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(54) Title: RNA INTERFERENCE MEDIATED INHIBITION OF GENE EXPRESSION USING CHEMICALLY MODIFIED SHORT INTERFERING NUCLEIC ACID (siNA)

(57) Abrégé/Abstract:
The present invention concerns methods and reagents useful in modulating gene expression in a variety of applications, including use in therapeutic, cosmetic, cosmeceutical, prophylactic, diagnostic, target validation, and genomic discovery applications. Specifically, the invention relates to synthetic chemically modified small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA interference (RNAi) against target nucleic acid sequences. The small nucleic acid molecules are useful in the treatment of any disease (e.g., cancer, proliferative, inflammatory, metabolic, autoimmune, nuerologic, ocular diseases), condition, trait (e.g., hair growth and removal), genotype or phenotype that responds to modulation of gene expression or activity in a cell, tissue, or organism. Such small nucleic acid molecules can be administered systemically, locally, or topically.

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(54) Title: RNA INTERFERENCE MEDIATED INHIBITION OF GENE EXPRESSION USING CHEMICALLY MODIFIED SHORT INTERFERING NUCLEIC ACID (siNA)

(57) Abstract: The present invention concerns methods and reagents useful in modulating gene expression in a variety of applications, including use in therapeutic, cosmetic, cosmeceutical, prophylactic, diagnostic, target validation, and genomic discovery applications. Specifically, the invention relates to synthetic chemically modified small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA interference (RNAi) against target nucleic acid sequences. The small nucleic acid molecules are useful in the treatment of any disease (e.g., cancer, proliferative, inflammatory, metabolic, autoimmune, nuerologic, ocular diseases), condition, trait (e.g., hair growth and removal), genotype or phenotype that responds to modulation of gene expression or activity in a cell, tissue, or organism. Such small nucleic acid molecules can be administered systemically, locally, or topically.

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RNA INTERFERENCE MEDIATED INHIBITION OF GENE EXPRESSION USING CHEMICALLY MODIFIED SHORT INTERFERING NUCLEIC ACID (siNA)

Field of the Invention

The present invention comprises methods and reagents useful in modulating gene expression in a variety of applications, including use in therapeutic, cosmetic, cosmeceutical, prophylactic, diagnostic, target validation, and genomic discovery.

applications. Specifically, the invention comprises synthetic small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA interference (RNAi).

Background Of The Invention

The following is a discussion of relevant art pertaining to RNAi. The discussion is provided only for understanding of the invention that follows. The summary is not an admission that any of the work described below is prior art to the claimed invention. Applicant demonstrates herein that chemically modified short interfering nucleic acids possess the same capacity to mediate RNAi as do siRNA molecules and are expected to possess improved stability and activity in vivo; therefore, this discussion is not meant to be limiting only to siRNA and can be applied to siNA as a whole.

RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Zamore *et al.,* 2000, Cell, 101, 25-33; Fire *et al.,* 1998, *Nature,* 391, 806; Hamilton *et al.,* 1999, *Science*, 286, 950-951; Lin *et al.,* 1999, *Nature,* 402, 128-129; and Strauss, 1999, *Science*, 286, 886). The corresponding process in plants (Heifetz *et al.,* International PCT Publication No. WO 99/61631) is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla (Fire *et al.,* 1999, *Trends Genet.,* 15, 358). Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or from the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response through a mechanism that has yet to be fully characterized. This mechanism appears to be different from other known mechanisms involving double stranded RNA-specific ribonucleases, such as the interferon response that results from dsRNA-mediated activation of protein kinase PKR and 2',5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L (see for example US Patent Nos. 6,107,094;

5,898,031; Clemens *et al.,* 1997, *J. Interferon & Cytokine Res.,* 17, 503-524; Adah *et al.,* 2001, *Curr. Med. Chem.,* 8, 1189).

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer (Bass, 2000, Cell, 101, 235; Zamore *et al.,* 2000, Cell, 101, 25-33; Hammond et al., 2000, Nature, 404, 293). Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs) (Zamore *et al.,* 2000, Cell, 101, 25-33; Bass, 2000, Cell, 101, 235; Berstein *et al.,* 2001, *Nature,* 409, 363). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes (Zamore *et al.,* 2000, Cell, 101, 25-33; Elbashir *et al.,* 2001, *Genes Dev.,* 15, 188). Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner *et al.,* 2001, *Science,* 293, 834). The RNAi response also features an endonuclease complex, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex (Elbashir *et al.,* 2001, *Genes Dev.,* 15, 188).

RNAi has been studied in a variety of systems. Fire *et al.,* 1998, *Nature,* 391, 806, were the first to observe RNAi in *C. elegans*. Bahramian and Zarbl, 1999, *Molecular and Cellular Biology*, 19, 274-283 and Wianny and Goetz, 1999, *Nature Cell Biol.,* 2, 70, describe RNAi mediated by dsRNA in mammalian systems. Hammond *et al.,* 2000, *Nature,* 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir *et al.,* 2001, *Nature,* 411, 494 and Tuschl *et al.,* International PCT Publication No. WO 01/75164, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells. Recent work in Drosophila embryonic lysates (Elbashir *et al.,* 2001, *EMBO J.,* 20, 6877 and Tuschl *et al.,* International PCT Publication No. WO 01/75164) has revealed certain requirements for siRNA length, structure, chemical composition, and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21-nucleotide siRNA duplexes are most active when containing 3'-terminal dinucleotide overhangs. Furthermore, complete substitution of one or both siRNA strands with 2'-deoxy (2'-H) or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of the 3'-terminal

siRNA overhang nucleotides with 2'-deoxy nucleotides (2'-H) was shown to be tolerated. Single mismatch sequences in the center of the siRNA duplex were also shown to abolish RNAi activity. In addition, these studies also indicate that the position of the cleavage site in the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end of the guide sequence (Elbashir *et al.,* 2001, *EMBO J.,* 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA (Nykanen *et al.,* 2001, *Cell,* 107, 309).

Studies have shown that replacing the 3'-terminal nucleotide overhanging segments of a 21-mer siRNA duplex having two-nucleotide 3'-overhangs with deoxyribonucleotides does not have an adverse effect on RNAi activity. Replacing up to four nucleotides on each end of the siRNA with deoxyribonucleotides has been reported to be well tolerated, whereas complete substitution with deoxyribonucleotides results in no RNAi activity (Elbashir *et al.,* 2001, *EMBO J.*, 20, 6877 and Tuschl *et al.,* International PCT Publication No. WO 01/75164). In addition, Elbashir *et al., supra*, also report that substitution of siRNA with 2'-O-methyl nucleotides completely abolishes RNAi activity. Li *et al.,* International PCT Publication No. WO 00/44914, and Beach *et al.,* International PCT Publication No. WO 01/68836 preliminarily suggest that siRNA may include modifications to either the phosphate-sugar backbone or the nucleoside to include at least one of a nitrogen or sulfur heteroatom, however, neither application postulates to what extent such modifications would be tolerated in siRNA molecules, nor provides any further guidance or examples of such modified siRNA. Kreutzer *et al.*, Canadian Patent Application No. 2,359,180, also describe certain chemical modifications for use in dsRNA constructs in order to counteract activation of double-stranded RNA-dependent protein kinase PKR, specifically 2'-amino or 2'-O-methyl nucleotides, and nucleotides containing a 2'-O or 4'-C methylene bridge. However, Kreutzer *et al.* similarly fails to provide examples or guidance as to what extent these modifications would be tolerated in dsRNA molecules.

Parrish *et al.,* 2000, *Molecular Cell*, 6, 1077-1087, tested certain chemical modifications targeting the unc-22 gene in *C. elegans* using long (>25 nt) siRNA transcripts. The authors describe the introduction of thiophosphate residues into these siRNA transcripts by incorporating thiophosphate nucleotide analogs with T7 and T3 RNA polymerase and observed that RNAs with two phosphorothioate modified bases also had

substantial decreases in effectiveness as RNAi. Further, Parrish *et al.* reported that phosphorothioate modification of more than two residues greatly destabilized the RNAs *in vitro* such that interference activities could not be assayed. *Id.* at 1081. The authors also tested certain modifications at the 2'-position of the nucleotide sugar in the long siRNA transcripts and found that substituting deoxynucleotides for ribonucleotides produced a substantial decrease in interference activity, especially in the case of Uridine to Thymidine and/or Cytidine to deoxy-Cytidine substitutions. *Id.* In addition, the authors tested certain base modifications, including substituting, in sense and antisense strands of the siRNA, 4-thiouracil, 5-bromouracil, 5-iodouracil, and 3-(aminoallyl)uracil for uracil, and inosine for guanosine. Whereas 4-thiouracil and 5-bromouracil substitution appeared to be tolerated, Parrish reported that inosine produced a substantial decrease in interference activity when incorporated in either strand. Parrish also reported that incorporation of 5-iodouracil and 3-(aminoallyl)uracil in the antisense strand resulted in a substantial decrease in RNAi activity as well.

The use of longer dsRNA has been described. For example, Beach *et al.*, International PCT Publication No. WO 01/68836, describes specific methods for attenuating gene expression using endogenously-derived dsRNA. Tuschl *et al.*, International PCT Publication No. WO 01/75164, describe a *Drosophila in vitro* RNAi system and the use of specific siRNA molecules for certain functional genomic and certain therapeutic applications; although Tuschl, 2001, *Chem. Biochem.*, 2, 239-245, doubts that RNAi can be used to cure genetic diseases or viral infection due to the danger of activating interferon response. Li *et al.*, International PCT Publication No. WO 00/44914, describe the use of specific long (141 bp-488 bp) enzymatically synthesized or vector expressed dsRNAs for attenuating the expression of certain target genes. Zernicka-Goetz *et al.*, International PCT Publication No. WO 01/36646, describe certain methods for inhibiting the expression of particular genes in mammalian cells using certain long (550 bp-714 bp), enzymatically synthesized or vector expressed dsRNA molecules. Fire *et al.*, International PCT Publication No. WO 99/32619, describe particular methods for introducing certain long dsRNA molecules into cells for use in inhibiting gene expression in nematodes. Plaetinck *et al.*, International PCT Publication No. WO 00/01846, describe certain methods for identifying specific genes responsible for conferring a particular phenotype in a cell using specific long dsRNA molecules. Mello *et al.*, International PCT Publication No. WO

01/29058, describe the identification of specific genes involved in dsRNA-mediated RNAi. Pachuck et al., International PCT Publication No. WO 00/63364, describe certain long (at least 200 nucleotide) dsRNA constructs. Deschamps Depaillette *et al.,* International PCT Publication No. WO 99/07409, describe specific compositions consisting of particular dsRNA molecules combined with certain anti-viral agents. Waterhouse *et al.,* International PCT Publication No. 99/53050 and 1998, *PNAS*, 95, 13959-13964, describe certain methods for decreasing the phenotypic expression of a nucleic acid in plant cells using certain dsRNAs. Driscoll *et al.,* International PCT Publication No. WO 01/49844, describe specific DNA expression constructs for use in facilitating gene silencing in targeted organisms.

