duplex agents to improve the gene silencing efficacy of siRNA gene therapeutics. This invention is directed to that need.

SUMMARY

This invention provides effective nucleotide or chemical motifs for dsRNA agents optionally conjugated to at least one ligand, which are advantageous for inhibition of target gene expression, as well as RNAi compositions suitable for therapeutic use.

The inventors surprisingly discovered that introducing one or more motifs of three identical modifications on three consecutive nucleotides at or near the cleavage site of a dsRNA agent that is comprised of modified sense and antisense strands enhances the gene silencing acitivity of the dsRNA agent.

In one aspect, the invention relates to a double-stranded RNAi (dsRNA) agent capable of inhibiting the expression of a target gene. The dsRNA agent comprises a sense strand and an antisense strand, each strand having 14 to 30 nucleotides. The dsRNA duplex is represented by formula (III):

sense: 5' n_p -N_a -(X X X) $_i$-N_b -Y Y Y -N_b -(Z Z Z)$_j$ -N_a - n_q 3'

antisense: 3' n_p'-N_a'-(X'X'X')$_k$-N_b'-Y'Y'Y'-N_b'-(Z'Z'Z')$_l$-N_a'- n_q' 5'

(III),

In formula (III), i, j, k, and l are each independently 0 or 1; p and q are each independently 0-6; n represents a nucleotide; each N_a and N_a' independently represents an oligonucleotide sequence comprising 0-25 nucleotides which are either modified or unmodified or combinations thereof, each sequence comprising at least two differently modified nucleotides; each N_b and N_b' independently represents an oligonucleotide sequence comprising 0-10 nucleotides which are either modified or unmodified or combinations thereof; each n_p and n_q independently represents an overhang nucleotide sequence comprising 0-6 nucleotides; and XXX, YYY, ZZZ, X'X'X', Y'Y'Y', and Z'Z'Z' each independently represent one motif of three identical modifications on three consecutive nucleotides; wherein the modifications on Nb is different than the modification on Y and the modifications on Nb' is different than the modification on Y'. At least one of the Y nucleotides forms a base pair with its complementary Y' nucleotides, and wherein the modification on the Y nucleotide is different than the modification on the Y' nucleotide.

Each n_p and n_q independently represents an overhang nucleotide sequence comprising 0-6 nucleotides; each n and n' represents an overhang nucleotide; and p and q are each independently 0-6.

In another aspect, the invention relates to a dsRNA agent capable of inhibiting the expression of a target gene. The dsRNA agent comprises a sense strand and an antisense strand, each strand having 14 to 30 nucleotides. The sense strand contains at least two motifs of three identical modifications on three consecutive nucleotides, where at least one of the motifs occurs at or near the cleavage site within the strand and at least one of the motifs occurs at another portion of the strand that is separated from the motif at the cleavage site by at least one nucleotide. The antisense strand contains at least one motif of three identical modifications on three consecutive nucleotides, where at least one of the motifs occurs at or near the cleavage site within the strand and at least one of the motifs occurs at another portion of the strand that is separated from the motif at or near cleavage site by at least one nucleotide. The modification in the motif occurring at or near the cleavage site in the sense strand is different than the modification in the motif occurring at or near the cleavage site in the antisense strand.

In another aspect, the invention relates to a dsRNA agent capable of inhibiting the expression of a target gene. The dsRNA agent comprises a sense strand and an antisense strand, each strand having 14 to 30 nucleotides. The sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides, where at least one of the motifs occurs at or near the cleavage site in the strand. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at or near the cleavage site.

In another aspect, the invention relates to a dsRNA agent capable of inhibiting the expression of a target gene. The dsRNA agent comprises a sense strand and an antisense strand, each strand having 14 to 30 nucleotides. The sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 9,10,11 from the 5'end. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11,12,13 from the 5'end.

In another aspect, the invention further provides a method for delivering the dsRNA to a specific target in a subject by subcutaneous or intravenenuous administration.

DETAILED DESCRIPTION

A superior result may be obtained by introducing one or more motifs of three identical modifications on three consecutive nucleotides into a sense strand and/or antisense strand of a dsRNA agent, particularly at or near the cleavage site. The sense strand and antisense strand of the dsRNA agent may otherwise be completely modified. The introduction of these motifs interrupts the modification pattern, if present, of the sense and/or antisense strand. The dsRNA agent optionally conjugates with a GalNAc derivative ligand, for instance on the sense strand. The resulting dsRNA agents present superior gene silencing activity.

The inventors surprisingly discovered that having one or more motifs of three identical modifications on three consecutive nucleotides at or near the cleavage site of at least one strand of a dsRNA agent superiorly enhanced the gene silencing acitivity of the dsRNA agent.

Accordingly, the invention provides a double-stranded RNAi (dsRNA) agent capable of inhibiting the expression of a target gene. The dsRNA agent comprises a sense strand and an antisense strand. Each strand of the dsRNA agent can range from 12-30 nucleotides in length. For example, each strand can be between 14-30 nucleotides in length, 17-30 nucleotides in length, 25-30 nucleotides in length, 27-30 nucleotides in length, 17-23 nucleotides in length, 17-21 nucleotides in length, 17-19 nucleotides in length, 19-25 nucleotides in length, 19-23 nucleotides in length, 19-21 nucleotides in length, 21-25 nucleotides in length, or 21-23 nucleotides in length.

The sense strand and antisense strand typically form a duplex dsRNA. The duplex region of a dsRNA agent may be 12-30 nucleotide pairs in length. For example, the duplex region can be between 14-30 nucleotide pairs in length, 17-30 nucleotide pairs in length, 25-30 nucleotides in length, 27-30 nucleotide pairs in length, 17 - 23 nucleotide pairs in length, 17-21 nucleotide pairs in length, 17-19 nucleotide pairs in length, 19-25 nucleotide pairs in length, 19-23 nucleotide pairs in length, 19- 21 nucleotide pairs in

length, 21-25 nucleotide pairs in length, or 21-23 nucleotide pairs in length. In another example, the duplex region is selected from 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, and 27.

In one embodiment, the dsRNA agent of the invention comprises may contain one or more overhang regions and/or capping groups of dsRNA agent at the 3'-end, or 5'-end or both ends of a strand. The overhang can be 1-6 nucleotides in length, for instance 2-6 nucleotides in length, 1-5 nucleotides in length, 2-5 nucleotides in length, 1-4 nucleotides in length, 2-4 nucleotides in length, 1-3 nucleotides in length, 2-3 nucleotides in length, or 1-2 nucleotides in length. The overhangs can be the result of one strand being longer than the other, or the result of two strands of the same length being staggered. The overhang can form a mismatch with the target mRNA or it can be complementary to the gene sequences being targeted or can be other sequence. The first and second strands can also be joined, e.g., by additional bases to form a hairpin, or by other non-base linkers.

In one embodiment, the nucleotides in the overhang region of the dsRNA agent of the invention can each independently be a modified or unmodified nucleotide including, but no limited to 2'-sugar modified, such as, 2-F 2'-Omethyl, thymidine (T), 2'-O-methoxyethyl-5-methyluridine (Teo), 2'-O-methoxyethyladenosine (Aeo), 2'-O-methoxyethyl-5-methylcytidine (m5Ceo), and any combinations thereof. For example, TT can be an overhang sequence for either end on either strand. The overhang can form a mismatch with the target mRNA or it can be complementary to the gene sequences being targeted or can be other sequence.

The 5'- or 3'- overhangs at the sense strand, antisense strand or both strands of the dsRNA agent of the invention may be phosphorylated. In some embodiments, the overhang region contains two nucleotides having a phosphorothioate between the two nucleotides, where the two nucleotides can be the same or different. In one embodiment, the overhang is present at the 3'-end of the sense strand, antisense strand or both strands. In one embodiment, this 3'-overhang is present in the antisense strand. In one embodiment, this 3'-overhang is present in the sense strand.

The dsRNA agent of the invention comprises only single overhang, which can strengthen the interference activity of the dsRNA, without affecting its overall stability. For example, the single-stranded overhang is located at the 3'-terminal end of the sense

strand or, alternatively, at the 3'-terminal end of the antisense strand. The dsRNA may also have a blunt end, located at the 5'-end of the antisense strand (or the 3'-end of the sense strand) or vice versa. Generally, the antisense strand of the dsRNA has a nucleotide overhang at the 3'-end, and the 5'-end is blunt. While not bound by theory, the asymmetric blunt end at the 5'-end of the antisense strand and 3'-end overhang of the antisense strand favor the guide strand loading into RISC process.

In one embodiment, the dsRNA agent of the invention may also have two blunt ends, at both ends of the dsRNA duplex.

In one embodiment, the dsRNA agent of the invention is a double ended bluntmer of 19 nt in length, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 7,8,9 from the 5'end. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11,12,13 from the 5'end.

In one embodiment, the dsRNA agent of the invention is a double ended bluntmer of 20 nt in length, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 8,9,10 from the 5'end. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11,12,13 from the 5'end.

In one embodiment, the dsRNA agent of the invention is a double ended bluntmer of 21 nt in length, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 9,10,11 from the 5'end. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11,12,13 from the 5'end.

In one embodiment, the dsRNA agent of the invention comprises a 21 nucleotides (nt) sense strand and a 23 nucleotides (nt) antisense, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 9,10,11 from the 5'end; the antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11,12,13 from the 5'end, wherein one end of the dsRNA is blunt, while the other end is comprises a 2 nt overhang. Preferably, the 2 nt overhang is at the 3'-end of the antisense. Optionally, the dsRNA further comprises a ligand (preferably $GalNAc_3$).

In one embodiment, the dsRNA agent of the invention comprising a sense and antisense strands, wherein: the sense strand is 25-30 nucleotide residues in length, wherein starting from the 5' terminal nucleotide (position 1) positions 1 to 23 of said first strand comprise at least 8 ribonucleotides; antisense strand is 36-66 nucleotide residues in length and, starting from the 3' terminal nucleotide, comprises at least 8 ribonucleotides in the positions paired with positions 1- 23 of sense strand to form a duplex; wherein at least the 3 ' terminal nucleotide of antisense strand is unpaired with sense strand, and up to 6 consecutive 3' terminal nucleotides are unpaired with sense strand, thereby forming a 3' single stranded overhang of 1-6 nucleotides; wherein the 5' terminus of antisense strand comprises from 10-30 consecutive nucleotides which are unpaired with sense strand, thereby forming a 10-30 nucleotide single stranded 5' overhang; wherein at least the sense strand 5' terminal and 3' terminal nucleotides are base paired with nucleotides of antisense strand when sense and antisense strands are aligned for maximum complementarity, thereby forming a substantially duplexed region between sense and antisense strands; and antisense strand is sufficiently complementary to a target RNA along at least 19 ribonucleotides of antisense strand length to reduce target gene expression when said double stranded nucleic acid is introduced into a mammalian cell; and wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides, where at least one of the motifs occurs at or near the cleavage site. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at or near the cleavage site.

In one embodiment, the dsRNA agent of the invention comprising a sense and antisense strands, wherein said dsRNA agent comprises a first strand having a length which is at least 25 and at most 29 nucleotides and a second strand having a length which is at most 30 nucleotides with at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at position 11,12,13 from the 5' end; wherein said 3' end of said first strand and said 5' end of said second strand form a blunt end and said second strand is 1-4 nucleotides longer at its 3' end than the first strand, wherein the duplex region region which is at least 25 nucleotides in length, and said second strand is sufficiently complemenatary to a target mRNA along at least 19 nt of said second strand length to reduce target gene expression when said dsRNA agent is

introduced into a mammalian cell, and wherein dicer cleavage of said dsRNA preferentially results in an siRNA comprising said 3' end of said second strand, thereby reducing expression of the target gene in the mammal. Optionally, the dsRNA agent further comprises a ligand.

In one embodiment, the sense strand of the dsRNA agent contains at least one motif of three identical modifications on three consecutive nucleotides, where one of the motifs occurs at the cleavage site in the sense strand.

In one embodiment, the antisense strand of the dsRNA agent can also contain at least one motif of three identical modifications on three consecutive nucleotides, where one of the motifs occurs at or near the cleavage site in the antisense strand.

For dsRNA agent having a duplex region of 17-23 nt in length, the cleavage site of the antisense strand is typically around the 10, 11 and 12 positions from the 5'-end. Thus the motifs of three identical modifications may occur at the 9, 10, 11 positions; 10, 11, 12 positions; 11, 12, 13 positions; 12, 13, 14 positions; or 13, 14, 15 positions of the antisense strand, the count starting from the 1st nucleotide from the 5'-end of the antisense strand, or, the count starting from the 1st paired nucleotide within the duplex region from the 5'- end of the antisense strand. The cleavage site in the antisense strand may also change according to the length of the duplex region of the dsRNA from the 5'-end.

The sense strand of the dsRNA agent comprises at least one motif of three identical modifications on three consecutive nucleotides at the cleavage site of the strand; and the antisense strand may have at least one motif of three identical modifications on three consecutive nucleotides at or near the cleavage site of the strand. When the sense strand and the antisense strand form a dsRNA duplex, the sense strand and the antisense strand can be so aligned that one motif of the three nucleotides on the sense strand and one motif of the three nucleotides on the antisense strand have at least one nucleotide overlap, i.e., at least one of the three nucleotides of the motif in the sense strand forms a base pair with at least one of the three nucleotides of the motif in the antisense strand. Alternatively, at least two nucleotides of the motifs from both strands may overlap, or all three nucleotides may overlap.

In one embodiment, the sense strand of the dsRNA agent comprises more than one motif of three identical modifications on three consecutive nucleotides. The first motif should occur at or near the cleavage site of the strand and the other motifs may be a wing modifications. The term "wing modification" herein refers to a motif occurring at another portion of the strand that is separated from the motif at or near the cleavage site of the same strand. The wing modification is either adajacent to the first motif or is separated by at least one or more nucleotides. When the motifs are immediately adjacent to each other the chemistry of the motifs are distinct from each other and when the motifs are separated by one or more nucleotide the chemistries of the motifs can be the same or different. Two or more wing modifications may be present. For instance, when two wing modifications are present, the wing modifications may both occur at one end of the duplex region relative to the first motif which is at or near the cleavage site or each of the wing modifications may occur on either side of the first motif.

Like the sense strand, the antisense strand of the dsRNA agent comprises at least two motifs of three identical modifications on three consecutive nucleotides, with at least one of the motifs occurring at or near the cleavage site of the strand. This antisense strand may also contain one or more wing modifications in an alignment similar to the wing modifications that is present on the sense strand.

In one embodiment, the wing modification on the sense strand, antisense strand, or both strands of the dsRNA agent typically does not include the first one or two terminal nucleotides at the 3'-end, 5'-end or both ends of the strand.

In another embodiment, the wing modification on the sense strand, antisense strand, or both strands of the dsRNA agent typically does not include the first one or two paired nucleotides within the duplex region at the 3'-end, 5'-end or both ends of the strand.

When the sense strand and the antisense strand of the dsRNA agent each contain at least one wing modification, the wing modifications may fall on the same end of the duplex region, and have an overlap of one, two or three nucleotides.

When the sense strand and the antisense strand of the dsRNA agent each contain at least two wing modifications, the sense strand and the antisense strand can be aligned so that two wing modifications each from one strand fall on one end of the duplex region,

having an overlap of one, two or three nucleotides; two modifications each from one strand fall on the other end of the duplex region, having an overlap of one, two or three nucleotides.

In one embodiment, every nucleotide in the sense strand and antisense strand of the dsRNA agent, including the nucleotides that are part of the motifs, may be modified. Each nucleotide may be modified with the same or different modification which can include one or more alteration of one or both of the non-linking phosphate oxygens and/or of one or more of the linking phosphate oxygens; alteration of a constituent of the ribose sugar, *e.g.*, of the 2′ hydroxyl on the ribose sugar; wholesale replacement of the phosphate moiety with "dephospho" linkers; modification or replacement of a naturally occurring base; and replacement or modification of the ribose-phosphate backbone.

As nucleic acids are polymers of subunits, many of the modifications occur at a position which is repeated within a nucleic acid, *e.g.*, a modification of a base, or a phosphate moiety, or a non-linking O of a phosphate moiety. In some cases the modification will occur at all of the subject positions in the nucleic acid but in many cases it will not. By way of example, a modification may only occur at a 3' or 5' terminal position, may only occur in a terminal region, *e.g.,* at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand. A modification may occur in a double strand region, a single strand region, or in both. A modification may occur only in the double strand region of a RNA or may only occur in a single strand region of a RNA. *E.g.*, a phosphorothioate modification at a non-linking O position may only occur at one or both termini, may only occur in a terminal region, *e.g.*, at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand, or may occur in double strand and single strand regions, particularly at termini. The 5' end or ends can be phosphorylated.

It may be possible, *e.g.*, to enhance stability, to include particular bases in overhangs, or to include modified nucleotides or nucleotide surrogates, in single strand overhangs, *e.g.*, in a 5' or 3' overhang, or in both. *E.g.*, it can be desirable to include purine nucleotides in overhangs. In some embodiments all or some of the bases in a 3' or 5' overhang may be modified, *e.g.*, with a modification described herein. Modifications can include, *e.g.*, the use of modifications at the 2' position of the ribose sugar with

modifications that are known in the art, *e.g.*, the use of deoxyribonucleotides, 2'-deoxy-2'-fluoro (2'-F) or 2'-O-methyl modified instead of the ribosugar of the nucleobase, and modifications in the phosphate group, *e.g.*, phosphorothioate modifications. Overhangs need not be homologous with the target sequence.

In one embodiment, each residue of the sense strand and antisense strand is independently modified with LNA, HNA, CeNA, 2'-methoxyethyl, 2'- O-methyl, 2'-O-allyl, 2'-C- allyl, 2'-deoxy, or 2'-fluoro. The strands can contain more than one modification. In one embodiment, each residue of the sense strand and antisense strand is independently modified with 2'- O-methyl or 2'-fluoro.

At least two different modifications are typically present on the sense strand and antisense strand. Those two modifications may be the 2'- O-methyl or 2'-fluoro modifications, or others.

In one embodiment, the sense strand and antisense strand each contains two differently modified nucleotides selected from 2'-O-methyl or 2'-fluoro.

In one embodiment, each residue of the sense strand and antisense strand is independently modified with 2'-O-methyl nucleotide, 2′-deoxyfluoro nucleotide, 2'-O-N-methylacetamido (2'-O-NMA) nucleotide, a 2'-O-dimethylaminoethoxyethyl (2'-O-DMAEOE) nucleotide, 2'-O-aminopropyl (2'-O-AP) nucleotide, or 2'-ara-F nucleotide.

In one embodiment, the N_a and/or N_b comprise modifications of an alternating pattern. The term "alternating motif" or "alternative pattern" as used herein refers to a motif having one or more modifications, each modification occurring on alternating nucleotides of one strand. The alternating nucleotide may refer to one per every other nucleotide or one per every three nucleotides, or a similar pattern. For example, if A, B and C each represent one type of modification to the nucleotide, the alternating motif can be "ABABABABABAB…," "AABBAABBAABB…," "AABAABAABAAB…," "AAABAAABAAAB…," "AAABBBAAABBB…," or "ABCABCABCABC…," etc.

In one embodiment, the N_a' and/or N_b' comprise modifications of an alternating pattern. The term "alternating motif" or "alternative pattern" as used herein refers to a motif having one or more modifications, each modification occurring on alternating nucleotides of one strand. The alternating nucleotide may refer to one per every other nucleotide or one per every three nucleotides, or a similar pattern. For example, if A, B

and C each represent one type of modification to the nucleotide, the alternating motif can be "ABABABABABAB…," "AABBAABBAABB…," "AABAABAABAAB…," "AAABAAABAAAB…," "AAABBBAAABBB…," or "ABCABCABCABC…," etc.

The type of modifications contained in the alternating motif may be the same or different. For example, if A, B, C, D each represent one type of modification on the nucleotide, the alternating pattern, i.e., modifications on every other nucleotide, may be the same, but each of the sense strand or antisense strand can be selected from several possibilities of modifications within the alternating motif such as "ABABAB…", "ACACAC…" "BDBDBD…" or "CDCDCD…," etc.

In one embodiment, the dsRNA agent of the invnetion comprises the modification pattern for the alternating motif on the sense strand relative to the modification pattern for the alternating motif on the antisense strand is shifted.The shift may be such that the modified group of nucleotides of the sense strand corresponds to a differently modified group of nucleotides of the antisense strand and vice versa. For example, the sense strand when paired with the antisense strand in the dsRNA duplex, the alternating motif in the sense strand may start with "ABABAB" from 5'-3' of the strand and the alternating motif in the antisense strand may start with "BABABA" from 3'-5'of the strand within the duplex region. As another example, the alternating motif in the sense strand may start with "AABBAABB" from 5'-3' of the strand and the alternating motif in the antisenese strand may start with "BBAABBAA" from 3'-5'of the strand within the duplex region, so that there is a complete or partial shift of the modification patterns between the sense strand and the antisense strand.

In one embodiment, the dsRNA agent of the invention comprises the pattern of the alternating motif of 2'-O-methyl modification and 2'-F modification on the sense strand initially has a shift relative to the pattern of the alternating motif of 2'-O-methyl modification and 2'-F modification on the antisense strand initially, i.e., the 2'-O-methyl modified nucleotide on the sense strand base pairs with a 2'-F modified nucleotide on the antisense strand and vice versa. The 1 position of the sense strand may start with the 2'-F modification, and the 1 position of the antisense strand may start with the 2'- O-methyl modification.

The introduction of one or more motifs of three identical modifications on three consecutive nucleotides to the sense strand and/or antisense strand interrupts the initial modification pattern present in the sense strand and/or antisense strand. This interruption of the modification pattern of the sense and/or antisense strand by introducing one or more motifs of three identical modifications on three consecutive nucleotides to the sense and/or antisense strand surprisingly enhances the gene silencing acitivty to the target gene.

In one embodiment, when the motif of three identical modifications on three consecutive nucleotides is introduced to any of the strands, the modification of the nucleotide next to the motif is a different modification than the modification of the motif. For example, the portion of the sequence containing the motif is "...N_aYYYN_b...," where "Y" represents the modification of the motif of three identical modifications on three consecutive nucleotide, and "N_a" and "N_b" represent a modification to the nucleotide next to the motif "YYY" that is different than the modification of Y, and where N_a and N_b can be the same or different modifications. Altnernatively, N_a and/or N_b may be present or absent when there is a wing modification present.

The dsRNA agent of the invention may further comprise at least one phosphorothioate or methylphosphonate internucleotide linkage. The phosphorothioate or methylphosphonate internucleotide linkage modification may occur on any nucleotide of the sense strand or antisense strand or both in any position of the strand. For instance, the internucleotide linkage modification may occur on every nucleotide on the sense strand and/or antisense strand; each internucleotide linkage modification may occur in an alternating pattern on the sense strand or antisense strand; or the sense strand or antisense strand comprises both internucleotide linkage modifications in an alternating pattern. The alternating pattern of the internucleotide linkage modification on the sense strand may be the same or different from the antisense strand, and the alternating pattern of the internucleotide linkage modification on the sense strand may have a shift relative to the alternating pattern of the internucleotide linkage modification on the antisense strand.

In one embodiment, the dsRNA comprises the phosphorothioate or methylphosphonate internucleotide linkage modification in the overhang region. For example, the overhang region comprises two nucleotides having a phosphorothioate or

methylphosphonate internucleotide linkage between the two nucleotides. Internucleotide linkage modifications also may be made to link the overhang nucleotides with the terminal paired nucleotides within duplex region. For example, at least 2, 3, 4, or all the overhang nucleotides may be linked through phosphorothioate or methylphosphonate internucleotide linkage, and optionally, there may be additional phosphorothioate or methylphosphonate internucleotide linkages linking the overhang nucleotide with a paired nucleotide that is next to the overhang nucleotide. For instance, there may be at least two phosphorothioate internucleotide linkages between the terminal three nucleotides, in which two of the three nucleotides are overhang nucleotides, and the third is a paried nucleotide next to the overhang nucleotide. Preferably, these terminal three nucleotides may be at the 3'-end of the antisense strand.

In one embodiment the sense strand of the dsRNA comprises 1-10 blocks of two to ten phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said sense strand is paired with an antisense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphophonate or phosphate linkage.

In one embodiment the antisense strand of the dsRNA comprises two blocks of two phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphophonate or phosphate linkage.

In one embodiment the antisense strand of the dsRNA comprises two blocks of three phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at

any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphophonate or phosphate linkage.

In one embodiment the antisense strand of the dsRNA comprises two blocks of four phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphophonate or phosphate linkage.

In one embodiment the antisense strand of the dsRNA comprises two blocks of five phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphophonate or phosphate linkage.

In one embodiment the antisense strand of the dsRNA comprises two blocks of six phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphophonate or phosphate linkage.

In one embodiment the antisense strand of the dsRNA comprises two blocks of seven phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7 or 8 phosphate internucleotide linkages, wherein one of the phosphorothioate

or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphophonate or phosphate linkage.

In one embodiment the antisense strand of the dsRNA comprises two blocks of eight phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5 or 6 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphophonate or phosphate linkage.

In one embodiment the antisense strand of the dsRNA comprises two blocks of nine phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3 or 4 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphophonate or phosphate linkage.

In one embodiment, the dsRNA of the invention further comprises one or more phosphorothioate or methylphosphonate internucleotide linkage modification within 1-10 of the termini position(s) of the sense and/or antisense strand. For example, at least 2, 3, 4, 5, 6, 7, 8, 9 or 10 nucleotides may be linked through phosphorothioate or methylphosphonate internucleotide linkage at one end or both ends of the sense and/or antisense strand.

In one embodiment, the dsRNA of the invention further comprises one or more phosphorothioate or methylphosphonate internucleotide linkage modification within 1-10 of the internal region of the duplex of each of the sense and/or antisense strand. For example, at least 2, 3, 4, 5, 6, 7, 8, 9 or 10 nucleotides may be linked through

phosphorothioate methylphosphonate internucleotide linkage at position 8-16 of the duplex region counting from the 5'-end of the sense strand; the dsRNA can optionally further comprise one or more phosphorothioate or methylphosphonate internucleotide linkage modification within 1-10 of the termini position(s).

In one embodiment, the dsRNA of the invention further comprises one to five phosphorothioate or methylphosphonate internucleotide linkage modification(s) within position 1-5 and one to five phosphorothioate or methylphosphonate internucleotide linkage modification(s) within position 18-23 of the sense strand (counting from the 5'-end), and one to five phosphorothioate or methylphosphonate internucleotide linkage modification at positions 1 and 2 and one to five within positions 18-23 of the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises one phosphorothioate internucleotide linkage modification within position 1-5 and one phosphorothioate or methylphosphonate internucleotide linkage modification within position 18-23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and 2 and two phosphorothioate or methylphosphonate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and one phosphorothioate internucleotide linkage modification within position 18-23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and two phosphorothioate internucleotide linkage modifications within position 18-23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and two phosphorothioate internucleotide linkage modifications within position 18-23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and 2 and one phosphorothioate internucleotide linkage modification within positions 18-23 of the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises one phosphorothioate internucleotide linkage modification within position 1-5 and one phosphorothioate internucleotide linkage modification within position 18-23 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises one phosphorothioate internucleotide linkage modification within position 1-5 and one within position 18-23 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modification at positions 1 and 2 and one phosphorothioate internucleotide linkage modification within positions 18-23 of the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises one phosphorothioate internucleotide linkage modification within position 1-5 (counting from the 5'-end), and two phosphorothioateinternucleotide linkage modifications at positions 1 and 2 and one phosphorothioate internucleotide linkage modification within positions 18-23 of the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 (counting from the 5'-end), and one phosphorothioateinternucleotide linkage modification at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and one within position 18-23 of the sense strand (counting from the 5'-end), and two

phosphorothioate internucleotide linkage modifications at positions 1 and 2 and one phosphorothioate internucleotide linkage modification within positions 18-23 of the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and one phosphorothioate internucleotide linkage modification within position 18-23 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and one phosphorothioate internucleotide linkage modification within position 18-23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises two phosphorothioate internucleotide linkage modifications at position 1 and 2, and two phosphorothioate internucleotide linkage modifications at position 20 and 21 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and one at position 21 of the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises one phosphorothioate internucleotide linkage modification at position 1, and one phosphorothioate internucleotide linkage modification at position 21 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications at positions 20 and 21 the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises two phosphorothioate internucleotide linkage modifications at position 1 and 2, and two phosphorothioate internucleotide linkage modifications at position 21 and 22 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage

modification at positions 1 and one phosphorothioate internucleotide linkage modification at position 21 of the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises one phosphorothioate internucleotide linkage modification at position 1, and one phosphorothioate internucleotide linkage modification at position 21 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications at positions 21 and 22 the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises two phosphorothioate internucleotide linkage modifications at position 1 and 2, and two phosphorothioate internucleotide linkage modifications at position 22 and 23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and one phosphorothioate internucleotide linkage modification at position 21 of the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA of the invention further comprises one phosphorothioate internucleotide linkage modification at position 1, and one phosphorothioate internucleotide linkage modification at position 21 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications at positions 23 and 23 the antisense strand (counting from the 5'-end).

In one embodiment, the dsRNA agent of the invention comprises mismatch(es) with the target, within the duplex, or combinations thereof. The mistmatch can occur in the overhang region or the duplex region. The base pair can be ranked on the basis of their propensity to promote dissociation or melting (e.g., on the free energy of association or dissociation of a particular pairing, the simplest approach is to examine the pairs on an individual pair basis, though next neighbor or similar analysis can also be used). In terms of promoting dissociation: A:U is preferred over G:C; G:U is preferred over G:C; and I:C is preferred over G:C (I=inosine). Mismatches, e.g., non-canonical or other than canonical pairings (as described elsewhere herein) are preferred over canonical (A:T, A:U, G:C) pairings; and pairings which include a universal base are preferred over canonical pairings.

In one embodiment, the dsRNA agent of the invention comprises at least one of the first 1, 2, 3, 4, or 5 base pairs within the duplex regions from the 5'- end of the antisense strand can be chosen independently from the group of: A:U, G:U, I:C, and mismatched pairs, e.g., non-canonical or other than canonical pairings or pairings which include a universal base, to promote the dissociation of the antisense strand at the 5'-end of the duplex.

In one embodiment, the nucleotide at the 1 position within the duplex region from the 5'-end in the antisense strand is selected from the group consisting of A, dA, dU, U, and dT. Alternatively, at least one of the first 1, 2 or 3 base pair within the duplex region from the 5'- end of the antisense strand is an AU base pair. For example, the first base pair within the duplex region from the 5'- end of the antisense strand is an AU base pair.

In one embodiment, the sense strand sequence may be represented by formula (I):

$5'\ n_p\text{-}N_a\text{-}(X\ X\ X)_i\text{-}N_b\text{-}Y\ Y\ Y\text{-}N_b\text{-}(Z\ Z\ Z)_j\text{-}N_a\text{-}n_q\ 3'$ (I)

wherein:

i and j are each independently 0 or 1;

p and q are each independently 0-6;

each N_a independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

each n_p and n_q independently represent an overhang nucleotide;

wherein N_b and Y do not have the same modification; and

XXX, YYY and ZZZ each independently represent one motif of three identical modifications on three consecutive nucleotides. Preferably YYY is all 2'-F modified nucleotides.

In one embodiment, the N_a and/or N_b comprise modifications of alternating pattern.

In one embodiment, the YYY motif occurs at or near the cleavage site of the sense strand. For example, when the dsRNA agent has a duplex region of 17-23 nucleotide pairs in length, the YYY motif can occur at or the vicinity of the cleavage site

(e.g.: can occur at positions 6, 7, 8, 7, 8, 9, 8, 9, 10, 9, 10, 11, 10, 11,12 or 11, 12, 13) of - the sense strand, the count starting from the 1st nucleotide, from the 5'-end; or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end.

In one embodiment, i is 1 and j is 0, or i is 0 and j is 1, or both i and j are 1. The sense strand can therefore be represented by the following formulas:

5' n_p-N_a-YYY-N_b-ZZZ-N_a-n_q 3' (Ia);

5' n_p-N_a-XXX-N_b-YYY-N_a-n_q 3' (Ib); or

5' n_p-N_a-XXX-N_b-YYY-N_b-ZZZ-N_a-n_q 3' (Ic).

When the sense strand is represented by formula (Ia), N_b represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a independently can represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the sense strand is represented as formula (Ib), N_b represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a can independently represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the sense strand is represented as formula (Ic), each N_b independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Preferably, N_b is 0, 1, 2, 3, 4, 5 or 6 Each N_a can independently represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

Each of X, Y and Z may be the same or different from each other.

In one embodiment, the antisense strand sequence of the dsRNA may be represented by formula (II):

5' n_q'-N_a'-$(Z'Z'Z')_k$-N_b'-$Y'Y'Y'$-N_b'-$(X'X'X')_l$-N'_a-n_p' 3' (II)

wherein:

k and l are each independently 0 or 1;

p and q are each independently 0-6;

each N_a' independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b' independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

each n_p' and n_q' independently represent an overhang nucleotide comprising 0-6 nucleotides;

wherein N_b' and Y' do not have the same modification;

and

X′X′X′, Y′Y′Y′ and Z′Z′Z′ each independently represent one motif of three identical modifications on three consecutive nucleotides.

In one embodiment, the N_a' and/or N_b' comprise modifications of alternating pattern.

The Y′Y′Y′ motif occurs at or near the cleavage site of the antisense strand. For example, when the dsRNA agent has a duplex region of 17-23 nt in length, the Y′Y′Y′ motif can occur at positions 9, 10, 11;10, 11, 12; 11, 12, 13; 12, 13, 14 ; or 13, 14, 15 of the antisense strand, with the count starting from the 1st nucleotide, from the 5'-end; or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end. Preferably, the Y′Y′Y′ motif occurs at positions 11, 12, 13.

In one embodiment, Y′Y′Y′ motif is all 2'-OMe modified nucleotides.

In one embodiment, k is 1 and l is 0, or k is 0 and l is 1, or both k and l are 1.

The antisense strand can therefore be represented by the following formulas:

5' n_q'-N_a'-Z′Z′Z′-N_b'-Y′Y′Y′-N_a'-n_p' 3' (IIa);

5' n_q'-N_a'-Y′Y′Y′-N_b'-X′X′X′-n_p' 3' (IIb); or

5' n_q'-N_a'- Z′Z′Z′-N_b'-Y′Y′Y′-N_b'- X′X′X′-N_a'-n_p' 3' (IIc).