Others have reported on various RNAi and gene-silencing systems. For example, Parrish *et al.,* 2000, *Molecular Cell*, 6, 1077-1087, describe specific chemically-modified dsRNA constructs targeting the unc-22 gene of *C. elegans*. Grossniklaus, International PCT Publication No. WO 01/38551, describes certain methods for regulating polycomb gene expression in plants using certain dsRNAs. Churikov *et al.,* International PCT Publication No. WO 01/42443, describe certain methods for modifying genetic characteristics of an organism using certain dsRNAs. Cogoni *et al.,* International PCT Publication No. WO 01/53475, describe certain methods for isolating a Neurospora silencing gene and uses thereof. Reed *et al.,* International PCT Publication No. WO 01/68836, describe certain methods for gene silencing in plants. Honer *et al.,* International PCT Publication No. WO 01/70944, describe certain methods of drug screening using transgenic nematodes as Parkinson's Disease models using certain dsRNAs. Deak *et al.,* International PCT Publication No. WO 01/72774, describe certain *Drosophila*-derived gene products that may be related to RNAi in *Drosophila*. Arndt *et al.,* International PCT Publication No. WO 01/92513 describe certain methods for mediating gene suppression by using factors that enhance RNAi. Tuschl *et al.,* International PCT Publication No. WO 02/44321, describe certain synthetic siRNA constructs. Pachuk *et al.,* International PCT Publication No. WO 00/63364, and Satishchandran *et al.,* International PCT Publication No. WO 01/04313, describe certain methods and compositions for inhibiting the function of certain polynucleotide sequences using certain long (over 250 bp), vector expressed dsRNAs. Echeverri *et al.,* International PCT Publication No. WO 02/38805, describe certain *C. elegans* genes identified via RNAi. Kreutzer *et al.,* International PCT

Publications Nos. WO 02/055692, WO 02/055693, and EP 1144623 B1 describes certain methods for inhibiting gene expression using dsRNA. Graham *et al.,* International PCT Publications Nos. WO 99/49029 and WO 01/70949, and AU 4037501 describe certain vector expressed siRNA molecules. Fire *et al.,* US 6,506,559, describe certain methods for inhibiting gene expression in vitro using certain long dsRNA (299 bp-1033 bp) constructs that mediate RNAi. Martinez *et al.,* 2002, *Cell*, 110, 563-574, describe certain single stranded siRNA constructs, including certain 5'-phosphorylated single stranded siRNAs that mediate RNA interference in Hela cells. Harborth *et al.,* 2003, *Antisense & Nucleic Acid Drug Development,* 13, 83-105, describe certain chemically and structurally modified siRNA molecules. Chiu and Rana, 2003, *RNA*, 9, 1034-1048, describe certain chemically and structurally modified siRNA molecules. Woolf *et al.,* International PCT Publication Nos. WO 03/064626 and WO 03/064625 describe certain chemically modified dsRNA constructs.

SUMMARY OF THE INVENTION

This invention comprises compounds, compositions, and methods useful for modulating RNA function and/or gene expression in a cell. Specifically, the instant invention features synthetic small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of modulating gene expression in cells by RNA inference (RNAi). The siNA molecules of the invention can be chemically modified. The use of chemically modified siNA can improve various properties of native siRNA molecules through increased resistance to nuclease degradation *in vivo* and/or improved cellular uptake. The chemically modified siNA molecules of the instant invention provide useful reagents and methods for a variety of therapeutic, cosmetic, cosmeceutical, prophylactic, diagnostic, agricultural, target validation, genomic discovery, genetic engineering and pharmacogenomic applications.

In one embodiment, the invention features compounds, compositions, and methods useful for modulating the expression of genes associated with the maintenance or development of a disease, condition, or trait in a cell, organism, or subject, for example genes and variants thereof, including polymorphic variants such as single nucleotide polymorphism (SNP) variants associated with one or more diseases, conditions, or traits

using short interfering nucleic acid (siNA) molecules. This invention also relates to compounds, compositions, and methods useful for modulating the expression and activity of genes associated with the maintenance or development of a disease, condition, or trait in a cell, organism, or subject by RNA interference (RNAi) using small nucleic acid molecules. In particular, the instant invention features small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules and methods used to modulate the expression of genes and/or gene alleles associated with the development or maintenance of a disease, condition, or trait in a cell, organism, or subject.

In a non-limiting example, the introduction of chemically modified nucleotides into nucleic acid molecules provides a powerful tool in overcoming potential limitations of *in vivo* stability and bioavailability inherent to native RNA molecules that are delivered exogenously. For example, the use of chemically modified nucleic acid molecules can enable a lower dose of a particular nucleic acid molecule for a given therapeutic effect since chemically modified nucleic acid molecules tend to have a longer half-life in serum. Furthermore, certain chemical modifications can improve the bioavailability of nucleic acid molecules by targeting particular cells or tissues and/or improving cellular uptake of the nucleic acid molecule. Therefore, even if the activity of a chemically modified nucleic acid molecule is reduced as compared to a native nucleic acid molecule, for example when compared to an all RNA nucleic acid molecule, the overall activity of the modified nucleic acid molecule can be greater than the native molecule due to improved stability and/or delivery of the molecule. Unlike native unmodified siRNA, chemically modified siNA can also minimize the possibility of activating interferon activity in humans.

In one embodiment, the invention features one or more siNA molecules and methods that independently or in combination modulate the expression of genes encoding proteins that are associated with the maintenance and/or development of a disease, condition, or trait in a cell, organism, or subject, Such genes include those encoding sequences comprising those sequences referred to by GenBank Accession Nos. described herein and in Table V of PCT/US03/05028 (International PCT Publication No. WO 03/74654), all of which genes are included within in the defintion of gene(s) herein. The description below of the various aspects and embodiments of the invention is provided with reference to such exemplary genes. However, the various aspects and embodiments are also directed to other genes,

such as gene mutations, althernative splice variants, allelic variants and polymorphisms such as single nucleotide polymorphisms (SNPs) associated with the development or maintenance of a disease, condition, or trait in a cell, organism, or subject. These additional genes can be analyzed for target sites using the methods generally described for genes herein. Thus, the modulation of other genes and the effects of such modulation of the other genes can be performed, determined, and measured as described herein.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a gene, wherein said siNA molecule comprises about 19 to about 21 base pairs.

In one embodiment, the invention features a siNA molecule that down-regulates expression of a gene, for example, wherein the gene comprises a protein encoding sequence. In one embodiment, the invention features a siNA molecule that down-regulates expression of a gene, for example, wherein the gene comprises non-coding sequence or regulatory elements involved in gene expression.

In one embodiment, the invention features a siNA molecule having RNAi activity against a RNA, wherein the siNA molecule comprises a sequence complementary to any RNA having coding or non-encoding sequence, such as those sequences having GenBank Accession Nos. shown in **Table I** or sequences referred to by GenBank Accession Nos. described herein and in Table V of PCT/US03/05028 (International PCT Publication No. WO 03/74654) or otherwise known in the art. In another embodiment, the invention features a siNA molecule having RNAi activity against a RNA, wherein the siNA molecule comprises a sequence complementary to an RNA having variant (e.g., mutant, polymorphism, alternative splice variant) encoding sequence, for example other mutant genes not shown in **Table I** but known in the art to be associated with the maintenance and/or development of a disease, condition, or trait. Chemical modifications as shown in **Tables III and IV** or otherwise described herein can be applied to any siNA construct of the invention. In another embodiment, a siNA molecule of the invention includes a nucleotide sequence that can interact with nucleotide sequence of a gene and thereby mediate silencing of gene expression, for example, wherein the siNA mediates regulation of gene expression by cellular processes that modulate the chromatin structure or methylation patterns of the gene and prevent transcription of the gene.

In one embodiment, the nucleic acid molecules of the invention that act as mediators of the RNA interference gene silencing response are chemically modified double stranded nucleic acid molecules. As in their native double stranded RNA counterparts, these siNA molecules typically consist of duplexes containing about 19 base pairs between oligonucleotides comprising about 19 to about 25 nucleotides. The most active siRNA molecules are thought to have such duplexes with overhanging ends of 1-3 nucleotides, for example 21 nucleotide duplexes with 19 base pairs and 2 nucleotide 3'-overhangs. These overhanging segments are readily hydrolyzed by endonucleases *in vivo*. Studies have shown that replacing the 3'-overhanging segments of a 21-mer siRNA duplex having 2 nucleotide 3' overhangs with deoxyribonucleotides does not have an adverse effect on RNAi activity. Replacing up to 4 nucleotides on each end of the siRNA with deoxyribonucleotides has been reported to be well tolerated whereas complete substitution with deoxyribonucleotides results in no RNAi activity (Elbashir et al., 2001, EMBO J., 20, 6877). In addition, Elbashir *et al, supra*, also report that substitution of siRNA with 2'-O-methyl nucleotides completely abolishes RNAi activity. Li *et al.,* International PCT Publication No. WO 00/44914, and Beach *et al.,* International PCT Publication No. WO 01/68836 both suggest that siRNA may include modifications to either the phosphate-sugar back bone or the nucleoside to include at least one of a nitrogen or sulfur heteroatom, however neither application teaches to what extent these modifications are tolerated in siRNA molecules nor provide any examples of such modified siRNA. Kreutzer and Limmer, Canadian Patent Application No. 2,359,180, also describe certain chemical modifications for use in dsRNA constructs in order to counteract activation of double stranded-RNA-dependent protein kinase PKR, specifically 2'-amino or 2'-O-methyl nucleotides, and nucleotides containing a 2'-O or 4'-C methylene bridge. However, Kreutzer and Limmer similarly fail to show to what extent these modifications are tolerated in siRNA molecules nor provide any examples of such modified siRNA.

In one embodiment, the invention features chemically modified siNA constructs having specificity for target nucleic acid molecules in a cell. Non-limiting examples of such chemical modifications include without limitation phosphorothioate internucleotide linkages, 2'-O-methyl ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, 2'-deoxy ribonucleotides, "universal base" nucleotides, 5-C-methyl nucleotides, and inverted deoxyabasic residue incorporation. These chemical modifications, when used in various

siNA constructs, are shown to preserve RNAi activity in cells while at the same time, dramatically increasing the serum stability of these compounds. Furthermore, contrary to the data published by Parrish *et al., supra,* applicant demonstrates that multiple (greater than one) phosphorothioate substitutions are well-tolerated and confer substantial increases in serum stability for modified siNA constructs.

In one embodiment, the chemically-modified siNA molecules of the invention comprise a duplex having two strands, one or both of which can be chemically-modified, wherein each strand is about 19 to about 29 (e.g., about 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30) nucleotides. In one embodiment, the chemically-modified siNA molecules of the invention comprise a duplex having two strands, one or both of which can be chemically-modified, wherein each strand is about 19 to about 23 (e.g., about 18, 19, 20, 21, 22, 23 or 24) nucleotides. In one embodiment, a siNA molecule of the invention comprises modified nucleotides while maintaining the ability to mediate RNAi. The modified nucleotides can be used to improve *in vitro* or *in vivo* characteristics such as stability, activity, and/or bioavailability. For example, a siNA molecule of the invention can comprise modified nucleotides as a percentage of the total number of nucleotides present in the siNA molecule. As such, a siNA molecule of the invention can generally comprise modified nucleotides from about 5 to about 100% of the nucleotide positions (e.g., 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% of the nucleotide positions). The actual percentage of modified nucleotides present in a given siNA molecule depends on the total number of nucleotides present in the siNA. If the siNA molecule is single stranded, the percent modification can be based upon the total number of nucleotides present in the single stranded siNA molecules. Likewise, if the siNA molecule is double stranded, the percent modification can be based upon the total number of nucleotides present in the sense strand, antisense strand, or both the sense and antisense strands. In addition, the actual percentage of modified nucleotides present in a given siNA molecule can also depend on the total number of purine and pyrimidine nucleotides present in the siNA, for example, wherein all pyrimidine nucleotides and/or all purine nucleotides present in the siNA molecule are modified.