When the antisense strand is represented by formula (IIa), N_b' represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the antisense strand is represented as formula (IIb), N_b' represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the antisense strand is represented as formula (IIc), each N_b' independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides. Preferably, N_b is 0, 1, 2, 3, 4, 5 or 6.

Each of X′, Y′ and Z′ may be the same or different from each other.

Each nucleotide of the sense strand and antisense strand may be independently modified with LNA, HNA, CeNA, 2'-methoxyethyl, 2'-O-methyl, 2'-O-allyl, 2'-C- allyl, or 2'-fluoro. For example, each nucleotide of the sense strand and antisense strand is independently modified with 2'-O-methyl or 2'-fluoro. Each X, Y, Z, X′, Y′ and Z′, in particular, may represent a 2'-O-methyl modification or a 2'-fluoro modification.

In one embodiment, the sense strand of the dsRNA agent comprisesYYY motif occurring at 9, 10 and 11 positions of the strand when the duplex region is 21 nt, the count starting from the 1st nucleotide from the 5'-end, or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end; and Y represents 2'-F modification. The sense strand may additionally contain XXX motif or ZZZ motifs as wing modifications at the opposite end of the duplex region; and XXX and ZZZ each independently represents a 2'-OMe modification or 2'-F modification.

In one embodiment the antisense strand may contain Y′Y′Y′ motif occurring at positions 11, 12, 13 of the strand, the count starting from the 1st nucleotide from the 5'-end, or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end; and Y′ represents 2'-O-methyl modification. The antisense strand may additionally contain X′X′X′ motif or Z′Z′Z′ motifs as wing modifications at the opposite end of the duplex region; and X′X′X′ and Z′Z′Z′ each independently represents a 2'-OMe modification or 2'-F modification.

The sense strand represented by any one of the above formulas (Ia), (Ib) and (Ic) forms a duplex with a antisense strand being represented by any one of formulas (IIa), (IIb) and (IIc), respectively.

Accordingly, the dsRNA agent may comprise a sense strand and an antisense strand, each strand having 14 to 30 nucleotides, the dsRNA duplex represented by formula (III):

sense: 5' n_p -N_a-(X X X)$_i$ -N_b- Y Y Y -N_b -(Z Z Z)$_j$-N_a-n_q 3'

antisense: 3' n_p'-N_a'-(X'X'X')$_k$-N_b'-Y'Y'Y'-N_b'-(Z'Z'Z')$_l$-N_a'-n_q' 5'

(III)

wherein:

i, j, k, and l are each independently 0 or 1;

p and q are each independently 0-6;

each N_a and N_a' independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b and N_b' independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

wherein

each n_p', n_p, n_q', and n_q independently represents an overhang nucleotide sequence; and

XXX, YYY, ZZZ,X'X'X', Y'Y'Y', and Z'Z'Z' each independently represent one motif of three identical modifications on three consecutive nucleotides.

In one embodiment, i is 1 and j is 0; or i is 0 and j is 1; or both i and j are 1.In another embodiment, k is 1 and l is 0; k is 0 and l is 1; or both k and l are 1.

In one embodiment, the dsRNA agent of the invention comprises a sense strand and an antisense strand, each strand having 14 to 30 nucleotides, the dsRNA duplex represented by formula (V):

sense: 5' N_a-(X X X)$_i$-N_b-Y Y Y -N_b-(Z Z Z)$_j$-N_a-n_q 3'

antisense: 3' n_p'-N_a'-(X'X'X')$_k$-N_b'-Y'Y'Y'-N_b'-(Z'Z'Z')$_l$-N_a' 5'

(V)

wherein:

i, j, k, and lare each independently 0 or 1;

p and q are each independently 2;

each N_a and N_a' independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b and N_b' independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

wherein

each n_p', and n_q independently represents an overhang nucleotide sequence; and

XXX, YYY, ZZZ, X'X'X', Y'Y'Y', and Z'Z'Z' each independently represent one motif of three identical modifications on three consecutive nucleotides.

In one embodiment, i is 1 and j is 0; or i is 0 and j is 1; or both i and j are 1. In another embodiment, k is 1 and l is 0; k is 0 and l is 1; or both k and l are 1.

In one embodiment, the dsRNA agent of the invention comprises a sense strand and an antisense strand, each strand having 14 to 30 nucleotides, the dsRNA duplex represented by formula (Va):

sense: 5' N_a-(X X X)$_i$-N_b-Y Y Y -N_b-(Z Z Z)$_j$-N_a 3'

antisense: 3' n_p'-N_a'-(X'X'X')$_k$-N_b'-Y'Y'Y'-N_b'-(Z'Z'Z')$_l$-N_a' 5'

(Va)

wherein:

i, j, k, and lare each independently 0 or 1;

p and q are each independently 2;

each N_a and N_a' independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b and N_b' independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

wherein

n_p' represents an overhang nucleotide sequence; and

XXX, YYY, ZZZ, X'X'X', Y'Y'Y', and Z'Z'Z' each independently represent one motif of three identical modifications on three consecutive nucleotides.

Exemplary combinations of the sense strand and antisense strand forming a dsRNA duplex include the formulas below:

5' n_p -N_a -Y Y Y -N_b -Z Z Z -N_a-n_q 3'

3' n_p'-N_a'-Y'Y'Y'-N_b'-Z'Z'Z'-$N_a'n_q'$ 5'

(IIIa)

5' n_p-N_a- X X X -N_b -Y Y Y - N_a-n_q 3'

3' n_p'-N_a'-X'X'X'-N_b'-Y'Y'Y'-N_a'-n_q' 5'

(IIIb)

$5'\ n_p\text{-}N_a\text{-}X\ X\ X\text{-}N_b\text{-}Y\ Y\ Y\text{-}N_b\text{-}\ Z\ Z\ Z\text{-}N_a\text{-}n_q\ 3'$

$3'\ n_p'\text{-}N_a'\text{-}X'X'X'\text{-}N_b'\text{-}Y'Y'Y'\text{-}N_b'\text{-}Z'Z'Z'\text{-}N_a\text{-}n_q'\ 5'$

(IIIc)

When the dsRNA agent is represented by formula (IIIa), each N_b and N_b' independently represents an oligonucleotide sequence comprising 1-10, 1-7, 1-5 or 1-4 modified nucleotides. Each N_a and N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the dsRNA agent is represented as formula (IIIb), each N_b and N_b' independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0modified nucleotides. Each N_a and N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the dsRNA agent is represented as formula (IIIc), each N_b and N_b' independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a and N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides. Each of N_a, N_a', N_b and N_b' independently comprises modifications of alternating pattern.

Each of X, Y and Z in formulas (III), (IIIa), (IIIb) and (IIIc) may be the same or different from each other.

When the dsRNA agent is represented by formula (III), (IIIa), (IIIb) or (IIIc), at least one of the Y nucleotides may form a base pair with one of the Y′ nucleotides. Alternatively, at least two of the Y nucleotides form base pairs with the corresponding Y′ nucleotides; or all three of the Y nucleotides all form base pairs with the corresponding Y′ nucleotides.

It is understood that N_a nucleotides from base pair with N_a', N_b nucleotides from base pair with N_b', X nucleotides from base pair with X', Y nucleotides from base pair with Y', and Z nucleotides from base pair with Z'.

When the dsRNA agent is represented by formula (IIIa) or (IIIc), at least one of the Z nucleotides may form a base pair with one of the Z′ nucleotides. Alternatively, at least two of the Z nucleotides form base pairs with the corresponding Z′ nucleotides; or all three of the Z nucleotides all form base pairs with the corresponding Z′ nucleotides.

When the dsRNA agent is represented as formula (IIIb) or (IIIc), at least one of the X nucleotides may form a base pair with one of the X′ nucleotides. Alternatively, at least two of the X nucleotides form base pairs with the corresponding X′ nucleotides; or all three of the X nucleotides all form base pairs with the corresponding X′ nucleotides.

In one embodiment, the modification on the Y nucleotide is different than the modification on the Y' nucleotide, the modification on the Z nucleotide is different than the modification on the Z' nucleotide, and/or the modification on the X nucleotide is different than the modification on the X' nucleotide.

In one embodiment, the dsRNA agent is a multimer containing at least two duplexes represented by formula (III), (IIIa), (IIIb) or (IIIc), wherein said duplexes are connected by a linker. The linker can be cleavable or non-cleavable. Optionally, said multimer further comprise a ligand. Each of the dsRNA can target the same gene or two different genes; or each of the dsRNA can target same gene at two different target sites.

In one embodiment, the dsRNA agent is a multimer containing three, four, five, six or more duplexes represented by formula (III), (IIIa), (IIIb) or (IIIc), wherein said duplexes are connected by a linker. The linker can be cleavable or non-cleavable. Optionally, said multimer further comprises a ligand. Each of the dsRNA can target the same gene or two different genes; or each of the dsRNA can target same gene at two different target sites.

In one embodiment, two dsRNA agent represented by formula (III), (IIIa), (IIIb) or (IIIc) are linked to each other at the 5' end, and one or both of the 3' ends of the are optionally conjugated to to a ligand. Each of the dsRNA can target the same gene or two different genes; or each of the dsRNA can target same gene at two different target sites.

Various publications described multimeric siRNA and can all be used with the dsRNA of the invention. Such publications include WO2007/091269, US Patent No. 7858769, WO2010/141511, WO2007/117686, WO2009/014887 and WO2011/031520 which are hereby incorporated by their entirely.

The dsRNA agent that contains conjugations of one or more carbohydrate moieties to a dsRNA agent can optimize one or more properties of the dsRNA agent. In many cases, the carbohydrate moiety will be attached to a modified subunit of the dsRNA agent. E.g., the ribose sugar of one or more ribonucleotide subunits of a dsRNA agent

can be replaced with another moiety, e.g., a non-carbohydrate (preferably cyclic) carrier to which is attached a carbohydrate ligand. A ribonucleotide subunit in which the ribose sugar of the subunit has been so replaced is referred to herein as a ribose replacement modification subunit (RRMS). A cyclic carrier may be a carbocyclic ring system, i.e., all ring atoms are carbon atoms, or a heterocyclic ring system, i.e., one or more ring atoms may be a heteroatom, e.g., nitrogen, oxygen, sulfur. The cyclic carrier may be a monocyclic ring system, or may contain two or more rings, e.g. fused rings. The cyclic carrier may be a fully saturated ring system, or it may contain one or more double bonds.

The ligand may be attached to the polynucleotide via a carrier. The carriers include (i) at least one "backbone attachment point," preferably two "backbone attachment points" and (ii) at least one "tethering attachment point." A "backbone attachment point" as used herein refers to a functional group, e.g. a hydroxyl group, or generally, a bond available for, and that is suitable for incorporation of the carrier into the backbone, e.g., the phosphate, or modified phosphate, e.g., sulfur containing, backbone, of a ribonucleic acid. A "tethering attachment point" (TAP) in some embodiments refers to a constituent ring atom of the cyclic carrier, e.g., a carbon atom or a heteroatom (distinct from an atom which provides a backbone attachment point), that connects a selected moiety. The moiety can be, e.g., a carbohydrate, e.g. monosaccharide, disaccharide, trisaccharide, tetrasaccharide, oligosaccharide and polysaccharide. Optionally, the selected moiety is connected by an intervening tether to the cyclic carrier. Thus, the cyclic carrier will often include a functional group, e.g., an amino group, or generally, provide a bond, that is suitable for incorporation or tethering of another chemical entity, e.g., a ligand to the constituent ring.

In embodimentn the dsRNA of the invention is conjugated to a ligand via a carrier, wherein the carrier can be cyclic group or acyclic group; preferably, the cyclic group is selected from pyrrolidinyl, pyrazolinyl, pyrazolidinyl, imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, [1,3]dioxolane, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, quinoxalinyl, pyridazinonyl, tetrahydrofuryl and and decalin; preferably, the acyclic group is selected from serinol backbone or diethanolamine backbone.

The double-stranded RNA (dsRNA) agent of the invention may optionally be conjugated to one or more ligands. The ligand can be attached to the sense strand, antisense strand or both strands, at the 3'-end, 5'-end or both ends. For instance, the ligand may be conjugated to the sense strand, in particular, the 3'-end of the sense strand.

Ligands

A wide variety of entities can be coupled to the oligonucleotides of the present invention. Preferred moieties are ligands, which are coupled, preferably covalently, either directly or indirectly via an intervening tether.

In preferred embodiments, a ligand alters the distribution, targeting or lifetime of the molecule into which it is incorporated. In preferred embodiments a ligand provides an enhanced affinity for a selected target, *e.g.*, molecule, cell or cell type, compartment, receptor *e.g.*, a cellular or organ compartment, tissue, organ or region of the body, as, e.g., compared to a species absent such a ligand. Ligands providing enhanced affinity for a selected target are also termed targeting ligands.

Some ligands can have endosomolytic properties. The endosomolytic ligands promote the lysis of the endosome and/or transport of the composition of the invention, or its components, from the endosome to the cytoplasm of the cell. The endosomolytic ligand may be a polyanionic peptide or peptidomimetic which shows pH-dependent membrane activity and fusogenicity. In one embodiment, the endosomolytic ligand assumes its active conformation at endosomal pH. The "active" conformation is that conformation in which the endosomolytic ligand promotes lysis of the endosome and/or transport of the composition of the invention, or its components, from the endosome to the cytoplasm of the cell. Exemplary endosomolytic ligands include the GALA peptide (Subbarao et al., Biochemistry, 1987, 26: 2964-2972), the EALA peptide (Vogel et al., J. Am. Chem. Soc., 1996, 118: 1581-1586), and their derivatives (Turk et al., Biochem. Biophys. Acta, 2002, 1559: 56-68). In one embodiment, the endosomolytic component may contain a chemical group (e.g., an amino acid) which will undergo a change in charge or protonation in response to a change in pH. The endosomolytic component may be linear or branched.

Ligands can improve transport, hybridization, and specificity properties and may also improve nuclease resistance of the resultant natural or modified oligoribonucleotide, or a polymeric molecule comprising any combination of monomers described herein and/or natural or modified ribonucleotides.

Ligands in general can include therapeutic modifiers, e.g., for enhancing uptake; diagnostic compounds or reporter groups e.g., for monitoring distribution; cross-linking agents; and nuclease-resistance conferring moieties. General examples include lipids, steroids, vitamins, sugars, proteins, peptides, polyamines, and peptide mimics.

Ligands can include a naturally occurring substance, such as a protein (e.g., human serum albumin (HSA), low-density lipoprotein (LDL), high-density lipoprotein (HDL), or globulin); a carbohydrate (e.g., a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); or a lipid. The ligand may also be a recombinant or synthetic molecule, such as a synthetic polymer, e.g., a synthetic polyamino acid, an oligonucleotide (e.g. an aptamer). Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacryllic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

Ligands can also include targeting groups, e.g., a cell or tissue targeting agent, e.g., a lectin, glycoprotein, lipid or protein, e.g., an antibody, that binds to a specified cell type such as a kidney cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid,

folate, vitamin B12, biotin, an RGD peptide, an RGD peptide mimetic or an aptamer. Table 2 shows some examples of targeting ligands and their associated receptors.

Other examples of ligands include dyes, intercalating agents (*e.g.* acridines), cross-linkers (*e.g.* psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial endonucleases or a chelator (*e.g.* EDTA), lipophilic molecules, e.g, cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid,O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine)and peptide conjugates (*e.g.*, antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, $[MPEG]_2$, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.* biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu3+ complexes of tetraazamacrocycles), dinitrophenyl, HRP, or AP.

Ligands can be proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies *e.g.*, an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Ligands may also include hormones and hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, or aptamers. The ligand can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF-κB.

The ligand can be a substance, e.g, a drug, which can increase the uptake of the iRNA agent into the cell, for example, by disrupting the cell's cytoskeleton, e.g., by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin.

The ligand can increase the uptake of the oligonucleotide into the cell by activating an inflammatory response, for example. Exemplary ligands that would have

such an effect include tumor necrosis factor alpha (TNFalpha), interleukin-1 beta, or gamma interferon.

In one aspect, the ligand is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule preferably binds a serum protein, e.g., human serum albumin (HSA). An HSA binding ligand allows for distribution of the conjugate to a target tissue, e.g., a non-kidney target tissue of the body. For example, the target tissue can be the liver, including parenchymal cells of the liver. Other molecules that can bind HSA can also be used as ligands. For example, naproxen or aspirin can be used. A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, and/or (c) can be used to adjust binding to a serum protein, e.g., HSA.

A lipid based ligand can be used to modulate, e.g., control the binding of the conjugate to a target tissue. For example, a lipid or lipid-based ligand that binds to HSA more strongly will be less likely to be targeted to the kidney and therefore less likely to be cleared from the body. A lipid or lipid-based ligand that binds to HSA less strongly can be used to target the conjugate to the kidney.

In a preferred embodiment, the lipid based ligand binds HSA. Preferably, it binds HSA with a sufficient affinity such that the conjugate will be preferably distributed to a non-kidney tissue. However, it is preferred that the affinity not be so strong that the HSA-ligand binding cannot be reversed.

In another preferred embodiment, the lipid based ligand binds HSA weakly or not at all, such that the conjugate will be preferably distributed to the kidney. Other moieties that target to kidney cells can also be used in place of or in addition to the lipid based ligand.

In another aspect, the ligand is a moiety, e.g., a vitamin, which is taken up by a target cell, e.g., a proliferating cell. These are particularly useful for treating disorders characterized by unwanted cell proliferation, e.g., of the malignant or non-malignant type, e.g., cancer cells. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include B vitamins, e.g., folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by cancer cells. Also included are HAS, low density lipoprotein (LDL) and high-density lipoprotein (HDL).

In another aspect, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennopedia. If the agent is a peptide, it can be modified, including a peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is preferably an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase.

The ligand can be a peptide or peptidomimetic. A peptidomimetic (also referred to herein as an oligopeptidomimetic) is a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide. The peptide or peptidomimetic moiety can be about 5-50 amino acids long, *e.g.*, about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long. A peptide or peptidomimetic can be, for example, a cell permeation peptide, cationic peptide, amphipathic peptide, or hydrophobic peptide (e.g., consisting primarily of Tyr, Trp or Phe). The peptide moiety can be a dendrimer peptide, constrained peptide or crosslinked peptide. In another alternative, the peptide moiety can include a hydrophobic membrane translocation sequence (MTS). An exemplary hydrophobic MTS-containing peptide is RFGF having the amino acid sequence AAVALLPAVLLALLAP. An RFGF analogue (e.g., amino acid sequence AALLPVLLAAP) containing a hydrophobic MTS can also be a targeting moiety. The peptide moiety can be a "delivery" peptide, which can carry large polar molecules including peptides, oligonucleotides, and protein across cell membranes. For example, sequences from the HIV Tat protein (GRKKRRQRRRPPQ) and the Drosophila Antennapedia protein (RQIKIWFQNRRMKWKK) have been found to be capable of functioning as delivery peptides. A peptide or peptidomimetic can be encoded by a random sequence of DNA, such as a peptide identified from a phage-display library, or one-bead-one-compound (OBOC) combinatorial library (Lam et al., Nature, 354:82-84, 1991). Preferably the peptide or peptidomimetic tethered to an iRNA agent via an incorporated monomer unit is a cell targeting peptide such as an arginine-glycine-aspartic acid (RGD)-peptide, or RGD mimic. A peptide moiety can range in length from about 5 amino acids to about 40 amino acids. The peptide moieties can have a structural modification, such as to increase stability or direct conformational properties. Any of the structural modifications described below can be utilized.An RGD peptide moiety can be

used to target a tumor cell, such as an endothelial tumor cell or a breast cancer tumor cell (Zitzmann et al., Cancer Res., 62:5139-43, 2002). An RGD peptide can facilitate targeting of an iRNA agent to tumors of a variety of other tissues, including the lung, kidney, spleen, or liver (Aoki et al., Cancer Gene Therapy 8:783-787, 2001). Preferably, the RGD peptide will facilitate targeting of an iRNA agent to the kidney. The RGD peptide can be linear or cyclic, and can be modified, e.g., glycosylated or methylated to facilitate targeting to specific tissues. For example, a glycosylated RGD peptide can deliver an iRNA agent to a tumor cell expressing $\alpha_V\beta_3$ (Haubner et al., Jour. Nucl. Med., 42:326-336, 2001). Peptides that target markers enriched in proliferating cells can be used. E.g., RGD containing peptides and peptidomimetics can target cancer cells, in particular cells that exhibit an integrin. Thus, one could use RGD peptides, cyclic peptides containing RGD, RGD peptides that include D-amino acids, as well as synthetic RGD mimics. In addition to RGD, one can use other moieties that target the integrin ligand. Generally, such ligands can be used to control proliferating cells and angiogeneis. Preferred conjugates of this type lignads that targets PECAM-1, VEGF, or other cancer gene, e.g., a cancer gene described herein.

A "cell permeation peptide" is capable of permeating a cell, e.g., a microbial cell, such as a bacterial or fungal cell, or a mammalian cell, such as a human cell. A microbial cell-permeating peptide can be, for example, an α-helical linear peptide (e.g., LL-37 or Ceropin P1), a disulfide bond-containing peptide (e.g., α -defensin, β-defensin or bactenecin), or a peptide containing only one or two dominating amino acids (e.g., PR-39 or indolicidin). A cell permeation peptide can also include a nuclear localization signal (NLS). For example, a cell permeation peptide can be a bipartite amphipathic peptide, such as MPG, which is derived from the fusion peptide domain of HIV-1 gp41 and the NLS of SV40 large T antigen (Simeoni et al., Nucl. Acids Res. 31:2717-2724, 2003).

In one embodiment, a targeting peptide can be an amphipathic α-helical peptide. Exemplary amphipathic α-helical peptides include, but are not limited to, cecropins, lycotoxins, paradaxins, buforin, CPF, bombinin-like peptide (BLP), cathelicidins, ceratotoxins, S. clava peptides, hagfish intestinal antimicrobial peptides (HFIAPs), magainines, brevinins-2, dermaseptins, melittins, pleurocidin, H_2A peptides, Xenopus peptides, esculentinis-1, and caerins. A number of factors will preferably be considered

to maintain the integrity of helix stability. For example, a maximum number of helix stabilization residues will be utilized (e.g., leu, ala, or lys), and a minimum number helix destabilization residues will be utilized (e.g., proline, or cyclic monomeric units. The capping residue will be considered (for example Gly is an exemplary N-capping residue and/or C-terminal amidation can be used to provide an extra H-bond to stabilize the helix. Formation of salt bridges between residues with opposite charges, separated by i ± 3, or i ± 4 positions can provide stability. For example, cationic residues such as lysine, arginine, homo-arginine, ornithine or histidine can form salt bridges with the anionic residues glutamate or aspartate.

Peptide and peptidomimetic ligands include those having naturally occurring or modified peptides, e.g., D or L peptides; α, β, or γ peptides; N-methyl peptides; azapeptides; peptides having one or more amide, i.e., peptide, linkages replaced with one or more urea, thiourea, carbamate, or sulfonyl urea linkages; or cyclic peptides.

The targeting ligand can be any ligand that is capable of targeting a specific receptor. Examples are: folate, GalNAc, galactose, mannose, mannose-6P, clusters of sugars such as GalNAc cluster, mannose cluster, galactose cluster, or an apatamer. A cluster is a combination of two or more sugar units. The targeting ligands also include integrin receptor ligands, Chemokine receptor ligands, transferrin, biotin, serotonin receptor ligands, PSMA, endothelin, GCPII, somatostatin, LDL and HDL ligands. The ligands can also be based on nucleic acid, e.g., an aptamer. The aptamer can be unmodified or have any combination of modifications disclosed herein.

Endosomal release agents include imidazoles, poly or oligoimidazoles, PEIs, peptides, fusogenic peptides, polycaboxylates, polyacations, masked oligo or poly cations or anions, acetals, polyacetals, ketals/polyketyals, orthoesters, polymers with masked or unmasked cationic or anionic charges, dendrimers with masked or unmasked cationic or anionic charges.

PK modulator stands for pharmacokinetic modulator. PK modulator include lipophiles, bile acids, steroids, phospholipid analogues, peptides, protein binding agents, PEG, vitamins etc. Examplary PK modulator include, but are not limited to, cholesterol, fatty acids, cholic acid, lithocholic acid, dialkylglycerides, diacylglyceride, phospholipids, sphingolipids, naproxen, ibuprofen, vitamin E, biotin etc.

Oligonucleotides that comprise a number of phosphorothioate linkages are also known to bind to serum protein, thus short oligonucleotides, e.g. oligonucleotides of about 5 bases, 10 bases, 15 bases or 20 bases, comprising multiple of phosphorothioate linkages in the backbaone are also amenable to the present invention as ligands (e.g. as PK modulating ligands).

In addition, aptamers that bind serum components (e.g. serum proteins) are also amenable to the present invention as PK modulating ligands.

Other ligand conjugates amenable to the invention are described in U.S. Patent Applications USSN: 10/916,185, filed August 10, 2004; USSN: 10/946,873, filed September 21, 2004; USSN: 10/833,934, filed August 3, 2007; USSN: 11/115,989 filed April 27, 2005 and USSN: 11/944,227 filed November 21, 2007, which are incorporated by reference in their entireties for all purposes.

When two or more ligands are present, the ligands can all have same properties, all have different properties or some ligands have the same properties while others have different properties. For example, a ligand can have targeting properties, have endosomolytic activity or have PK modulating properties. In a preferred embodiment, all the ligands have different properties.

Ligands can be coupled to the oligonucleotides at various places, for example, 3'-end, 5'-end, and/or at an internal position. In preferred embodiments, the ligand is attached to the oligonucleotides *via* an intervening tether, e.g. a carrier described herein. The ligand or tethered ligand may be present on a monomer when said monomer is incorporated into the growing strand. In some embodiments, the ligand may be incorporated via coupling to a "precursor" monomer after said "precursor" monomer has been incorporated into the growing strand. For example, a monomer having, e.g., an amino-terminated tether (i.e., having no associated ligand), e.g., TAP-$(CH_2)_nNH_2$ may be incorporated into a growing oligonucelotide strand. In a subsequent operation, i.e., after incorporation of the precursor monomer into the strand, a ligand having an electrophilic group, e.g., a pentafluorophenyl ester or aldehyde group, can subsequently be attached to the precursor monomer by coupling the electrophilic group of the ligand with the terminal nucleophilic group of the precursor monomer's tether.

In another example, a monomer having a chemical group suitable for taking part in Click Chemistry reaction may be incorporated e.g., an azide or alkyne terminated tether/linker. In a subsequent operation, i.e., after incorporation of the precursor monomer into the strand, a ligand having complementary chemical group, e.g. an alkyne or azide can be attached to the precursor monomer by coupling the alkyne and the azide together.

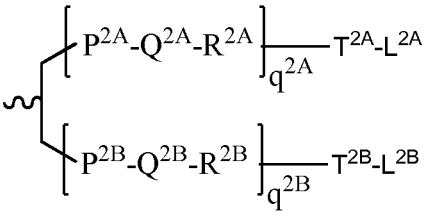
For double- stranded oligonucleotides, ligands can be attached to one or both strands. In some embodiments, a double-stranded iRNA agent contains a ligand conjugated to the sense strand. In other embodiments, a double-stranded iRNA agent contains a ligand conjugated to the antisense strand.

In some embodiments, ligand can be conjugated to nucleobases, sugar moieties, or internucleosidic linkages of nucleic acid molecules. Conjugation to purine nucleobases or derivatives thereof can occur at any position including, endocyclic and exocyclic atoms. In some embodiments, the 2-, 6-, 7-, or 8-positions of a purine nucleobase are attached to a conjugate moiety. Conjugation to pyrimidine nucleobases or derivatives thereof can also occur at any position. In some embodiments, the 2-, 5-, and 6-positions of a pyrimidine nucleobase can be substituted with a conjugate moiety. Conjugation to sugar moieties of nucleosides can occur at any carbon atom. Example carbon atoms of a sugar moiety that can be attached to a conjugate moiety include the 2', 3', and 5' carbon atoms. The 1' position can also be attached to a conjugate moiety, such as in an abasic residue. Internucleosidic linkages can also bear conjugate moieties. For phosphorus-containing linkages (e.g., phosphodiester, phosphorothioate, phosphorodithiotate, phosphoroamidate, and the like), the conjugate moiety can be attached directly to the phosphorus atom or to an O, N, or S atom bound to the phosphorus atom. For amine- or amide-containing internucleosidic linkages (e.g., PNA), the conjugate moiety can be attached to the nitrogen atom of the amine or amide or to an adjacent carbon atom.

Any suitable ligand in the field of RNA interference may be used, although the ligand is typically a carbohydrate e.g. monosaccharide (such as GalNAc), disaccharide, trisaccharide, tetrasaccharide, polysaccharide.

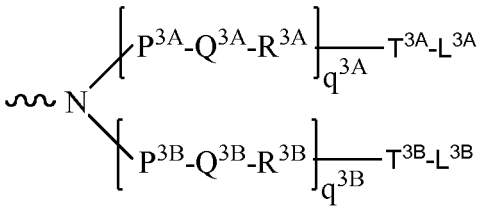
Linkers that conjugate the ligand to the nucleic acid include those discussed above. For example, the ligand can be one or more GalNAc (*N*-acetylglucosamine) derivatives attached through a bivalent or trivalent branched linker.

In one embodiment, the dsRNA of the invention is conjugated to a bivalent and trivalent branched linkers include the structures shown in any of formula (IV) – (VII):



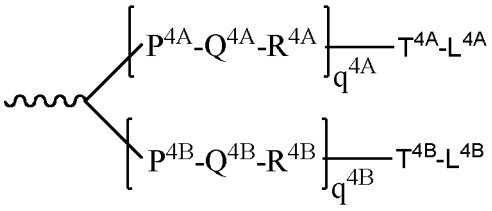
Formula (IV)

,



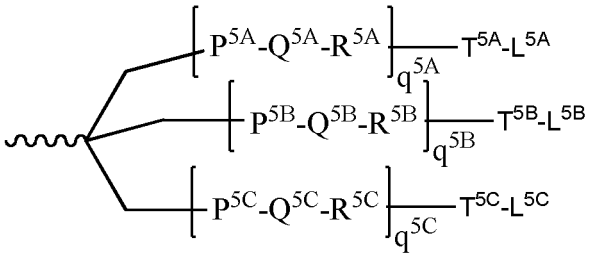
Formula (V)

,



Formula (VI)

, or



Formula (VII)

;

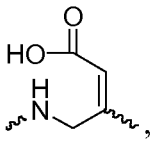
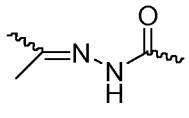
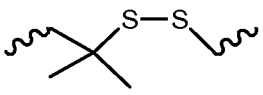
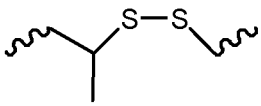
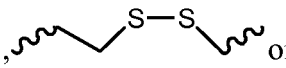
wherein:

q^{2A}, q^{2B}, q^{3A}, q^{3B}, $q4^{A}$, q^{4B}, q^{5A}, q^{5B} and q^{5C} represent independently for each occurrence 0-20 and wherein the repeating unit can be the same or different;

P^{2A}, P^{2B}, P^{3A}, P^{3B}, P^{4A}, P^{4B}, P^{5A}, P^{5B}, P^{5C}, T^{2A}, T^{2B}, T^{3A}, T^{3B}, T^{4A}, T^{4B}, T^{4A}, T^{5B}, T^{5C} are each independently for each occurrence absent, CO, NH, O, S, OC(O), NHC(O), CH_2, CH_2NH or CH_2O;

Q^{2A}, Q^{2B}, Q^{3A}, Q^{3B}, Q^{4A}, Q^{4B}, Q^{5A}, Q^{5B}, Q^{5C} are independently for each occurrence absent, alkylene, substituted alkylene wherin one or more methylenes can be interrupted or terminated by one or more of O, S, S(O), SO_2, $N(R^N)$, C(R')=C(R''), C≡C or C(O);

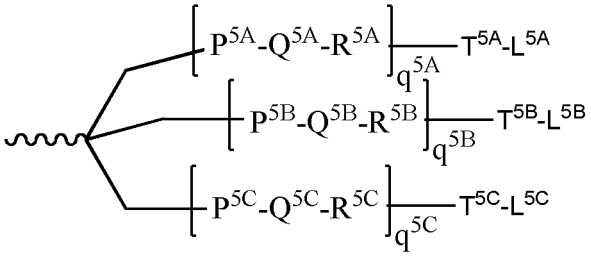
R^{2A}, R^{2B}, R^{3A}, R^{3B}, R^{4A}, R^{4B}, R^{5A}, R^{5B}, R^{5C} are each independently for each occurrence absent, NH, O, S, CH_2, C(O)O, C(O)NH, $NHCH(R^a)C(O)$, $-C(O)-CH(R^a)-$

NH-, CO, CH=N-O, , , , ,  or heterocyclyl;

L^{2A}, L^{2B}, L^{3A}, L^{3B}, L^{4A}, L^{4B}, L^{5A}, L^{5B} and L^{5C} represent the ligand; i.e. each independently for each occurrence a monosaccharide (such as GalNAc), disaccharide, trisaccharide, tetrasaccharide, oligosaccharide, or polysaccharide; and

R^a is H or amino acid side chain.

Trivalent conjugating GalNAc derivatives are particularly useful for use with RNAi agents for inhibiting the expression of a target gene, such as those of formula (VII):

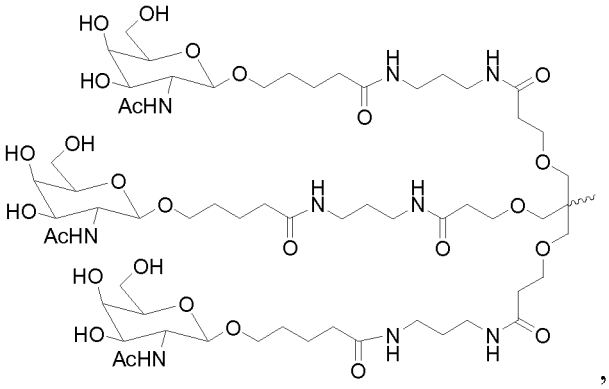


Formula (VII)

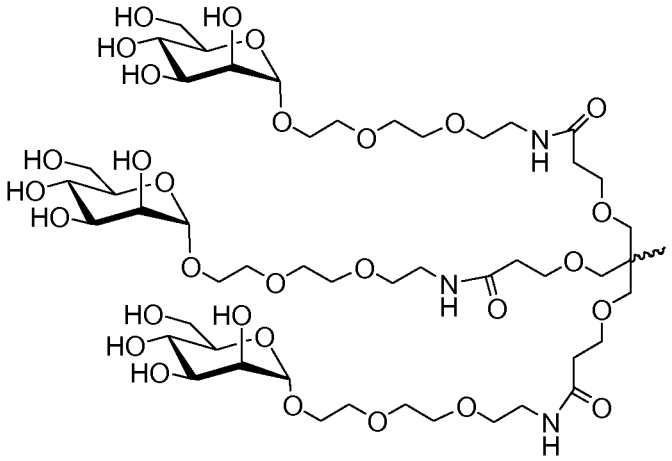
,

wherein L^{5A}, L^{5B} and L^{5C} represent a monosaccharide, such as GalNAc derivative.

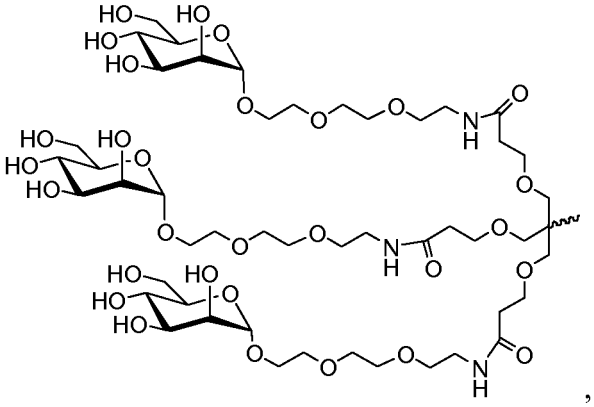
Examples of suitable bivalent and trivalent branched linker groups conjugating GalNAc derivatives include, but are not limited to, the following compounds:



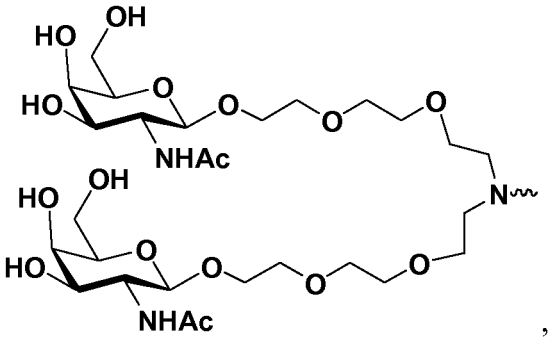
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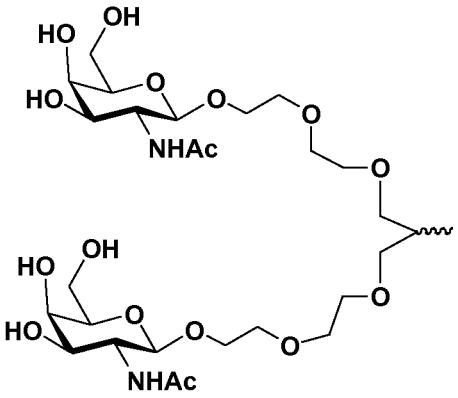
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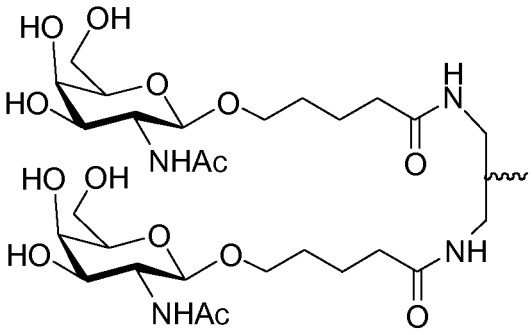
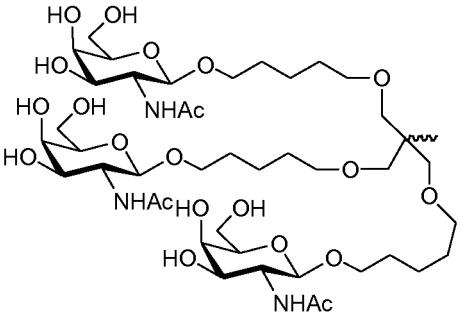
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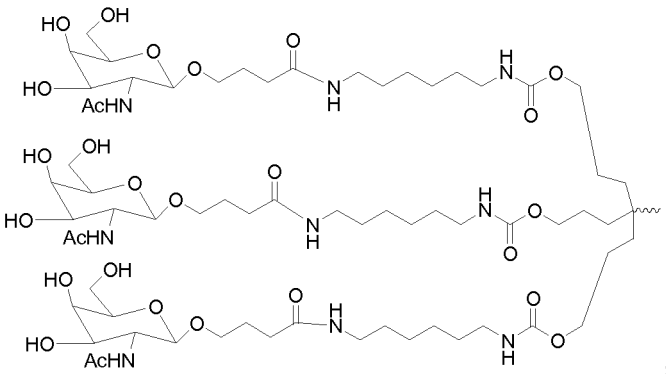


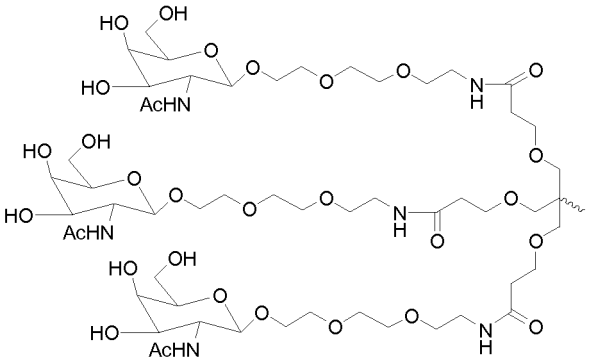
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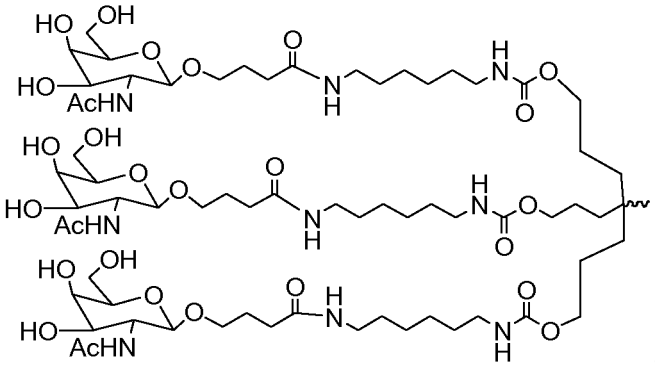


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, or


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Definitions

As used herein, the terms "dsRNA", "siRNA", and "iRNA agent" are used interchangeably to agents that can mediate silencing of a target RNA, *e.g.*, mRNA, *e.g.*, a transcript of a gene that encodes a protein. For convenience, such mRNA is also referred to herein as mRNA to be silenced. Such a gene is also referred to as a target gene. In general, the RNA to be silenced is an endogenous gene or a pathogen gene. In addition, RNAs other than mRNA, *e.g.*, tRNAs, and viral RNAs, can also be targeted.

As used herein, the phrase "mediates RNAi" refers to the ability to silence, in a sequence specific manner, a target RNA. While not wishing to be bound by theory, it is believed that silencing uses the RNAi machinery or process and a guide RNA, *e.g.*, an siRNA agent of 21 to 23 nucleotides.

As used herein, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between a compound of the invention and a target RNA molecule. Specific binding requires a sufficient degree of complementarity to avoid non-specific binding of the oligomeric compound to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed. The non-target sequences typically differ by at least 5 nucleotides.

In one embodiment, a dsRNA agent of the invention is "sufficiently complementary" to a target RNA, *e.g.*, a target mRNA, such that the dsRNA agent silences production of protein encoded by the target mRNA. In another embodiment, the dsRNA agent of the invention is "exactly complementary" to a target RNA, *e.g.*, the target RNA and the dsRNA duplex agent anneal, for example to form a hybrid made exclusively of Watson-Crick base pairs in the region of exact complementarity. A "sufficiently complementary" target RNA can include an internal region (*e.g.*, of at least 10 nucleotides) that is exactly complementary to a target RNA. Moreover, in some embodiments, the dsRNA agent of the invention specifically discriminates a single-nucleotide difference. In this case, the dsRNA agent only mediates RNAi if exact complementary is found in the region (*e.g.*, within 7 nucleotides of) the single-nucleotide difference.

As used herein, the term "oligonucleotide" refers to a nucleic acid molecule (RNA or DNA) for example of length less than 100, 200, 300, or 400 nucleotides.

The term "halo" refers to any radical of fluorine, chlorine, bromine or iodine. The term "alkyl" refers to saturated and unsaturated non-aromatic hydrocarbon chains that may be a straight chain or branched chain, containing the indicated number of carbon atoms (these include without limitation propyl, allyl, or propargyl), which may be optionally inserted with N, O, or S. For example, C_1-C_{10} indicates that the group may have from 1 to 10 (inclusive) carbon atoms in it. The term "alkoxy" refers to an -O-alkyl radical. The term "alkylene" refers to a divalent alkyl (*i.e.*, -R-). The term "alkylenedioxo" refers to a divalent species of the structure -O-R-O-, in which R represents an alkylene. The term "aminoalkyl" refers to an alkyl substituted with an aminoThe term "mercapto" refers to an -SH radical. The term "thioalkoxy" refers to an -S-alkyl radical.

The term "aryl" refers to a 6-carbon monocyclic or 10-carbon bicyclic aromatic ring system wherein 0, 1, 2, 3, or 4 atoms of each ring may be substituted by a substituent. Examples of aryl groups include phenyl, naphthyl and the like. The term "arylalkyl" or the term "aralkyl" refers to alkyl substituted with an aryl. The term "arylalkoxy" refers to an alkoxy substituted with aryl.

The term "cycloalkyl" as employed herein includes saturated and partially unsaturated cyclic hydrocarbon groups having 3 to 12 carbons, for example, 3 to 8 carbons, and, for example, 3 to 6 carbons, wherein the cycloalkyl group additionally may be optionally substituted. Cycloalkyl groups include, without limitation, cyclopropyl, cyclobutyl, cyclopentyl, cyclopentenyl, cyclohexyl, cyclohexenyl, cycloheptyl, and cyclooctyl.

The term "heteroaryl" refers to an aromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (*e.g.*, carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein 0, 1, 2, 3, or 4 atoms of each ring may be substituted by a substituent. Examples of heteroaryl groups include pyridyl, furyl or furanyl, imidazolyl, benzimidazolyl, pyrimidinyl, thiophenyl or thienyl,

quinolinyl, indolyl, thiazolyl, and the like. The term "heteroarylalkyl" or the term "heteroaralkyl" refers to an alkyl substituted with a heteroaryl. The term "heteroarylalkoxy" refers to an alkoxy substituted with heteroaryl.

The term "heterocyclyl" refers to a nonaromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (*e.g.*, carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein 0, 1, 2 or 3 atoms of each ring may be substituted by a substituent. Examples of heterocyclyl groups include trizolyl, tetrazolyl, piperazinyl, pyrrolidinyl, dioxanyl, morpholinyl, tetrahydrofuranyl, and the like.

The term "oxo" refers to an oxygen atom, which forms a carbonyl when attached to carbon, an N-oxide when attached to nitrogen, and a sulfoxide or sulfone when attached to sulfur.

The term "acyl" refers to an alkylcarbonyl, cycloalkylcarbonyl, arylcarbonyl, heterocyclylcarbonyl, or heteroarylcarbonyl substituent, any of which may be further substituted by substituents.

The term "substituted" refers to the replacement of one or more hydrogen radicals in a given structure with the radical of a specified substituent including, but not limited to: halo, alkyl, alkenyl, alkynyl, aryl, heterocyclyl, thiol, alkylthio, arylthio, alkylthioalkyl, arylthioalkyl, alkylsulfonyl, alkylsulfonylalkyl, arylsulfonylalkyl, alkoxy, aryloxy, aralkoxy, aminocarbonyl, alkylaminocarbonyl, arylaminocarbonyl, alkoxycarbonyl, aryloxycarbonyl, haloalkyl, amino, trifluoromethyl, cyano, nitro, alkylamino, arylamino, alkylaminoalkyl, arylaminoalkyl, aminoalkylamino, hydroxy, alkoxyalkyl, carboxyalkyl, alkoxycarbonylalkyl, aminocarbonylalkyl, acyl, aralkoxycarbonyl, carboxylic acid, sulfonic acid, sulfonyl, phosphonic acid, aryl, heteroaryl, heterocyclic, and aliphatic. It is understood that the substituent can be further substituted.

Cleavable Linking Groups

A cleavable linking group is one which is sufficiently stable outside the cell, but which upon entry into a target cell is cleaved to release the two parts the linker is holding

together. In a preferred embodiment, the cleavable linking group is cleaved at least 10 times or more, preferably at least 100 times faster in the target cell or under a first reference condition (which can, e.g., be selected to mimic or represent intracellular conditions) than in the blood of a subject, or under a second reference condition (which can, e.g., be selected to mimic or represent conditions found in the blood or serum).

Cleavable linking groups are susceptible to cleavage agents, e.g., pH, redox potential or the presence of degradative molecules. Generally, cleavage agents are more prevalent or found at higher levels or activities inside cells than in serum or blood. Examples of such degradative agents include: redox agents which are selected for particular substrates or which have no substrate specificity, including, e.g., oxidative or reductive enzymes or reductive agents such as mercaptans, present in cells, that can degrade a redox cleavable linking group by reduction; esterases; endosomes or agents that can create an acidic environment, e.g., those that result in a pH of five or lower; enzymes that can hydrolyze or degrade an acid cleavable linking group by acting as a general acid, peptidases (which can be substrate specific), and phosphatases.

A cleavable linkage group, such as a disulfide bond can be susceptible to pH. The pH of human serum is 7.4, while the average intracellular pH is slightly lower, ranging from about 7.1-7.3. Endosomes have a more acidic pH, in the range of 5.5-6.0, and lysosomes have an even more acidic pH at around 5.0. Some linkers will have a cleavable linking group that is cleaved at a preferred pH, thereby releasing the cationic lipid from the ligand inside the cell, or into the desired compartment of the cell.

A linker can include a cleavable linking group that is cleavable by a particular enzyme. The type of cleavable linking group incorporated into a linker can depend on the cell to be targeted. For example, liver targeting ligands can be linked to the cationic lipids through a linker that includes an ester group. Liver cells are rich in esterases, and therefore the linker will be cleaved more efficiently in liver cells than in cell types that are not esterase-rich. Other cell-types rich in esterases include cells of the lung, renal cortex, and testis.

Linkers that contain peptide bonds can be used when targeting cell types rich in peptidases, such as liver cells and synoviocytes.

In general, the suitability of a candidate cleavable linking group can be evaluated by testing the ability of a degradative agent (or condition) to cleave the candidate linking group. It will also be desirable to also test the candidate cleavable linking group for the ability to resist cleavage in the blood or when in contact with other non-target tissue. Thus one can determine the relative susceptibility to cleavage between a first and a second condition, where the first is selected to be indicative of cleavage in a target cell and the second is selected to be indicative of cleavage in other tissues or biological fluids, e.g., blood or serum. The evaluations can be carried out in cell free systems, in cells, in cell culture, in organ or tissue culture, or in whole animals. It may be useful to make initial evaluations in cell-free or culture conditions and to confirm by further evaluations in whole animals. In preferred embodiments, useful candidate compounds are cleaved at least 2, 4, 10 or 100 times faster in the cell (or under in vitro conditions selected to mimic intracellular conditions) as compared to blood or serum (or under in vitro conditions selected to mimic extracellular conditions).

Redox cleavable linking groups

One class of cleavable linking groups are redox cleavable linking groups that are cleaved upon reduction or oxidation. An example of reductively cleavable linking group is a disulphide linking group (-S-S-). To determine if a candidate cleavable linking group is a suitable "reductively cleavable linking group," or for example is suitable for use with a particular iRNA moiety and particular targeting agent one can look to methods described herein. For example, a candidate can be evaluated by incubation with dithiothreitol (DTT), or other reducing agent using reagents know in the art, which mimic the rate of cleavage which would be observed in a cell, e.g., a target cell. The candidates can also be evaluated under conditions which are selected to mimic blood or serum conditions. In a preferred embodiment, candidate compounds are cleaved by at most 10% in the blood. In preferred embodiments, useful candidate compounds are degraded at least 2, 4, 10 or 100 times faster in the cell (or under in vitro conditions selected to mimic intracellular conditions) as compared to blood (or under in vitro conditions selected to mimic extracellular conditions). The rate of cleavage of candidate compounds can be determined using standard enzyme kinetics assays under conditions chosen to

mimic intracellular media and compared to conditions chosen to mimic extracellular media.

Phosphate-based cleavable linking groups

Phosphate-based cleavable linking groups are cleaved by agents that degrade or hydrolyze the phosphate group. An example of an agent that cleaves phosphate groups in cells are enzymes such as phosphatases in cells. Examples of phosphate-based linking groups are -O-P(O)(ORk)-O-, -O-P(S)(ORk)-O-, -O-P(S)(SRk)-O-, -S-P(O)(ORk)-O-, -O-P(O)(ORk)-S-, -S-P(O)(ORk)-S-, -O-P(S)(ORk)-S-, -S-P(S)(ORk)-O-, -O-P(O)(Rk)-O-, -O-P(S)(Rk)-O-, -S-P(O)(Rk)-O-, -S-P(S)(Rk)-O-, -S-P(O)(Rk)-S-, -O-P(S)(Rk)-S-. Preferred embodiments are -O-P(O)(OH)-O-, -O-P(S)(OH)-O-, -O-P(S)(SH)-O-, -S-P(O)(OH)-O-, -O-P(O)(OH)-S-, -S-P(O)(OH)-S-, -O-P(S)(OH)-S-, -S-P(S)(OH)-O-, -O-P(O)(H)-O-, -O-P(S)(H)-O-, -S-P(O)(H)-O-, -S-P(S)(H)-O-, -S-P(O)(H)-S-, -O-P(S)(H)-S-. A preferred embodiment is -O-P(O)(OH)-O-. These candidates can be evaluated using methods analogous to those described above.

Acid cleavable linking groups

Acid cleavable linking groups are linking groups that are cleaved under acidic conditions. In preferred embodiments acid cleavable linking groups are cleaved in an acidic environment with a pH of about 6.5 or lower (e.g., about 6.0, 5.5, 5.0, or lower), or by agents such as enzymes that can act as a general acid. In a cell, specific low pH organelles, such as endosomes and lysosomes can provide a cleaving environment for acid cleavable linking groups. Examples of acid cleavable linking groups include but are not limited to hydrazones, esters, and esters of amino acids. Acid cleavable groups can have the general formula -C=NN-, C(O)O, or -OC(O). A preferred embodiment is when the carbon attached to the oxygen of the ester (the alkoxy group) is an aryl group, substituted alkyl group, or tertiary alkyl group such as dimethyl pentyl or t-butyl. These candidates can be evaluated using methods analogous to those described above.

Ester-based linking groups

Ester-based cleavable linking groups are cleaved by enzymes such as esterases and amidases in cells. Examples of ester-based cleavable linking groups include but are not limited to esters of alkylene, alkenylene and alkynylene groups. Ester cleavable

linking groups have the general formula -C(O)O-, or -OC(O)-. These candidates can be evaluated using methods analogous to those described above.

Peptide-based cleaving groups

Peptide-based cleavable linking groups are cleaved by enzymes such as peptidases and proteases in cells. Peptide-based cleavable linking groups are peptide bonds formed between amino acids to yield oligopeptides (e.g., dipeptides, tripeptides etc.) and polypeptides. Peptide-based cleavable groups do not include the amide group (-C(O)NH-). The amide group can be formed between any alkylene, alkenylene or alkynelene. A peptide bond is a special type of amide bond formed between amino acids to yield peptides and proteins. The peptide based cleavage group is generally limited to the peptide bond (i.e., the amide bond) formed between amino acids yielding peptides and proteins and does not include the entire amide functional group. Peptide-based cleavable linking groups have the general formula – $NHCHR^{A}C(O)NHCHR^{B}C(O)$-, where R^{A} and R^{B} are the R groups of the two adjacent amino acids. These candidates can be evaluated using methods analogous to those described above.As used herein, "carbohydrate" refers to a compound which is either a carbohydrate per se made up of one or more monosaccharide units having at least 6 carbon atoms (which may be linear, branched or cyclic) with an oxygen, nitrogen or sulfur atom bonded to each carbon atom; or a compound having as a part thereof a carbohydrate moiety made up of one or more monosaccharide units each having at least six carbon atoms (which may be linear, branched or cyclic), with an oxygen, nitrogen or sulfur atom bonded to each carbon atom. Representative carbohydrates include the sugars (mono-, di-, tri- and oligosaccharides containing from about 4-9 monosaccharide units), and polysaccharides such as starches, glycogen, cellulose and polysaccharide gums. Specific monosaccharides include C_5 and above (preferably C_5 -C_8) sugars; di- and trisaccharides include sugars having two or three monosaccharide units (preferably C_5 -C_8).

Alternative embodiments

In another embodiment, the invention relates to a dsRNA agent capable of inhibiting the expression of a target gene. The dsRNA agent comprises a sense strand and an antisense strand, each strand having 14 to 30 nucleotides. The sense strand

contains at least one motif of three identical modifications on three consecutive nucleotides, where at least one of the motifs occurs at or near the cleavage site in the antisense strand. Every nucleotide in the sense strand and antisense strand has been modified. The modifications on sense strand and antisense strand each independently comprises at least two different modifications.

In another embodiment, the invention relates to a dsRNA agent capable of inhibiting the expression of a target gene. The dsRNA agent comprises a sense strand and an antisense strand, each strand having 14 to 30 nucleotides. The sense strand contains at least one motif of three identical modifications on three consecutive nucleotides, where at least one of the motifs occurs at or near the cleavage site in the antisense strand. The antisense strand contains at least one motif of three identical modifications on three consecutive nucleotides. The modification pattern of the antisense strand is shifted by one or more nucleotides relative to the modification pattern of the sense strand.

In another embodiment, the invention relates to a dsRNA agent capable of inhibiting the expression of a target gene. The dsRNA agent comprises a sense strand and an antisense strand, each strand having 14 to 30 nucleotides. The sense strand contains at least two motifs of three identical modifications on three consecutive nucleotides, when at least one of the motifs occurs at the cleavage site in the strand and at least one of the motifs occurs at another portion of the strand that is separated from the motif at the cleavage site by at least one nucleotide. The antisense strand contains at least one motif of three identical modifications on three consecutive nucleotides, where at least one of the motifs occurs at or near the cleavage site in the strand and at least one of the motifs occurs at another portion of the strand that is separated from the motif at or near cleavage site by at least one nucleotide.

In another embodiment, the invention relates to a dsRNA agent capable of inhibiting the expression of a target gene. The dsRNA agent comprises a sense strand and an antisense strand, each strand having 14 to 30 nucleotides. The sense strand contains at least two motifs of three identical modifications on three consecutive nucleotides, where at least one of the motifs occurs at the cleavage site in the strand and at least one of the motifs occurs at another portion of the strand that is separated from the

motif at the cleavage site by at least one nucleotide. The antisense strand contains at least one motif of three identical modifications on three consecutive nucleotides, where at least one of the motifs occurs at or near the cleavage site in the strand and at least one of the motifs occurs at another portion of the strand that is separated from the motif at or near cleavage site by at least one nucleotide. The modification in the motif occurring at the cleavage site in the sense strand is different than the modification in the motif occurring at or near the cleavage site in the antisense strand. In another embodiment, the invention relates to a dsRNA agent capable of inhibiting the expression of a target gene. The dsRNA agent comprises a sense strand and an antisense strand, each strand having 12 to 30 nucleotides. The sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides, where at least one of the motifs occurs at the cleavage site in the strand. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides.

The sense strand may further comprises one or more motifs of three identical modifications on three consecutive nucleotides, where the one or more additional motifs occur at another portion of the strand that is separated from the three 2'-F modifications at the cleavage site by at least one nucleotide. The antisense strand may further comprises one or more motifs of three identical modifications on three consecutive nucleotides, where the one or more additional motifs occur at another portion of the strand that is separated from the three 2'-O-methyl modifications by at least one nucleotide. At least one of the nucleotides having a 2'-F modification may form a base pair with one of the nucleotides having a 2'-O-methyl modification.

In one embodiment, the dsRNA of the invention is administered in buffer.

In one embodiment, siRNA compounds described herein can be formulated for administration to a subject. A formulated siRNA composition can assume a variety of states. In some examples, the composition is at least partially crystalline, uniformly crystalline, and/or anhydrous (*e.g.*, less than 80, 50, 30, 20, or 10% water). In another example, the siRNA is in an aqueous phase, *e.g.*, in a solution that includes water.

The aqueous phase or the crystalline compositions can, *e.g.*, be incorporated into a delivery vehicle, *e.g.*, a liposome (particularly for the aqueous phase) or a particle (*e.g.*, a microparticle as can be appropriate for a crystalline composition). Generally, the siRNA

composition is formulated in a manner that is compatible with the intended method of administration, as described herein. For example, in particular embodiments the composition is prepared by at least one of the following methods: spray drying, lyophilization, vacuum drying, evaporation, fluid bed drying, or a combination of these techniques; or sonication with a lipid, freeze-drying, condensation and other self-assembly.

A siRNA preparation can be formulated in combination with another agent, *e.g.*, another therapeutic agent or an agent that stabilizes a siRNA, *e.g.*, a protein that complexes with siRNA to form an iRNP. Still other agents include chelators, *e.g.*, EDTA (*e.g.*, to remove divalent cations such as Mg^{2+}), salts, RNAse inhibitors (*e.g.*, a broad specificity RNAse inhibitor such as RNAsin) and so forth.

In one embodiment, the siRNA preparation includes another siNA compound, *e.g.*, a second siRNA that can mediate RNAi with respect to a second gene, or with respect to the same gene. Still other preparation can include at least 3, 5, ten, twenty, fifty, or a hundred or more different siRNA species. Such siRNAs can mediate RNAi with respect to a similar number of different genes.

In one embodiment, the siRNA preparation includes at least a second therapeutic agent (*e.g.*, an agent other than a RNA or a DNA). For example, a siRNA composition for the treatment of a viral disease, *e.g.,* HIV, might include a known antiviral agent (*e.g.*, a protease inhibitor or reverse transcriptase inhibitor). In another example, a siRNA composition for the treatment of a cancer might further comprise a chemotherapeutic agent.

Exemplary formulations are discussed below.

Liposomes. For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to unmodified siRNA compounds. It may be understood, however, that these formulations, compositions and methods can be practiced with other siRNA compounds, *e.g.*, modified siRNAs, and such practice is within the invention. An siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof)

preparation can be formulated for delivery in a membranous molecular assembly, *e.g.*, a liposome or a micelle. As used herein, the term "liposome" refers to a vesicle composed of amphiphilic lipids arranged in at least one bilayer, *e.g.*, one bilayer or a plurality of bilayers. Liposomes include unilamellar and multilamellar vesicles that have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the siRNA composition. The lipophilic material isolates the aqueous interior from an aqueous exterior, which typically does not include the siRNA composition, although in some examples, it may. Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomal bilayer fuses with bilayer of the cellular membranes. As the merging of the liposome and cell progresses, the internal aqueous contents that include the siRNA are delivered into the cell where the siRNA can specifically bind to a target RNA and can mediate RNAi. In some cases the liposomes are also specifically targeted, *e.g.*, to direct the siRNA to particular cell types.

A liposome containing a siRNA can be prepared by a variety of methods. In one example, the lipid component of a liposome is dissolved in a detergent so that micelles are formed with the lipid component. For example, the lipid component can be an amphipathic cationic lipid or lipid conjugate. The detergent can have a high critical micelle concentration and may be nonionic. Exemplary detergents include cholate, CHAPS, octylglucoside, deoxycholate, and lauroyl sarcosine. The siRNA preparation is then added to the micelles that include the lipid component. The cationic groups on the lipid interact with the siRNA and condense around the siRNA to form a liposome. After condensation, the detergent is removed, *e.g.,* by dialysis, to yield a liposomal preparation of siRNA.

If necessary a carrier compound that assists in condensation can be added during the condensation reaction, *e.g.*, by controlled addition. For example, the carrier compound can be a polymer other than a nucleic acid (*e.g.*, spermine or spermidine). pH can also adjusted to favor condensation.

Further description of methods for producing stable polynucleotide delivery vehicles, which incorporate a polynucleotide/cationic lipid complex as structural

components of the delivery vehicle, are described in, *e.g.*, WO 96/37194. Liposome formation can also include one or more aspects of exemplary methods described in Felgner, P. L. *et al.*, *Proc. Natl. Acad. Sci.*, USA 8:7413-7417, 1987; U.S. Pat. No. 4,897,355; U.S. Pat. No. 5,171,678; Bangham, *et al. M. Mol. Biol.* 23:238, 1965; Olson, *et al. Biochim. Biophys. Acta* 557:9, 1979; Szoka, *et al. Proc. Natl. Acad. Sci.* 75: 4194, 1978; Mayhew, *et al. Biochim. Biophys. Acta* 775:169, 1984; Kim, *et al. Biochim. Biophys. Acta* 728:339, 1983; and Fukunaga, *et al. Endocrinol.* 115:757, 1984. Commonly used techniques for preparing lipid aggregates of appropriate size for use as delivery vehicles include sonication and freeze-thaw plus extrusion (see, *e.g.*, Mayer, *et al. Biochim. Biophys. Acta* 858:161, 1986). Microfluidization can be used when consistently small (50 to 200 nm) and relatively uniform aggregates are desired (Mayhew, *et al. Biochim. Biophys. Acta* 775:169, 1984). These methods are readily adapted to packaging siRNA preparations into liposomes.

Liposomes that are pH-sensitive or negatively-charged entrap nucleic acid molecules rather than complex with them. Since both the nucleic acid molecules and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some nucleic acid molecules are entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al.*, *Journal of Controlled Release*, 19, (1992) 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Examples of other methods to introduce liposomes into cells *in vitro* and include U.S. Pat. No. 5,283,185; U.S. Pat. No. 5,171,678; WO 94/00569; WO 93/24640; WO 91/16024; Felgner, *J. Biol. Chem.* 269:2550, 1994; Nabel, *Proc. Natl. Acad. Sci.* 90:11307, 1993; Nabel, *Human Gene Ther.* 3:649, 1992; Gershon, *Biochem.* 32:7143, 1993; and Strauss *EMBO J.* 11:417, 1992.

In one embodiment, cationic liposomes are used. Cationic liposomes possess the advantage of being able to fuse to the cell membrane. Non-cationic liposomes, although not able to fuse as efficiently with the plasma membrane, are taken up by macrophages *in vivo* and can be used to deliver siRNAs to macrophages.

Further advantages of liposomes include: liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated siRNAs in their internal compartments from metabolism and degradation (Rosoff, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

A positively charged synthetic cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) can be used to form small liposomes that interact spontaneously with nucleic acid to form lipid-nucleic acid complexes which are capable of fusing with the negatively charged lipids of the cell membranes of tissue culture cells, resulting in delivery of siRNA (see, *e.g.*, Felgner, P. L. *et al.*, Proc. Natl. Acad. Sci., USA 8:7413-7417, 1987 and U.S. Pat. No. 4,897,355 for a description of DOTMA and its use with DNA).

A DOTMA analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonia)propane (DOTAP) can be used in combination with a phospholipid to form DNA-complexing vesicles. Lipofectin™ Bethesda Research Laboratories, Gaithersburg, Md.) is an effective agent for the delivery of highly anionic nucleic acids into living tissue culture cells that comprise positively charged DOTMA liposomes which interact spontaneously with negatively charged polynucleotides to form complexes. When enough positively charged liposomes are used, the net charge on the resulting complexes is also positive. Positively charged complexes prepared in this way spontaneously attach to negatively

charged cell surfaces, fuse with the plasma membrane, and efficiently deliver functional nucleic acids into, for example, tissue culture cells. Another commercially available cationic lipid, 1,2-bis(oleoyloxy)-3,3-(trimethylammonia)propane ("DOTAP") (Boehringer Mannheim, Indianapolis, Indiana) differs from DOTMA in that the oleoyl moieties are linked by ester, rather than ether linkages.

Other reported cationic lipid compounds include those that have been conjugated to a variety of moieties including, for example, carboxyspermine which has been conjugated to one of two types of lipids and includes compounds such as 5-carboxyspermylglycine dioctaoleoylamide ("DOGS") (Transfectam™, Promega, Madison, Wisconsin) and dipalmitoylphosphatidylethanolamine 5-carboxyspermyl-amide ("DPPES") (see, *e.g.*, U.S. Pat. No. 5,171,678).

Another cationic lipid conjugate includes derivatization of the lipid with cholesterol ("DC-Chol") which has been formulated into liposomes in combination with DOPE (See, Gao, X. and Huang, L., Biochim. Biophys. Res. Commun. 179:280, 1991). Lipopolylysine, made by conjugating polylysine to DOPE, has been reported to be effective for transfection in the presence of serum (Zhou, X. *et al.*, Biochim. Biophys. Acta 1065:8, 1991). For certain cell lines, these liposomes containing conjugated cationic lipids, are said to exhibit lower toxicity and provide more efficient transfection than the DOTMA-containing compositions. Other commercially available cationic lipid products include DMRIE and DMRIE-HP (Vical, La Jolla, California) and Lipofectamine (DOSPA) (Life Technology, Inc., Gaithersburg, Maryland). Other cationic lipids suitable for the delivery of oligonucleotides are described in WO 98/39359 and WO 96/37194.

Liposomal formulations are particularly suited for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer siRNA, into the skin. In some implementations, liposomes are used for delivering siRNA to epidermal cells and also to enhance the penetration of siRNA into dermal tissues, *e.g.*, into skin. For example, the liposomes can be applied topically. Topical delivery of drugs formulated as liposomes to the skin has been documented (see, *e.g.*, Weiner *et al.*, *Journal of Drug Targeting*, 1992, vol. 2,405-410 and du Plessis *et al.*,

Antiviral Research, 18, 1992, 259-265; Mannino, R. J. and Fould-Fogerite, S., Biotechniques 6:682-690, 1988; Itani, T. *et al.* Gene 56:267-276. 1987; Nicolau, C. *et al.* Meth. Enz. 149:157-176, 1987; Straubinger, R. M. and Papahadjopoulos, D. Meth. Enz. 101:512-527, 1983; Wang, C. Y. and Huang, L., Proc. Natl. Acad. Sci. USA 84:7851-7855, 1987).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome II (glyceryl distearate/ cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver a drug into the dermis of mouse skin. Such formulations with siRNA are useful for treating a dermatological disorder.