The antisense region of a siNA molecule of the invention can comprise a phosphorothioate internucleotide linkage at the 3'-end of said antisense region. The

antisense region can comprise about one to about five phosphorothioate internucleotide linkages at the 5'-end of said antisense region. The 3'-terminal nucleotide overhangs of a siNA molecule of the invention can comprise ribonucleotides or deoxyribonucleotides that are chemically-modified at a nucleic acid sugar, base, or backbone. The 3'-terminal nucleotide overhangs can comprise one or more universal base ribonucleotides. The 3'-terminal nucleotide overhangs can comprise one or more acyclic nucleotides. The 3'-terminal nucleotide overhangs can comprise one or more cap moieties, such as cap moieties shown in **Figure 22**.

In one embodiment, a siNA molecule of the invention comprises blunt ends, i.e., the ends do not include any overhanging nucleotides. For example, a siNA molecule of the invention comprising modifications described herein (e.g., comprising nucleotides having Formulae I-VII or siNA constructs comprising "Stab00"-"Stab25" (**Table IV**) or any combination thereof) and/or any length described herein can comprise blunt ends or ends with no overhanging nucleotides.

In one embodiment, any siNA molecule of the invention can comprise one or more blunt ends, i.e. where a blunt end does not have any overhanging nucleotides. In a non-limiting example, a blunt ended siNA molecule has a number of base pairs equal to the number of nucleotides present in each strand of the siNA molecule. In another example, a siNA molecule comprises one blunt end, for example wherein the 5'-end of the antisense strand and the 3'-end of the sense strand do not have any overhanging nucleotides. In another example, a siNA molecule comprises one blunt end, for example wherein the 3'-end of the antisense strand and the 5'-end of the sense strand do not have any overhanging nucleotides. In another example, a siNA molecule comprises two blunt ends, for example wherein the 3'-end of the antisense strand and the 5'-end of the sense strand as well as the 5'-end of the antisense strand and 3'-end of the sense strand do not have any overhanging nucleotides. A blunt ended siNA molecule can comprise, for example, from about 18 to about 30 nucleotides (e.g., about 18, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides). Other nucleotides present in a blunt ended siNA molecule can comprise mismatches, bulges, loops, or wobble base pairs, for example, to modulate the activity of the siNA molecule to mediate RNA interference.

By "blunt ends" is meant symmetric termini or termini of a double stranded siNA molecule having no overhainging nucleotides. The two strands of a double stranded siNA molecule align with each other without over-hanging nucleotides at the termini. For example, a blunt ended siNA construct comprises terminal nucleotides that are complimentary between the sense and antisense regions of the siNA molecule.

In one embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siNA) molecule to down-regulate expression of a target gene, wherein the siNA molecule comprises one or more chemical modifications and each strand of the double-stranded siNA is about 19 to about 23 nucleotides (e.g., about 19, 20, 21, 22, or 23 nucleotides) long.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene, wherein the siNA molecule comprises no ribonucleotides and each strand of the double-stranded siNA comprises about 19 to about 23 nucleotides (e.g., about 19, 20, 21, 22, or 23 nucleotides).

In one embodiment, one of the strands of a double-stranded siNA molecule of the invention comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of a target gene, and wherein the second strand of a double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of the target gene.

In one embodiment, a siNA molecule of the invention comprises about 19 to about 23 nucleotides (e.g., about 19, 20, 21, 22, or 23 nucleotides), and each strand comprises at least about 19 nucleotides that are complementary to the nucleotides of the other strand.

In one embodiment, a siNA molecule of the invention comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of a target gene, and the siNA further comprises a sense region, wherein the sense region comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of the target gene. The antisense region and the sense region each comprise about 19 to about 23 nucleotides (e.g., about 19, 20, 21, 22, or 23 nucleotides), and the antisense region comprises at least about 19 nucleotides that are complementary to nucleotides of the sense region.

In one embodiment, a siNA molecule of the invention comprises a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of RNA encoded by a target gene and the sense region comprises a nucleotide sequence that is complementary to the antisense region.

In one embodiment, a siNA molecule of the invention is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule. In another embodiment, the sense region is connected to the antisense region via a linker molecule, which can be a polynucleotide linker or a non-nucleotide linker.

In one embodiment, a siNA molecule of the invention comprises a sense region and antisense region, wherein pyrimidine nucleotides in the sense region compries 2'-O-methyl pyrimidine nucleotides and purine nucleotides in the sense region comprise 2'-deoxy purine nucleotides. In one embodiment, a siNA molecule of the invention comprises a sense region and antisense region, wherein pyrimidine nucleotides present in the sense region comprise 2'-deoxy-2'-fluoro pyrimidine nucleotides and wherein purine nucleotides present in the sense region comprise 2'-deoxy purine nucleotides.

In one embodiment, a siNA molecule of the invention comprises a sense region and antisense region, wherein the pyrimidine nucleotides when present in said antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides when present in said antisense region are 2'-O-methyl purine nucleotides.

In one embodiment, a siNA molecule of the invention comprises a sense region and antisense region, wherein the pyrimidine nucleotides when present in said antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and wherein the purine nucleotides when present in said antisense region comprise 2'-deoxy- purine nucleotides.

In one embodiment, a siNA molecule of the invention comprises a sense region and antisense region, wherein the sense region includes a terminal cap moiety at the 5'-end, the 3'-end, or both of the 5' and 3' ends of the sense region. In another embodiment, the terminal cap moiety is an inverted deoxy abasic moiety or any other cap moiety such as those shown in **Figure 22**.

In one embodiment, a siNA molecule of the invention has RNAi activity that modulates expression of RNA encoded by a gene. Because many genes can share some degree of sequence homology with each other, siNA molecules can be designed to target a class of genes (and associated receptor or ligand genes) or alternately specific genes by selecting sequences that are either shared amongst different gene targets or alternatively that are unique for a specific gene target. Therefore, in one embodiment, the siNA molecule can be designed to target conserved regions of a RNA sequence having sequence homology between several genes so as to target several genes or gene families (e.g., different gene isoforms, splice variants, mutant genes etc.) with one siNA molecule. In another embodiment, the siNA molecule can be designed to target a sequence that is unique to a specific RNA sequence of a specific gene due to the high degree of specificity that the siNA molecule requires to mediate RNAi activity.

In one embodiment, a siNA of the invention is used to inhibit the expression of genes or a gene family, wherein the genes or gene family sequences share sequence homology. Such homologous sequences can be identified as is known in the art, for example using sequence alignments. siNA molecules can be designed to target such homologous sequences, for example using perfectly complementary sequences or by incorporating non-canonical base pairs (*e.g.*, mismatches and/or wobble base pairs, that can provide additional target sequences. In instances where mismatches are identified, non-canonical base pairs (*e.g.*, mismatches and/or wobble bases) can be used to generate siNA molecules that target more than one gene sequence. In a non-limiting example, non-canonical base pairs such as UU and CC base pairs are used to generate siNA molecules that are capable of targeting differing VEGF and/or VEGFR sequences (e.g., VEGFR1 and VEGFR2). As such, one advantage of using siNAs of the invention is that a single siNA can be designed to include a nucleic acid sequence that is complementary to the nucleotide sequence that is conserved between the VEGF receptors (i.e., VEGFR1, VEGFR2, and/or VEGFR3) such that the siNA can interact with RNAs of the receptors and mediate RNAi to achieve inhibition of expression of the VEGF receptors. In this approach, a single siNA can be used to inhibit expression of more than one VEGF receptor instead of using more than one siNA molecule to target the different receptors.

In one embodiment, the invention features a siNA molecule having RNAi activity against a target RNA, wherein the siNA molecule comprises a sequence complementary to

any RNA having target gene encoding sequence, such as those sequences having GenBank Accession Nos. referred to herein. In another embodiment, the invention features a siNA molecule having RNAi activity against a target RNA, wherein the siNA molecule comprises a sequence complementary to an RNA having other sequences, for example mutant genes as are known in the art to be associated with a disease, condition, trait, genotype or phenotype. Chemical modifications as shown in **Tables I and IV** or otherwise described herein can be applied to any siNA construct of the invention. In another embodiment, a siNA molecule of the invention includes nucleotide sequence that can interact with nucleotide sequence of a target gene and thereby mediate silencing of target gene expression, for example, wherein the siNA mediates regulation of target gene expression by cellular processes that modulate the chromatin structure or methylation patterns of the target gene and prevent transcription of the target gene.

In one embodiment, siNA molecules of the invention are used to down regulate or inhibit the expression of target proteins arising from haplotype polymorphisms that are associated with a disease, condition, trait, genotype or phenotype, (e.g., associated with a gain of function). Analysis of target genes, or target protein or RNA levels can be used to identify subjects with such polymorphisms or those subjects who are at risk of developing a disease, condition, trait, genotype or phenotype. These subjects are amenable to treatment, for example, treatment with siNA molecules of the invention and any other composition useful in treating a diseases, conditions, traits, genotypes or phenotypes related to target gene expression or expressed protein activity. As such, analysis of target protein or RNA levels can be used to determine treatment type and the course of therapy in treating a subject. Monitoring of protein or RNA levels can be used to predict treatment outcome and to determine the efficacy of compounds and compositions that modulate the level and/or activity of certain proteins associated with a disease, condition, trait, genotype or phenotype.

In one embodiment, the antisense region of a siNA molecule of the invention comprises sequence complementary to a portion of a gene transcript having sequence unique to a particular disease, condition, trait, genotype or phenotype related allele, such as sequence comprising a SNP associated with the disease, condition, trait, genotype or phenotype specific allele. As such, the antisense region of a siNA molecule of the invention can comprise sequence complementary to sequences that are unique to a

particular allele to provide specificity in mediating selective RNAi againt the disease, condition, trait, genotype or phenotype related allele.

In another embodiment, the invention features a siNA molecule comprising nucleotide sequence, for example, nucleotide sequence in the antisense region of the siNA molecule that is complementary to a nucleotide sequence or portion of sequence of a target gene. In another embodiment, the invention features a siNA molecule comprising a region, for example, the antisense region of the siNA construct, complementary to a sequence comprising a target gene sequence or a portion thereof.

In one embodiment of the invention a siNA molecule comprises an antisense strand having about 19 to about 29 (e.g., about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29) nucleotides, wherein the antisense strand is complementary to a RNA sequence encoding a target protein, and wherein said siNA further comprises a sense strand having about 19 to about 29 (e.g., about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29) nucleotides, and wherein said sense strand and said antisense strand are distinct nucleotide sequences with at least about 19 complementary nucleotides.

In another embodiment of the invention a siNA molecule of the invention comprises an antisense region having about 19 to about 29 (e.g., about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29) nucleotides, wherein the antisense region is complementary to a RNA sequence encoding a target protein, and wherein said siNA further comprises a sense region having about 19 to about 29 or more (e.g., about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or more) nucleotides, wherein said sense region and said antisense region comprise a linear molecule with at least about 19 complementary nucleotides.

In one embodiment of the invention a siNA molecule comprises an antisense strand comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a target protein. The siNA further comprises a sense strand, wherein said sense strand comprises a nucleotide sequence of a target gene or a portion thereof.