Liposomes that include siRNA can be made highly deformable. Such deformability can enable the liposomes to penetrate through pore that are smaller than the average radius of the liposome. For example, transfersomes are a type of deformable liposomes. Transferosomes can be made by adding surface edge activators, usually surfactants, to a standard liposomal composition. Transfersomes that include siRNA can be delivered, for example, subcutaneously by infection in order to deliver siRNA to keratinocytes in the skin. In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. In addition, due to the lipid properties, these transferosomes can be self-optimizing (adaptive to the shape of pores, *e.g.*, in the skin), self-repairing, and can frequently reach their targets without fragmenting, and often self-loading.

Other formulations amenable to the present invention are described in United States provisional application serial nos. 61/018,616, filed January 2, 2008; 61/018,611, filed January 2, 2008; 61/039,748, filed March 26, 2008; 61/047,087, filed April 22, 2008 and 61/051,528, filed May 8, 2008. PCT application no PCT/US2007/080331, filed October 3, 2007 also describes formulations that are amenable to the present invention.

Surfactants. For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to unmodified siRNA compounds. It may be understood, however, that these formulations, compositions and methods can be practiced with other siRNA compounds, *e.g.*, modified siRNA compounds, and such practice is within the scope of the invention. Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes (see above). siRNA (or a precursor, *e.g.*, a larger dsiRNA which can be processed into a siRNA, or a DNA which encodes a siRNA or precursor) compositions can include a surfactant. In one embodiment, the siRNA is formulated as an emulsion that includes a surfactant. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in "Pharmaceutical Dosage Forms," Marcel Dekker, Inc., New York, NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in "Pharmaceutical Dosage Forms," Marcel Dekker, Inc., New York, NY, 1988, p. 285).

Micelles and other Membranous Formulations. For ease of exposition the micelles and other formulations, compositions and methods in this section are discussed largely with regard to unmodified siRNA compounds. It may be understood, however, that these micelles and other formulations, compositions and methods can be practiced with other siRNA compounds, *e.g.*, modified siRNA compounds, and such practice is within the invention. The siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof)) composition can be provided as a micellar formulation. "Micelles" are defined herein as a particular type of molecular assembly in which amphipathic molecules are arranged in a spherical structure such that all the hydrophobic portions of the molecules are directed inward, leaving the hydrophilic portions in contact with the surrounding aqueous phase. The converse arrangement exists if the environment is hydrophobic.

A mixed micellar formulation suitable for delivery through transdermal membranes may be prepared by mixing an aqueous solution of the siRNA composition, an alkali metal C_8 to C_{22} alkyl sulphate, and a micelle forming compounds. Exemplary micelle forming compounds include lecithin, hyaluronic acid, pharmaceutically acceptable salts of hyaluronic acid, glycolic acid, lactic acid, chamomile extract, cucumber extract, oleic acid, linoleic acid, linolenic acid, monoolein, monooleates, monolaurates, borage oil, evening of primrose oil, menthol, trihydroxy oxo cholanyl

glycine and pharmaceutically acceptable salts thereof, glycerin, polyglycerin, lysine, polylysine, triolein, polyoxyethylene ethers and analogues thereof, polidocanol alkyl ethers and analogues thereof, chenodeoxycholate, deoxycholate, and mixtures thereof. The micelle forming compounds may be added at the same time or after addition of the alkali metal alkyl sulphate. Mixed micelles will form with substantially any kind of mixing of the ingredients but vigorous mixing in order to provide smaller size micelles.

In one method a first micellar composition is prepared which contains the siRNA composition and at least the alkali metal alkyl sulphate. The first micellar composition is then mixed with at least three micelle forming compounds to form a mixed micellar composition. In another method, the micellar composition is prepared by mixing the siRNA composition, the alkali metal alkyl sulphate and at least one of the micelle forming compounds, followed by addition of the remaining micelle forming compounds, with vigorous mixing.

Phenol and/or m-cresol may be added to the mixed micellar composition to stabilize the formulation and protect against bacterial growth. Alternatively, phenol and/or m-cresol may be added with the micelle forming ingredients. An isotonic agent such as glycerin may also be added after formation of the mixed micellar composition.

For delivery of the micellar formulation as a spray, the formulation can be put into an aerosol dispenser and the dispenser is charged with a propellant. The propellant, which is under pressure, is in liquid form in the dispenser. The ratios of the ingredients are adjusted so that the aqueous and propellant phases become one, *i.e.,* there is one phase. If there are two phases, it is necessary to shake the dispenser prior to dispensing a portion of the contents, *e.g.,* through a metered valve. The dispensed dose of pharmaceutical agent is propelled from the metered valve in a fine spray.

Propellants may include hydrogen-containing chlorofluorocarbons, hydrogen-containing fluorocarbons, dimethyl ether and diethyl ether. In certain embodiments, HFA 134a (1,1,1,2 tetrafluoroethane) may be used.

The specific concentrations of the essential ingredients can be determined by relatively straightforward experimentation. For absorption through the oral cavities, it is often desirable to increase, *e.g.,* at least double or triple, the dosage for through injection or administration through the gastrointestinal tract.

Particles. For ease of exposition the particles, formulations, compositions and methods in this section are discussed largely with regard to modified siRNA compounds. It may be understood, however, that these particles, formulations, compositions and methods can be practiced with other siRNA compounds, *e.g.*, unmodified siRNA compounds, and such practice is within the invention. In another embodiment, an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof) preparations may be incorporated into a particle, *e.g.*, a microparticle. Microparticles can be produced by spray-drying, but may also be produced by other methods including lyophilization, evaporation, fluid bed drying, vacuum drying, or a combination of these techniques.

Pharmaceutical compositions

The iRNA agents of the invention may be formulated for pharmaceutical use. Pharmaceutically acceptable compositions comprise a therapeutically-effective amount of one or more of the the dsRNA agents in any of the preceding embodiments, taken alone or formulated together with one or more pharmaceutically acceptable carriers (additives), excipient and/or diluents.

The pharmaceutical compositions may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, e.g., those targeted for buccal, sublingual, and systemic absorption, boluses, powders, granules, pastes for application to the tongue; (2) parenteral administration, for example, by subcutaneous, intramuscular, intravenous or epidural injection as, for example, a sterile solution or suspension, or sustained-release formulation; (3) topical application, for example, as a cream, ointment, or a controlled-release patch or spray applied to the skin; (4) intravaginally or intrarectally, for example, as a pessary, cream or foam; (5) sublingually; (6) ocularly; (7) transdermally; or (8) nasally. Delivery using subcutaneous or intravenous methods can be particularly advantageous.

The phrase "therapeutically-effective amount" as used herein means that amount of a compound, material, or composition comprising a compound of the invention which

is effective for producing some desired therapeutic effect in at least a sub-population of cells in an animal at a reasonable benefit/risk ratio applicable to any medical treatment.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically-acceptable carrier" as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, manufacturing aid (e.g., lubricant, talc magnesium, calcium or zinc stearate, or steric acid), or solvent encapsulating material, involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) lubricating agents, such as magnesium state, sodium lauryl sulfate and talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates and/or polyanhydrides; (22) bulking agents, such as polypeptides and amino acids (23) serum component, such as serum albumin, HDL and LDL; and (22) other non-toxic compatible substances employed in pharmaceutical formulations.

The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active

ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 0.1 per cent to about ninety-nine percent of active ingredient, preferably from about 5 per cent to about 70 per cent, most preferably from about 10 per cent to about 30 per cent.

In certain embodiments, a formulation of the present invention comprises an excipient selected from the group consisting of cyclodextrins, celluloses, liposomes, micelle forming agents, e.g., bile acids, and polymeric carriers, e.g., polyesters and polyanhydrides; and a compound of the present invention. In certain embodiments, an aforementioned formulation renders orally bioavailable a compound of the present invention.

iRNA agent preparation can be formulated in combination with another agent, *e.g.*, another therapeutic agent or an agent that stabilizes a iRNA, *e.g.*, a protein that complexes with iRNA to form an iRNP. Still other agents include chelators, *e.g.*, EDTA (*e.g.*, to remove divalent cations such as Mg^{2+}), salts, RNAse inhibitors (*e.g.*, a broad specificity RNAse inhibitor such as RNAsin) and so forth.

Methods of preparing these formulations or compositions include the step of bringing into association a compound of the present invention with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a compound of the present invention with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form.

Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

The compounds according to the invention may be formulated for administration in any convenient way for use in human or veterinary medicine, by analogy with other pharmaceuticals.

The term "treatment" is intended to encompass also prophylaxis, therapy and cure. The patient receiving this treatment is any animal in need, including primates, in particular humans, and other mammals such as equines, cattle, swine and sheep; and poultry and pets in general.

Double-stranded RNAi agents are produced in a cell *in vivo*, *e.g.*, from exogenous DNA templates that are delivered into the cell. For example, the DNA templates can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (U.S. Pat. No. 5,328,470), or by stereotactic injection (see, *e.g.*, Chen *et al.* (1994) Proc. Natl. Acad. Sci. USA 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. The DNA templates, for example, can include two transcription units, one that produces a transcript that includes the top strand of a dsRNA agent and one that produces a transcript that includes the bottom strand of a dsRNA agent. When the templates are transcribed, the dsRNA agent is produced, and processed into siRNA agent fragments that mediate gene silencing.

Routes of Delivery

A composition that includes an iRNA can be delivered to a subject by a variety of routes. Exemplary routes include: intravenous, subcutaneous, topical, rectal, anal, vaginal, nasal, pulmonary, ocular.

The iRNA molecules of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically include one or more species of iRNA and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and

absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

The compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, or intrathecal or intraventricular administration.

The route and site of administration may be chosen to enhance targeting. For example, to target muscle cells, intramuscular injection into the muscles of interest would be a logical choice. Lung cells might be targeted by administering the iRNA in aerosol form. The vascular endothelial cells could be targeted by coating a balloon catheter with the iRNA and mechanically introducing the DNA.

Dosage

In one aspect, the invention features a method of administering a dsRNA agent, *e.g.*, a siRNA agent, to a subject (*e.g.*, a human subject). The method includes administering a unit dose of the dsRNA agent, *e.g.*, a siRNA agent, *e.g.*, double stranded siRNA agent that (a) the double-stranded part is 14-30 nucleotides (nt) long, for example, 21-23 nt, (b) is complementary to a target RNA (*e.g.*, an endogenous or pathogen target RNA), and, optionally, (c) includes at least one 3' overhang 1-5 nucleotide long. In one embodiment, the unit dose is less than 10 mg per kg of bodyweight, or less than 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005 or 0.00001 mg per kg of bodyweight, and less than 200 nmole of RNA agent (*e.g.,* about 4.4×10^{16} copies) per kg of bodyweight, or less than 1500, 750, 300, 150, 75, 15, 7.5, 1.5, 0.75, 0.15, 0.075, 0.015, 0.0075, 0.0015, 0.00075, 0.00015 nmole of RNA agent per kg of bodyweight.

The defined amount can be an amount effective to treat or prevent a disease or disorder, *e.g.*, a disease or disorder associated with the target RNA. The unit dose, for example, can be administered by injection (*e.g.*, intravenous, subcutaneous or

intramuscular), an inhaled dose, or a topical application. In some ebmodiments dosages may be less than 10, 5, 2, 1, or 0.1 mg/kg of body weight.

In some embodiments, the unit dose is administered less frequently than once a day, *e.g.*, less than every 2, 4, 8 or 30 days. In another embodiment, the unit dose is not administered with a frequency (*e.g.*, not a regular frequency). For example, the unit dose may be administered a single time.

In one embodiment, the effective dose is administered with other traditional therapeutic modalities. In one embodiment, the subject has a viral infection and the modality is an antiviral agent other than a dsRNA agent, *e.g.*, other than a siRNA agent. In another embodiment, the subject has atherosclerosis and the effective dose of a dsRNA agent, *e.g.*, a siRNA agent, is administered in combination with, *e.g.*, after surgical intervention, *e.g.*, angioplasty.

In one embodiment, a subject is administered an initial dose and one or more maintenance doses of a dsRNA agent, *e.g.*, a siRNA agent, (*e.g.*, a precursor, *e.g.*, a larger dsRNA agent which can be processed into a siRNA agent, or a DNA which encodes a dsRNA agent, *e.g.*, a siRNA agent, or precursor thereof). The maintenance dose or doses can be the same or lower than the initial dose, *e.g.*, one-half less of the initial dose. A maintenance regimen can include treating the subject with a dose or doses ranging from 0.01 μg to 15 mg/kg of body weight per day, *e.g.*, 10, 1, 0.1, 0.01, 0.001, or 0.00001 mg per kg of bodyweight per day. The maintenance doses are, for example, administered no more than once every 2, 5, 10, or 30 days. Further, the treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease, its severity and the overall condition of the patient. In certain embodiments the dosage may be delivered no more than once per day, e.g., no more than once per 24, 36, 48, or more hours, e.g., no more than once for every 5 or 8 days. Following treatment, the patient can be monitored for changes in his condition and for alleviation of the symptoms of the disease state. The dosage of the compound may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disease state is observed, if the disease state has been ablated, or if undesired side-effects are observed.

The effective dose can be administered in a single dose or in two or more doses, as desired or considered appropriate under the specific circumstances. If desired to facilitate repeated or frequent infusions, implantation of a delivery device, e.g., a pump, semi-permanent stent (e.g., intravenous, intraperitoneal, intracisternal or intracapsular), or reservoir may be advisable.

In one embodiment, the composition includes a plurality of dsRNA agent species. In another embodiment, the dsRNA agent species has sequences that are non-overlapping and non-adjacent to another species with respect to a naturally occurring target sequence. In another embodiment, the plurality of dsRNA agent species is specific for different naturally occurring target genes. In another embodiment, the dsRNA agent is allele specific.

The dsRNA agents of the invention described herein can be administered to mammals, particularly large mammals such as nonhuman primates or humans in a number of ways.

In one embodiment, the administration of the dsRNA agent, *e.g.*, a siRNA agent, composition is parenteral, *e.g.,* intravenous (*e.g.*, as a bolus or as a diffusible infusion), intradermal, intraperitoneal, intramuscular, intrathecal, intraventricular, intracranial, subcutaneous, transmucosal, buccal, sublingual, endoscopic, rectal, oral, vaginal, topical, pulmonary, intranasal, urethral or ocular. Administration can be provided by the subject or by another person, *e.g.*, a health care provider. The medication can be provided in measured doses or in a dispenser which delivers a metered dose. Selected modes of delivery are discussed in more detail below.

The invention provides methods, compositions, and kits, for rectal administration or delivery of dsRNA agents described herein

Methods of inhibiting expression of the target gene

Embodiments of the invention also relate to methods for inhibiting the expression of a target gene. The method comprises the step of administering the dsRNA agents in any of the preceding embodiments, in an amount sufficient to inhibit expression of the target gene.

Another aspect the invention relates to a method of modulating the expression of a target gene in a cell, comprising providing to said cell a dsRNA agent of this invention.

In one embodiment, the target gene is selected from the group consisting of Factor VII, Eg5, PCSK9, TPX2, apoB, SAA, TTR, RSV, PDGF beta gene, Erb-B gene, Src gene, CRK gene, GRB2 gene, RAS gene, MEKK gene, JNK gene, RAF gene, Erk1/2 gene, PCNA(p21) gene, MYB gene, JUN gene, FOS gene, BCL-2 gene, hepciden, Activated Protein C, Cyclin D gene, VEGF gene, EGFR gene, Cyclin A gene, Cyclin E gene, WNT-1 gene, beta-catenin gene, c-MET gene, PKC gene, NFKB gene, STAT3 gene, survivin gene, Her2/Neu gene, topoisomerase I gene, topoisomerase II alpha gene, mutations in the p73 gene, mutations in the p21(WAF1/CIP1) gene, mutations in the p27(KIP1) gene, mutations in the PPM1D gene, mutations in the RAS gene, mutations in the caveolin I gene, mutations in the MIB I gene, mutations in the MTAI gene, mutations in the M68 gene, mutations in tumor suppressor genes, and mutations in the p53 tumor suppressor gene.

The invention is further illustrated by the following examples, which should not be construed as further limiting. The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference.

EXAMPLES

Example 1. *In vitro* screening of siRNA duplexes

Cell culture and transfections:

Human Hep3B cells or rat H.II.4.E cells (ATCC, Manassas, VA) were grown to near confluence at 37 °C in an atmosphere of 5% CO_2 in RPMI (ATCC) supplemented with 10% FBS, streptomycin, and glutamine (ATCC) before being released from the plate by trypsinization. Transfection was carried out by adding 14.8 µl of Opti-MEM plus 0.2 µl of Lipofectamine RNAiMax per well (Invitrogen, Carlsbad CA. cat # 13778-150) to 5 µl of siRNA duplexes per well into a 96-well plate and incubated at room temperature for 15 minutes. 80 µl of complete growth media without antibiotic containing ~2 x10^4 Hep3B cells were then added to the siRNA mixture. Cells were incubated for either 24 or 120 hours prior to RNA purification. Single dose experiments were performed at 10nM and 0.1nM final duplex concentration and dose response

experiments were done using 8, 4 fold serial dilutions with a maximum dose of 10nM final duplex concentration.

Total RNA isolation using DYNABEADS mRNA Isolation Kit (Invitrogen, part #: 610-12):

Cells were harvested and lysed in 150 µl of Lysis/Binding Buffer then mixed for 5 minute at 850rpm using an Eppendorf Thermomixer (the mixing speed was the same throughout the process). Ten microliters of magnetic beads and 80 µl Lysis/Binding Buffer mixture were added to a round bottom plate and mixed for 1 minute. Magnetic beads were captured using magnetic stand and the supernatant was removed without disturbing the beads. After removing supernatant, the lysed cells were added to the remaining beads and mixed for 5 minutes. After removing supernatant, magnetic beads were washed 2 times with 150 µl Wash Buffer A and mixed for 1 minute. Beads were capture again and supernatant removed. Beads were then washed with 150 µl Wash Buffer B, captured and supernatant was removed. Beads were next washed with 150 µl Elution Buffer, captured and supernatant removed. Beads were allowed to dry for 2 minutes. After drying, 50 µl of Elution Buffer was added and mixed for 5 minutes at 70°C. Beads were captured on magnet for 5 minutes. 40 µl of supernatant was removed and added to another 96 well plate.

cDNA synthesis using ABI High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, Cat #4368813):

A master mix of 1 µl 10X Buffer, 0.4µl 25X dNTPs, 1µl Random primers, 0.5 µl Reverse Transcriptase, 0.5 µl RNase inhibitor and 1.6µl of H_2O per reaction were added into 5 µl total RNA. cDNA was generated using a Bio-Rad C-1000 or S-1000 thermal cycler (Hercules, CA) through the following steps: 25 °C 10 min, 37 °C 120 min, 85 °C 5 sec, 4 °C hold.

Real time PCR:

2µl of cDNA were added to a master mix containing 0.5µl GAPDH TaqMan Probe (Applied Biosystems Cat #4326317E (human) Cat # 4308313 (rodent)), 0.5µl TTR

TaqMan probe (Applied Biosystems cat # HS00174914 _m1 (human) cat # Rn00562124_m1 (rat)) and 5µl Lightcycler 480 probe master mix (Roche Cat #04887301001) per well in a 384 well plate (Roche cat # 04887301001). Real time PCR was done in a Roche LC 480 Real Time PCR machine (Roche). Each duplex was tested in at least two independent transfections and each transfection was assayed in duplicate, unless otherwise noted.

To calculate relative fold change, real time data were analyzed using the ΔΔCt method and normalized to assays performed with cells transfected with 10nM AD-1955, or mock transfected cells. IC_{50}s were calculated using a 4 parameter fit model using XLFit and normalized to cells transfected with AD-1955 or naïve cells over the same dose range, or to its own lowest dose. IC_{50}s were calculated for each individual transfection as well as in combination, where a single IC_{50} was fit to the data from both transfections.

The results of gene silencing of the exemplary siRNA duplex with various motif modifications of the invention are shown in the table below.

Example 2. RNA Synthesis and Duplex Annealing

1. Oligonucleotide Synthesis:

All oligonucleotides were synthesized on an AKTAoligopilot synthesizer or an ABI 394 synthsizer. Commercially available controlled pore glass solid support (dT-CPG, 500Å, Prime Synthesis) and RNA phosphoramidites with standard protecting groups, 5'-*O*-dimethoxytrityl N6-benzoyl-2'-*t*-butyldimethylsilyl-adenosine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-dimethoxytrityl-N4-acetyl-2'-*t*-butyldimethylsilyl-cytidine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-dimethoxytrityl-N2--isobutryl-2'-*t*-butyldimethylsilyl-guanosine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, and 5'-*O*-dimethoxytrityl-2'-*t*-butyldimethylsilyl-uridine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite (Pierce Nucleic Acids Technologies) were used for the oligonucleotide synthesis unless otherwise specified. The 2'-F phosphoramidites, 5'-*O*-dimethoxytrityl-N4-acetyl-2'-fluro-cytidine-3'-*O*-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite and 5'-*O*-dimethoxytrityl-2'-fluro-uridine-3'-O-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite were purchased from (Promega). All phosphoramidites were used at a concentration of 0.2M in acetonitrile (CH_3CN) except for guanosine which was used at 0.2M concentration in 10% THF/ANC (v/v). Coupling/recycling time of 16 minutes was used. The activator was 5-ethyl thiotetrazole (0.75M, American International Chemicals), for the PO-oxidation Iodine/Water/Pyridine was used and the PS-oxidation PADS (2 %) in 2,6-lutidine/ACN (1:1 v/v) was used. .

Ligand conjugated strands were synthesized using solid support containing the corresponding ligand. For example, the introduction of carbohydrate moiety/ligand (for e.g., GalNAc) at the 3'-end of a sequence was achieved by starting the synthesis with the corresponding carbohydrate solid support. Similarly a cholesterol moiety at the 3'-end was introduced by starting the synthesis on the cholesterol support. In general, the ligand moiety was tethered to *trans*-4-hydroxyprolinol via a tether of choice as described in the previous examples to obtain a hydroxyprolinol-ligand moiety. The hydroxyprolinol-ligand moiety was then coupled to a solid support via a succinate linker or was converted to phosphoramidite via standard phosphitylation conditions to obtain the desired carbohydrate conjugate building blocks. Fluorophore labeled siRNAs were synthesized

from the corresponding phosphoramidite or solid support, purchased from Biosearch Technologies. The oleyl lithocholic $(GalNAc)_3$ polymer support made in house at a loading of 38.6 μmol/gram. The Mannose $(Man)_3$ polymer support was also made in house at a loading of 42.0 μmol/gram.

Conjugation of the ligand of choice at desired position, for example at the 5'-end of the sequence, was achieved by coupling of the corresponding phosphoramidite to the growing chain under standard phosphoramidite coupling conditions unless otherwise specified. An extended 15 min coupling of 0.1M solution of phosphoramidite in anhydrous CH_3CN in the presence of 5-(ethylthio)-1*H*-tetrazole activator to a solid bound oligonucleotide. Oxidation of the internucleotide phosphite to the phosphate was carried out using standard iodine-water as reported (1) or by treatment with *tert*-butyl hydroperoxide/acetonitrile/water (10: 87: 3) with 10 min oxidation wait time conjugated oligonucleotide. Phosphorothioate was introduced by the oxidation of phosphite to phosphorothioate by using a sulfur transfer reagent such as DDTT (purchased from AM Chemicals), PADS and or Beaucage reagent The cholesterol phosphoramidite was synthesized in house, and used at a concentration of 0.1 M in dichloromethane. Coupling time for the cholesterol phosphoramidite was 16 minutes.

2. Deprotection- I (Nucleobase Deprotection)

After completion of synthesis, the support was transferred to a 100 ml glass bottle (VWR). The oligonucleotide was cleaved from the support with simultaneous deprotection of base and phosphate groups with 80 mL of a mixture of ethanolic ammonia [ammonia: ethanol (3:1)] for 6.5h at 55°C. The bottle was cooled briefly on ice and then the ethanolic ammonia mixture was filtered into a new 250 ml bottle. The CPG was washed with 2 x 40 mL portions of ethanol/water (1:1 v/v). The volume of the mixture was then reduced to ~ 30 ml by roto-vap. The mixture was then frozen on dry ice and dried under vacuum on a speed vac.

3. Deprotection-II (Removal of 2' TBDMS group)

The dried residue was resuspended in 26 ml of triethylamine, triethylamine trihydrofluoride (TEA.3HF) or pyridine-HF and DMSO (3:4:6) and heated at 60°C for 90 minutes to remove the *tert*-butyldimethylsilyl (TBDMS) groups at the 2' position. The

reaction was then quenched with 50 ml of 20mM sodium acetate and pH adjusted to 6.5, and stored in freezer until purification.

4. Analysis

The oligoncuelotides were analyzed by high-performance liquid chromatography (HPLC) prior to purification and selection of buffer and column depends on nature of the sequence and or conjugated ligand.

5. HPLC Purification

The ligand conjugated oligonucleotides were purified reverse phase preparative HPLC. The unconjugated oligonucleotides were purified by anion-exchange HPLC on a TSK gel column packed in house. The buffers were 20 mM sodium phosphate (pH 8.5) in 10% CH_3CN (buffer A) and 20 mM sodium phosphate (pH 8.5) in 10% CH_3CN, 1M NaBr (buffer B). Fractions containing full-length oligonucleotides were pooled, desalted, and lyophilized. Approximately 0.15 OD of desalted oligonucleotidess were diluted in water to 150 µl and then pipetted in special vials for CGE and LC/MS analysis. Compounds were finally analyzed by LC-ESMS and CGE.

6. siRNA preparation

For the preparation of siRNA, equimolar amounts of sense and antisense strand were heated in 1xPBS at 95°C for 5 min and slowly cooled to room temperature. Integrity of the duplex was confirmed by HPLC analysis.

Table 2. ANGPTL3 modified duplex

Duplex ID	S ID	Sense strand (S)	AS ID	Antisense strand (AS)	% of mRNA remained conc. of siRNA			IC50 (nM)
					1 nM	0.1 nM	0.01 nM	
D1000	S1000	AfuGfuAfaCfcAfAfGfaGfuAfuUfcCfasu	AS1000	AfUfgGfaAfuAfcUfcuuGfgUfuAfcAfusGfsa	0.03	0.1	0.47	0.006
D1001	S1001	AfsuGfuAfaCfcAfAfGfaGfuAfuucCfasUf	AS1001	aUfsgGfAfAfuAfcUfcuuGfgUfuAfcAfusGfsa	0.03	0.10	0.49	0.0065
D1002	S1002	AfuGfuAfaCfcAfAfGfaGfuAfuucCfasUf	AS1002	aUfgGfAfAfuAfcUfcuuGfgsUfuAfcAfusGfsa	0.04	0.10	0.46	0.0068
D1003	S1003	AfuGfuAfaCfcAfAfGfaGfuAfuucCfasUf	AS1003	aUfgGfAfAfuAfcUfcuuGfgUfsuAfcAfusGfsa	0.05	0.12	0.56	0.0073
D1004	S1004	aUGuaACccAGagUAuuCCasu	AS1004	AUggAAuaCUcuUGguUAcaUsGsa	0.07	0.13	0.44	0.008
D1005	S1005	AfuGfuAfaCfcAfAfGfaGfuAfuucCfasUf	AS1005	aUfgGfAfAfuAfcUfcuuGfgsUfsuAfcAfusGfsa	0.06	0.11	0.53	0.0093
D1006	S1006	AfuGfuAfAfccAfAfGfaGfuAfuUfcCfasUf	AS1006	aUfgGfaAfuAfcUfcuuGfGfuuAfcAfusGfsa	0.05	0.16	0.55	0.0095
D1007	S1007	AfuGfuAfAfCfcAfAfGfaGfuAfuUfcCfasUf	AS1007	aUfgGfaAfuAfcUfcuuGfguuAfcAfusGfsa	0.05	0.14	0.48	0.0098
D1008	S1008	auguaaccaadGadGudAudAcdGasu	AS1008	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.07	0.11	0.33	0.010
D1009	S1009	UfgGfGfAfuUfuCfAfUfgUfaAfcCfAfAfgsAf	AS1009	uCfuugGfuUfaCfaugAfaAfuccCfasUfsc	0.03	0.14	0.56	0.0101
D1010	S1010	UfgGfgauUfuCfAfUfgUfaAfcCfaAfgsAf	AS1010	uCfuUfgGfuUfaCfaugAfaAfUfCfcCfasUfsc	0.03	0.14	0.65	0.0101
D1011	S1011	aUfGfuAfAfccAfAfGfaGfuAfuUfcCfasUf	AS1011	aUfgGfaAfuAfcUfcuuGfGfuuAfcaUfsgsa	0.06	0.10	0.55	0.011
D1012	S1012	UfgGfgAfuUfuCfAfUfgUfaacCfaAfgsAf	AS1012	uCfuUfgGfUfUfaCfaugAfaAfuCfcCfasUfsc	0.04	0.13	0.54	0.0114
D1013	S1013	auguaaccaadGadGudAudAcdGasu	AS1013	aUfgGfaAfuAfcUfcUfugdGudTadCadTsgsa	0.11	0.19	0.49	0.011
D1014	S1014	AfuGfuaaCfcAfAfGfaGfuAfuUfcCfasUf	AS1014	aUfgGfaAfuAfcUfcuuGfgUfUfAfcAfusGfsa	0.04	0.16	0.59	0.013
D1015	S1015	AfuguAfaccAfaGfdAGfdTAdTudCcdAsu	AS1015	dAUdGgdAadTAfdCUfcUfuGfgUfuAfcAfusGfsa	0.07	0.15	0.51	0.013
D1016	S1016	auGfuAfaCfcAfAfGfaGfuAfuUfcCfasUf	AS1016	aUfgGfaAfuAfcUfcuuGfgUfuAfcAfUfsGfsa	0.05	0.14	0.64	0.013
D1017	S1017	UfGfggAfuUfuCfAfUfgUfAfAfcCfaAfgsAf	AS1017	uCfuUfgGfuuaCfaugAfaAfuCfCfcasUfsc	0.09	0.41	0.74	0.0133
D1018	S1018	AfuguAfaCfcAfAfGfaGfuAfuUfcCfasUf	AS1018	aUfgGfaAfuAfcUfcuuGfgUfuAfCfAfusGfsa	0.03	0.14	0.61	0.014
D1019	S1019	AfuGfuAfaccAfAfGfaGfuAfuUfcCfasUf	AS1019	aUfgGfaAfuAfcUfcuuGfGfUfuAfcAfusGfsa	0.02	0.2	0.7	0.014
D1020	S1020	AfsuGfuAfaCfcAfAfGfaGfuAfuucCfasUf	AS1020	asUfsgGfAfAfuAfcUfcuuGfgUfuAfcAfusGfsa	0.04	0.16	0.67	0.0156
D1021	S1021	aUfguAfAfccAfAfgagUfaUfuCfcasUf	AS1021	aUfGfgAfaUfaCfUfCfuuGfGfuuAfCfaUfsgsa	0.11	0.24	0.64	0.016

D1022	S1022	dTdGggdAdTuudCdAugdTdAacdCdAagsdA	AS1022	udCdTugdGdTuadCdAugdAdAaudCdCcasdTsc	0.08	0.27	0.64	0.0161
D1023	S1023	AfsuGfuAfaCfcAfAfGfaGfuAfuucCfasUf	AS1023	aUfgsGfAfAfuAfcUfcuuGfgUfuAfcAfusGfsa	0.03	0.19	0.63	0.0163
D1024	S1024	UfgGfgAfuUfuCfAfUfguaAfcCfaAfgsAf	AS1024	uCfuUfgGfuUfAfCfaugAfaAfuCfcCfasUfsc	0.05	0.25	0.69	0.0164
D1025	S1025	UfgGfgAfuUfuCfAfUfgUfAfAfcCfaAfgsAf	AS1025	uCfuUfgGfuuaCfaugAfaAfuCfcCfasUfsc	0.04	0.18	0.75	0.0166
D1026	S1026	UfgGfgAfuUfuCfAfUfgUfaAfcCfaAfgsAf	AS1026	uCfuUfgGfuUfaCfaugAfaAfuCfcCfasUfsc	0.04	0.19	0.66	0.0178
D1027	S1027	UfgGfgAfuUfuCfAfUfgUfaAfccaAfgsAf	AS1027	uCfuUfGfGfuUfaCfaugAfaAfuCfcCfasUfsc	0.04	0.19	0.69	0.018
D1028	S1028	dAdTgudAdAccdAdAgadGdTaudTdCcasdT	AS1028	adTdGgadAdTacdTdCuudGdGuudAdCausdGsa	0.15	0.29	0.72	0.018
D1029	S1029	AdTGdTAdACdCAdAGdAGdTAdTUdCCdAsU	AS1029	dAUdGGdAAdTAdCUdCUdTGdGUdTAdCAdTsGsdA	0.1	0.27	0.61	0.018
D1030	S1030	UfgGfGfAfuuuCfAfUfgUfaAfcCfaAfgsAf	AS1030	uCfuUfgGfuUfaCfaugAfAfAfuccCfasUfsc	0.04	0.21	0.64	0.0187
D1031	S1031	AfuGfuAfAfccAfAfGfAfGfuAfuuccAfsu	AS1031	AfUfGfGfAfAfuAfCfUfCfUfuGfGfuuAfcAfusGfsa	0.06	0.15	0.62	0.019
D1032	S1032	AfsuGfuAfaCfcAfAfGfaGfuAfuucCfasUf	AS1032	asUfgGfAfAfuAfcUfcuuGfgUfsuAfcAfusGfsa	0.09	0.34	0.78	0.021
D1033	S1033	UfgGfgAfuUfuCfaUfGfUfaacCfaAfgsAf	AS1033	uCfuUfgGfUfUfacaUfgAfaAfuCfcCfasUfsc	0.06	0.26	0.57	0.0212
D1034	S1034	AfuGfuAfAfccAfaGfaGfuAfuUfcCfasUf	AS1034	aUfgGfaAfuAfcUfcUfuGfGfuuAfcAfusGfsa	0.11	0.39	0.82	0.0216
D1035	S1035	UfgGfgAfuuuCfAfUfgUfaAfcCfaAfgsAf	AS1035	uCfuUfgGfuUfaCfaugAfAfAfuCfcCfasUfsc	0.04	0.16	0.56	0.0222
D1036	S1036	UfgGfGfAfuUfuCfaUfgUfaAfcCfAfAfgsAf	AS1036	uCfuugGfuUfaCfaUfgAfaAfuccCfasUfsc	0.06	0.31	0.78	0.0234
D1037	S1037	UfgGfGfAfuUfuCfAfUfgUfaAfcCfaAfgsAf	AS1037	uCfuUfgGfuUfaCfaugAfaAfuccCfasUfsc	0.03	0.14	0.62	0.0235
D1038	S1038	UfGfggAfUfuuCfAfugUfAfacCfAfagsAf	AS1038	uCfUfugGfUfuaCfAfugAfAfauCfCfcasUfsc	0.09	0.39	0.78	0.0239
D1039	S1039	AfuGfuAfaCfcAfAfGfaGfuAfuucCfasUf	AS1039	aUfgGfAfAfuAfcUfcuuGfgUfuAfcAfusGfsa	0.03	0.14	0.59	0.025
D1040	S1040	AfuGfuAfaCfcAfAfGfaGfuAfuUfccasUf	AS1040	aUfGfGfaAfuAfcUfcuuGfgUfuAfcAfusGfsa	0.03	0.13	0.56	0.025
D1041	S1041	AfsuGfuAfaCfcAfAfGfaGfuAfuucCfasUf	AS1041	asUfgGfAfAfuAfcUfcuuGfgUfuAfcAfusGfsa	0.06	0.27	0.79	0.0252
D1042	S1042	UfgGfgAfuuuCfAfUfgUfAfAfcCfaAfgsAf	AS1042	uCfuUfgGfuuaCfaugAfAfAfuCfcCfasUfsc	0.05	0.27	0.67	0.0259
D1043	S1043	AfuGfuAfaCfcAfAfGfaGfuauUfcCfasUf	AS1043	aUfgGfaAfUfAfcUfcuuGfgUfuAfcAfusGfsa	0.02	0.16	0.63	0.027
D1044	S1044	AfsuGfuAfaCfcAfAfGfaGfuAfuucCfasUf	AS1044	asUfgGfAfAfuAfcUfcuuGfgsUfsuAfcAfusGfsa	0.06	0.30	0.81	0.0271
D1045	S1045	aUfguAfAfccAfAfgaGfGfauUfCfcasUf	AS1045	aUfGfgaAfUfacUfCfuuGfGfuuAfCfaUfsgsa	0.12	0.29	0.8	0.028
D1046	S1046	AfuGfuAfaCfcAfAfGfaguAfuUfcCfasUf	AS1046	aUfgGfaAfuAfCfUfcuuGfgUfuAfcAfusGfsa	0.03	0.15	0.59	0.030
D1047	S1047	UfgGfGfAfuUfuCfaUfgUfAfAfcCfaAfgsAf	AS1047	uCfuUfgGfuuaCfaUfgAfaAfuccCfasUfsc	0.08	0.44	0.83	0.0324
D1048	S1048	AfuGfuAfaCfcAfAfGfaGfuAfuUfcCfasUf	AS1048	aUfgGfaAfuAfcUfcuuGfgUfuAfcAfusGfsa	0.07	0.23	0.67	0.036
D1049	S1049	AfuGfuAfAfccAfAfGfAfGfuAfuuccAfsu	AS1049	AfUfGfGfAfAfuAfCfUfCfUfUfGfGfUfuAfCfAfusGfsa	0.08	0.23	0.73	0.037

D1050	S1050	UfgGfgAfuuuCfaUfgUfaAfcCfAfAfgsAf	AS1050	uCfuugGfuUfaCfaUfgAfAfAfuCfcCfasUfsc	0.06	0.29	0.78	0.0372
D1051	S1051	AfuGfuAfaccaagaguAfuUfcCfasUf	AS1051	aUfgGfaAfudAcdTcdTudGgdTuAfcAfusgsa	0.12	0.41	0.86	0.040
D1052	S1052	AfuguAfaccAfaGfdAGfdTAdTUdCcdAsu	AS1052	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.1	0.22	0.72	0.042
D1053	S1053	AfuguAfaccAfaGfdAGfdTAdTUdCcdAsu	AS1053	dAUdGGdAAfuAfcUfcUfuGfGfUfuAfCfAfusGfsa	0.09	0.31	0.69	0.044
D1054	S1054	AfuGfuAfaCfcAfaGfadGdTAfuUfcdCdAsUf	AS1054	adTdGGfaAfudAdCUfcUfuGfgUfuAfcAfusGfsa	0.1	0.45	0.75	0.047
D1055	S1055	AfuguAfaccAfaGfaGfdTAdTUdCcdAsu	AS1055	dAUdGGdAadTAfcUfcUfuGfgUfuAfcAfusGfsa	0.12	0.26	0.7	0.049
D1056	S1056	AuGuAaCcAaGaGuAuUcCasU	AS1056	aUgGaAuAcUcUuGgUuAcAusGsa	0.08	0.24	0.65	0.050
D1057	S1057	AfuguAfaccAfagaGfuauUfccasUf	AS1057	aUfGfGfaAfUfAfcUfCfUfuGfGfUfuAfCfAfusGfsa	0.14	0.42	0.62	0.051
D1058	S1058	AfuGfuAfaccaagaguAfuUfcCfasUf	AS1058	aUfgGfaAfudAcdTcdTudGgdTuAfcAfusGfsa	0.12	0.36	0.86	0.053
D1059	S1059	AfuguAfaccAfaGfdAGfdTAdTUdCcdAsu	AS1059	dAUdGGdAadTAfdCUfcUfuGfgUfuAfcAfusGfsa	0.09	0.27	0.7	0.054
D1060	S1060	adTgudAdAccdAdAgagdTadTudCcasdT	AS1060	adTdGgdAadTadCdTdCuudGdGuudAdCadTsgsa	0.11	0.37	0.66	0.056
D1061	S1061	AfuGfuAfaCfcAfaGfdAdGuAfuUfcdCdAsUf	AS1061	adTdGGfaAfuAfdCdTcUfuGfgUfuAfcAfusGfsa	0.1	0.31	0.77	0.059
D1062	S1062	AfuguAfaccAfaGfdAGfdTAdTudCcdAsu	AS1062	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.1	0.27	0.65	0.059
D1063	S1063	adTdGuadAdCccdAdGagdTdAuudCdCasu	AS1063	dAdTggdAdAuadCdTcudTdGgudTdAcadTsdGsa	0.12	0.44	0.82	0.064
D1064	S1064	AfuGfuAfaCfcAfaGfaGfdTdAuUfcdCdAsUf	AS1064	adTdGGfaAfdTdAcUfcUfuGfgUfuAfcAfusGfsa	0.12	0.32	0.83	0.064
D1065	S1065	AfuguAfaccAfaGfaGfdTAdTudCcdAsu	AS1065	dAUdGgdAadTAfcUfcUfuGfgUfuAfcAfusGfsa	0.13	0.34	0.72	0.066
D1066	S1066	AfuGfuAfaCfcAfaGfaGfudAdTUfcdCdAsUf	AS1066	adTdGGfadAdTAfcUfcUfuGfgUfuAfcAfusGfsa	0.11	0.33	0.72	0.067
D1067	S1067	AfuguAfaccAfaGfaGfdTAdTUdCcdAsu	AS1067	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.11	0.37	0.62	0.070
D1068	S1068	AfuguAfaccAfaGfaGfdTAdTUdCcdAsu	AS1068	dAUdGGdAAuAfcUfcUfuGfGfUfuAfCfAfusGfsa	0.16	0.33	0.64	0.072
D1069	S1069	aUfGfuaAfCfccAfGfagUfAfuuCfCfasu	AS1069	AfUfggAfAfuaCfUfcuUfGfguUfAfcaUfsGfsa	0.14	0.43	0.73	0.074
D1070	S1070	AfuGfuAfaCfCfAfaGfaguAfuUfcCfasUf	AS1070	aUfgGfaAfuAfCfUfcUfuggUfuAfcAfusGfsa	0.08	0.42	0.94	0.075
D1071	S1071	UfgGfgAfuuuCfaUfgUfaAfcCfaAfgsAf	AS1071	uCfuUfgGfuUfaCfaUfgAfAfAfuCfcCfasUfsc	0.14	0.28	0.83	0.0797
D1072	S1072	AfuGfuAfaCfcAfaGfAfGfuauUfcCfasUf	AS1072	aUfgGfaAfUfAfcucUfuGfgUfuAfcAfusGfsa	0.05	0.26	0.8	0.082
D1073	S1073	AfuGfuAfaCfcAfaGfadGdTdAdTUfcCfasUf	AS1073	aUfgGfadAdTdAdCUfcUfuGfgUfuAfcAfusGfsa	0.12	0.41	0.73	0.083
D1074	S1074	AfUfguAfAfccAfAfgaGfUfauUfCfcasUf	AS1074	aUfGfgaAfUfacUfCfuuGfGfuuAfCfausGfsa	0.14	0.44	0.75	0.086
D1075	S1075	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1075	aUfgGfdAdAdTdAcUfcUfuGfgUfuAfcAfusGfsa	0.1	0.41	0.72	0.088
D1076	S1076	AfuGfuAfaCfcAfaGfaGfudAdTdTdCCfasUf	AS1076	aUfgdGdAdAdTAfcUfcUfuGfgUfuAfcAfusGfsa	0.15	0.45	0.86	0.088

D1077	S1077	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasu	AS1077	AfUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.08	0.46	0.95	0.092
D1078	S1078	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1078	dAUdGGdAadTAfcUfcUfuGfgUfuAfcAfusGfsa	0.09	0.32	0.76	0.093
D1079	S1079	AfuguAfaccAfaGfaGfdTadTudCcdAsu	AS1079	dAudGgdAadTAfcUfcUfuGfgUfuAfcAfusGfsa	0.14	0.38	0.76	0.095
D1080	S1080	AfuGfuAfaCfcAfaGfAfGfuAfuucCfasUf	AS1080	aUfgGfAfAfuAfcucUfuGfgUfuAfcAfusGfsa	0.05	0.42	0.86	0.099
D1081	S1081	AfuGfuAfaCfcAfaGfaGfuAfuUfdCdCdAsdT	AS1081	dAdTdGdGaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.17	0.47	0.9	0.105
D1082	S1082	AfuGfuAfaccaagaguAfuUfcCfasUf	AS1082	aUfgGfaAfudACfudCUfudGGfudTAfcAfusgsa	0.12	0.44	0.83	0.106
D1083	S1083	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1083	adTdGGfaAfdTdAcUfcUfuGfgUfuAfcAfusGfsa	0.11	0.34	0.74	0.109
D1084	S1084	AfuGfuAfAfCfcAfaGfaGfuauUfcCfasUf	AS1084	aUfgGfaAfUfAfcUfcUfuGfguuAfcAfusGfsa	0.1	0.45	0.93	0.117
D1085	S1085	AfuGfUfAfaCfcAfaGfaGfuauUfcCfasUf	AS1085	aUfgGfaAfUfAfcUfcUfuGfgUfuacAfusGfsa	0.07	0.42	0.78	0.120
D1086	S1086	aUfguAfAfccAfAfgaGfuAfuUfcCfasUf	AS1086	aUfgGfaAfuAfcUfCfuuGfGfuuAfCfaUfsgsa	0.17	0.45	0.83	0.1197
D1087	S1087	AfuGfuAfaCfcAfaGfaGfUfAfuUfcCfasu	AS1087	AfUfgGfaAfuacUfcUfuGfgUfuAfcAfusGfsa	0.05	0.3	0.7	0.120
D1088	S1088	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1088	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusgsa	0.11	0.46	0.8	0.120
D1089	S1089	AfuGfuAfaCfcAfaGfaGfUfAfuUfcCfasUf	AS1089	aUfgGfaAfuacUfcUfuGfgUfuAfcAfusGfsa	0.14	0.49	0.85	0.122
D1090	S1090	AfuGfuAfaCfcAfaGfaGfuauUfcCfasUf	AS1090	aUfgGfaAfUfAfcUfcUfuGfgUfuAfcAfusGfsa	0.1	0.41	0.85	0.125
D1091	S1091	AfuguAfaccAfaGfaGfdTAdTudCcdAsu	AS1091	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.16	0.38	0.77	0.125
D1092	S1092	AfuGfuAfaCfcAfaGfAfGfuAfuUfcCfasu	AS1092	AfUfgGfaAfuAfcucUfuGfgUfuAfcAfusGfsa	0.05	0.31	0.93	0.126
D1093	S1093	auGfuAfaCfcAfaGfAfGfuAfuUfcCfasUf	AS1093	aUfgGfaAfuAfcucUfuGfgUfuAfcAfUfsGfsa	0.06	0.33	0.9	0.135
D1094	S1094	AfuGfuAfaCfcAfaGfaGfUfAfuUfccasUf	AS1094	aUfGfGfaAfuacUfcUfuGfgUfuAfcAfusGfsa	0.07	0.39	0.85	0.142
D1095	S1095	AfuGfuAfaCfcAfaGfAfGfuAfuUfcCfasUf	AS1095	aUfgGfaAfuAfcucUfuGfgUfuAfcAfusGfsa	0.09	0.39	0.76	0.146
D1096	S1096	AfuGfuAfaCfcAfaGfaGfUfAfuucCfasUf	AS1096	aUfgGfAfAfuacUfcUfuGfgUfuAfcAfusGfsa	0.06	0.38	0.85	0.147
D1097	S1097	AfuGfUfAfaCfcAfaGfaGfuAfuucCfasUf	AS1097	aUfgGfAfAfuAfcUfcUfuGfgUfuacAfusGfsa	0.12	0.47	0.87	0.147
D1098	S1098	AfuGfuAfaCfcAfaGfaGfuAfUfUfccasUf	AS1098	aUfGfGfaauAfcUfcUfuGfgUfuAfcAfusGfsa	0.06	0.42	0.85	0.151
D1099	S1099	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1099	dAUdGGdAadTAfdCUfcUfuGfgUfuAfcAfusGfsa	0.16	0.41	0.85	0.152
D1100	S1100	AfuguAfaccAfaGfaGfuAfuUfcCfasUf	AS1100	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.15	0.48	0.72	0.152
D1101	S1101	AfuGfuAfaCfcAfaGfAfGfuAfuUfccasUf	AS1101	aUfGfGfaAfuAfcucUfuGfgUfuAfcAfusGfsa	0.06	0.38	0.94	0.158
D1102	S1102	AfuGfuAfaccaagaguAfuUfcCfasUf	AS1102	aUfgGfaAfuAfdCuCfdTuGfdGuUfacAfusGfsa	0.21	0.45	0.89	0.162
D1103	S1103	AfuGfuaaCfCfAfaGfaGfuAfuUfcCfasUf	AS1103	aUfgGfaAfuAfcUfcUfuggUfUfAfcAfusGfsa	0.14	0.49	0.95	0.163

D1104	S1104	AfuGfuAfaccAfaGfaGfUfAfuUfcCfasUf	AS1104	aUfgGfaAfuacUfcUfuGfGfUfuAfcAfusGfsa	0.06	0.36	0.92	0.163
D1105	S1105	AfuGfuAfaCfcAfaGfaGfuAfuucCfasUf	AS1105	aUfgGfAfAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.1	0.45	0.84	0.167
D1106	S1106	AfuGfuaaCfcAfaGfAfGfuAfuUfcCfasUf	AS1106	aUfgGfaAfuAfcucUfuGfgUfUfAfcAfusGfsa	0.09	0.43	0.91	0.170
D1107	S1107	AfuGfuAfaccAfaGfAfGfuAfuUfcCfasUf	AS1107	aUfgGfaAfuAfcucUfuGfGfUfuAfcAfusGfsa	0.09	0.46	1	0.171
D1108	S1108	AfuguAfaccAfaGfaGfdTadTudCcdAsu	AS1108	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.11	0.39	0.71	0.176
D1109	S1109	AfuGfUfAfaCfcAfaGfaGfuAfuUfccasUf	AS1109	aUfGfGfaAfuAfcUfcUfuGfgUfuacAfusGfsa	0.1	0.43	0.9	0.180
D1110	S1110	AfuGfuAfaCfcAfaGfaguAfUfUfcCfasUf	AS1110	aUfgGfaauAfCfUfcUfuGfgUfuAfcAfusGfsa	0.06	0.42	0.88	0.182
D1111	S1111	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1111	dAUdGGdAAuAfcUfcUfuGfGfUfuAfCfAfusGfsa	0.18	0.49	0.79	0.183
D1112	S1112	AfuGfUfAfaccAfaGfaGfuAfuUfcCfasUf	AS1112	aUfgGfaAfuAfcUfcUfuGfGfUfuacAfusGfsa	0.14	0.48	0.85	0.195
D1113	S1113	AfuGfuAfaCfcAfaGfaguAfuUfcCfasUf	AS1113	aUfgGfaAfuAfCfUfcUfuGfgUfuAfcAfusGfsa	0.09	0.41	0.85	0.201
D1114	S1114	auGfuAfaCfcAfaGfaGfUfAfuUfcCfasUf	AS1114	aUfgGfaAfuacUfcUfuGfgUfuAfcAfUfsGfsa	0.05	0.44	0.94	0.201
D1115	S1115	AfuguAfaCfcAfaGfaGfUfAfuUfcCfasUf	AS1115	aUfgGfaAfuacUfcUfuGfgUfuAfCfAfusGfsa	0.08	0.41	0.96	0.204
D1116	S1116	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1116	adTdGGfadAdTAfcUfcUfuGfgUfuAfcAfusGfsa	0.15	0.47	0.79	0.208
D1117	S1117	AfuGfuaaCfcAfaGfaGfUfAfuUfcCfasUf	AS1117	aUfgGfaAfuacUfcUfuGfgUfUfAfcAfusGfsa	0.08	0.42	0.92	0.224
D1118	S1118	auguaaccaagaguauuccasu	AS1118	AfUfGfGfAfAfUfAfCfUfCfUfUfGfGfUfUfAfCfAfUfsgsa	0.19	0.5	0.87	0.303
D1119	S1119	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1119	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.14	0.55	0.89	
D1120	S1120	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1120	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.19	0.63	0.72	
D1121	S1121	AfuGfuAfaccAfaGfaGfuAfuUfcCfasUf	AS1121	aUfgGfaAfuAfcUfcUfuGfGfUfuAfcAfusGfsa	0.14	0.61	0.91	
D1122	S1122	AfUfGfuAfaCfcAfaGfaGfuAfuUfccasUf	AS1122	aUfGfGfaAfuAfcUfcUfuGfgUfuAfcausGfsa	0.14	0.54	0.95	
D1123	S1123	auGfuAfAfCfcAfaGfaGfuAfuUfcCfasUf	AS1123	aUfgGfaAfuAfcUfcUfuGfguuAfcAfUfsGfsa	0.13	0.61	0.97	
D1124	S1124	AfuGfuAfaCfcAfaGfaGfuAfUfUfcCfasUf	AS1124	aUfgGfaauAfcUfcUfuGfgUfuAfcAfusGfsa	0.14	0.56	0.94	
D1125	S1125	AfuGfuAfaCfcaaGfaGfuAfuUfcCfasUf	AS1125	aUfgGfaAfuAfcUfcUfUfGfgUfuAfcAfusGfsa	0.21	0.74	0.95	
D1126	S1126	AfUfGfuAfaCfcAfaGfaGfuAfuucCfasUf	AS1126	aUfgGfAfAfuAfcUfcUfuGfgUfuAfcausGfsa	0.2	0.69	0.91	
D1127	S1127	AfuguAfAfCfcAfaGfaGfuAfuUfcCfasUf	AS1127	aUfgGfaAfuAfcUfcUfuGfguuAfCfAfusGfsa	0.17	0.7	0.96	
D1128	S1128	AfUfGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1128	aUfgGfaAfuAfcUfcUfuGfgUfuAfcausGfsa	0.19	0.62	0.85	
D1129	S1129	AfuGfuAfaCfcAfaGfaGfuAfuUfCfCfasUf	AS1129	aUfggaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.23	0.76	0.98	
D1130	S1130	AfuGfuAfaCfcAfagaGfuAfuUfcCfasUf	AS1130	aUfgGfaAfuAfcUfCfUfuGfgUfuAfcAfusGfsa	0.21	0.64	0.9	

D1131	S1131	AfuGfuAfAfCfcaaGfaGfuAfuUfcCfasUf	AS1131	aUfgGfaAfuAfcUfcUfUfGfguuAfcAfusGfsa	0.17	0.7	1.01	
D1132	S1132	AfuGfUfAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1132	aUfgGfaAfuAfcUfcUfuGfgUfuacAfusGfsa	0.17	0.58	0.87	
D1133	S1133	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfAfsUf	AS1133	augGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.33	0.89	1.05	
D1134	S1134	AfUfGfuAfaCfcAfaGfaguAfuUfcCfasUf	AS1134	aUfgGfaAfuAfCfUfcUfuGfgUfuAfcausGfsa	0.16	0.64	0.96	
D1135	S1135	AfuGfUfAfaCfcAfaGfaguAfuUfcCfasUf	AS1135	aUfgGfaAfuAfCfUfcUfuGfgUfuacAfusGfsa	0.12	0.53	0.96	
D1136	S1136	AfuGfuAfAfCfcAfagaGfuAfuUfcCfasUf	AS1136	aUfgGfaAfuAfcUfCfUfuGfguuAfcAfusGfsa	0.16	0.58	0.98	
D1137	S1137	AfuGfuAfAfCfcAfaGfaGfuAfuUfcCfasUf	AS1137	aUfgGfaAfuAfcUfcUfuGfguuAfcAfusGfsa	0.16	0.6	0.91	
D1138	S1138	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1138	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsAf	0.1	0.54	0.91	
D1139	S1139	AfUfGfuAfaCfcAfagaGfuAfuUfcCfasUf	AS1139	aUfgGfaAfuAfcUfCfUfuGfgUfuAfcausGfsa	0.24	0.68	0.98	
D1140	S1140	AfuGfUfAfaCfcAfagaGfuAfuUfcCfasUf	AS1140	aUfgGfaAfuAfcUfCfUfuGfgUfuacAfusGfsa	0.13	0.75	0.9	
D1141	S1141	AfuGfuAfAfCfcAfaGfaguAfuUfcCfasUf	AS1141	aUfgGfaAfuAfCfUfcUfuGfguuAfcAfusGfsa	0.15	0.52	1.05	
D1142	S1142	AfuGfuAfaCfCfAfaGfaGfuAfuUfcCfasUf	AS1142	aUfgGfaAfuAfcUfcUfuggUfuAfcAfusGfsa	0.16	0.66	0.89	
D1143	S1143	auGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1143	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfUfsGfsa	0.12	0.51	0.89	
D1144	S1144	AfUfGfuAfaCfcaaGfaGfuAfuUfcCfasUf	AS1144	aUfgGfaAfuAfcUfcUfUfGfgUfuAfcausGfsa	0.25	0.71	0.95	
D1145	S1145	AfuGfUfAfaCfcaaGfaGfuAfuUfcCfasUf	AS1145	aUfgGfaAfuAfcUfcUfUfGfgUfuacAfusGfsa	0.17	0.74	0.98	
D1146	S1146	AfuguAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1146	aUfgGfaAfuAfcUfcUfuGfgUfuAfCfAfusGfsa	0.11	0.51	0.86	
D1147	S1147	AfuGfuAfaCfcAfaGfaGfuAfuUfccasUf	AS1147	aUfGfGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.1	0.52	0.83	
D1148	S1148	AfUfGfuAfaccAfaGfaGfuAfuUfcCfasUf	AS1148	aUfgGfaAfuAfcUfcUfuGfGfUfuAfcausGfsa	0.14	0.63	0.98	
D1149	S1149	AfuGfuAfAfCfcAfaGfaGfuAfuucCfasUf	AS1149	aUfgGfAfAfuAfcUfcUfuGfguuAfcAfusGfsa	0.13	0.58	0.88	
D1150	S1150	AfuGfuaaCfcAfaGfaGfuAfuUfcCfasUf	AS1150	aUfgGfaAfuAfcUfcUfuGfgUfUfAfcAfusGfsa	0.15	0.62	0.94	
D1151	S1151	AfUfGfuaaCfcAfaGfaGfuAfuUfcCfasUf	AS1151	aUfgGfaAfuAfcUfcUfuGfgUfUfAfcausGfsa	0.18	0.73	0.94	
D1152	S1152	auGfUfAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1152	aUfgGfaAfuAfcUfcUfuGfgUfuacAfUfsGfsa	0.13	0.53	0.97	
D1153	S1153	AfuGfuAfAfCfcAfaGfaGfuAfuUfccasUf	AS1153	aUfGfGfaAfuAfcUfcUfuGfguuAfcAfusGfsa	0.13	0.53	0.98	
D1154	S1154	UfgGfgAfuUfuCfaUfgUfaAfcCfaAfgsAf	AS1154	uCfuUfgGfuUfaCfaUfgAfaAfuCfcCfasUfsc	0.09	0.5	0.78	
D1155	S1155	UfgGfGfAfuuuCfaUfgUfAfAfcCfaAfgsAf	AS1155	uCfuUfgGfuuaCfaUfgAfAfAfuccCfasUfsc	0.13	0.62	0.89	
D1156	S1156	UfgGfgAfuuuCfaUfGfUfaAfcCfaAfgsAf	AS1156	uCfuUfgGfuUfacaUfgAfAfAfuCfcCfasUfsc	0.12	0.65	0.85	
D1157	S1157	UfgGfgAfuUfuCfaUfgUfAfAfcCfaAfgsAf	AS1157	uCfuUfgGfuuaCfaUfgAfaAfuCfcCfasUfsc	0.11	0.54	0.85	

D1158	S1158	UfgGfgAfuuuCfaUfgUfAfAfcCfaAfgsAf	AS1158	uCfuUfgGfuuaCfaUfgAfAfAfuCfcCfasUfsc	0.13	0.53	0.8	
D1159	S1159	UfGfggAfUfuUfcAfuGfuAfAfccAfAfgsAf	AS1159	uCfuuGfGfuuAfcAfuGaAfauCfCfcasUfsc	0.59	0.89	0.81	
D1160	S1160	UfGfggAfUfuuCfaUfgUfAfAfcCfaAfgsAf	AS1160	uCfuUfgGfuuaCfaUfgAfAfauCfCfcasUfsc	0.16	0.72	0.9	
D1161	S1161	UfgGfgAfuUfucaUfGfUfaAfcCfaAfgsAf	AS1161	uCfuUfgGfuUfacaUfGfAfaAfuCfcCfasUfsc	0.27	0.69	0.86	
D1162	S1162	AfuGfuAfaCfcaaGfaGfUfAfuUfcCfasUf	AS1162	aUfgGfaAfuacUfcUfUfGfgUfuAfcAfusGfsa	0.12	0.6	0.95	
D1163	S1163	AfuGfuAfaccAfaGfaGfuAfUfUfcCfasUf	AS1163	aUfgGfaauAfcUfcUfuGfGfUfuAfcAfusGfsa	0.05	0.56	1.02	
D1164	S1164	AfuGfuAfaCfcAfagaGfUfAfuUfcCfasUf	AS1164	aUfgGfaAfuacUfCfUfuGfgUfuAfcAfusGfsa	0.13	0.55	1	
D1165	S1165	AfuGfuAfaCfcaaGfaGfuAfUfUfcCfasUf	AS1165	aUfgGfaauAfcUfcUfUfGfgUfuAfcAfusGfsa	0.09	0.6	0.97	
D1166	S1166	AfuguAfaCfCfAfaGfaGfuAfuUfcCfasUf	AS1166	aUfgGfaAfuAfcUfcUfuggUfuAfCfAfusGfsa	0.15	0.59	0.91	
D1167	S1167	AfuGfuAfaCfcAfagaGfuAfUfUfcCfasUf	AS1167	aUfgGfaauAfcUfCfUfuGfgUfuAfcAfusGfsa	0.11	0.59	1	
D1168	S1168	AfuGfuAfaCfCfAfagaGfuAfuUfcCfasUf	AS1168	aUfgGfaAfuAfcUfCfUfuggUfuAfcAfusGfsa	0.13	0.57	0.94	
D1169	S1169	auGfuAfaCfcAfaGfaGfuAfUfUfcCfasUf	AS1169	aUfgGfaauAfcUfcUfuGfgUfuAfcAfUfsGfsa	0.08	0.5	0.9	
D1170	S1170	AfuguAfaCfcAfaGfaGfuAfUfUfcCfasUf	AS1170	aUfgGfaauAfcUfcUfuGfgUfuAfCfAfusGfsa	0.06	0.53	0.91	
D1171	S1171	auGfuAfaCfcAfaGfaGfuAfuUfCfCfasUf	AS1171	aUfggaAfuAfcUfcUfuGfgUfuAfcAfUfsGfsa	0.07	0.56	0.89	
D1172	S1172	AfuGfuAfaCfCfAfaGfaGfuAfuucCfasUf	AS1172	aUfgGfAfAfuAfcUfcUfuggUfuAfcAfusGfsa	0.13	0.59	0.98	
D1173	S1173	AfuGfuAfaCfcaaGfAfGfuAfuUfcCfasUf	AS1173	aUfgGfaAfuAfcucUfUfGfgUfuAfcAfusGfsa	0.2	0.65	1.03	
D1174	S1174	AfuGfuaaCfcAfaGfaGfuAfUfUfcCfasUf	AS1174	aUfgGfaauAfcUfcUfuGfgUfUfAfcAfusGfsa	0.07	0.51	0.95	
D1175	S1175	AfuguAfaCfcAfaGfaGfuAfuUfCfCfasUf	AS1175	aUfggaAfuAfcUfcUfuGfgUfuAfCfAfusGfsa	0.2	0.53	0.76	
D1176	S1176	auGfuAfaCfcAfaGfaGfuAfuUfcCfAfsUf	AS1176	augGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.74	0.98	0.81	
D1177	S1177	AfuGfuAfaCfcAfaGfaGfuAfuucCfAfsUf	AS1177	augGfAfAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.43	0.64	0.88	
D1178	S1178	auguaaccAfaGfaGfuAfuUfcCfasUf	AS1178	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.17	0.49	0.81	
D1179	S1179	AfuGfuaaCfcAfaGfaGfuAfuUfCfCfasUf	AS1179	aUfggaAfuAfcUfcUfuGfgUfUfAfcAfusGfsa	0.22	0.65	0.73	
D1180	S1180	AfuguAfaCfcAfaGfaGfuAfuUfcCfAfsUf	AS1180	augGfaAfuAfcUfcUfuGfgUfuAfcAfUfsGfsa	0.6	1.09	0.8	
D1181	S1181	auGfuAfaCfcAfaGfaGfuAfuUfccasu	AS1181	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.3	0.78	0.78	
D1182	S1182	auguaaccaaGfaGfuAfuUfcCfasUf	AS1182	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.35	0.73	0.84	
D1183	S1183	AfuGfuAfaccAfaGfaGfuAfuUfCfCfasUf	AS1183	aUfggaAfuAfcUfcUfuGfGfUfuAfcAfusGfsa	0.19	0.6	0.94	
D1184	S1184	AfuGfuaaCfcAfaGfaGfuAfuUfcCfAfsUf	AS1184	augGfaAfuAfcUfcUfuGfgUfuAfCfAfusGfsa	0.61	1.08	0.8	

D1185	S1185	auGfuAfaCfcAfaGfaGfuAfuuccasu	AS1185	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.16	0.52	0.72	
D1186	S1186	auguaaccaagaGfuAfuUfcCfasUf	AS1186	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.2	0.53	0.74	
D1187	S1187	AfuGfuAfaCfcaaGfaGfuAfuUfCfCfasUf	AS1187	aUfggaAfuAfcUfcUfUfGfgUfuAfcAfusGfsa	0.34	0.66	0.85	
D1188	S1188	AfuGfuAfaccAfaGfaGfuAfuUfcCfAfsUf	AS1188	augGfaAfuAfcUfcUfuGfgUfUfAfcAfusGfsa	0.61	0.98	1.02	
D1189	S1189	AfuGfuAfaCfcAfaGfaGfuAfuuccasu	AS1189	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.3	0.73	0.85	
D1190	S1190	auguaaccaagaguauuccasu	AS1190	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.28	0.69	0.78	
D1191	S1191	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1191	aUfgGfaAfuAfcUfcUfugdGudTadCadTsgsa	0.33	0.88	0.64	
D1192	S1192	AfuGfuAfaCfcAfagaGfuAfuUfCfCfasUf	AS1192	aUfggaAfuAfcUfCfUfuGfgUfuAfcAfusGfsa	0.31	0.64	0.83	
D1193	S1193	AfuGfuAfaCfcaaGfaGfuAfuUfcCfAfsUf	AS1193	augGfaAfuAfcUfcUfuGfGfUfuAfcAfusGfsa	0.64	0.82	0.92	
D1194	S1194	AfuGfuAfaCfcAfaGfaGfuauuccasu	AS1194	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.21	0.62	0.77	
D1195	S1195	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1195	aUfgGfaAfuAfcUfcUfuGfGfUfuAfCfAfusGfsa	0.17	0.7	0.95	
D1196	S1196	AfuGfuAfaCfcAfaGfaguAfuUfCfCfasUf	AS1196	aUfggaAfuAfCfUfcUfuGfgUfuAfcAfusGfsa	0.19	0.71	0.65	
D1197	S1197	AfuGfuAfaCfcAfagaGfuAfuUfcCfAfsUf	AS1197	augGfaAfuAfcUfcUfUfGfgUfuAfcAfusGfsa	0.64	0.82	0.93	
D1198	S1198	auguAfaCfcAfaGfaGfuAfuUfccasu	AS1198	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.19	0.65	0.72	
D1199	S1199	AfuGfuAfaCfcAfaGfaGfuauUfCfCfasUf	AS1199	aUfggaAfUfAfcUfcUfuGfgUfuAfcAfusGfsa	0.15	0.52	0.64	
D1200	S1200	AfuGfuAfaCfcAfaGfaguAfuUfcCfAfsUf	AS1200	augGfaAfuAfcUfCfUfuGfgUfuAfcAfusGfsa	0.48	0.74	0.92	
D1201	S1201	auguAfaCfcAfaGfaGfuAfuUfcCfasu	AS1201	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.17	0.71	0.77	
D1202	S1202	AfuGfuAfaCfcAfaGfaGfuauUfcCfAfsUf	AS1202	augGfaAfuAfCfUfcUfuGfgUfuAfcAfusGfsa	0.43	0.69	0.85	
D1203	S1203	auguaaCfcAfaGfaGfuAfuUfcCfasUf	AS1203	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.14	0.61	0.76	
D1204	S1204	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1204	adTdGGfaAfudAdCUfcUfuGfgUfuAfcAfusGfsa	0.16	0.56	0.89	
D1205	S1205	AfuGfuAfaCfcAfaGfaGfdTdAdTdTcCfasUf	AS1205	aUfgGfdAdAdTdAcUfcUfuGfgUfuAfcAfusGfsa	0.13	0.57	0.9	
D1206	S1206	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1206	adTdGdGdAAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.29	0.73	0.89	
D1207	S1207	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1207	adTdGGfaAfuAfdCdTcUfuGfgUfuAfcAfusGfsa	0.16	0.56	0.78	
D1208	S1208	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1208	aUfdGdGdAdAuAfcUfcUfuGfgUfuAfcAfusGfsa	0.22	0.67	0.89	
D1209	S1209	AfuguAfaccAfaGfaGfuAfuUfcCfasUf	AS1209	aUfgGfaAfuAfcUfcUfuGfGfUfuAfCfAfusGfsa	0.14	0.55	0.78	
D1210	S1210	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1210	aUfgdGdAdAdTAfcUfcUfuGfgUfuAfcAfusGfsa	0.14	0.5	0.84	
D1211	S1211	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1211	aUfgGfadAdTdAdCUfcUfuGfgUfuAfcAfusGfsa	0.14	0.59	0.72	