In another embodiment, a siNA molecule comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence encoding a target

protein or a portion thereof. The siNA molecule further comprises a sense region, wherein said sense region comprises a nucleotide sequence of a target gene or a portion thereof.

In one embodiment, a siNA molecule of the invention has RNAi activity that modulates expression of RNA encoded by a target gene. Because certain genes can share some degree of sequence homology with each other, siNA molecules can be designed to target a class of genes or alternately specific genes (e.g., polymorphic variants) by selecting sequences that are either shared amongst different targets or alternatively that are unique for a specific target. Therefore, in one embodiment, the siNA molecule can be designed to target conserved regions of RNA sequence having homology between several gene variants so as to target a class of genes with one siNA molecule. Accordingly, in one embodiment, the siNA molecule of the invention modulates the expression of one or both alleles of a target gene in a subject. In another embodiment, the siNA molecule can be designed to target a sequence that is unique to a specific target RNA sequence (e.g., a single allele or associated SNP) due to the high degree of specificity that the siNA molecule requires to mediate RNAi activity.

In one embodiment, nucleic acid molecules of the invention that act as mediators of the RNA interference gene silencing response are double-stranded nucleic acid molecules. In another embodiment, the siNA molecules of the invention consist of duplexes containing about 19 base pairs between oligonucleotides comprising about 19 to about 25 (*e.g.*, about 19, 20, 21, 22, 23, 24, or 25) nucleotides. In yet another embodiment, siNA molecules of the invention comprise duplexes with overhanging ends of about about 1 to about 3 (*e.g.*, about 1, 2, or 3) nucleotides, for example, about 21-nucleotide duplexes with about 19 base pairs and 3'-terminal mononucleotide, dinucleotide, or trinucleotide overhangs.

One aspect of the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene. In one embodiment, a double stranded siNA molecule comprises one or more chemical modifications and each strand of the double-stranded siNA is about 21 nucleotides long. In one embodiment, the double-stranded siNA molecule does not contain any ribonucleotides. In another embodiment, the double-stranded siNA molecule comprises one or more ribonucleotides. In one embodiment, each strand of the double-stranded siNA molecule comprises about 19 to about 29 (e.g., about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29) nucleotides, wherein

each strand comprises about 19 nucleotides that are complementary to the nucleotides of the other strand. In one embodiment, one of the strands of the double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of the target gene, and the second strand of the double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence of the target gene or a portion thereof.

In another embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene comprising an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of the target gene or a portion thereof, and a sense region, wherein the sense region comprises a nucleotide sequence substantially similar to the nucleotide sequence of the target gene or a portion thereof. In one embodiment, the antisense region and the sense region each comprise about 19 to about 23 (e.g., about 19, 20, 21, 22, or 23) nucleotides, wherein the antisense region comprises about 19 nucleotides that are complementary to nucleotides of the sense region.

In another embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene comprising a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by the target gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region.

In one embodiment, a siNA of the invention is used to inhibit the expression of more than one gene, wherein the genes share some degree of sequence homology. Such homologous sequences can be identified as is known in the art, for example using sequence alignments. siNA molecules can be designed to target such homologous sequences, for example using perfectly complementary sequences or by incorporating mismatches and/or wobble base pairs that can provide additional target sequences One advantage of using siNAs of the invention is that a single siNA can be designed to include nucleic acid sequence that is complementary to a nucleotide sequence that is conserved between the genes such that the siNA can interact with RNA transcripts of the genes and mediate RNAi to achieve inhibition of expression of the genes. In this approach, a single siNA can be used

to inhibit expression of more than one gene, thereby obviating the need to use more than one siNA molecule to target the different genes. The different genes can comprise, for example, a cytokine and its corresponding receptor(s).

In one embodiment, the invention features a method of designing a single siNA to inhibit the expression of two or more genes comprising designing a siNA having nucleotide sequence that is complementary to nucleotide sequence encoded by or present in the genes or a portion thereof, wherein the siNA mediates RNAi to inhibit the expression of the genes. For example, a single siNA can inhibit the expression of two genes by binding to conserved or homologous sequence present in RNA encoded by both genes or a portion thereof.

In one embodiment, nucleic acid molecules of the invention that act as mediators of the RNA interference gene silencing response are double-stranded nucleic acid molecules. In another embodiment, the siNA molecules of the invention consist of duplexes containing about 19 base pairs between oligonucleotides comprising about 19 to about 25 (*e.g.*, about 19, 20, 21, 22, 23, 24, or 25) nucleotides. In yet another embodiment, siNA molecules of the invention comprise duplexes with overhanging ends of about 1 to about 3 (*e.g.*, about 1, 2, or 3) nucleotides, for example, about 21-nucleotide duplexes with about 19 base pairs and 3'-terminal mononucleotide, dinucleotide, or trinucleotide overhangs.

In one embodiment, the invention features one or more chemically-modified siNA constructs having specificity for nucleic acid molecules that express or encode a protein sequence, such as RNA or DNA encoding a protein sequence. Non-limiting examples of such chemical modifications include without limitation phosphorothioate internucleotide linkages, 2'-deoxyribonucleotides, 2'-O-methyl ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, "universal base" nucleotides, "acyclic" nucleotides, 5-C-methyl nucleotides, and terminal glyceryl and/or inverted deoxy abasic residue incorporation. These chemical modifications, when used in various siNA constructs, are shown to preserve RNAi activity in cells while at the same time, dramatically increasing the serum stability of these compounds.

In one embodiment, a siNA molecule of the invention does not contain any ribonucleotides. In another embodiment, a siNA molecule of the invention comprises one or more ribonucleotides.

In one embodiment, the invention features the use of compounds or compositions that inhibit the activity of double stranded RNA binding proteins (dsRBPs, see for example Silhavy *et al.,* 2003, *Journal of General Virology*, 84, 975-980). Non-limiting examples of compounds and compositions that can be used to inhibit the activity of dsRBPs include but are not limited to small molecules and nucleic acid aptamers that bind to or interact with the dsRBPs and consequently reduce dsRBP activity and/or siNA molecules that target nucleic acid sequences encoding dsRBPs. The use of such compounds and compositions is expected to improve the activity of siNA molecules in biological systems in which dsRBPs can abrogate or suppress the efficacy of siNA mediated RNA interference, such as where dsRBPs are expressed during viral infection of a cell to escape RNAi surveillance. Therefore, the use of agents that inhibit dsRBP activity is preferred in those instances where RNA interference activity can be improved via the abrogation or suppression of dsRBP activity. Such anti-dsRBP agents can be administered alone or can be co-administered with siNA molecules of the invention, or can be used to pretreat cells or a subject before siNA administration. In another embodiment, anti-dsRBP agents are used to treat viral infection, such as HCV, HBV, or HIV infection with or without siNA molecules of the invention.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a gene, wherein one of the strands of the double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence of the gene or RNA encoded by the gene or a portion thereof, and wherein the second strand of the double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence of the gene or RNA encoded by the gene or a portion thereof.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a gene, wherein each strand of the siNA molecule comprises about 19 to about 23 nucleotides, and wherein each strand comprises at least about 19 nucleotides that are complementary to the nucleotides of the other strand.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a gene, wherein the siNA molecule

comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence of the gene or RNA encoded by the gene or a portion thereof, and wherein the siNA further comprises a sense region, wherein the sense region comprises a nucleotide sequence substantially similar to the nucleotide sequence of the gene or RNA encoded by the gene or a portion thereof.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a target gene by mediating RNA interference (RNAi) process, wherein the siNA molecule comprises no ribonucleotides and wherein each strand of the double-stranded siNA molecule comprises about 21 nucleotides.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the replication of a virus (e.g, as mammalian virus, plant virus, hepatitis C virus, human immunodeficiency virus, hepatitis B virus, herpes simplex virus, cytomegalovirus, human papilloma virus, respiratory syncytial virus, or influenza virus), wherein the siNA molecule does not require the presence of a ribonucleotide within the siNA molecule for the inhibition of replication of the virus and each strand of the double-stranded siNA molecule comprises about 21 nucleotides.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a gene, wherein the siNA molecule comprises a sense region and an antisense region and wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of RNA encoded by the gene and the sense region comprises a nucleotide sequence that is complementary to the antisense region or a portion thereof, and wherein the purine nucleotides present in the antisense region comprise 2'-deoxy- purine nucleotides. In another embodiment, the purine nucleotides present in the antisense region comprise 2'-O-methyl purine nucleotides. In either of the above embodiments, the antisense region comprises a phosphorothioate internucleotide linkage at the 3' end of the antisense region. In an alternative embodiment, the antisense region comprises a glyceryl modification at the 3' end of the antisense region. In another embodiment of any of the above described siNA molecules, any nucleotides present in a non-complementary region of the antisense strand (e.g., overhang region) are 2'-deoxy nucleotides.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a gene, wherein the siNA molecule is assembled from two separate oligonucleotide fragments each comprising 21 nucleotides, wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule, and wherein about 19 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule and wherein at least two 3' terminal nucleotides of each fragment of the siNA molecule are not base-paired to the nucleotides of the other fragment of the siNA molecule. In one embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule is a 2'-deoxy-pyrimidine nucleotide, such as 2'-deoxy-thymidine. In another embodiment, all 21 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule. In another embodiment, about 19 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the gene. In another embodiment, 21 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the gene. In any of the above embodiments, the 5'-end of the fragment comprising said antisense region can optionally include a phosphate group.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a RNA sequence (e.g., wherein said target RNA sequence is encoded by a gene or a gene involved in a pathway of gene expression), wherein the siNA molecule does not contain any ribonucleotides and wherein each strand of the double-stranded siNA molecule is about 21 nucleotides long. Examples of non-ribonucleotide containing siNA constructs are combinations of stabilization chemistries shown in **Table IV** in any combination of Sense/Antisense chemistries, e.g., Stab 7/8, Stab 7/11, Stab 8/8, Stab 18/8, Stab 18/11, Stab 12/13, Stab 7/13, Stab 18/13, Stab 7/19, Stab 8/19, Stab 18/19, Stab 7/20, Stab 8/20, or Stab 18/20.

In one embodiment, the invention features a chemically synthesized double stranded RNA molecule that directs cleavage of a target RNA via RNA interference, wherein each strand of said RNA molecule is about 21 to about 23 nucleotides in length; one strand of the RNA molecule comprises nucleotide sequence having sufficient complementarity to the target RNA for the RNA molecule to direct cleavage of the target RNA via RNA

interference; and wherein at least one strand of the RNA molecule comprises one or more chemically modified nucleotides described herein, such as deoxynucleotides, 2'-O-methyl nucleotides, 2'-deoxy-2'-fluoro nucloetides, 2'-O-methoxyethyl nucleotides etc.

In one embodiment, the invention features a medicament comprising a siNA molecule of the invention.

In one embodiment, the invention features an active ingredient comprising a siNA molecule of the invention.

In one embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siNA) molecule to down-regulate expression of a target gene, wherein the siNA molecule comprises one or more chemical modifications and each strand of the double-stranded siNA is about 21 nucleotides long.

In one embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siNA) molecule to down-regulate expression of a target gene, wherein the siNA molecule comprises one or more chemical modifications and each strand of the double-stranded siNA is about 18 to about 28 or more (e.g., 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 or more) nucleotides long.

The invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of a RNA encoded by the gene or a portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification.

In one embodiment, the nucleotide sequence of the antisense strand of a siNA molecule of the invention is complementary to the nucleotide sequence of a RNA which encodes a protein or a portion thereof. In one embodiment, each strand of the siNA molecule comprises about 19 to about 29 (e.g., about 18, 19, 20, 21, 22, 23, 24, 25, 26, 27,

28, 29 or 30) nucleotides, and each strand comprises at least about 19 nucleotides that are complementary to the nucleotides of the other strand.

In one embodiment, a siNA molecule of the invention is assembled from two oligonucleotide fragments, wherein one fragment comprises the nucleotide sequence of the antisense strand of the siNA molecule and a second fragment comprises nucleotide sequence of the sense region of the siNA molecule. In another embodiment, the sense strand is connected to the antisense strand via a linker molecule, such as a polynucleotide linker or a non-nucleotide linker.

In one embodiment, of a siNA molecule of the invention, the pyrimidine nucleotides present in the sense strand are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-deoxy purine nucleotides. In another embodiment, the pyrimidine nucleotides present in the sense strand are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides. In one embodiment, the sense strand comprises a 3'-end and a 5'-end, wherein a terminal cap moiety (e.g., an inverted deoxy abasic moiety) is present at the 5'-end, the 3'-end, or both of the 5' and 3' ends of the sense strand. In one embodiment, the antisense strand comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides and one or more 2'-O-methyl purine nucleotides. In one embodiment, the pyrimidine nucleotides present in the antisense strand are 2'-deoxy-2'-fluoro pyrimidine nucleotides and any purine nucleotides present in the antisense strand are 2'-O-methyl purine nucleotides. In one embodiment, the antisense strand comprises a phosphorothioate internucleotide linkage at the 3' end of the antisense strand. In another embodiment, the antisense strand comprises a glyceryl modification at the 3' end. In another embodiment, the 5'-end of the antisense strand optionally includes a phosphate group.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of RNA encoded by a gene or a portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a

sugar modification, and wherein the nucleotide sequence of the antisense strand is complementary to a nucleotide sequence of the 5'-untranslated region or a portion thereof of the RNA. In another embodiment, the nucleotide sequence of the antisense strand is complementary to a nucleotide sequence of the RNA or a portion thereof.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of a RNA or a portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification, and wherein each of the two strands of the siNA molecule comprises 21 nucleotides. In one embodiment, about 19 nucleotides of each strand of the siNA molecule are base-paired to the complementary nucleotides of the other strand of the siNA molecule and at least two 3' terminal nucleotides of each strand of the siNA molecule are not base-paired to the nucleotides of the other strand of the siNA molecule. In one embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule are 2'-deoxy-pyrimidines, such as 2'-deoxy-thymidine. In another embodiment, each strand of the siNA molecule is base-paired to the complementary nucleotides of the other strand of the siNA molecule. In one embodiment, about 19 nucleotides of the antisense strand are base-paired to the nucleotide sequence of the RNA or a portion thereof. In another embodiment, 21 nucleotides of the antisense strand are base-paired to the nucleotide sequence of the RNA or a portion thereof.

In one embodiment, the invention features a composition comprising a siNA molecule of the invention and a pharmaceutically acceptable carrier or diluent.

In one embodiment, the invention features a method of increasing the stability of a siNA molecule against cleavage by ribonucleases comprising introducing at least one modified nucleotide into the siNA molecule, wherein the modified nucleotide is a 2'-deoxy-2'-fluoro nucleotide. In another embodiment, all pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides. In another embodiment, the modified nucleotides in the siNA include at least one 2'-deoxy-2'-fluoro cytidine or

2'-deoxy-2'-fluoro uridine nucleotide. In another embodiment, the modified nucleotides in the siNA include at least one 2'-fluoro cytidine and at least one 2'-deoxy-2'-fluoro uridine nucleotides. In another embodiment, all uridine nucleotides present in the siNA are 2'-deoxy-2'-fluoro uridine nucleotides. In another embodiment, all cytidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro cytidine nucleotides. In another embodiment, all adenosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro adenosine nucleotides. In another embodiment, all guanosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro guanosine nucleotides. The siNA can further comprise at least one modified internucleotidic linkage, such as phosphorothioate linkage. In another embodiment, the 2'-deoxy-2'-fluoronucleotides are present at specifically selected locations in the siNA that are sensitive to cleavage by ribonucleases, such as locations having pyrimidine nucleotides.

In one embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a gene, wherein one of the strands of the double-stranded siNA molecule is an antisense strand which comprises nucleotide sequence that is complementary to nucleotide sequence of a RNA or a portion thereof, the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand and wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification.

In one embodiment, the invention features a short interfering nucleic acid (siNA) molecule comprising a double-stranded structure that down-regulates expression of a target nucleic acid, wherein the siNA molecule does not require a 2'-hydroxyl group containing ribonucleotide, each strand of the double-stranded structure of the siNA molecule comprises about 21 nucleotides and the siNA molecule comprises nucleotide sequence having complementarity to nucleotide sequence of the target nucleic acid or a portion thereof. The target nucleic acid can be an endogenous gene, an exogenous gene, a viral nucleic acid, or a RNA, such as a mammalian gene, plant gene, viral gene, fungal gene, bacterial gene, plant viral gene, or mammalian viral gene. Examples of mammalian viral gene include hepatitis C virus, human immunodeficiency virus, hepatitis B virus, herpes simplex virus, cytomegalovirus, human papilloma virus, respiratory syncytial virus, influenza virus, and severe acute respiratory syndrome virus (SARS).

In one embodiment, a siNA molecule of the invention comprises a sense region and an antisense region wherein the antisense region comprises the nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of the target nucleic acid and the sense region comprises a nucleotide sequence that is complementary to nucleotide sequence of the antisense region or a portion thereof.

In one embodiment, a siNA molecule of the invention is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule. The sense region can be connected to the antisense region via a linker molecule, such as a polynucleotide linker or non-nucleotide linker. In another embodiment, each sense region and antisense region comprise about 21 nucleotides in length. In another embodiment, about 19 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule and at least two 3' terminal nucleotides of each fragment of the siNA molecule are not base-paired to the nucleotides of the other fragment of the siNA molecule. In another embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule are 2'-deoxy-pyrimidines, such as the thymidine. In another embodiment, all 21 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule. In another embodiment, about 19 nucleotides of the antisense region of the siNA molecule are base-paired to the nucleotide sequence or a portion thereof of the the target nucleic acid. In another embodiment, 21 nucleotides of the antisense region of the siNA molecule are base-paired to the nucleotide sequence or a portion thereof of the target nucleic acid. In another embodiment, the 5'-end of the fragment comprising the antisense region optionally includes a phosphate group.

In one embodiment, a siNA molecule of the invention comprises nucleotide sequence having complementarity to nucleotide sequence of RNA or a portion thereof encoded by the target nucleic acid or a portion thereof.

In one embodiment, a siNA molecule of the invention comprises a sense region and an antisense region, wherein the pyrimidine nucleotides when present in the sense region are 2'-O-methyl pyrimidine nucleotides and wherein the purine nucleotides when present in the sense region are 2'-deoxy purine nucleotides.

In one embodiment, a siNA molecule of the invention comprises a sense region and an antisense region, wherein the pyrimidine nucleotides when present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and wherein the purine nucleotides when present in the sense region are 2'-deoxy purine nucleotides.

In one embodiment, a siNA molecule of the invention comprises a sense region and an antisense region, wherein the sense region includes a terminal cap moiety at the 5'-end, the 3'-end, or both of the 5' and 3' ends. The cap moiety can be an inverted deoxy abasic moiety, an inverted deoxy thymidine moiety, or a thymidine moiety.

In one embodiment, a siNA molecule of the invention comprises a sense region and an antisense region, wherein the pyrimidine nucleotides when present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides when present in the antisense region are 2'-O-methyl purine nucleotides.

In one embodiment, a siNA molecule of the invention comprises a sense region and an antisense region, wherein the pyrimidine nucleotides when present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and wherein the purine nucleotides when present in the antisense region comprise 2'-deoxy- purine nucleotides.

In one embodiment, a siNA molecule of the invention comprises a sense region and an antisense region, wherein the antisense region comprises a phosphate backbone modification at the 3' end of the antisense region. The phosphate backbone modification can be a phosphorothioate.

In one embodiment, a siNA molecule of the invention comprises a sense region and an antisense region, wherein the antisense region comprises a glyceryl modification at the 3' end of the antisense region.

In one embodiment, a siNA molecule of the invention comprises a sense region and an antisense region, wherein each of sense and the antisense regions of the siNA molecule comprise about 21 nucleotides.

In a non-limiting example, the introduction of chemically-modified nucleotides into nucleic acid molecules provides a powerful tool in overcoming potential limitations of *in vivo* stability and bioavailability inherent to native RNA molecules that are delivered

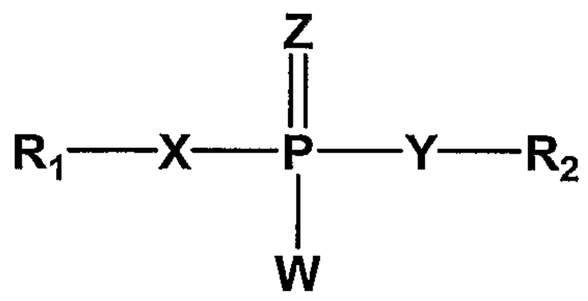
exogenously. For example, the use of chemically-modified nucleic acid molecules can enable a lower dose of a particular nucleic acid molecule for a given therapeutic effect since chemically-modified nucleic acid molecules tend to have a longer half-life in serum. Furthermore, certain chemical modifications can improve the bioavailability of nucleic acid molecules by targeting particular cells or tissues and/or improving cellular uptake of the nucleic acid molecule. Therefore, even if the activity of a chemically-modified nucleic acid molecule is reduced as compared to a native nucleic acid molecule, for example, when compared to an all-RNA nucleic acid molecule, the overall activity of the modified nucleic acid molecule can be greater than that of the native molecule due to improved stability and/or delivery of the molecule. Unlike native unmodified siNA, chemically-modified siNA can also minimize the possibility of activating interferon activity in humans.

In any of the embodiments of siNA molecules described herein, the antisense region of a siNA molecule of the invention can comprise a phosphorothioate internucleotide linkage at the 3'-end of said antisense region. In any of the embodiments of siNA molecules described herein, the antisense region can comprise about one to about five phosphorothioate internucleotide linkages at the 5'-end of said antisense region. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs of a siNA molecule of the invention can comprise ribonucleotides or deoxyribonucleotides that are chemically-modified at a nucleic acid sugar, base, or backbone. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs can comprise one or more universal base ribonucleotides. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs can comprise one or more acyclic nucleotides.