D1212	S1212	auguaaccaaGfaGfuAfuUfcCfasUf	AS1212	aUfgGfaAfuAfcUfcUfugdGudTadCadTsgsa	0.21	0.74	0.77	
D1213	S1213	AfuGfuAfaCfcAfaGfaGfuAfudTdCdCdAsUf	AS1213	adTdGdGdAAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.15	0.53	0.91	
D1214	S1214	aUfgUfaAfcCfaAfgAfgUfaUfuCfcAfsu	AS1214	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.12	0.71	0.87	
D1215	S1215	AfuGfuAfaCfcAfaGfaGfuAfdTdTdCdCasUf	AS1215	aUfdGdGdAdAuAfcUfcUfuGfgUfuAfcAfusGfsa	0.18	0.67	0.97	
D1216	S1216	AfuGfuAfaccaagaguAfuUfcCfasUf	AS1216	aUfgGfaAfuacucuuggUfuAfcAfusgsa	0.36	0.87	1.07	
D1217	S1217	AfuGfuAfaccaagaguAfuUfcCfasUf	AS1217	aUfgGfaAfuAfCfUfCfUfuGfGfuuAfcAfusgsa	0.37	0.73	1.03	
D1218	S1218	AfUfguAfAfccAfAfgaGfUfauUfCfcasUf	AS1218	aUfGfgaAfUfacUfCfuuGfGfuuAfCfausGfsa	0.23	0.42	0.84	
D1219	S1219	AfuGfuAfaccaagaguAfuUfcCfasUf	AS1219	aUfgGfaAfuaCfUfcUfUfgGfuuAfcAfusgsa	0.43	0.71	1.03	
D1220	S1220	AfuGfuAfaccaagaguAfuUfcCfasUf	AS1220	aUfgGfaAfuAfcUfCfUfuGfGfuuAfcAfusgsa	0.37	0.63	0.99	
D1221	S1221	AfuGfuAfaccaagaguAfuUfcCfasUf	AS1221	aUfgGfaAfuAfcUfCfUfuGfGfuUfacAfusgsa	0.29	0.84	0.88	
D1222	S1222	AfuGfuAfaccaagaguAfuUfcCfasUf	AS1222	aUfgGfaAfuaCfuCfuUfgGfuuAfcAfusgsa	0.31	0.8	0.99	
D1223	S1223	auGfuAfAfccAfaGfagUfaUfUfcCfasUf	AS1223	aUfgGfaaUfaCfUfcUfuGfGfuuAfcAfAfsgsa	0.09	0.52	0.82	
D1224	S1224	AfuGfuAfaccaagaguAfuUfcCfasUf	AS1224	aUfgGfaAfuadCudCudTgdGuuAfcAfusgsa	0.22	0.79	1	
D1225	S1225	auGfuaAfccAfagAfguAfuuCfcasUf	AS1225	aUfGfgAfAfuAfCfuCfUfuGfGfuUfAfcAfUfsGfsa	0.31	0.76	0.84	
D1226	S1226	AfuGfuAfaccaagaguAfuUfcCfasUf	AS1226	aUfgGfaAfuadCUfcdTUfgdGuuAfcAfusgsa	0.26	0.64	0.87	
D1227	S1227	augUfaacCfaagAfguaUfuccAfsu	AS1227	aUfgGfAfaUfAfCfuCfUfUfgGfUfUfaCfAfUfsGfsa	0.33	0.79	0.81	
D1228	S1228	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1228	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.464	0.932	0.978	
D1229	S1229	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1229	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.453	1.047	1.178	
D1230	S1230	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1230	aUfgGfaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.831	0.967	1.151	
D1231	S1231	auGfuAfAfCfcAfaGfaGfuAfuUfcCfasu	AS1231	AfUfgGfaAfuAfcUfcUfuGfguuAfcAfUfsGfsa	0.09	0.5	1.07	
D1232	S1232	AfuGfuAfaCfCfAfaGfaGfuAfuUfcCfasu	AS1232	AfUfgGfaAfuAfcUfcUfuggUfuAfcAfusGfsa	0.11	0.54	1.1	
D1233	S1233	AfuGfuAfaCfcAfaGfaGfuAfuUfCfCfasu	AS1233	AfUfggaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.19	0.61	0.74	
D1234	S1234	aUfgUfaAfcCfaAfgAfgUfaUfuCfcAfsu	AS1234	AfuGfgAfaUfaCfuCfuUfgGfuUfaCfaUfsgsAf	0.22	0.61	0.98	
D1235	S1235	aUfgUfaAfcCfaAfgAfgUfaUfuCfcAfsu	AS1235	AfuGfgAfaUfaCfuCfuUfgGfuUfaCfaUfsgsAf	0.27	0.69	0.92	
D1236	S1236	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1236	AfuGfgAfaUfaCfuCfuUfgGfuUfaCfaUfsgsAf	0.54	1.08	0.8	
D1237	S1237	augUfaAfccaAfgAfguaUfuCfcasu	AS1237	AfUfGfgAfaUfAfCfuCfuUfGfGfuUfaCfAfUfsgsa	0.29	0.61	0.79	
D1238	S1238	AfugUfaAfccaAfgAfguaUfuCfcasu	AS1238	AfUfGfgAfaUfAfCfuCfuUfGfGfuUfaCfAfusgsa	0.31	0.6	0.88	

D1239	S1239	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1239	dAUdGGdAauAfcUfcUfuGfgUfuAfcAfusGfsa	0.2	0.67	0.85	
D1240	S1240	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1240	dAUdGgdAauAfcUfcUfuGfgUfuAfcAfusGfsa	0.23	0.58	0.68	
D1241	S1241	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1241	dAudGgdAauAfcUfcUfuGfgUfuAfcAfusGfsa	0.25	0.65	0.78	
D1242	S1242	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1242	dAUdGgdAadTAfcUfcUfuGfgUfuAfcAfusGfsa	0.18	0.64	0.84	
D1243	S1243	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1243	dAUdGGdAAfuAfcUfcUfuGfGfUfuAfCfAfusGfsa	0.19	0.72	0.87	
D1244	S1244	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1244	dAUdGgdAadTAfdCUfcUfuGfgUfuAfcAfusGfsa	0.16	0.55	0.8	
D1245	S1245	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1245	dAUdGGdAAuAfcUfcUfuGfgUfuAfcAfusGfsa	0.22	0.51	0.9	
D1246	S1246	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1246	dAudGgdAadTAfcUfcUfuGfgUfuAfcAfusGfsa	0.27	0.78	0.66	
D1247	S1247	AfuGfuAfaCfcAfaGfaGfuAfuUfcCfasUf	AS1247	dAdTdGdGaAfuAfcUfcUfuGfgUfuAfcAfusGfsa	0.16	0.57	0.97	
D1248	S1248	AfacaAfuguUfcUfuGfdCUdCudAudAsa	AS1248	dTUdAudAgdAGfcAfaGfaAfcAfcUfgUfusUfsu	0.06	0.09	0.36	0.0047
D1249	S1249	AfaCfaGfuGfuUfcUfuGfCfUfcUfaUfasa	AS1249	UfUfaUfaGfagcAfaGfaAfcAfcUfgUfusUfsu	0.06	0.10	0.47	0.005
D1250	S1250	AfaCfaGfuGfuUfcUfugcUfcUfAfUfasAf	AS1250	uUfauaGfaGfCfAfaGfaAfcAfcUfgUfusUfsu	0.07	0.14	0.55	0.005
D1251	S1251	AfaCfaGfuGfuUfcUfuGfcucUfAfUfasAf	AS1251	uUfauaGfAfGfcAfaGfaAfcAfcUfgUfusUfsu	0.07	0.14	0.49	0.006
D1252	S1252	cAGuGuucuuGcucuAuAAdTdT	AS1252	UuAuAGAGcAAGAAcACUGdTdT				0.006
D1253	S1253	AfaCfaGfuGfuUfcUfugcUfCfUfaUfasAf	AS1253	uUfaUfagaGfCfAfaGfaAfcAfcUfgUfusUfsu	0.05	0.12	0.43	0.006
D1254	S1254	AfaCfaGfuGfuUfCfUfuGfcUfcUfaUfasa	AS1254	UfUfaUfaGfaGfcAfagaAfcAfcUfgUfusUfsu	0.06	0.13	0.39	0.006
D1255	S1255	AfaCfaGfuGfuUfcUfuGfcUfCfUfaUfasa	AS1255	UfUfaUfagaGfcAfaGfaAfcAfcUfgUfusUfsu	0.08	0.17	0.48	0.007
D1256	S1256	AfaCfaGfuGfuUfcUfUfGfcUfcUfaUfasa	AS1256	UfUfaUfaGfaGfcaaGfaAfcAfcUfgUfusUfsu	0.08	0.14	0.40	0.007
D1257	S1257	AfaCfaGfuGfuUfcUfuGfcUfCfUfaUfasAf	AS1257	uUfaUfagaGfcAfaGfaAfcAfcUfgUfusUfsUf	0.07	0.12	0.40	0.007
D1258	S1258	AfaCfaguGfuUfCfUfuGfcUfcUfaUfasAf	AS1258	uUfaUfaGfaGfcAfagaAfcAfCfUfgUfusUfsu	0.08	0.13	0.41	0.007
D1259	S1259	AfaCfAfGfuGfuUfcUfuGfcucUfaUfasAf	AS1259	uUfaUfaGfAfGfcAfaGfaAfcAfcugUfusUfsu	0.05	0.11	0.35	0.008
D1260	S1260	AfacaGfuGfuUfCfUfuGfcUfcUfaUfasAf	AS1260	uUfaUfaGfaGfcAfagaAfcAfcUfGfUfusUfsu	0.06	0.12	0.40	0.008
D1261	S1261	AfacaGfuGfuUfcUfuGfcUfCfUfaUfasAf	AS1261	uUfaUfagaGfcAfaGfaAfcAfcUfGfUfusUfsu	0.06	0.13	0.42	0.008
D1262	S1262	AfaCfaGfuGfuUfcUfuGfcucUfaUfasAf	AS1262	uUfaUfaGfAfGfcAfaGfaAfcAfcUfgUfusUfsu	0.06	0.13	0.37	0.008
D1263	S1263	cAGuGuucuuGcucuAuAAdTdT	AS1263	UuAuAGAGcAAGAAcACUGdTdT				0.008
D1264	S1264	AfaCfaGfuGfuUfcUfuGfCfUfcUfauasAf	AS1264	uUfAfUfaGfagcAfaGfaAfcAfcUfgUfusUfsu	0.07	0.12	0.50	0.008
D1265	S1265	AfaCfaGfuguUfCfUfuGfcUfcUfaUfasAf	AS1265	uUfaUfaGfaGfcAfagaAfCfAfcUfgUfusUfsu	0.12	0.13	0.48	0.009

D1266	S1266	AfacaGfuGfuUfcUfuGfcUfcUfAfUfasAf	AS1266	uUfauaGfaGfcAfaGfaAfcAfcUfGfUfusUfsu	0.07	0.15	0.51	0.009
D1267	S1267	AfacaAfuguUfcUfuGfdCudCudAudAsa	AS1267	dTudAudAgdAGfcAfaGfaAfcAfcAfgUfusUfsu	0.06	0.14	0.48	0.0088
D1268	S1268	AfaCfaGfuGfuUfCfUfuGfcucUfaUfasAf	AS1268	uUfaUfaGfAfGfcAfagaAfcAfcUfgUfusUfsu	0.05	0.09	0.35	0.009
D1269	S1269	cAGuGuucuuGcucuAuAAdTdT	AS1269	UuAuAGAGcAAGAAcACUGdTdT				0.009
D1270	S1270	aaCfaGfuGfuUfcUfuGfcUfCfUfaUfasAf	AS1270	uUfaUfagaGfcAfaGfaAfcAfcUfgUfUfsUfsu	0.07	0.14	0.49	0.009
D1271	S1271	AfaCfaGfUfGfuUfcUfuGfcucUfaUfasAf	AS1271	uUfaUfaGfAfGfcAfaGfaAfcacUfgUfusUfsu	0.06	0.10	0.36	0.009
D1272	S1272	cAGuGuucuuGcucuAuAAdTdT	AS1272	UuAuAGAGcAAGAAcACUGdTdT				0.009
D1273	S1273	AfaCfaGfUfGfuUfcUfuGfcUfcUfaUfasAf	AS1273	uUfaUfaGfaGfcAfaGfaAfcacUfgUfusUfsUf	0.06	0.13	0.51	0.009
D1274	S1274	AfaCfaGfuGfuUfCfUfuGfcUfcuaUfasAf	AS1274	uUfaUfAfGfaGfcAfagaAfcAfcUfgUfusUfsu	0.06	0.12	0.46	0.010
D1275	S1275	cAGuGuucuuGcucuAuAAdTdT	AS1275	UuAuAGAGcAAGAAcACUGdTdT				0.010
D1276	S1276	AfaCfaGfuGfuUfCfUfuGfcUfcUfauasAf	AS1276	uUfAfUfaGfaGfcAfagaAfcAfcUfgUfusUfsu	0.06	0.14	0.47	0.010
D1277	S1277	AfaCfaguGfuUfcUfuGfcUfCfUfaUfasAf	AS1277	uUfaUfagaGfcAfaGfaAfcAfCfUfgUfusUfsu	0.07	0.15	0.50	0.010
D1278	S1278	AfaCfaGfuGfuUfCfUfugcUfcUfaUfasAf	AS1278	uUfaUfaGfaGfCfAfagaAfcAfcUfgUfusUfsu	0.06	0.13	0.43	0.010
D1279	S1279	cAGuGuucuuGcucuAuAAdTdT	AS1279	UuAuAGAGcAAGAAcACUGdTdT				0.010
D1280	S1280	AfaCfaGfuGfuUfcUfuGfcUfcUfaUfasa	AS1280	UfUfaUfaGfaGfcAfaGfaAfcAfcUfgUfususu	0.06	0.14	0.45	0.010
D1281	S1281	AfaCfAfGfuGfuUfcUfuGfcUfcUfaUfasa	AS1281	UfUfaUfaGfaGfcAfaGfaAfcAfcugUfusUfsu	0.07	0.18	0.46	0.011
D1282	S1282	AfaCfaGfuGfuUfcUfuGfcUfcUfaUfasAf	AS1282	uUfaUfaGfaGfcAfaGfaAfcAfcUfgUfusUfsu	0.07	0.15	0.55	0.011
D1283	S1283	AfaCfaGfuGfuUfcUfuGfcucUfaUfasAf	AS1283	uUfaUfaGfAfGfcAfaGfaAfcAfcUfgUfususu	0.07	0.12	0.45	0.011
D1284	S1284	AfacaGfuGfuUfcUfuGfcUfcUfaUfasAf	AS1284	uUfaUfaGfaGfcAfaGfaAfcAfcUfGfUfusUfsu	0.06	0.13	0.48	0.011
D1285	S1285	AfAfCfaGfuGfuUfcUfuGfcucUfaUfasAf	AS1285	uUfaUfaGfAfGfcAfaGfaAfcAfcUfguusUfsu	0.06	0.11	0.40	0.011
D1286	S1286	AfaCfAfGfuGfuUfcUfuGfcUfcUfauasAf	AS1286	uUfAfUfaGfaGfcAfaGfaAfcAfcugUfusUfsu	0.06	0.16	0.47	0.011
D1287	S1287	AfaCfaGfuGfuUfcUfugcUfcUfaUfasAf	AS1287	uUfaUfaGfaGfCfAfaGfaAfcAfcUfgUfususu	0.07	0.19	0.46	0.012
D1288	S1288	AfaCfaGfuGfuUfcUfugcUfcUfaUfasAf	AS1288	uUfaUfaGfaGfCfAfaGfaAfcAfcUfgUfusUfsu	0.06	0.17	0.46	0.012
D1289	S1289	AfaCfaGfuGfuUfcUfUfGfcucUfaUfasAf	AS1289	uUfaUfaGfAfGfcaaGfaAfcAfcUfgUfusUfsu	0.05	0.09	0.31	**0.012**
D1290	S1290	AfAfCfaGfuGfuUfcUfuGfcUfcUfaUfasa	AS1290	UfUfaUfaGfaGfcAfaGfaAfcAfcUfguusUfsu	0.06	0.16	0.49	0.013
D1291	S1291	AfaCfaGfuGfuUfCfUfuGfcUfcUfaUfasAf	AS1291	uUfaUfaGfaGfcAfagaAfcAfcUfgUfusUfsUf	0.06	0.11	0.32	0.013
D1292	S1292	AfaCfAfGfuGfuUfcUfugcUfcUfaUfasAf	AS1292	uUfaUfaGfaGfCfAfaGfaAfcAfcugUfusUfsu	0.06	0.14	0.44	0.013

D1293	S1293	AfaCfaGfUfGfuUfcUfuGfcUfcUfaUfasa	AS1293	UfUfaUfaGfaGfcAfaGfaAfcacUfgUfusUfsu	0.07	0.16	0.39	0.013
D1294	S1294	AfaCfAfGfuGfuUfcUfuGfcUfcuaUfasAf	AS1294	uUfaUfAfGfaGfcAfaGfaAfcAfcugUfusUfsu	0.07	0.18	0.41	0.014
D1295	S1295	AfaCfaGfUfGfuUfcUfuGfcUfcuaUfasAf	AS1295	uUfaUfAfGfaGfcAfaGfaAfcacUfgUfusUfsu	0.07	0.18	0.47	0.014
D1296	S1296	adAdCagdTdGuudCdTugdCdTcudAdTasa	AS1296	dTdTaudAdGagdCdAagdAdAcadCdTgudTsdTsu	0.12	0.21	0.68	0.0146
D1297	S1297	AfacaGfUfGfuUfcUfuGfcUfcUfaUfasAf	AS1297	uUfaUfaGfaGfcAfaGfaAfcacUfGfUfusUfsu	0.06	0.15	0.50	0.016
D1298	S1298	AfaCfaGfUfGfuUfcUfuGfcUfcUfauasAf	AS1298	uUfAfUfaGfaGfcAfaGfaAfcacUfgUfusUfsu	0.08	0.17	0.50	0.016
D1299	S1299	AfaCfaguGfuUfcUfuGfcUfcUfaUfasAf	AS1299	uUfaUfaGfaGfcAfaGfaAfcAfCfUfgUfususu	0.07	0.16	0.50	0.018
D1300	S1300	AfaCfaGfuGfuUfcUfUfGfcUfcUfauasAf	AS1300	uUfAfUfaGfaGfcaaGfaAfcAfcUfgUfusUfsu	0.06	0.12	0.43	0.020
D1301	S1301	AfaCfaGfUfGfuUfcUfugcUfcUfaUfasAf	AS1301	uUfaUfaGfaGfCfAfaGfaAfcacUfgUfusUfsu	0.07	0.17	0.45	0.021
D1302	S1302	AfaCfaGfuguUfcUfUfGfcUfcUfaUfasAf	AS1302	uUfaUfaGfaGfcaaGfaAfCfAfcUfgUfusUfsu	0.06	0.14	0.49	0.021
D1303	S1303	AfAfCfaguGfuUfcUfuGfcUfcUfaUfasAf	AS1303	uUfaUfaGfaGfcAfaGfaAfcAfCfUfguusUfsu	0.07	0.24	0.51	0.022
D1304	S1304	AfaCfaGfuGfuucUfuGfcUfcUfaUfasAf	AS1304	uUfaUfaGfaGfcAfaGfAfAfcAfcUfgUfususu	0.09	0.27	0.47	0.033
D1305	S1305	aadCdAgudGdTucdTdTgcdTdCuadTdAsa	AS1305	udTadTdAgadGdCaadGdAacdAdCugdTdTsusu	0.19	0.36	0.86	0.045
D1306	S1306	AfacaGfuguUfcUfuGfdCUdCUdAudAsa	AS1306	dTUdAUdAGfaGfcAfaGfaAfCfAfcUfGfUfusUfsu	0.08	0.22	0.61	
D1307	S1307	AfacaGfuguUfcUfdTGfdCUdCUdAudAsa	AS1307	dTUdAUdAGfaGfcAfaGfaAfCfAfcUfGfUfusUfsu	0.13	0.39	0.84	
D1308	S1308	AfacaGfuguUfcUfuGfdCUdCUdAudAsa	AS1308	dTUdAUdAgdAGfcAfaGfaAfcAfcUfgUfusUfsu	0.09	0.13	0.48	
D1309	S1309	AfacaGfuguUfcUfdTGfdCUdCUdAudAsa	AS1309	dTUdAUdAgdAGfdCAfaGfaAfcAfcUfgUfusUfsu	0.07	0.13	0.58	
D1310	S1310	AfacaAfuguUfcUfdTGfdCUdCudAudAsa	AS1310	dTUdAudAgdAGfdCAfaGfaAfcAfcAfgUfusUfsu	0.07	0.14	0.55	
D1311	S1311	AfaCfaAfuGfuUfcUfuGfcUfcUfdAdTdAsdA	AS1311	dTdTdAdTaGfaGfcAfaGfaAfcAfcAfgUfusUfsu	0.10	0.30	0.66	
D1312	S1312	AfacaGfuguUfcUfuGfdCUdCUdAudAsa	AS1312	dTUdAUdAgdAGfcAfaGfaAfcAfcUfgUfusUfsu	0.09	0.13	0.48	
D1313	S1313	AfAfCfaGfuGfuucUfuGfcUfcUfaUfasAf	AS1313	uUfaUfaGfaGfcAfaGfAfAfcAfcUfguusUfsu	0.14	0.38	0.74	
D1314	S1314	AfaCfaGfuGfuUfcUfuGfcUfcUfaUfasAf	AS1314	uUfaUfaGfaGfcAfaGfaAfcAfcUfgUfusUfsu	0.07	0.19	0.54	
D1315	S1315	AfaCfaGfuGfuUfcUfuGfcUfcUfaUfasAf	AS1315	uUfaUfaGfaGfcAfaGfaAfcAfcUfgUfusUfsu	0.07	0.15	0.55	
D1316	S1316	AfaCfaGfuGfuUfcUfuGfcUfcUfauasAf	AS1316	uUfAfUfaGfaGfcAfaGfaAfcAfcUfgUfususu	0.07	0.16	0.53	
D1317	S1317	AfacaGfuGfuUfcUfuGfcUfcUfaUfasAf	AS1317	uUfaUfaGfaGfcAfaGfaAfcAfcUfGfUfususu	0.07	0.16	0.55	
D1318	S1318	AfAfCfaGfuguUfcUfuGfcUfcUfaUfasAf	AS1318	uUfaUfaGfaGfcAfaGfaAfCfAfcUfguusUfsu	0.10	0.32	0.61	
D1319	S1319	AfaCfaGfuGfuUfcUfuGfcUfcUfaUfasAf	AS1319	uUfaUfaGfaGfcAfaGfaAfcAfcUfgUfususu	0.08	0.16	0.53	

D1320	S1320	AfaCfaGfuGfuUfcUfuGfcUfcUfaUfasAf	AS1320	uUfaUfaGfaGfcAfaGfaAfcAfcUfgUfususu	0.08	0.16	0.61	
D1321	S1321	AfaCfaGfuGfuUfcUfuGfcUfCfUfaUfasAf	AS1321	uUfaUfagaGfcAfaGfaAfcAfcUfgUfusUfsu	0.06	0.14	0.58	
D1322	S1322	AfaCfaGfuGfuUfcuuGfcUfcUfaUfasAf	AS1322	uUfaUfaGfaGfcAfAfGfaAfcAfcUfgUfusUfsu	0.15	0.49	0.84	
D1323	S1323	AfaCfaGfuGfuUfcUfuGfcUfcuaUfasAf	AS1323	uUfaUfAfGfaGfcAfaGfaAfcAfcUfgUfususu	0.07	0.20	0.62	
D1324	S1324	AfAfCfaGfuGfuUfcUfuGfcUfcUfaUfasAf	AS1324	uUfaUfaGfaGfcAfaGfaAfcAfcUfguusUfsu	0.08	0.25	0.78	
D1325	S1325	AfAfCfaGfuGfuUfcUfuGfcUfcUfaUfasAf	AS1325	uUfaUfaGfaGfcAfaGfaAfcAfcUfguusUfsu	0.08	0.18	0.80	
D1326	S1326	AfaCfaGfuGfuUfcUfuGfcUfcUfAfUfasAf	AS1326	uUfauaGfaGfcAfaGfaAfcAfcUfgUfusUfsu	0.07	0.21	0.66	
D1327	S1327	AfaCfaGfuGfuucUfuGfcUfcUfaUfasAf	AS1327	uUfaUfaGfaGfcAfaGfAfAfcAfcUfgUfusUfsu	0.10	0.31	0.70	
D1328	S1328	AfAfCfaGfuGfuUfcUfuGfcUfcUfauasAf	AS1328	uUfAfUfaGfaGfcAfaGfaAfcAfcUfguusUfsu	0.07	0.15	0.55	
D1329	S1329	AfaCfAfGfuGfuUfcUfuGfcUfcUfaUfasAf	AS1329	uUfaUfaGfaGfcAfaGfaAfcAfcugUfusUfsu	0.08	0.19	0.71	
D1330	S1330	AfaCfaGfuGfuUfcUfuGfcUfcUfaUfAfsAf	AS1330	uuaUfaGfaGfcAfaGfaAfcAfcUfgUfusUfsu	0.09	0.27	0.76	
D1331	S1331	AfaCfaGfuguUfcUfuGfcUfcUfaUfasAf	AS1331	uUfaUfaGfaGfcAfaGfaAfCfAfcUfgUfusUfsu	0.07	0.21	0.65	
D1332	S1332	AfAfCfaGfuGfuUfcUfuGfcUfcuaUfasAf	AS1332	uUfaUfAfGfaGfcAfaGfaAfcAfcUfguusUfsu	0.07	0.17	0.53	
D1333	S1333	AfaCfaGfUfGfuUfcUfuGfcUfcUfaUfasAf	AS1333	uUfaUfaGfaGfcAfaGfaAfcacUfgUfusUfsu	0.08	0.25	0.73	
D1334	S1334	AfaCfaguGfuUfcUfuGfcUfcUfaUfasAf	AS1334	uUfaUfaGfaGfcAfaGfaAfcAfCfUfgUfusUfsu	0.07	0.18	0.54	
D1335	S1335	AfaCfaGfuGfuUfcuuGfcUfcUfaUfasAf	AS1335	uUfaUfaGfaGfcAfAfGfaAfcAfcUfgUfususu	0.14	0.38	0.57	
D1336	S1336	AfaCfaGfuGfUfUfcUfuGfcUfcUfaUfasAf	AS1336	uUfaUfaGfaGfcAfaGfaacAfcUfgUfusUfsu	0.16	0.50	0.96	
D1337	S1337	AfaCfaGfuGfuUfcUfuGfcUfcUfauasAf	AS1337	uUfAfUfaGfaGfcAfaGfaAfcAfcUfgUfusUfsu	0.08	0.19	0.54	
D1338	S1338	AfAfCfaGfuGfuUfcUfugcUfcUfaUfasAf	AS1338	uUfaUfaGfaGfCfAfaGfaAfcAfcUfguusUfsu	0.08	0.20	0.69	
D1339	S1339	AfaCfaGfuGfuUfCfUfuGfcUfcUfaUfasAf	AS1339	uUfaUfaGfaGfcAfagaAfcAfcUfgUfusUfsu	0.07	0.16	0.55	
D1340	S1340	AfaCfaGfuGfuUfcUfuGfcUfcuaUfasAf	AS1340	uUfaUfAfGfaGfcAfaGfaAfcAfcUfgUfusUfsu	0.08	0.17	0.57	
D1341	S1341	AfaCfaGfuguUfcUfuGfcUfcUfaUfasAf	AS1341	uUfaUfaGfaGfcAfaGfaAfCfAfcUfgUfususu	0.08	0.22	0.63	
D1342	S1342	AfAfCfaGfuGfuUfcuuGfcUfcUfaUfasAf	AS1342	uUfaUfaGfaGfcAfAfGfaAfcAfcUfguusUfsu	0.21	0.56	0.86	
D1343	S1343	AfacaGfuGfUfUfcUfuGfcUfcUfaUfasAf	AS1343	uUfaUfaGfaGfcAfaGfaacAfcUfGfUfusUfsu	0.14	0.37	0.73	
D1344	S1344	AfaCfaGfuGfuucUfUfGfcUfcUfaUfasAf	AS1344	uUfaUfaGfaGfcaaGfAfAfcAfcUfgUfusUfsu	0.08	0.20	0.66	
D1345	S1345	AfaCfAfGfuGfuUfcuuGfcUfcUfaUfasAf	AS1345	uUfaUfaGfaGfcAfAfGfaAfcAfcugUfusUfsu	0.12	0.34	0.73	
D1346	S1346	AfaCfaGfuGfUfUfcUfuGfcUfcUfauasAf	AS1346	uUfAfUfaGfaGfcAfaGfaacAfcUfgUfusUfsu	0.16	0.42	0.90	

D1347	S1347	AfaCfaGfuGfUfUfcUfuGfcUfcUfaUfasAf	AS1347	uUfaUfaGfaGfcAfaGfaacAfcUfgUfusUfsUf	0.17	0.43	0.85	
D1348	S1348	AfaCfAfGfuGfuucUfuGfcUfcUfaUfasAf	AS1348	uUfaUfaGfaGfcAfaGfAfAfcAfcugUfusUfsu	0.08	0.21	0.58	
D1349	S1349	AfaCfaGfuGfUfUfcUfuGfcUfcuaUfasAf	AS1349	uUfaUfAfGfaGfcAfaGfaacAfcUfgUfusUfsu	0.21	0.39	0.88	
D1350	S1350	AfaCfaguGfuUfcUfUfGfcUfcUfaUfasAf	AS1350	uUfaUfaGfaGfcaaGfaAfcAfCfUfgUfusUfsu	0.06	0.13	0.52	
D1351	S1351	AfaCfAfGfuguUfcUfuGfcUfcUfaUfasAf	AS1351	uUfaUfaGfaGfcAfaGfaAfCfAfcugUfusUfsu	0.08	0.21	0.58	
D1352	S1352	AfaCfaGfUfGfuUfcuuGfcUfcUfaUfasAf	AS1352	uUfaUfaGfaGfcAfAfGfaAfcacUfgUfusUfsu	0.18	0.49	0.84	
D1353	S1353	AfaCfaGfuGfUfUfcUfuGfcucUfaUfasAf	AS1353	uUfaUfaGfAfGfcAfaGfaacAfcUfgUfusUfsu	0.11	0.25	0.68	
D1354	S1354	AfacaGfuGfuUfcUfUfGfcUfcUfaUfasAf	AS1354	uUfaUfaGfaGfcaaGfaAfcAfcUfGfUfusUfsu	0.07	0.15	0.52	
D1355	S1355	AfaCfaGfUfGfuucUfuGfcUfcUfaUfasAf	AS1355	uUfaUfaGfaGfcAfaGfAfAfcacUfgUfusUfsu	0.10	0.26	0.63	
D1356	S1356	AfaCfaGfuGfUfUfcUfugcUfcUfaUfasAf	AS1356	uUfaUfaGfaGfCfAfaGfaacAfcUfgUfusUfsu	0.16	0.33	0.79	
D1357	S1357	AfaCfAfGfuGfuUfcUfuGfcUfcUfaUfasAf	AS1357	uUfaUfaGfaGfcAfaGfaAfcAfcugUfusUfsUf	0.09	0.19	0.51	
D1358	S1358	AfaCfaGfuGfUfUfcuuGfcUfcUfaUfasAf	AS1358	uUfaUfaGfaGfcAfAfGfaacAfcUfgUfusUfsu	0.22	0.48	0.71	
D1359	S1359	AfaCfaGfuGfuUfcUfUfGfcUfcUfaUfasAf	AS1359	uUfaUfaGfaGfcaaGfaAfcAfcUfgUfusUfsUf	0.10	0.17	0.61	
D1360	S1360	AfaCfaguGfUfUfcUfuGfcUfcUfaUfasAf	AS1360	uUfaUfaGfaGfcAfaGfaacAfCfUfgUfusUfsu	0.14	0.40	0.87	
D1361	S1361	AfaCfaGfuGfuUfcUfUfGfcUfcuaUfasAf	AS1361	uUfaUfAfGfaGfcaaGfaAfcAfcUfgUfusUfsu	0.07	0.14	0.52	
D1362	S1362	aaCfaGfuGfuUfcUfuGfCfUfcUfaUfasAf	AS1362	uUfaUfaGfagcAfaGfaAfcAfcUfgUfUfsUfsu	0.10	0.28	0.81	
D1363	S1363	AfaCfaGfuGfuucUfuGfcUfcUfAfUfasAf	AS1363	uUfauaGfaGfcAfaGfAfAfcAfcUfgUfusUfsu	0.06	0.16	0.68	
D1364	S1364	AfaCfaGfuGfuUfcUfugcUfcUfaUfAfsAf	AS1364	uuaUfaGfaGfCfAfaGfaAfcAfcUfgUfusUfsu	0.09	0.26	0.67	
D1365	S1365	aacaguguucuugcucuauasa	AS1365	uUfaUfaGfaGfcAfaGfaAfcAfcUfgUfusUfsu	0.20	0.59	0.95	
D1366	S1366	AfaCfaGfuGfuUfcUfuGfCfUfcUfauasAf	AS1366	uUfAfUfaGfagcAfaGfaAfcAfcUfgUfusUfsu	0.06	0.13	0.53	
D1367	S1367	AfaCfaGfuGfuUfcUfuGfCfUfcUfaUfasAf	AS1367	uUfaUfaGfagcAfaGfaAfcAfcUfgUfusUfsUf	0.08	0.16	0.53	
D1368	S1368	AfaCfaGfuguUfcUfuGfcUfcUfAfUfasAf	AS1368	uUfauaGfaGfcAfaGfaAfCfAfcUfgUfusUfsu	0.07	0.15	0.54	
D1369	S1369	AfaCfaGfuGfuUfcuuGfcUfcUfaUfAfsAf	AS1369	uuaUfaGfaGfcAfAfGfaAfcAfcUfgUfusUfsu	0.23	0.56	0.89	
D1370	S1370	AfaCfaGfuGfuUfcUfuGfCfUfcuaUfasAf	AS1370	uUfaUfAfGfagcAfaGfaAfcAfcUfgUfusUfsu	0.06	0.12	0.55	
D1371	S1371	AfaCfaGfuGfuUfcUfuGfCfUfcuaUfasAf	AS1371	uUfaUfAfGfagcAfaGfaAfcAfcUfgUfusUfsu	0.07	0.18	0.58	
D1372	S1372	AfaCfaguGfuUfcUfuGfcUfcUfAfUfasAf	AS1372	uUfauaGfaGfcAfaGfaAfcAfCfUfgUfusUfsu	0.06	0.15	0.56	
D1373	S1373	AfaCfaGfuGfuucUfuGfcUfcUfaUfAfsAf	AS1373	uuaUfaGfaGfcAfaGfAfAfcAfcUfgUfusUfsu	0.21	0.51	0.89	