One embodiment of the invention provides an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the invention in a manner that allows expression of the nucleic acid molecule. Another embodiment of the invention provides a mammalian cell comprising such an expression vector. The mammalian cell can be a human cell. The siNA molecule of the expression vector can comprise a sense region and an antisense region. The antisense region can comprise sequence complementary to an RNA or DNA sequence encoding a protein or polypeptide and the sense region can comprise sequence complementary to the antisense region. The siNA molecule can comprise two distinct strands having complementary sense and antisense regions. The

siNA molecule can comprise a single strand having complementary sense and antisense regions.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides comprising a backbone modified internucleotide linkage having Formula I:

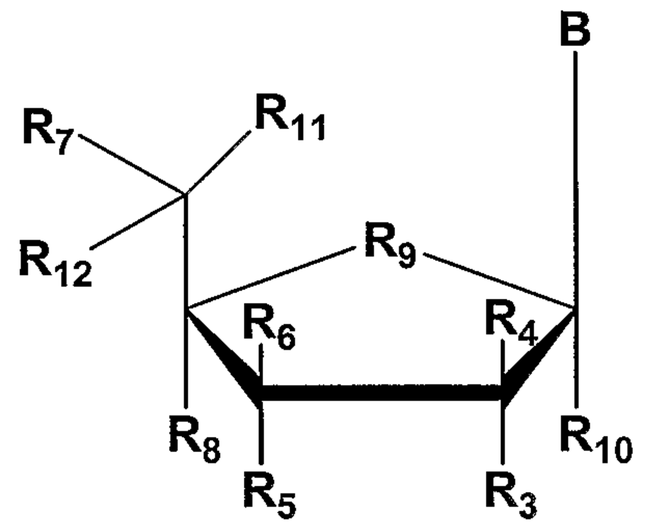


wherein each R_1 and R_2 is independently any nucleotide, non-nucleotide, or polynucleotide which can be naturally-occurring or chemically-modified, each X and Y is independently O, S, N, alkyl, or substituted alkyl, each Z and W is independently O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, or aralkyl, and wherein W, X, Y, and Z are optionally not all O. In another embodiment, a backbone modification of the invention comprises a phosphonoacetate and/or thiophosphonoacetate internucleotide linkage (see for example Sheehan et al., 2003, Nucleic Acids Research, 31, 4109-4118).

The chemically-modified internucleotide linkages having Formula I, for example, wherein any Z, W, X, and/or Y independently comprises a sulphur atom, can be present in one or both oligonucleotide strands of the siNA duplex, for example, in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) chemically-modified internucleotide linkages having Formula I at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified internucleotide linkages having Formula I at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) pyrimidine nucleotides with chemically-modified internucleotide linkages having Formula I in the sense strand, the

antisense strand, or both strands. In yet another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) purine nucleotides with chemically-modified internucleotide linkages having Formula I in the sense strand, the antisense strand, or both strands. In another embodiment, a siNA molecule of the invention having internucleotide linkage(s) of Formula I also comprises a chemically-modified nucleotide or non-nucleotide having any of Formulae I-VII.

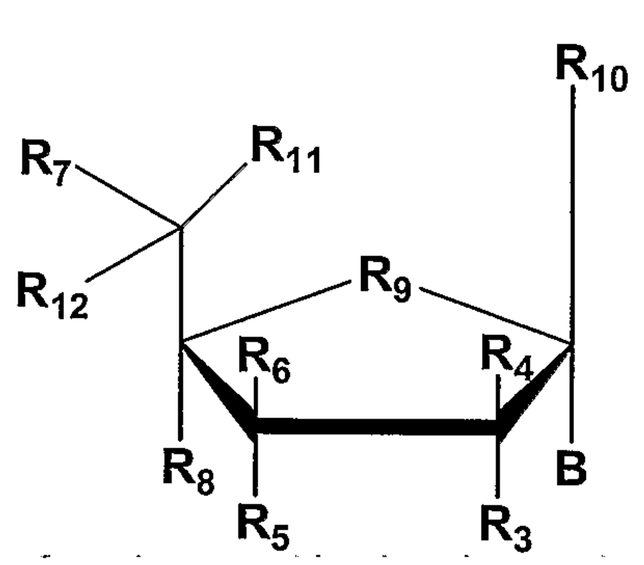
In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides or non-nucleotides having Formula II:



wherein each R_3, R_4, R_5, R_6, R_7, R_8, R_{10}, R_{11} and R_{12} is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF_3, OCF_3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO_2, NO_2, N_3, NH_2, aminoalkyl, aminoacid, aminoacyl, ONH_2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or group having Formula I or II; R_9 is O, S, CH_2, S=O, CHF, or CF_2, and B is a nucleosidic base such as adenine, guanine, uracil, cytosine, thymine, 2-aminoadenosine, 5-methylcytosine, 2,6-diaminopurine, or any other non-naturally occurring base that can be complementary or non-complementary to target RNA or a non-nucleosidic base such as phenyl, naphthyl, 3-nitropyrrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other non-naturally occurring universal base that can be complementary or non-complementary to target RNA.

The chemically-modified nucleotide or non-nucleotide of Formula II can be present in one or both oligonucleotide strands of the siNA duplex, for example in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more chemically-modified nucleotide or non-nucleotide of Formula II at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 5'-end of the sense strand, the antisense strand, or both strands. In anther non-limiting example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 3'-end of the sense strand, the antisense strand, or both strands.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides or non-nucleotides having Formula III:



wherein each R_3, R_4, R_5, R_6, R_7, R_8, R_{10}, R_{11} and R_{12} is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF_3, OCF_3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO_2, NO_2, N_3, NH_2, aminoalkyl, aminoacid, aminoacyl, ONH_2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or group having Formula I or II; R_9 is O, S, CH_2, S=O, CHF, or CF_2, and B is a nucleosidic base such as adenine, guanine, uracil, cytosine, thymine, 2-aminoadenosine,

5-methylcytosine, 2,6-diaminopurine, or any other non-naturally occurring base that can be employed to be complementary or non-complementary to target RNA or a non-nucleosidic base such as phenyl, naphthyl, 3-nitropyrrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other non-naturally occurring universal base that can be complementary or non-complementary to target RNA.

The chemically-modified nucleotide or non-nucleotide of Formula III can be present in one or both oligonucleotide strands of the siNA duplex, for example, in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more chemically-modified nucleotide or non-nucleotide of Formula III at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotide(s) or non-nucleotide(s) of Formula III at the 5'-end of the sense strand, the antisense strand, or both strands. In anther non-limiting example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotide or non-nucleotide of Formula III at the 3'-end of the sense strand, the antisense strand, or both strands.

In another embodiment, a siNA molecule of the invention comprises a nucleotide having Formula II or III, wherein the nucleotide having Formula II or III is in an inverted configuration. For example, the nucleotide having Formula II or III is connected to the siNA construct in a 3'-3', 3'-2', 2'-3', or 5'-5' configuration, such as at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both siNA strands.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises a 5'-terminal phosphate group having Formula IV:

wherein each X and Y is independently O, S, N, alkyl, substituted alkyl, or alkylhalo; wherein each Z and W is independently O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, aralkyl, or alkylhalo or acetyl; and/or wherein W, X, Y and Z are not all O.

In one embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the target-complementary strand, for example, a strand complementary to a target RNA, wherein the siNA molecule comprises an all RNA siNA molecule. In another embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the target-complementary strand wherein the siNA molecule also comprises about 1 to about 3 (*e.g.*, about 1, 2, or 3) nucleotide 3'-terminal nucleotide overhangs having about 1 to about 4 (*e.g.*, about 1, 2, 3, or 4) deoxyribonucleotides on the 3'-end of one or both strands. In another embodiment, a 5'-terminal phosphate group having Formula IV is present on the target-complementary strand of a siNA molecule of the invention, for example a siNA molecule having chemical modifications having any of Formulae I-VII.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more phosphorothioate, phosphonoacetate, and/or thiophosphonoacetate internucleotide linkages. For example, in a non-limiting example, the invention features a chemically-modified short interfering nucleic acid (siNA) having about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in one siNA strand. In yet another embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) individually having about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in both siNA strands. The phosphorothioate internucleotide linkages can be present in one or both oligonucleotide strands of the siNA duplex, for example in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more phosphorothioate, phosphonoacetate, and/or thiophosphonoacetate internucleotide linkages at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) consecutive phosphorothioate internucleotide linkages at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting

example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) pyrimidine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands. In yet another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) purine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands.

In one embodiment, the invention features a siNA molecule, wherein the sense strand comprises one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or about one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 10 or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

In another embodiment, the invention features a siNA molecule, wherein the sense strand comprises about 1 to about 5, specifically about 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5, or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7,

8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without about 1 to about 5 or more, for example about 1, 2, 3, 4, 5, or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

In one embodiment, the invention features a siNA molecule, wherein the antisense strand comprises one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or about one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 10 or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3' and 5'-ends, being present in the same or different strand.

In another embodiment, the invention features a siNA molecule, wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and

optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without about 1 to about 5, for example about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule having about 1 to about 5, specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages in each strand of the siNA molecule.

In another embodiment, the invention features a siNA molecule comprising 2'-5' internucleotide linkages. The 2'-5' internucleotide linkage(s) can be at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of one or both siNA sequence strands. In addition, the 2'-5' internucleotide linkage(s) can be present at various other positions within one or both siNA sequence strands, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a pyrimidine nucleotide in one or both strands of the siNA molecule can comprise a 2'-5' internucleotide linkage, or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a purine nucleotide in one or both strands of the siNA molecule can comprise a 2'-5' internucleotide linkage.

In another embodiment, a chemically-modified siNA molecule of the invention comprises a duplex having two strands, one or both of which can be chemically-modified, wherein each strand is about 18 to about 27 (*e.g.*, about 18, 19, 20, 21, 22, 23, 24, 25, 26, or 27) nucleotides in length, wherein the duplex has about 18 to about 23 (e.g., about 18, 19, 20, 21, 22, or 23) base pairs, and wherein the chemical modification comprises a structure having any of Formulae I-VII. For example, an exemplary chemically-modified siNA

molecule of the invention comprises a duplex having two strands, one or both of which can be chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein each strand consists of about 21 nucleotides, each having a 2-nucleotide 3'-terminal nucleotide overhang, and wherein the duplex has about 19 base pairs. In another embodiment, a siNA molecule of the invention comprises a single stranded hairpin structure, wherein the siNA is about 36 to about 70 (*e.g.*, about 36, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 18 to about 23 (e.g., about 18, 19, 20, 21, 22, or 23) base pairs, and wherein the siNA can include a chemical modification comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 42 to about 50 (*e.g.*, about 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms a hairpin structure having about 19 base pairs and a 2-nucleotide 3'-terminal nucleotide overhang. In another embodiment, a linear hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegradable. For example, a linear hairpin siNA molecule of the invention is designed such that degradation of the loop portion of the siNA molecule *in vivo* can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

In another embodiment, a siNA molecule of the invention comprises a hairpin structure, wherein the siNA is about 25 to about 50 (*e.g.*, about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides in length having about 3 to about 25 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 25 to about 35 (*e.g.*, about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35) nucleotides that is chemically-modified with one or more chemical modifications having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms a hairpin structure having about 3 to about 23 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or 23) base pairs and

a 5'-terminal phosphate group that can be chemically modified as described herein (for example a 5'-terminal phosphate group having Formula IV). In another embodiment, a linear hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegradable. In another embodiment, a linear hairpin siNA molecule of the invention comprises a loop portion comprising a non-nucleotide linker.

In another embodiment, a siNA molecule of the invention comprises an asymmetric hairpin structure, wherein the siNA is about 25 to about 50 (*e.g.*, about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides in length having about 3 to about 20 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) base pairs, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 25 to about 35 (e.g., about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35) nucleotides that is chemically-modified with one or more chemical modifications having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms an asymmetric hairpin structure having about 3 to about 18 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18) base pairs and a 5'-terminal phosphate group that can be chemically modified as described herein (for example a 5'-terminal phosphate group having Formula IV). In another embodiment, an asymmetric hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegradable. In another embodiment, an asymmetric hairpin siNA molecule of the invention comprises a loop portion comprising a non-nucleotide linker.