D1374	S1374	AfacaGfuguUfcUfuGfcUfcUfaUfasAf	AS1374	uUfaUfaGfaGfcAfaGfaAfCfAfcUfGfUfusUfsu	0.08	0.21	0.64	
D1375	S1375	AfaCfaGfuGfuUfcuuGfCfUfcUfaUfasAf	AS1375	uUfaUfaGfagcAfAfGfaAfcAfcUfgUfusUfsu	0.15	0.40	0.94	
D1376	S1376	AfaCfaGfuGfuUfcuuGfCfUfcUfaUfasAf	AS1376	uUfaUfaGfagcAfAfGfaAfcAfcUfgUfusUfsu	0.13	0.40	0.96	
D1377	S1377	AfaCfaGfuGfuUfcUfuGfcUfCfUfauasAf	AS1377	uUfAfUfagaGfcAfaGfaAfcAfcUfgUfusUfsu	0.08	0.17	0.64	
D1378	S1378	AfaCfaGfuguUfcUfuGfcUfcUfaUfAfsAf	AS1378	uuaUfaGfaGfcAfaGfaAfCfAfcUfgUfusUfsu	0.18	0.50	0.97	
D1379	S1379	AfaCfaGfuGfuucUfuGfCfUfcUfaUfasAf	AS1379	uUfaUfaGfagcAfaGfAfAfcAfcUfgUfusUfsu	0.08	0.24	0.79	
D1380	S1380	aaCfaGfuGfuUfcUfuGfcUfcUfAfUfasAf	AS1380	uUfauaGfaGfcAfaGfaAfcAfcUfgUfUfsUfsu	0.07	0.14	0.58	
D1381	S1381	AfaCfaguGfuUfcUfuGfcUfcUfaUfAfsAf	AS1381	uuaUfaGfaGfcAfaGfaAfcAfCfUfgUfusUfsu	0.11	0.34	0.96	
D1382	S1382	AfaCfaGfuguUfcUfuGfCfUfcUfaUfasAf	AS1382	uUfaUfaGfagcAfaGfaAfCfAfcUfgUfusUfsu	0.08	0.18	0.69	
D1383	S1383	AfaCfaGfuGfuUfcuuGfcUfCfUfaUfasAf	AS1383	uUfaUfagaGfcAfAfGfaAfcAfcUfgUfusUfsu	0.14	0.38	0.85	
D1384	S1384	AfaCfaGfuGfuUfcUfuGfcUfcUfAfUfasAf	AS1384	uUfauaGfaGfcAfaGfaAfcAfcUfgUfusUfsUf	0.07	0.16	0.54	
D1385	S1385	AfacaGfuGfuUfcUfuGfcUfcUfaUfAfsAf	AS1385	uuaUfaGfaGfcAfaGfaAfcAfcUfGfUfusUfsu	0.08	0.20	0.75	
D1386	S1386	aacaguguucUfuGfcUcUaudAsa	AS1386	uUfdAUdAGfaGfcAfaGfaadCadCudGdTdTsusu	0.25	0.56	0.90	
D1387	S1387	AfaCfaguGfuUfcUfuGfCfUfcUfaUfasAf	AS1387	uUfaUfaGfagcAfaGfaAfcAfCfUfgUfusUfsu	0.08	0.19	0.70	
D1388	S1388	AfaCfaGfuGfuucUfuGfcUfCfUfaUfasAf	AS1388	uUfaUfagaGfcAfaGfAfAfcAfcUfgUfusUfsu	0.08	0.14	0.60	
D1389	S1389	AfaCfaGfuGfuUfcUfuGfcUfcuaUfAfsAf	AS1389	uuaUfAfGfaGfcAfaGfaAfcAfcUfgUfusUfsu	0.08	0.19	0.62	
D1390	S1390	aaCfaGfuGfuUfcUfuGfcUfcUfaUfAfsAf	AS1390	uuaUfaGfaGfcAfaGfaAfcAfcUfgUfUfsUfsu	0.08	0.27	0.76	
D1391	S1391	aacaguguucdTudGcdTcdTadTasa	AS1391	uUfdAUdAGfaGfcAfaGfaadCadCudGudTsusu	0.18	0.36	0.81	
D1392	S1392	AfacaGfuGfuUfcUfuGfCfUfcUfaUfasAf	AS1392	uUfaUfaGfagcAfaGfaAfcAfcUfGfUfusUfsu	0.07	0.17	0.55	
D1393	S1393	AfaCfaGfuguUfcUfuGfcUfCfUfaUfasAf	AS1393	uUfaUfagaGfcAfaGfaAfCfAfcUfgUfusUfsu	0.07	0.15	0.57	
D1394	S1394	AfaCfaGfuGfuUfcuuGfcUfcUfAfUfasAf	AS1394	uUfauaGfaGfcAfAfGfaAfcAfcUfgUfusUfsu	0.26	0.68	1.06	
D1395	S1395	AfaCfaGfuGfuUfcUfuGfcucUfaUfAfsAf	AS1395	uuaUfaGfAfGfcAfaGfaAfcAfcUfgUfusUfsu	0.06	0.18	0.58	
D1396	S1396	AfaCfaGfuGfuUfcUfuGfcUfcUfaUfAfsAf	AS1396	uuaUfaGfaGfcAfaGfaAfcAfcUfgUfusUfsUf	0.09	0.27	0.73	
D1397	S1397	AfaCfaAfuGfuUfcUfuGfcdAdCdTdAUfasAf	AS1397	uUfadTdAdGdAGfcAfaGfaGfcAfcAfgUfusUfsu	0.20	0.51	0.73	
D1398	S1398	AfacaGfuguUfcuuGfcucUfauasAf	AS1398	uUfAfUfaGfAfGfcAfAfGfaAfCfAfcUfGfUfusUfsu	0.13	0.34	0.86	
D1399	S1399	dAacadGugudTcuudGcucdTauasdA	AS1399	udTdAdTadGdAdGcdAdAdGadAdCdAcdTdGdTusdTsu	0.24	0.42	0.82	
D1400	S1400	AfaCfaAfuGfuUfcUfuGfdCdAdCdTaUfasAf	AS1400	uUfaUfdAdGdAdGcAfaGfaGfcAfcAfgUfusUfsu	0.49	0.85	0.78	

D1401	S1401	AfaCfaAfuGfuUfcUfudGdCdAdCUfaUfasAf	AS1401	uUfaUfadGdAdGdCAfaGfaGfcAfcAfgUfusUfsu	0.67	0.83	0.85	
D1402	S1402	aaCfAfguGfUfucUfUfgcUfCfuaUfAfsa	AS1402	uUfaUfAfgaGfCfaaGfAfacAfCfugUfUfsusu	0.18	0.47	0.80	
D1403	S1403	AfaCfaAfuGfuUfcUfuGfcdAdCUfadTdAsAf	AS1403	udTdAUfadGdAGfcAfaGfaGfcAfcAfgUfusUfsu	0.73	0.89	0.77	
D1404	S1404	aacAgugUucuUgcuCuauAsa	AS1404	uUaUAgAGCaAGAaCACuGUUsusu	0.12	0.39	0.79	
D1405	S1405	AacaGuguUcuuGcucUauasA	AS1405	uUAUaGAGcAAGaACAcUGUusUsu	0.12	0.37	0.77	
D1406	S1406	AfaCfaAfuGfuUfcUfudGdCAfcUfadTdAsAf	AS1406	udTdAUfaGfadGdCAfaGfaGfcAfcAfgUfusUfsu	0.59	0.93	0.89	
D1407	S1407	aACagUGuuCUugCUcuAUasa	AS1407	UUauAGagCAagAAcaCUguUsUsu	0.09	0.16	0.55	
D1408	S1408	AfaCfaAfuGfuUfcUfuGfcAfcdTdAdTdAsAf	AS1408	udTdAdTdAGfaGfcAfaGfaAfcAfcAfgUfusUfsu	0.22	0.64	0.86	
D1409	S1409	aaCAguGUucUUgcUCuaUAsa	AS1409	uUaUAgaGCaaGAacACugUUsusu	0.13	0.31	0.76	
D1410	S1410	AfaCfaAfuGfuUfcUfuGfcAfdCdTdAdTdAsAf	AS1410	udTdAdTdAdGaGfcAfaGfaGfcAfcAfgUfusUfsu	0.77	0.94	0.93	
D1411	S1411	aacAfgugUfucuUfgcuCfuauAfsa	AS1411	uUfaUfAfgAfGfCfaAfGfAfaCfAfCfuGfUfUfsusu	0.23	0.53	1.04	
D1412	S1412	aacdAgugdTucudTgcudCuaudAsa	AS1412	udTadTdAgdAdGdCadAdGdAadCdAdCudGdTdTsusu	0.30	0.64	0.90	
D1413	S1413	AfaCfaGfuGfuUfcUfuGfcUfcUfaUfasa	AS1413	UfUfaUfaGfaGfcAfaGfaAfcAfcUfgUfusUfsu	0.09	0.19	0.63	
D1414	S1414	AfaCfaGfuGfUfUfcUfuGfcUfcUfaUfasa	AS1414	UfUfaUfaGfaGfcAfaGfaacAfcUfgUfusUfsu	0.11	0.28	0.66	
D1415	S1415	AfaCfaGfuGfuUfcUfuGfCfUfcUfaUfasa	AS1415	UfUfaUfaGfagcAfaGfaAfcAfcUfgUfusUfsu	0.06	0.13	0.53	
D1416	S1416	aacaguguucuugcucuauasa	AS1416	UfUfAfUfAfGfAfGfCfAfAfGfAfAfCfAfCfUfGfUfUfsusu	0.20	0.53	0.99	
D1417	S1417	AfaCfaGfuGfuUfcUfuGfcUfcUfAfUfasa	AS1417	UfUfauaGfaGfcAfaGfaAfcAfcUfgUfusUfsu	0.07	0.17	0.53	
D1418	S1418	aAfCfagUfGfuuCfUfugCfUfcuAfUfasa	AS1418	UfUfauAfGfagCfAfagAfAfcaCfUfguUfsUfsu	0.08	0.20	0.70	
D1419	S1419	AfaCfAfGfuGfuUfcUfuGfcUfcUfaUfasAf	AS1419	uUfaUfaGfaGfcAfaGfaAfcAfcugUfusUfsUf	0.08	0.20	0.70	

Example 3: In vitro silencing activity with various chemical modifications on TTR siRNA

The IC_{50} for each modified siRNA is determined in Hep3B cells by standard reverse transfection using Lipofectamine RNAiMAX. In brief, reverse transfection is carried out by adding 5 μL of Opti-MEM to 5 μL of siRNA duplex per well into a 96-well plate along with 10 μL of Opti-MEM plus 0.5 μL of Lipofectamine RNAiMax per well (Invitrogen, Carlsbad CA. cat # 13778-150) and incubating at room temperature for 15-20 minutes. Following incubation, 100 μL of complete growth media without antibiotic containing 12,000-15,000 Hep3B cells is then added to each well. Cells are incubated for 24 hours at 37°C in an atmosphere of 5% CO2 prior to lysis and analysis of ApoB and GAPDH mRNA by bDNA (Quantigene). Seven different siRNA concentrations ranging from 10nM to 0.6pM are assessed for IC_{50} determination and ApoB/GAPDH for ApoB transfected cells is normalized to cells transfected with 10nM Luc siRNA.

Abbreviation	Nucleotide(s)
Af	2'-F-adenosine
Cf	2'-F-cytidine
Gf	2'-F-guanosine
Uf	2'-F-uridine
A	adenosine
C	cytidine
G	guanosine
U	uridine
a	2'-O-methyladenosine
c	2'-O-methylcytidine
g	2'-O-methylguanosine
u	2'-O-methyluridine
dT	2'-deoxythymidine
s	phosphorothioate linkage

Table 3. ANGPTL3 modified duplex

Duplex ID	Sense ID	SS seq	AS ID	AS seq	RNAimax, Hep3b		
					10nM	0.1nM	0.025nM
D2000	S2000	UfcAfcAfaUfuAfAfGfcUfcCfuUfcUfuUf	A2000	aAfaGfaAfgGfaGfcuuAfaUfuGfuGfasAfsc	0.036	0.274	0.233
D2001	S2001	UfuAfuUfgUfuCfCfUfcUfaGfuUfaUfuUf	A2001	aAfaUfaAfcUfaGfaggAfaCfaAfuAfasAfsa	0.044	0.278	0.247
D2002	S2002	GfcUfaUfgUfuAfGfAfcGfaUfgUfaAfaAf	A2002	uUfuUfaCfaUfcGfucuAfaCfaUfaGfcsAfsa	0.062	0.474	0.449
D2003	S2003	GfgAfcAfuGfgUfCfUfuAfaAfgAfcUfuUf	A2003	aAfaGfuCfuUfuAfagaCfcAfuGfuCfcsCfsa	0.303	1.042	0.912
D2004	S2004	CfaAfaAfaCfuCfAfAfcAfuAfuUfuGfaUf	A2004	aUfcAfaAfuAfuGfuugAfgUfuUfuUfgsAfsa	0.102	0.623	0.499
D2005	S2005	AfcCfaGfuGfaAfAfUfcAfaAfgAfaGfaAf	A2005	uUfcUfuCfuUfuGfauuUfcAfcUfgGfusUfsu	0.124	0.901	0.756
D2006	S2006	CfaCfaAfuUfaAfGfCfuCfcUfuCfuUfuUf	A2006	aAfaAfgAfaGfgAfgcuUfaAfuUfgUfgsAfsa	0.069	0.269	0.244
D2007	S2007	CfuAfuGfuUfaGfAfCfgAfuGfuAfaAfaAf	A2007	uUfuUfuAfcAfuCfgucUfaAfcAfuAfgsCfsa	0.052	0.622	0.589
D2008	S2008	UfcAfaCfaUfaUfUfUfgAfuCfaGfuCfuUf	A2008	aAfgAfcUfgAfuCfaaaUfaUfgUfuGfasGfsu	0.133	0.798	0.785
D2009	S2009	AfaCfuGfaGfaAfGfAfaCfuAfcAfuAfuAf	A2009	uAfuAfuGfuAfgUfucuUfcUfcAfgUfusCfsc	0.097	0.671	0.528
D2010	S2010	AfcAfaUfuAfaGfCfUfcCfuUfcUfuUfuUf	A2010	aAfaAfaGfaAfgGfagcUfuAfaUfuGfusGfsa	0.145	0.308	0.293
D2011	S2011	CfuCfcAfgAfgCfCfAfaAfaUfcAfaGfaUf	A2011	aUfcUfuGfaUfuUfuggCfuCfuGfgAfgsAfsu	0.122	0.882	0.938
D2012	S2012	CfgAfuGfuAfaAfAfAfuUfuUfaGfcCfaAf	A2012	uUfgGfcUfaAfaAfuuuUfuAfcAfuCfgsUfsc	0.102	0.843	0.733
D2013	S2013	GfuCfuUfaAfaGfAfCfuUfuGfuCfcAfuAf	A2013	uAfuGfgAfcAfaAfgucUfuUfaAfgAfcsCfsa	1.133	1.105	1.022
D2014	S2014	CfaAfcAfuAfuUfUfGfaUfcAfgUfcUfuUf	A2014	aAfaGfaCfuGfaUfcaaAfuAfuGfuUfgsAfsg	0.077	0.413	0.450
D2015	S2015	AfcUfgAfgAfaGfAfAfcUfaCfaUfaUfaAf	A2015	uUfaUfaUfgUfaGfuucUfuCfuCfaGfusUfsc	0.055	0.293	0.364
D2016	S2016	CfcAfgAfgCfcAfAfAfaUfcAfaGfaUfuUf	A2016	aAfaUfcUfuGfaUfuuuGfgCfuCfuGfgsAfsg	0.080	0.650	0.499
D2017	S2017	GfaUfgUfaAfaAfAfUfuUfuAfgCfcAfaUf	A2017	aUfuGfgCfuAfaAfauuUfuUfaCfaUfcsGfsu	0.076	0.605	0.579
D2018	S2018	UfcUfuAfaAfgAfCfUfuUfgUfcCfaUfaAf	A2018	uUfaUfgGfaCfaAfaguCfuUfuAfaGfasCfsc	1.326	1.098	0.927
D2019	S2019	AfaCfaUfaUfuUfGfAfuCfaGfuCfuUfuUf	A2019	aAfaAfgAfcUfgAfucaAfaUfaUfgUfusGfsa	0.047	0.560	0.477
D2020	S2020	CfuGfaGfaAfgAfAfCfuAfcAfuAfuAfaAf	A2020	uUfuAfuAfuGfuAfguuCfuUfcUfcAfgsUfsu	0.066	0.690	0.681
D2021	S2021	AfaUfuAfaGfcUfCfCfuUfcUfuUfuUfaUf	A2021	aUfaAfaAfaGfaAfggaGfcUfuAfaUfusGfsu	0.041	0.611	0.251
D2022	S2022	AfaAfuCfaAfgAfUfUfuGfcUfaUfgUfuAf	A2022	uAfaCfaUfaGfcAfaauCfuUfgAfuUfusUfsg	0.053	0.555	0.516
D2023	S2023	UfuCfaGfuUfgGfGfAfcAfuGfgUfcUfuAf	A2023	uAfaGfaCfcAfuGfuccCfaAfcUfgAfasGfsg	0.779	1.045	0.963

D2024	S2024	GfgGfcCfaAfaUfUfAfaUfgAfcAfuAfuUf	A2024	aAfuAfuGfuCfaUfuaaUfuUfgGfcCfcsUfsu	1.487	0.949	0.883
D2025	S2025	AfcAfuAfuUfuGfAfUfcAfgUfcUfuUfuUf	A2025	aAfaAfaGfaCfuGfaucAfaAfuAfuGfusUfsg	0.043	0.432	0.477
D2026	S2026	AfgAfaCfuAfcAfUfAfuAfaAfcUfaCfaAf	A2026	uUfgUfaGfuUfuAfuauGfuAfgUfuCfusUfsc	0.324	1.042	0.905
D2027	S2027	AfuUfaAfgCfuCfCfUfuCfuUfuUfuAfuUf	A2027	aAfuAfaAfaAfgAfaggAfgCfuUfaAfusUfsg	0.042	0.283	0.224
D2028	S2028	AfgAfuUfuGfcUfAfUfgUfuAfgAfcGfaUf	A2028	aUfcGfuCfuAfaCfauaGfcAfaAfuCfusUfsg	0.349	0.936	0.896
D2029	S2029	UfcAfgUfuGfgGfAfCfaUfgGfuCfuUfaAf	A2029	uUfaAfgAfcCfaUfgucCfcAfaCfuGfasAfsg	0.914	0.907	0.944
D2030	S2030	GfgCfcAfaAfuUfAfAfuGfaCfaUfaUfuUf	A2030	aAfaUfaUfgUfcAfuuaAfuUfuGfgCfcsCfsu	0.047	0.353	0.326
D2031	S2031	CfaUfaUfuUfgAfUfCfaGfuCfuUfuUfuAf	A2031	uAfaAfaAfgAfcUfgauCfaAfaUfaUfgsUfsu	0.110	0.867	0.842
D2032	S2032	UfaCfaUfaUfaAfAfCfuAfcAfaGfuCfaAf	A2032	uUfgAfcUfuGfuAfguuUfaUfaUfgUfasGfsu	0.200	0.699	0.656
D2033	S2033	UfuUfuAfuUfgUfUfCfcUfcUfaGfuUfaUf	A2033	aUfaAfcUfaGfaGfgaaCfaAfuAfaAfasAfsg	0.050	0.218	0.192
D2034	S2034	UfuGfcUfaUfgUfUfAfgAfcGfaUfgUfaAf	A2034	uUfaCfaUfcGfuCfuaaCfaUfaGfcAfasAfsu	0.096	0.792	0.640
D2035	S2035	CfaGfuUfgGfgAfCfAfuGfgUfcUfuAfaAf	A2035	uUfuAfaGfaCfcAfuguCfcCfaAfcUfgsAfsa	0.127	0.936	0.890
D2036	S2036	AfaAfuUfaAfuGfAfCfaUfaUfuUfcAfaAf	A2036	uUfuGfaAfaUfaUfgucAfuUfaAfuUfusGfsg	0.061	0.683	0.668
D2037	S2037	GfaUfcAfgUfcUfUfUfuUfaUfgAfuCfuAf	A2037	uAfgAfuCfaUfaAfaaaGfaCfuGfaUfcsAfsa	0.157	1.010	0.723
D2038	S2038	AfcAfuAfuAfaAfCfUfaCfaAfgUfcAfaAf	A2038	uUfuGfaCfuUfgUfaguUfuAfuAfuGfusAfsg	0.047	0.532	0.525
D2039	S2039	UfuUfaUfuGfuUfCfCfuCfuAfgUfuAfuUf	A2039	aAfuAfaCfuAfgAfggaAfcAfaUfaAfasAfsa	0.031	0.505	0.238
D2040	S2040	UfgCfuAfuGfuUfAfGfaCfgAfuGfuAfaAf	A2040	uUfuAfcAfuCfgUfcuaAfcAfuAfgCfasAfsa	0.056	0.484	0.408
D2041	S2041	GfgGfaCfaUfgGfUfCfuUfaAfaGfaCfuUf	A2041	aAfgUfcUfuUfaAfgacCfaUfgUfcCfcsAfsa	0.570	0.999	0.994
D2042	S2042	UfgAfcAfuAfuUfUfCfaAfaAfaCfuCfaAf	A2042	uUfgAfgUfuUfuUfgaaAfuAfuGfuCfasUfsu	0.065	0.870	0.728
D2043	S2043	AfuCfaGfuCfuUfUfUfuAfuGfaUfcUfaUf	A2043	aUfaGfaUfcAfuAfaaaAfgAfcUfgAfusCfsa	0.048	0.362	0.282
D2044	S2044	CfaUfaUfaAfaCfUfAfcAfaGfuCfaAfaAf	A2044	uUfuUfgAfcUfuGfuagUfuUfaUfaUfgsUfsa	0.314	0.904	0.937
D2045	S2045	CfuUfgAfaCfuCfAfAfcUfcAfaAfaCfuUf	A2045	aAfgUfuUfuGfaGfuugAfgUfuCfaAfgsUfsg	0.060	0.295	0.251
D2046	S2046	CfuAfcUfuCfaAfCfAfaAfaAfgUfgAfaAf	A2046	uUfuCfaCfuUfuUfuguUfgAfaGfuAfgsAfsa	0.052	0.570	0.599
D2047	S2047	AfaGfaGfcAfaCfUfAfaCfuAfaCfuUfaAf	A2047	uUfaAfgUfuAfgUfuagUfuGfcUfcUfusCfsu	0.028	0.369	0.381
D2048	S2048	AfaAfcAfaGfaUfAfAfuAfgCfaUfcAfaAf	A2048	uUfuGfaUfgCfuAfuuaUfcUfuGfuUfusUfsu	0.039	0.227	0.204
D2049	S2049	GfcAfuAfgUfcAfAfAfuAfaAfaGfaAfaUf	A2049	aUfuUfcUfuUfuAfuuuGfaCfuAfuGfcsUfsg	0.032	0.437	0.422
D2050	S2050	AfuAfuAfaAfcUfAfCfaAfgUfcAfaAfaAf	A2050	uUfuUfuGfaCfuUfguaGfuUfuAfuAfusGfsu	0.297	0.946	0.850

D2051	S2051	GfaAfcUfcAfaCfUfCfaAfaAfcUfuGfaAf	A2051	uUfcAfaGfuUfuUfgagUfuGfaGfuUfcsAfsa	0.179	0.929	0.884
D2052	S2052	UfaCfuUfcAfaCfAfAfaAfaGfuGfaAfaUf	A2052	aUfuUfcAfcUfuUfuugUfuGfaAfgUfasGfsa	0.091	0.536	0.524
D2053	S2053	AfgAfgCfaAfcUfAfAfcUfaAfcUfuAfaUf	A2053	aUfuAfaGfuUfaGfuuaGfuUfgCfuCfusUfsc	0.086	0.611	0.621
D2054	S2054	GfaUfaAfuAfgCfAfUfcAfaAfgAfcCfuUf	A2054	aAfgGfuCfuUfuGfaugCfuAfuUfaUfcsUfsu	0.058	0.676	0.591
D2055	S2055	CfaUfaGfuCfaAfAfUfaAfaAfgAfaAfuAf	A2055	uAfuUfuCfuUfuUfauuUfgAfcUfaUfgsCfsu	0.048	0.630	0.674
D2056	S2056	UfaUfaAfaCfuAfCfAfaGfuCfaAfaAfaUf	A2056	aUfuUfuUfgAfcUfuguAfgUfuUfaUfasUfsg	0.072	0.534	0.459
D2057	S2057	AfaCfuCfaAfcUfCfAfaAfaCfuUfgAfaAf	A2057	uUfuCfaAfgUfuUfugaGfuUfgAfgUfusCfsa	0.161	0.864	0.775
D2058	S2058	AfcUfuCfaAfcAfAfAfaAfgUfgAfaAfuAf	A2058	uAfuUfuCfaCfuUfuuuGfuUfgAfaGfusAfsg	0.198	0.969	0.865
D2059	S2059	GfaGfcAfaCfuAfAfCfuAfaCfuUfaAfuUf	A2059	aAfuUfaAfgUfuAfguuAfgUfuGfcUfcsUfsu	0.031	0.253	0.210
D2060	S2060	AfaCfcAfaCfaGfCfAfuAfgUfcAfaAfuAf	A2060	uAfuUfuGfaCfuAfugcUfgUfuGfgUfusUfsa	0.035	0.561	0.569
D2061	S2061	AfgUfcAfaAfuAfAfAfaGfaAfaUfaGfaAf	A2061	uUfcUfaUfuUfcUfuuuAfuUfuGfaCfusAfsu	0.057	0.668	0.386
D2062	S2062	AfgUfcAfaAfaAfUfGfaAfgAfgGfuAfaAf	A2062	uUfuAfcCfuCfuUfcauUfuUfuGfaCfusUfsg	0.720	1.017	0.924
D2063	S2063	CfuUfgAfaAfgCfCfUfcCfuAfgAfaGfaAf	A2063	uUfcUfuCfuAfgGfaggCfuUfuCfaAfgsUfsu	0.324	1.020	0.963
D2064	S2064	CfuUfcAfaCfaAfAfAfaGfuGfaAfaUfaUf	A2064	aUfaUfuUfcAfcUfuuuUfgUfuGfaAfgsUfsa	0.048	0.549	0.531
D2065	S2065	CfaAfcUfaAfcUfAfAfcUfuAfaUfuCfaAf	A2065	uUfgAfaUfuAfaGfuuaGfuUfaGfuUfgsCfsu	0.046	0.739	0.649
D2066	S2066	AfcCfaAfcAfgCfAfUfaGfuCfaAfaUfaAf	A2066	uUfaUfuUfgAfcUfaugCfuGfuUfgGfusUfsu	0.076	0.840	0.777
D2067	S2067	GfaAfcCfcAfcAfGfAfaAfuUfuCfuCfuAf	A2067	uAfgAfgAfaAfuUfucuGfuGfgGfuUfcsUfsu	0.103	0.916	0.808
D2068	S2068	GfaAfuAfuGfuCfAfCfuUfgAfaCfuCfaAf	A2068	uUfgAfgUfuCfaAfgugAfcAfuAfuUfcsUfsu	0.046	0.532	0.520
D2069	S2069	UfgAfaAfgCfcUfCfCfuAfgAfaGfaAfaAf	A2069	uUfuUfcUfuCfuAfggaGfgCfuUfuCfasAfsg	0.067	0.894	0.822
D2070	S2070	UfuCfaAfcAfaAfAfAfgUfgAfaAfuAfuUf	A2070	aAfuAfuUfuCfaCfuuuUfuGfuUfgAfasGfsu	0.052	0.557	0.395
D2071	S2071	AfaCfuAfaCfuAfAfCfuUfaAfuUfcAfaAf	A2071	uUfuGfaAfuUfaAfguuAfgUfuAfgUfusGfsc	0.025	0.220	0.232
D2072	S2072	CfcAfaCfaGfcAfUfAfgUfcAfaAfuAfaAf	A2072	uUfuAfuUfuGfaCfuauGfcUfgUfuGfgsUfsu	0.293	0.923	0.899
D2073	S2073	AfaCfcCfaCfaGfAfAfaUfuUfcUfcUfaUf	A2073	aUfaGfaGfaAfaUfuucUfgUfgGfgUfusCfsu	0.021	0.375	0.356
D2074	S2074	UfgUfcAfcUfuGfAfAfcUfcAfaCfuCfaAf	A2074	uUfgAfgUfuGfaGfuucAfaGfuGfaCfasUfsa	0.052	0.402	0.513
D2075	S2075	GfaAfaGfcCfuCfCfUfaGfaAfgAfaAfaAf	A2075	uUfuUfuCfuUfcUfaggAfgGfcUfuUfcsAfsa	0.171	0.904	0.893
D2076	S2076	AfaUfaUfuUfaGfAfAfgAfgCfaAfcUfaAf	A2076	uUfaGfuUfgCfuCfuucUfaAfaUfaUfusUfsc	0.142	0.614	0.688
D2077	S2077	AfcUfaAfcUfaAfCfUfuAfaUfuCfaAfaAf	A2077	uUfuUfgAfaUfuAfaguUfaGfuUfaGfusUfsg	0.020	0.312	0.316

D2078	S2078	CfaAfcAfgCfaUfAfGfuCfaAfaUfaAfaAf	A2078	uUfuUfaUfuUfgAfcuaUfgCfuGfuUfgsGfsu	0.026	0.313	0.393
D2079	S2079	CfcAfcAfgAfaAfUfUfuCfuCfuAfuCfuUf	A2079	aAfgAfuAfgAfgAfaauUfuCfuGfuGfgsGfsu	0.012	0.596	0.345
D2080	S2080	GfuCfaCfuUfgAfAfCfuCfaAfcUfcAfaAf	A2080	uUfuGfaGfuUfgAfguuCfaAfgUfgAfcsAfsu	0.054	0.503	0.456
D2081	S2081	CfuCfcUfaGfaAfGfAfaAfaAfaUfuCfuAf	A2081	uAfgAfaUfuUfuUfucuUfcUfaGfgAfgsGfsc	0.050	0.596	0.531
D2082	S2082	AfuUfuAfgAfaGfAfGfcAfaCfuAfaCfuAf	A2082	uAfgUfuAfgUfuGfcucUfuCfuAfaAfusAfsu	0.064	0.806	0.928
D2083	S2083	CfuAfaCfuAfaCfUfUfaAfuUfcAfaAfaUf	A2083	aUfuUfuGfaAfuUfaagUfuAfgUfuAfgsUfsu	0.056	0.844	0.761
D2084	S2084	CfaGfcAfuAfgUfCfAfaAfuAfaAfaGfaAf	A2084	uUfcUfuUfuAfuUfugaCfuAfuGfcUfgsUfsu	0.046	0.859	0.756
D2085	S2085	GfaAfaUfaAfgAfAfAfuGfuAfaAfaCfaUf	A2085	aUfgUfuUfuAfcAfuuuCfuUfaUfuUfcsAfsu	0.039	0.615	0.612
D2086	S2086	UfcAfcUfuGfaAfCfUfcAfaCfuCfaAfaAf	A2086	uUfuUfgAfgUfuGfaguUfcAfaGfuGfasCfsa	0.057	0.724	0.663
D2087	S2087	UfcUfaCfuUfcAfAfCfaAfaAfaGfuGfaAf	A2087	uUfcAfcUfuUfuUfguuGfaAfgUfaGfasAfsu	0.732	1.028	0.915
D2088	S2088	UfuUfaGfaAfgAfGfCfaAfcUfaAfcUfaAf	A2088	uUfaGfuUfaGfuUfgcuCfuUfcUfaAfasUfsa	0.061	0.795	0.785
D2089	S2089	AfaAfaCfaAfgAfUfAfaUfaGfcAfuCfaAf	A2089	uUfgAfuGfcUfaUfuauCfuUfgUfuUfusUfsc	0.330	1.017	0.865
D2090	S2090	AfgCfaUfaGfuCfAfAfaUfaAfaAfgAfaAf	A2090	uUfuCfuUfuUfaUfuugAfcUfaUfgCfusGfsu	0.038	0.606	0.589
D2091	S2091	AfgAfcCfcAfgCfAfAfcUfcUfcAfaGfuUf	A2091	aAfcUfuGfaGfaGfuugCfuGfgGfuCfusGfsa	0.301	0.850	0.753
D2092	S2092	AfgUfcCfaUfgGfAfCfaUfuAfaUfuCfaAf	A2092	uUfgAfaUfuAfaUfgucCfaUfgGfaCfusAfsc	0.407	0.791	0.726
D2093	S2093	GfaUfgGfaUfcAfCfAfaAfaCfuUfcAfaUf	A2093	aUfuGfaAfgUfuUfuguGfaUfcCfaUfcsUfsa	0.120	0.658	0.654
D2094	S2094	CfuAfgAfgAfaGfAfUfaUfaCfuCfcAfuAf	A2094	uAfuGfgAfgUfaUfaucUfuCfuCfuAfgsGfsc	0.071	0.610	0.645
D2095	S2095	AfaAfgAfcAfaCfAfAfaCfaUfuAfuAfuUf	A2095	aAfuAfuAfaUfgUfuugUfuGfuCfuUfusCfsc	0.029	0.306	0.461
D2096	S2096	CfaUfuAfuAfuUfGfAfaUfaUfuCfuUfuUf	A2096	aAfaAfgAfaUfaUfucaAfuAfuAfaUfgsUfsu	0.031	0.510	0.595
D2097	S2097	GfaCfcCfaGfcAfAfCfuCfuCfaAfgUfuUf	A2097	aAfaCfuUfgAfgAfguuGfcUfgGfgUfcsUfsg	0.075	0.697	0.845
D2098	S2098	GfgAfuCfaCfaAfAfAfcUfuCfaAfuGfaAf	A2098	uUfcAfuUfgAfaGfuuuUfgUfgAfuCfcsAfsu	0.130	0.831	0.951
D2099	S2099	GfaAfgAfuAfuAfCfUfcCfaUfaGfuGfaAf	A2099	uUfcAfcUfaUfgGfaguAfuAfuCfuUfcsUfsc	0.058	0.828	0.938
D2100	S2100	GfaCfaAfcAfaAfCfAfuUfaUfaUfuGfaAf	A2100	uUfcAfaUfaUfaAfuguUfuGfuUfgUfcsUfsu	0.026	0.564	0.856
D2101	S2101	GfgGfaAfaUfcAfCfGfaAfaCfcAfaCfuAf	A2101	uAfgUfuGfgUfuUfcguGfaUfuUfcCfcsAfsa	0.314	0.948	1.033
D2102	S2102	AfcCfcAfgCfaAfCfUfcUfcAfaGfuUfuUf	A2102	aAfaAfcUfuGfaGfaguUfgCfuGfgGfusCfsu	0.033	0.448	0.675
D2103	S2103	GfgAfcAfuUfaAfUfUfcAfaCfaUfcGfaAf	A2103	uUfcGfaUfgUfuGfaauUfaAfuGfuCfcsAfsu	0.156	0.897	0.912
D2104	S2104	GfaUfcAfcAfaAfAfCfuUfcAfaUfgAfaAf	A2104	uUfuCfaUfuGfaAfguuUfuGfuGfaUfcsCfsa	0.056	0.619	0.769

D2105	S2105	AfcUfcCfaUfaGfUfGfaAfgCfaAfuCfuAf	A2105	uAfgAfuUfgCfuUfcacUfaUfgGfaGfusAfsu	0.100	0.823	0.925
D2106	S2106	AfcAfaCfaAfaCfAfUfuAfuAfuUfgAfaUf	A2106	aUfuCfaAfuAfuAfaugUfuUfgUfuGfusCfsu	0.035	0.565	0.843
D2107	S2107	GfgAfaAfuCfaCfGfAfaAfcCfaAfcUfaUf	A2107	aUfaGfuUfgGfuUfucgUfgAfuUfuCfcsCfsa	0.076	0.701	0.890
D2108	S2108	CfcCfaGfcAfaCfUfCfuCfaAfgUfuUfuUf	A2108	aAfaAfaCfuUfgAfgagUfuGfcUfgGfgsUfsc	0.057	0.626	0.884
D2109	S2109	GfaCfaUfuAfaUfUfCfaAfcAfuCfgAfaUf	A2109	aUfuCfgAfuGfuUfgaaUfuAfaUfgUfcsCfsa	0.160	0.873	1.012
D2110	S2110	AfaCfgUfgGfgAfGfAfaCfuAfcAfaAfuAf	A2110	uAfuUfuGfuAfgUfucuCfcCfaCfgUfusUfsc	0.101	0.881	0.981
D2111	S2111	CfuCfcAfuAfgUfGfAfaGfcAfaUfcUfaAf	A2111	uUfaGfaUfuGfcUfucaCfuAfuGfgAfgsUfsa	0.026	0.435	0.691
D2112	S2112	CfaAfcAfaAfcAfUfUfaUfuUfuGfaAfuAf	A2112	uAfuUfcAfaUfaUfaauGfuUfuGfuUfgsUfsc	0.154	0.882	1.091
D2113	S2113	GfaAfaUfcAfcGfAfAfaCfcAfaCfuAfuAf	A2113	uAfuAfgUfuGfgUfuucGfuGfaUfuUfcsCfsc	0.045	0.764	1.004
D2114	S2114	CfuCfuCfaAfgUfUfUfuUfcAfuGfuCfuAf	A2114	uAfgAfcAfuGfaAfaaaCfuUfgAfgAfgsUfsu	0.105	0.925	0.988
D2115	S2115	AfcAfuUfaAfuUfCfAfaCfaUfcGfaAfuAf	A2115	uAfuUfcGfaUfgUfugaAfuUfaAfuGfusCfsc	0.114	0.919	0.905
D2116	S2116	GfgGfaGfaAfcUfAfCfaAfaUfuUfgGfuUf	A2116	aAfcCfaUfaUfuUfUfguaGfuUfUfcUfcCfcsAfsc	0.234	1.023	0.951
D2117	S2117	UfcCfaUfaGfuGfAfAfgCfaAfuCfuAfaUf	A2117	aUfuAfgAfuUfgCfuucAfcUfaUfgGfasGfsu	0.033	0.566	0.778
D2118	S2118	AfaCfaAfaCfaUfUfAfuAfuUfgAfaUfaUf	A2118	aUfaUfuCfaAfuAfuAaaUfgUfuUfgUfusGfsu	0.031	0.535	0.785
D2119	S2119	UfgGfcAfaUfgUfCfCfcCfaAfuGfcAfaUf	A2119	aUfuGfcAfuUfgGfggaCfaUfuGfcCfasGfsu	0.065	0.815	0.967
D2120	S2120	UfcAfgGfuAfgUfCfCfaUfgGfaCfaUfuAf	A2120	uAfaUfgUfcCfaUfggaCfuAfcCfuGfasUfsa	0.223	0.825	0.924
D2121	S2121	UfuAfaUfuCfaAfCfAfuCfgAfaUfaGfaUf	A2121	aUfcUfaUfuCfgAfuguUfgAfaUfuAfasUfsg	0.083	0.781	0.915
D2122	S2122	GfgAfgAfaCfuAfCfAfaAfuAfuGfgUfuUf	A2122	aAfaCfcAfuAfuUfuguAfgUfuCfuCfcsCfsa	0.079	0.680	0.767
D2123	S2123	CfcAfuAfgUfgAfAfGfcAfaUfcUfaAfuUf	A2123	aAfuUfaGfaUfuGfcuuCfaCfuAfuGfgsAfsg	0.026	0.537	0.793
D2124	S2124	AfcAfaAfcAfuUfAfUfaUfuGfaAfuAfuUf	A2124	aAfuAfuUfcAfaUfauaAfuGfuUfuGfusUfsg	0.044	0.680	0.828
D2125	S2125	AfaUfgCfaAfuCfCfCfgGfaAfaAfcAfaAf	A2125	uUfuGfuUfuUfcCfgggAfuUfgCfaUfusGfsg	0.349	0.971	1.005
D2126	S2126	CfaGfgUfaGfuCfCfAfuGfgAfcAfuUfaAf	A2126	uUfaAfuGfuCfcAfuggAfcUfaCfcUfgsAfsu	0.070	0.548	0.546
D2127	S2127	UfuCfaAfcAfuCfGfAfaUfaGfaUfgGfaUf	A2127	aUfcCfaUfcUfaUfucgAfuGfuUfgAfasUfsu	0.225	0.958	0.967
D2128	S2128	GfuUfgGfgCfcUfAfGfaGfaAfgAfuAfuAf	A2128	uAfuAfuCfuUfcUfcuaGfgCfcCfaAfcsCfsa	0.765	0.969	0.922
D2129	S2129	CfaUfaGfuGfaAfGfCfaAfuCfuAfaUfuAf	A2129	uAfaUfuAfgAfuUfgcuUfcAfcUfaUfgsGfsa	0.028	0.583	0.777
D2130	S2130	AfaCfaUfuAfuAfUfUfgAfaUfaUfuCfuUf	A2130	aAfgAfaUfaUfuCfaauAfuAfaUfgUfusUfsg	0.249	0.916	0.981
D2131	S2131	GfcAfaUfcCfcGfGfAfaAfaCfaAfaGfaUf	A2131	aUfcUfuUfgUfuUfuccGfgGfaUfuGfcsAfsu	0.435	1.002	1.019

D2132	S2132	GfgUfaGfuCfcAfUfGfgAfcAfuUfaAfuUf	A2132	aAfuUfaAfuGfuCfcauGfgAfcUfaCfcsUfsg	0.427	0.988	0.918
D2133	S2133	AfuCfgAfaUfaGfAfUfgGfaUfcAfcAfaAf	A2133	uUfuGfuGfaUfcCfaucUfaUfuCfgAfusGfsu	0.170	0.706	0.890
D2134	S2134	CfcUfaGfaGfaAfGfAfuAfuAfcUfcCfaUf	A2134	aUfgGfaGfuAfuAfucuUfcUfcUfaGfgsCfsc	0.033	0.543	0.733
D2135	S2135	GfuUfgGfaAfgAfcUfgGfaAfaGfaCfaAf	A2135	uUfgUfcUfuUfcCfaguCfuUfcCfaAfcsUfsc	0.137	0.975	0.944
D2136	S2136	AfcAfuUfaUfaUfUfGfaAfuAfuUfcUfuUf	A2136	aAfaGfaAfuAfuUfcaaUfaUfaAfuGfusUfsu	0.114	0.882	0.940
D2137	S2137	CfaAfuCfcCfgGfAfAfaAfcAfaAfgAfuUf	A2137	aAfuCfuUfuGfuUfuucCfgGfgAfuUfgsCfsa	0.155	0.755	0.686
D2138	S2138	CfuAfcUfuGfgGfaUfcAfcAfaAfgCfaAf	A2138	uUfgCfuUfuGfuGfaucCfcAfaGfuAfgsAfsa	0.196	0.825	0.658
D2139	S2139	AfcAfaCfcUfaAfAfUfgGfuAfaAfuAfuAf	A2139	uAfuAfuUfuAfcCfauuUfaGfgUfuGfusUfsu	0.133	0.704	0.671
D2140	S2140	AfuCfcAfuCfcAfAfCfaGfaUfuCfaGfaAf	A2140	uUfcUfgAfaUfcUfguuGfgAfuGfgAfusCfsa	0.184	0.775	0.658
D2141	S2141	AfaCfuGfaGfgCfAfAfaUfuUfuAfaAfgAf	A2141	uCfuUfuUfaAfaUfuugCfcUfcAfgUfusCfsa	0.076	0.682	0.777
D2142	S2142	AfgAfgUfaUfgUfGfUfaAfaAfaUfcUfgUf	A2142	aCfaGfaUfuUfuUfuUfacaCfaUfaCfuCfusGfsu	0.448	0.659	0.761
D2143	S2143	AfaUfcCfcGfgAfAfAfaCfaAfaGfaUfuUf	A2143	aAfaUfcUfuUfgUfuuuCfcGfgGfaUfusGfsc	0.097	0.844	0.924
D2144	S2144	UfaCfuUfgGfgAfUfCfaCfaAfaGfcAfaAf	A2144	uUfuGfcUfuUfgUfgauCfcCfaAfgUfasGfsa	0.084	0.875	0.947
D2145	S2145	CfaAfcCfuAfaAfUfGfgUfaAfaUfaUfaAf	A2145	uUfaUfaUfuUfaCfcauUfuAfgGfuUfgsUfsu	0.104	0.811	0.814
D2146	S2146	UfuGfaAfuGfaAfCfUfgAfgGfcAfaAfuUf	A2146	aAfuUfuGfcCfuCfaguUfcAfuUfcAfasAfsg	0.046	0.549	0.680
D2147	S2147	AfcUfgAfgGfcAfAfAfuUfuAfaAfaGfgAf	A2147	uCfcUfuUfuAfaAfuuuGfcCfuCfaGfusUfsc	0.079	0.890	1.005
D2148	S2148	GfaGfuAfuGfuGfUfAfaAfaAfuCfuGfuAf	A2148	uAfcAfgAfuUfuUfuacAfcAfuAfcUfcsUfsg	0.497	0.676	0.783
D2149	S2149	AfcUfuGfgGfaUfCfAfcAfaAfgCfaAfaAf	A2149	uUfuUfgCfuUfuGfugaUfcCfcAfaGfusAfsg	0.049	0.699	0.907
D2150	S2150	AfuGfgUfaAfaUfAfUfaAfcAfaAfcCfaAf	A2150	uUfgGfuUfuGfuUfauaUfuUfaCfcAfusUfsu	0.093	0.928	0.941
D2151	S2151	UfgAfaUfgAfaCfUfGfaGfgCfaAfaUfuUf	A2151	aAfaUfuUfgCfcUfcagUfuCfaUfuCfasAfsa	0.201	0.736	0.885
D2152	S2152	CfuGfaGfgCfaAfAfUfuUfaAfaAfgGfcAf	A2152	uGfcCfuUfuUfaAfauuUfgCfcUfcAfgsUfsu	0.071	0.938	0.872
D2153	S2153	AfgUfaUfgUfgUfAfAfaAfaUfcUfgUfaAf	A2153	uUfaCfaGfaUfuUfuuaCfaCfaUfaCfusCfsu	0.504	0.816	0.689
D2154	S2154	GfaAfaAfcAfaAfGfAfuUfuGfgUfgUfuUf	A2154	aAfaCfaCfcAfaAfucuUfuGfuUfuUfcsCfsg	0.061	0.723	0.922
D2155	S2155	AfgUfgUfgGfaGfAfAfaAfcAfaCfcUfaAf	A2155	uUfaGfgUfuGfuUfuucUfcCfaCfaCfusCfsa	0.071	0.689	0.869
D2156	S2156	GfuCfuCfaAfaAfUfGfgAfaGfgUfuAfuAf	A2156	uAfuAfaCfcUfuCfcauUfuUfgAfgAfcsUfsu	0.133	0.643	0.974
D2157	S2157	GfaAfuGfaAfcUfGfAfgGfcAfaAfuUfuAf	A2157	uAfaAfuUfuGfcCfucaGfuUfcAfuUfcsAfsa	0.204	0.751	1.008
D2158	S2158	UfgAfgGfcAfaAfUfUfuAfaAfaGfgCfaAf	A2158	uUfgCfcUfuUfuAfaauUfuGfcCfuCfasGfsu	0.089	0.820	0.937

D2159	S2159	GfuAfuGfuGfuAfAfAfaAfuCfuGfuAfaUf	A2159	aUfuAfcAfgAfuUfuuuAfcAfcAfuAfcsUfsc	0.535	0.697	0.788
D2160	S2160	AfaAfaCfaAfaGfAfUfuUfgGfuGfuUfuUf	A2160	aAfaAfcAfcCfaAfaucUfuUfgUfuUfusCfsc	0.297	0.954	1.004
D2161	S2161	GfuGfuGfgAfgAfAfAfaCfaAfcCfuAfaAf	A2161	uUfuAfgGfuUfgUfuuuCfuCfcAfcAfcsUfsc	0.178	0.872	0.918
D2162	S2162	AfuGfgAfaGfgUfUfAfuAfcUfcUfuAfuAfaAf	A2162	uUfaUfaGfaGfuAfuaaCfcUfuCfcAfusUfsu	0.026	0.489	0.890
D2163	S2163	AfaUfgAfaCfuGfAfGfgCfaAfaUfuUfaAf	A2163	uUfaAfaUfuUfgCfcucAfgUfuCfaUfusCfsa	0.111	0.789	0.859
D2164	S2164	GfaGfgCfaAfaUfUfUfaAfaAfgGfcAfaUf	A2164	aUfuGfcCfuUfuUfaaaUfuUfgCfcUfcsAfsg	0.241	0.956	0.869
D2165	S2165	UfaUfgUfgUfaAfAfAfaUfcUfgUfaAfuAf	A2165	uAfuUfaCfaGfaUfuuuUfaCfaCfaUfasCfsu	0.571	0.762	0.931
D2166	S2166	AfcAfaAfgAfuUfUfGfgUfgUfuUfuCfuAf	A2166	uAfgAfaAfaCfaCfcaaAfuCfuUfuGfusUfsu	0.106	0.981	0.924
D2167	S2167	UfgUfgGfaGfaAfAfAfcAfaCfcUfaAfaUf	A2167	aUfuUfaGfgUfuGfuuuUfcUfcCfaCfasCfsu	0.064	0.765	0.902
D2168	S2168	UfgGfaAfgGfuUfAfUfaCfuCfuAfuAfaAf	A2168	uUfuAfuAfgAfgUfauaAfcCfuUfcCfasUfsu	0.029	0.675	0.859
D2169	S2169	AfuGfaAfcUfgAfGfGfcAfaAfuUfuAfaAf	A2169	uUfuAfaAfuUfuGfccuCfaGfuUfcAfusUfsc	0.054	0.733	0.843
D2170	S2170	AfgGfcAfaAfuUfUfAfaAfaGfgCfaAfuAf	A2170	uAfuUfgCfcUfuUfuaaAfuUfuGfcCfusCfsa	0.075	0.754	0.881
D2171	S2171	AfaGfaUfuUfgGfUfGfuUfuUfcUfaCfuUf	A2171	aAfgUfaGfaAfaAfcacCfaAfaUfcUfusUfsg	0.303	1.065	0.977
D2172	S2172	AfaAfcAfaCfcUfAfAfaUfgGfuAfaAfuAf	A2172	uAfuUfuAfcCfaUfuuaGfgUfuGfuUfusUfsc	0.101	0.855	0.880
D2173	S2173	AfuAfcUfcUfaUfAfAfaAfuCfaAfcCfaAf	A2173	uUfgGfuUfgAfuUfuuaUfaGfaGfuAfusAfsa	0.107	0.961	0.960
D2174	S2174	UfgAfaCfuGfaGfGfCfaAfaUfuUfaAfaAf	A2174	uUfuUfaAfaUfuUfgccUfcAfgUfuCfasUfsu	0.078	0.714	0.878
D2175	S2175	GfgCfaAfaUfuUfAfAfaAfgGfcAfaUfaAf	A2175	uUfaUfuGfcCfuUfuuaAfaUfuUfgCfcsUfsc	0.054	0.767	0.918
D2176	S2176	UfuUfuCfuAfcUfUfGfgGfaUfcAfcAfaAf	A2176	uUfuGfuGfaUfcCfcaaGfuAfgAfaAfasCfsa	0.915	1.030	0.916
D2177	S2177	AfaCfaAfcCfuAfAfAfaUfgGfuAfaAfuAfuUf	A2177	aUfaUfuUfaCfcAfuuuAfgGfuUfgUfusUfsu	0.042	0.260	0.448
D2178	S2178	UfaCfuCfuAfuAfAfAfaUfcAfaCfcAfaAf	A2178	uUfuGfgUfuGfaUfuuuAfuAfgAfgUfasUfsa	0.063	0.897	0.869
D2179	S2179	GfaAfcUfgAfgGfCfAfaAfuUfuAfaAfaAf	A2179	uUfuUfuAfaAfuUfugcCfuCfaGfuUfcsAfsu	0.178	0.858	0.869
D2180	S2180	CfaGfaGfuAfuGfUfGfuAfaAfaAfuCfuUf	A2180	aAfgAfuUfuUfuAfcacAfuAfcUfcUfgsUfsg	0.436	0.677	0.813

Example 4: *In vitro* silencing activity with various chemical modifications on ANGPTL3 siRNA

Cell culture and transfections

Hep3B cells (ATCC, Manassas, VA) were grown to near confluence at 37°C in an atmosphere of 5% CO_2 in RPMI (ATCC) supplemented with 10% FBS, streptomycin, and glutamine (ATCC) before being released from the plate by trypsinization. Transfection was carried out by adding 14.8 µl of Opti-MEM plus 0.2µl of Lipofectamine RNAiMax per well (Invitrogen, Carlsbad CA. cat # 13778-150) to 5 µl of siRNA duplexes per well into a 96-well plate and incubated at room temperature for 15 minutes. 80 µl of complete growth media without antibiotic containing ~2 x10^4 Hep3B cells were then added to the siRNA mixture. Cells were incubated for either 24 or 120 hours prior to RNA purification. Single dose experiments were performed at 10nM and 0.1nM final duplex concentration and dose response experiments were done at 10, 1, 0.5, 0.1,0.05, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005 and 0.00001 nM final duplex concentration unless otherwise stated.

cDNA synthesis using ABI High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, Cat #4368813)

A master mix of 2µl 10X Buffer, 0.8 µl 25X dNTPs, 2µl Random primers, 1µl Reverse Transcriptase, 1 µl RNase inhibitor and 3.2µl of H_2O per reaction were added into 10µl total RNA. cDNA was generated using a Bio-Rad C-1000 or S-1000 thermal cycler (Hercules, CA) through the following steps: 25°C 10 min, 37°C 120 min, 85°C 5 sec, 4°C hold.

Real time PCR

2 µl of cDNA was added to a master mix containing 0.5µl GAPDH TaqMan Probe (Applied Biosystems Cat #4326317E), 0.5µl ANGPTL TaqMan probe (Applied Biosystems cat # Hs00205581_m1) and 5µl Lightcycler 480 probe master mix (Roche Cat #04887301001) per well in a 384 well 50 plates (Roche cat # 04887301001). Real time PCR was done in an ABI 7900HT Real Time PCR system (Applied Biosystems) using the ΔΔCt(RQ) assay. Each duplex was tested in two independent transfections, and each transfection was assayed in duplicate, unless otherwise noted in the summary tables.

To calculate relative fold change, real time data was analyzed using the ΔΔCt method and normalized to assays performed with cells transfected with 10nM AD-1955, or mock transfected cells. IC_{50}s were calculated using a 4 parameter fit model using XLFit and normalized to cells transfected with AD-1955 or naïve cells over the same dose range, or to its own lowest dose. AD-1955 sequence, used as a negative control, targets luciferase and has the following sequence:

sense: cuuAcGcuGAGuAcuucGAdTsdT;

antisense: UCGAAGuACUcAGCGuAAGdTsdT.

The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, foreign patents, foreign patent applications and non-patent publications referred to in this specification are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments.

These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

We claim:

1. A double-stranded RNAi agent capable of inhibiting the expression of a target gene, comprising a sense strand and an antisense strand, each strand having 14 to 30 nucleotides, wherein the duplex is represented by formula (III):

sense: 5' n_p -N_a -(X X X) $_i$-N_b -Y Y Y -N_b -(Z Z Z)$_j$ -N_a - n_q 3'

antisense: 3' n_p'-N_a'-(X'X'X')$_k$-N_b'-Y'Y'Y'-N_b'-(Z'Z'Z')$_l$-N_a'- n_q' 5'

(III)

wherein:

i, j, k, and l are each independently 0 or 1;

p and q are each independently 0-6;

each Na and Na′ independently represents an oligonucleotide sequence comprising 0-25 nucleotides which are either modified or unmodified or combinations thereof, each sequence comprising at least two differently modified nucleotides, each Nb and Nb′ independently represents an oligonucleotide sequence comprising 0-10 nucleotides which are either modified or unmodified or combinations thereof;

each n_p, n_p', n_q and n_q' independently represents an overhang nucleotide sequence comprising 0-6 nucleotides; and

XXX, YYY, ZZZ, X′X′X′, Y′Y′Y′, and Z′Z′Z′ each independently represent one motif of three identical modifications on three consecutive nucleotides; and

wherein the modification on Nb is different than the modification on Y and the modification on Nb' is different than the modification on Y'.

2. The double-stranded RNAi agent of claim 1, wherein i is 1; j is 1; or both i and j are 1.

3. The double-stranded RNAi agent of claim 1, wherein k is 1; l is 1; or both k and l are 1.

4. The double-stranded RNAi agent of claim 1, wherein the YYY motif occurs at or near the cleavage site of the sense strand.

5. The double-stranded RNAi agent of claim 1, wherein the Y'Y'Y' motif occurs at the 11, 12 and 13 positions of the antisense strand from the 5'-end.

6. The double-stranded RNAi agent of claim 5, wherein the Y' is 2'-OMe.

7. The double-stranded RNAi agent of claim 1, wherein formula (III) is represented as formula (IIIa):

$$5' \; n_p\text{-}N_a\text{-}Y\;Y\;Y\text{-}N_b\text{-}Z\;Z\;Z\text{-}N_a\text{-}n_q \; 3'$$
$$3' \; n_p'\text{-}N_a'\text{-}Y'Y'Y'\text{-}N_b'\text{-}Z'Z'Z'\text{-}N_a'n_q' \; 5'$$

(IIIa)

wherein each N_b and N_b' independently represents an oligonucleotide sequence comprising 1-5 modified nucleotides.

8. The double-stranded RNAi agent of claim 1, wherein formula (III) is represented as formula (IIIb):

$$5' \; n_p\text{-}N_a\text{-}X\;X\;X\text{-}N_b\text{-}Y\;Y\;Y\text{-}N_a\text{-}n_q \; 3'$$
$$3' \; n_p\text{-}N_a\text{-}X'X'X'\text{-}N_b\text{-}Y'Y'Y'\text{-}N_a\text{-}n_q \; 5'$$

(IIIb)

wherein each N_b and N_b' independently represents an oligonucleotide sequence comprising 1-5 modified nucleotides.

9. The double-stranded RNAi agent of claim 1, wherein formula (III) is represented as formula (IIIc):

$$5' \; n_p\text{-}N_a\text{-}X\;X\;X\text{-}N_b\text{-}Y\;Y\;Y\text{-}N_b\text{-}Z\;Z\;Z\text{-}N_a\text{-}n_q \; 3'$$
$$3' \; n_p\text{-}N_a\text{-}X'X'X'\text{-}N_b\text{-}Y'Y'Y'\text{-}N_b\text{-}Z'Z'Z'\text{-}N_a\text{-}n_q \; 5'$$

(IIIc)

wherein each N_b and N_b' independently represents an oligonucleotide sequence comprising 1-5 modified nucleotides and each N_a and N_a' independently represents an oligonucleotide sequence comprising 2-10 modified nucleotides.

10. The double-stranded RNAi agent of claim 1, wherein the duplex region is 17-30 nucleotide pairs in length.

11. The double-stranded RNAi agent of claim 10, wherein the duplex region is 17-19 nucleotide pairs in length.

12. The double-stranded RNAi agent of claim 10, wherein the duplex region is 27-30 nucleotide pairs in length.

13. The double-stranded RNAi agent of claim 1, wherein each strand has 17-30 nucleotides.

14. The double-stranded RNAi agent of claim 1, wherein the modifications on the nucleotides are selected from the group consisting of LNA, HNA, CeNA, 2′-methoxyethyl, 2′-O-alkyl, 2′-O-allyl, 2′-C- allyl, 2′-fluoro, 2'-deoxy, and combinations thereof.

15. The double-stranded RNAi agent of claim 14, wherein the nucleotides are modified with either 2'-OCH_3 or 2'-F.

16. The double-stranded RNAi agent of claim 1, further comprising at least one ligand.

17. The double-stranded RNAi agent of claim 16, wherein the ligand is a one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

18. The double-stranded RNAi agent of claim 1, wherein the modifications on the nucleotides are selected from the group consisting of 2'-O-methyl nucleotide, 2′-deoxyfluoro nucleotide, 2'-O-N-methylacetamido (2'-O-NMA) nucleotide, a 2'-O-dimethylaminoethoxyethyl (2'-O-DMAEOE) nucleotide, 2'-O-aminopropyl (2'-O-AP) nucleotide, 2'-ara-F, and combinations thereof.

19. The double-stranded RNAi agent of claim 14, wherein the ligand is attached to the 3' end of the sense strand.

20. The double-stranded RNAi agent of claim 1, further comprising at least one phosphorothioate or methylphosphonate internucleotide linkage.

21. The double-stranded RNAi agent of claim 1, wherein the nucleotide at the 1 position of the 5'-end of the duplex in the antisense strand is selected from the group consisting of A, dA, dU, U, and dT.

22. The double-stranded RNAi agent of claim 1, wherein the base pair at the 1 position of the 5'-end of the duplex is an AU base pair.

23. The double-stranded RNAi agent of claim 1, wherein the Y nucleotides contain a 2'-fluoro modification.

24. The double-stranded RNAi agent of claim 1, wherein the Y' nucleotides contain a 2'-O-methyl modification.

25. A double-stranded RNAi agent capable of inhibiting the expression of a target gene, comprising a sense strand and an antisense strand, each strand having 14 to 30 nucleotides,

wherein the sense strand contains at least two motifs of three identical modifications on three consecutive nucleotides, one of said motifs occurring at the cleavage site in the strand and at least one of said motifs occurring at another portion of the strand that is separated from the motif at the cleavage site by at least one nucleotide; and

wherein the antisense strand contains at least first motif of three identical modifications on three consecutive nucleotides, one of said motifs occurring at or near the cleavage site in the strand and a second moitif occurring at another portion of the strand that is separated from the first motif by at least one nucleotide;

wherein the modification in the motif occurring at the cleavage site in the sense strand is different than the modification in the motif occurring at or near the cleavage site in the antisense strand.

26. The double-stranded RNAi agent of claim 25, wherein at least one of the nucleotides occurring at the cleavage site in the sense strand forms a base pair with one of the nucleotides in the motif occurring at or near the cleavage site in the antisense strand.

27. The double-stranded RNAi agent of claim 25, wherein the duplex has 17-30 nucleotides.

28. The double-stranded RNAi agent of claim 25, wherein the duplex has 17-19 nucleotides.

29. The double-stranded RNAi agent of claim 25, wherein each strand has 17-23 nucleotides.

30. The double-stranded RNAi agent of claim 25, wherein the modifications on the nucleotides are selected from the group consisting of LNA, HNA, CeNA, 2′-methoxyethyl, 2′-O-alkyl, 2′-O-allyl, 2′-C- allyl, 2′-fluoro, 2’-deoxy, and combinations thereof.

31. The double-stranded RNAi agent of claim 30, wherein the modifications on the nucleotide are 2’-OCH_3 or 2’-F.

32. The double-stranded RNAi agent of claim 31, further comprising a ligand attached to the 3’ end of the sense strand.

33. A double-stranded RNAi agent capable of inhibiting the expression of a target gene, comprising a sense strand and an antisense strand, each strand having 14 to 30 nucleotides,

wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides, one of said motifs occurring at or near the cleavage site in the strand; and

wherein the antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides, one of said motifs occuring at or near the cleavage site.

34. The double-stranded RNAi agent of claim 33, wherein the sense strand comprises one or more motifs of three identical modifications on three consecutive nucleotides, said motifs occurring at another portion of the strand that is separated from the three 2'-F modifications at the cleavage site by at least one nucleotide.

35. The double-stranded RNAi agent of claim 33, wherein the antisense strand comprises one or more motifs of three identical modifications on three consecutive nucleotides, said motifs occurring at another portion of the strand that is separated from the three 2'-O-methyl modifications by at least one nucleotide.

36. The double-stranded RNAi agent of claim 33, wherein at least one of the nucleotides having a 2'-F modification forms a base pair with one of the nucleotides having a 2'-O-methyl modification.

37. The double-stranded RNAi agent of claim 33, wherein the duplex is 17-30 nucleotide pairs in length.

38. The double-stranded RNAi agent of claim 33, wherein the duplex is 17-19 nucleotide pairs in length.

39. The double-stranded RNAi agent of claim 33, wherein each strand has 17-23 nucleotides.

40. The double-stranded RNAi agent of claim 33, further comprising a ligand attached to the 3' end of the sense strand.

41. A pharmaceutical composition comprising the double-stranded RNAi agent according to any one of the preceding claims alone or in combination with a pharmaceutically acceptable carrier or excipient.

42. A method for inhibiting the expression of a target gene comprising the step of administering the double-stranded RNAi agent according to any one of the preceding claims, in an amount sufficient to inhibit expression of the target gene.

43. The method of claim 42, wherein the double-stranded RNAi agent is administered through subcutaneous or intravenous administration.

44. A method for delivering polynucleotide to specific target in a subject by administering said dsRNA agent represented by formula (III):

sense: 5' n_p -N_a -(X X X) $_i$-N_b -Y Y Y -N_b -(Z Z Z)$_j$ -N_a - n_q 3'

antisense: 3' n_p'-N_a'-(X'X'X')$_k$-N_b'-Y'Y'Y'-N_b'-(Z'Z'Z')$_l$-N_a'- n_q' 5'

(III)

wherein:

i, j, k, and l are each independently 0 or 1;

p and q are each independently 0-6;

each N_a and N_a' independently represents an oligonucleotide sequence comprising 0-25 nucleotides which are either modified or unmodified or combinations thereof, each sequence comprising at least two differently modified nucleotideseach N_b and N_b' independently represents an oligonucleotide sequence comprising 0-10 nucleotides which are either modified or unmodified or combinations thereof;

each n_p, n_p', n_q and n_q' independently represents an overhang nucleotide sequence comprising 0-6 nucleotide sequence; and

XXX, YYY, ZZZ, X'X'X', Y'Y'Y', and Z'Z'Z' each independently represent one motif of three identical modifications on three consecutive nucleotides; and

wherein the modifications on N_b is different than the modification on Y and the modification on N_b' is different than the modification on Y'.

45. The method of claim 44, wherein said administering step is carried out by an administration means comprising intramuscular, intrabronchial, intrapleural, intraperitoneal, intraarterial, lymphatic, intravenous, subcutaneous, cerebrospinal, or combinations thereof.

46. A method for delivering a polynucleotide to a specific target of a subject, the method comprising: delivering the dsRNA agent according to claim 1 by subcutaneous administration into the subject, such that the polynucleotide is delivered into specific target of the subject.