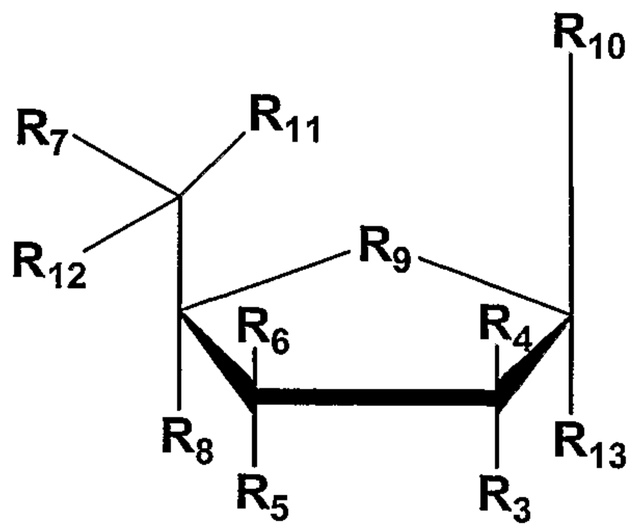
In another embodiment, a siNA molecule of the invention comprises an asymmetric double stranded structure having separate polynucleotide strands comprising sense and antisense regions, wherein the antisense region is about 16 to about 25 (*e.g.*, about 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides in length, wherein the sense region is about 3 to about 18 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18) nucleotides in length, wherein the sense region the antisense region have at least 3 complementary nucleotides, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. For

example, an exemplary chemically-modified siNA molecule of the invention comprises an asymmetric double stranded structure having separate polynucleotide strands comprising sense and antisense regions, wherein the antisense region is about 18 to about 22 (*e.g.*, about 18, 19, 20, 21, or 22) nucleotides in length and wherein the sense region is about 3 to about 15 (*e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15) nucleotides in length, wherein the sense region the antisense region have at least 3 complementary nucleotides, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. In another embodiment, the asymmetic double stranded siNA molecule can also have a 5'-terminal phosphate group that can be chemically modified as described herein (for example a 5'-terminal phosphate group having Formula IV).

In another embodiment, a siNA molecule of the invention comprises a circular nucleic acid molecule, wherein the siNA is about 38 to about 70 (*e.g.*, about 38, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 18 to about 23 (e.g., about 17, 18, 19, 20, 21, 22, 23 or 24) base pairs, and wherein the siNA can include a chemical modification, which comprises a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a circular oligonucleotide having about 42 to about 50 (*e.g.*, about 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein the circular oligonucleotide forms a dumbbell shaped structure having about 19 base pairs and 2 loops.

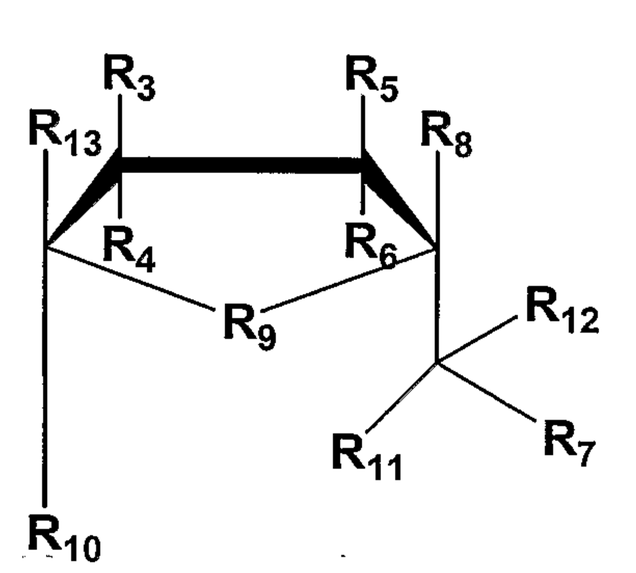
In another embodiment, a circular siNA molecule of the invention contains two loop motifs, wherein one or both loop portions of the siNA molecule is biodegradable. For example, a circular siNA molecule of the invention is designed such that degradation of the loop portions of the siNA molecule *in vivo* can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

In one embodiment, a siNA molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) abasic moiety, for example a compound having Formula V:



wherein each R_3, R_4, R_5, R_6, R_7, R_8, R_{10}, R_{11}, R_{12}, and R_{13} is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF_3, OCF_3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO_2, NO_2, N_3, NH_2, aminoalkyl, aminoacid, aminoacyl, ONH_2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or group having Formula I or II; R_9 is O, S, CH_2, S=O, CHF, or CF_2.

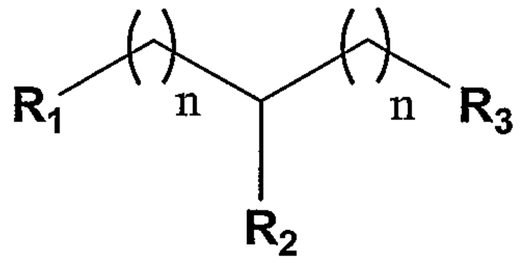
In one embodiment, a siNA molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) inverted abasic moiety, for example a compound having Formula VI:



wherein each R_3, R_4, R_5, R_6, R_7, R_8, R_{10}, R_{11}, R_{12}, and R_{13} is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF_3, OCF_3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO_2, NO_2, N_3, NH_2, aminoalkyl, aminoacid, aminoacyl, ONH_2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl,

or group having Formula I or II; R_9 is O, S, CH_2, S=O, CHF, or CF_2, and either R_3, R_5, R_8 or R_{13} serve as points of attachment to the siNA molecule of the invention.

In another embodiment, a siNA molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) substituted polyalkyl moieties, for example a compound having Formula VII:



wherein each n is independently an integer from 1 to 12, each R_1, R_2 and R_3 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF_3, OCF_3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO_2, NO_2, N_3, NH_2, aminoalkyl, aminoacid, aminoacyl, ONH_2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or a group having Formula I, and R_1, R_2 or R_3 serves as points of attachment to the siNA molecule of the invention.

In another embodiment, the invention features a compound having Formula VII, wherein R_1 and R_2 are hydroxyl (OH) groups, n = 1, and R_3 comprises O and is the point of attachment to the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both strands of a double-stranded siNA molecule of the invention or to a single-stranded siNA molecule of the invention. This modification is referred to herein as "glyceryl" (for example modification 6 in **Figure 22**).

In another embodiment, a moiety having any of Formula V, VI or VII of the invention is at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of a siNA molecule of the invention. For example, a moiety having Formula V, VI or VII can be present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense strand, the sense strand, or both antisense and sense strands of the siNA molecule. In addition, a moiety having Formula VII can be present at the 3'-end or the 5'-end of a hairpin siNA molecule as described herein.

In another embodiment, a siNA molecule of the invention comprises an abasic residue having Formula V or VI, wherein the abasic residue having Formula V or VI is connected to the siNA construct in a 3-3', 3-2', 2-3', or 5-5' configuration, such as at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both siNA strands.

In one embodiment, a siNA molecule of the invention comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) locked nucleic acid (LNA) nucleotides, for example at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

In another embodiment, a siNA molecule of the invention comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) acyclic nucleotides, for example at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

In one embodiment, the sense strand of a double stranded siNA molecule of the invention comprises a terminal cap moiety, (see for example **Figure 22**) such as an inverted deoxyabasic moiety or inverted nucleotide, at the 3'-end, 5'-end, or both 3' and 5'-ends of the sense strand.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises a sense region, where any (*e.g.,* one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where any (*e.g.,* one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides).

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises a sense region, where any (*e.g.,* one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and

where any (*e.g.,* one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides), wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises a sense region, where any (*e.g.,* one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where any (*e.g.,* one or more or all) purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides).

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises a sense region, where any (*e.g.,* one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where any (*e.g.,* one or more or all) purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises an antisense region, where any (*e.g.,* one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a

plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (*e.g.,* one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides).

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises an antisense region, where any (*e.g.,* one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (*e.g.,* one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said antisense region are 2'-deoxy nucleotides.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises an antisense region, where any (*e.g.,* one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where any (*e.g.,* one or more or all) purine nucleotides present in the antisense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides).

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system comprising a sense region and an antisense region. In one embodiment, the sense region comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine

nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides). The sense region can comprise inverted deoxy abasic modifications that are optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense region. The sense region can optionally further comprise a 3'-terminal overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxyribonucleotides. The antisense region comprisesone or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides). The antisense region can comprise a terminal cap modification, such as any modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence. The antisense region optionally further comprises a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides, wherein the overhang nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages. Non-limiting examples of these chemically-modified siNAs are shown in **Figures 18 and 19** and **Table IV** herein.

In another embodiment of the chemically-modified short interfering nucleic acid comprising a sense region and an antisense region, the sense region comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more purine ribonucleotides (e.g., wherein all purine nucleotides are purine ribonucleotides or alternately a plurality of purine nucleotides are purine ribonucleotides). The sense region can also compriseinverted deoxy abasic modifications that are optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense region. The sense region optionally further comprises a 3'-terminal overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxyribonucleotides. The antisense region comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are

2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides). The antisense region can also comprise a terminal cap modification, such as any modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence. The antisense region optionally further comprises a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides, wherein the overhang nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages. Non-limiting examples of these chemically-modified siNAs are shown in **Figures 18 and 19** and **Table IV** herein.

In another embodiment of the chemically-modified short interfering nucleic acid comprising a sense region and an antisense region, the sense region comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more purine nucleotides selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides (*e.g.*, wherein all purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides or alternately a plurality of purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides). The sense region can comprise inverted deoxy abasic modifications that are optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense region. The sense region can optionally further comprise a 3'-terminal overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxyribonucleotides. The antisense region comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more purine nucleotides selected from the group consisting of 2'-deoxy nucleotides, locked

nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides (e.g., wherein all purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides or alternately a plurality of purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides). The antisense can also comprise a terminal cap modification, such as any modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence. The antisense region optionally further comprises a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides, wherein the overhang nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages.

In another embodiment, any modified nucleotides present in the siNA molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also optionally in the sense and/or both antisense and sense strands, comprise modified nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag ed., 1984). As such, chemically modified nucleotides present in the siNA molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also optionally in the sense and/or both antisense and sense strands, are resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi. Non-limiting examples of nucleotides having a northern configuration include locked nucleic acid (LNA) nucleotides (e.g., 2′-O,4′-C-methylene-(D-ribofuranosyl) nucleotides); 2'-methoxyethoxy (MOE) nucleotides; 2'-methyl-thio-ethyl, 2'-deoxy-2'-fluoro nucleotides, 2'-deoxy-2'-chloro nucleotides, 2'-azido nucleotides, and 2'-O-methyl nucleotides.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid molecule (siNA) capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises a conjugate

attached to the chemically-modified siNA molecule. The conjugate can be attached to the chemically-modified siNA molecule via a covalent attachment. In one embodiment, the conjugate is attached to the chemically-modified siNA molecule via a biodegradable linker. In one embodiment, the conjugate molecule is attached at the 3'-end ofeither the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In another embodiment, the conjugate molecule is attached at the 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In yet another embodiment, the conjugate molecule is attached both the 3'-end and 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule, or any combination thereof. In one embodiment, the conjugate molecule of the invention comprises a molecule that facilitates delivery of a chemically-modified siNA molecule into a biological system, such as a cell. In another embodiment, the conjugate molecule attached to the chemically-modified siNA molecule is a poly ethylene glycol, human serum albumin, or a ligand for a cellular receptor that can mediate cellular uptake. Examples of specific conjugate molecules contemplated by the instant invention that can be attached to chemically-modified siNA molecules are described in Vargeese *et al.*, U.S. Serial No. 10/201,394. The type of conjugates
used and the extent of conjugation of siNA molecules of the invention can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of siNA constructs while at the same time maintaining the ability of the siNA to mediate RNAi activity. As such, one skilled in the art can screen siNA constructs that are modified with various conjugates to determine whether the siNA conjugate complex possesses improved properties while maintaining the ability to mediate RNAi, for example in animal models as are generally known in the art.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemically-modified siNA comprises a sense region, where one or more pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where one or more purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*,

wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides), and inverted deoxy abasic modifications that are optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense region, the sense region optionally further comprising a 3'-terminal overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxyribonucleotides; and wherein the chemically-modified short interfering nucleic acid molecule comprises an antisense region, where one or more pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein one or more purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the antisense region optionally further comprising a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides, wherein the overhang nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages. Non-limiting examples of these chemically-modified siNAs are shown in **Figures 18 and 19** and **Table IV** herein.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemically-modified siNA comprises a sense region, where one or more pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where one or more purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), and inverted deoxy abasic modifications that are optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense region, the sense region optionally further comprising a 3'-terminal

overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxyribonucleotides; and wherein the chemically-modified short interfering nucleic acid molecule comprises an antisense region, where one or more pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein one or more purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the antisense region optionally further comprising a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides, wherein the overhang nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages. Non-limiting examples of these chemically-modified siNAs are shown in **Figures 18 and 19** and **Table IV** herein.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the siNA comprises a sense region, where one or more pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where one or more purine nucleotides present in the sense region are purine ribonucleotides (e.g., wherein all purine nucleotides are purine ribonucleotides or alternately a plurality of purine nucleotides are purine ribonucleotides), and inverted deoxy abasic modifications that are optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense region, the sense region optionally further comprising a 3'-terminal overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxyribonucleotides; and wherein the siNA comprises an antisense region, where one or more pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine

nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the antisense region optionally further comprising a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides, wherein the overhang nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages. Non-limiting examples of these chemically-modified siNAs are shown in **Figures 18 and 19** and **Table IV** herein.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemically-modified siNA comprises a sense region, where one or more pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and for example where one or more purine nucleotides present in the sense region are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides (*e.g.*, wherein all purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides or alternately a plurality of purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides), and wherein inverted deoxy abasic modifications are optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense region, the sense region optionally further comprising a 3'-terminal overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxyribonucleotides; and wherein the chemically-modified short interfering nucleic acid molecule comprises an antisense region, where one or more pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine

nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein one or more purine nucleotides present in the antisense region are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides (e.g., wherein all purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides or alternately a plurality of purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the antisense region optionally further comprising a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides, wherein the overhang nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages.

In one embodiment, the invention features a short interfering nucleic acid (siNA) molecule of the invention, wherein the siNA further comprises a nucleotide, non-nucleotide, or mixed nucleotide/non-nucleotide linker that joins the sense region of the siNA to the antisense region of the siNA. In one embodiment, a nucleotide linker of the invention can be a linker of ≥ 2 nucleotides in length, for example 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. In another embodiment, the nucleotide linker can be a nucleic acid aptamer. By "aptamer" or "nucleic acid aptamer" as used herein is meant a nucleic acid molecule that binds specifically to a target molecule wherein the nucleic acid molecule has sequence that comprises a sequence recognized by the target molecule in its natural setting. Alternately, an aptamer can be a nucleic acid molecule that binds to a target molecule where the target molecule does not naturally bind to a nucleic acid. The target molecule can be any molecule of interest. For example, the aptamer can be used to bind to a ligand-binding domain of a protein, thereby preventing interaction of the naturally occurring ligand with the protein. This is a non-limiting example and those in the art will recognize that other embodiments can be readily generated using techniques generallyknown in the art (see, for example, Gold *et al.,* 1995, *Annu. Rev. Biochem.,* 64,

763; Brody and Gold, 2000, *J. Biotechnol.*, 74, 5; Sun, 2000, *Curr. Opin. Mol. Ther.*, 2, 100; Kusser, 2000, *J. Biotechnol.*, 74, 27; Hermann and Patel, 2000, *Science*, 287, 820; and Jayasena, 1999, *Clinical Chemistry*, 45, 1628.)

In yet another embodiment, a non-nucleotide linker of the invention comprises abasic nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, or other polymeric compounds (e.g., polyethylene glycols such as those having between 2 and 100 ethylene glycol units). Specific examples include those described by Seela and Kaiser, *Nucleic Acids Res.* 1990, *18*:6353 and *Nucleic Acids Res.* 1987, *15*:3113; Cload and Schepartz, *J. Am. Chem. Soc.* 1991, *113*:6324; Richardson and Schepartz, *J. Am. Chem. Soc.* 1991, *113*:5109; Ma *et al.*, *Nucleic Acids Res.* 1993, *21*:2585 and *Biochemistry* 1993, *32*:1751; Durand *et al.*, *Nucleic Acids Res.* 1990, *18*:6353; McCurdy *et al.*, *Nucleosides & Nucleotides* 1991, *10*:287; Jschke *et al.*, *Tetrahedron Lett.* 1993, *34*:301; Ono *et al.*, *Biochemistry* 1991, *30*:9914; Arnold *et al.*, International Publication No. WO 89/02439; Usman *et al.*, International Publication No. WO 95/06731; Dudycz *et al.*, International Publication No. WO 95/11910 and Ferentz and Verdine, *J. Am. Chem. Soc.* 1991, *113*:4000. A "non-nucleotide" further means any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound can be abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine, for example at the C1 position of the sugar.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid molecule (siNA) capable of mediating RNA interference (RNAi) against a target gene inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises a conjugate covalently attached to the chemically-modified siNA molecule. Non-limiting examples of conjugates contemplated by the invention include conjugates and ligands described in Vargeese *et al.*, USSN 10/427,160, filed April 30, 2003. In another embodiment, the conjugate is covalently attached to the chemically-modified siNA molecule via a biodegradable linker. In one embodiment, the conjugate molecule is attached at the 3'-end of either the sense strand, the antisense strand, or both strands of the

chemically-modified siNA molecule. In another embodiment, the conjugate molecule is attached at the 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In yet another embodiment, the conjugate molecule is attached both the 3'-end and 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule, or any combination thereof. In one embodiment, a conjugate molecule of the invention comprises a molecule that facilitates delivery of a chemically-modified siNA molecule into a biological system, such as a cell. In another embodiment, the conjugate molecule attached to the chemically-modified siNA molecule is a polyethylene glycol, human serum albumin, or a ligand for a cellular receptor that can mediate cellular uptake. Examples of specific conjugate molecules contemplated by the instant invention that can be attached to chemically-modified siNA molecules are described in Vargeese *et al.*, U.S. Serial No. 10/201,394.

The type of conjugates used and the extent of conjugation of siNA molecules of the invention can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of siNA constructs while at the same time maintaining the ability of the siNA to mediate RNAi activity. As such, one skilled in the art can screen siNA constructs that are modified with various conjugates to determine whether the siNA conjugate complex possesses improved properties while maintaining the ability to mediate RNAi, for example in animal models as are generally known in the art.

In one embodiment, the invention features a short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein one or both strands of the siNA molecule that are assembled from two separate oligonucleotides do not comprise any ribonucleotides. For example, a siNA molecule can be assembled from a single oligonculeotide where the sense and antisense regions of the siNA comprise separate oligonucleotides that do not have any ribonucleotides (e.g., nucleotides having a 2'-OH group) present in the oligonucleotides. In another example, a siNA molecule can be assembled from a single oligonculeotide where the sense and antisense regions of the siNA are linked or circularized by a nucleotide or non-nucleotide linker as described herein, wherein the oligonucleotide does not have any ribonucleotides (e.g., nucleotides having a 2'-OH group) present in the oligonucleotide. Applicant has surprisingly found that the presense of ribonucleotides (e.g., nucleotides having a 2'-hydroxyl group) within the siNA molecule is not required or essential to

support RNAi activity. As such, in one embodiment, all positions within the siNA can include chemically modified nucleotides and/or non-nucleotides such as nucleotides and or non-nucleotides having Formula I, II, III, IV, V, VI, or VII or any combination thereof to the extent that the ability of the siNA molecule to support RNAi activity in a cell is maintained.

In one embodiment, the invention features a siNA molecule that does not require the presence of a 2'-OH group (ribonucleotide) to be present withing the siNA molecule to support RNA interference.

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the siNA molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence. In another embodiment, the single stranded siNA molecule of the invention comprises a 5'-terminal phosphate group. In another embodiment, the single stranded siNA molecule of the invention comprises a 5'-terminal phosphate group and a 3'-terminal phosphate group (e.g., a 2',3'-cyclic phosphate). In another embodiment, the single stranded siNA molecule of the invention comprises about 19 to about 29 (e.g., about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29) nucleotides. In yet another embodiment, the single stranded siNA molecule of the invention comprises one or more chemically modified nucleotides or non-nucleotides described herein. For example, all the positions within the siNA molecule can include chemically-modified nucleotides such as nucleotides having any of Formulae I-VII, or any combination thereof to the extent that the ability of the siNA molecule to support RNAi activity in a cell is maintained.

In one embodiment, the single stranded siNA molecule having complementarity to a target nucleic acid sequence comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides). In another embodiment, the single stranded siNA molecule comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or

alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides). In another embodiment, the single stranded siNA molecule comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), wherein any purine nucleotides present in the antisense region are locked nucleic acid (LNA) nucleotides (e.g., wherein all purine nucleotides are LNA nucleotides or alternately a plurality of purine nucleotides are LNA nucleotides). In another embodiment, the single stranded siNA molecule comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more 2'-methoxyethyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-methoxyethyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-methoxyethyl purine nucleotides), the single stranded siNA can comprise a terminal cap modification, such as any modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence. The single stranded siNA optionally further comprises about 1 to about 4 (e.g., about 1, 2, 3, or 4) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages. The single stranded siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group.

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the siNA molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence, and wherein one or more pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality

of purine nucleotides are 2'-O-methyl purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence. The siNA optionally further comprises about 1 to about 4 or more (e.g., about 1, 2, 3, 4 or more) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, 4 or more) phosphorothioate, phosphonoacetate, and/or thiophosphonoacetate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group. In any of these embodiments, any purine nucleotides present in the antisense region are alternatively 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides). Also, in any of these embodiments, any purine nucleotides present in the siNA (i.e., purine nucleotides present in the sense and/or antisense region) can alternatively be locked nucleic acid (LNA) nucleotides (e.g., wherein all purine nucleotides are LNA nucleotides or alternately a plurality of purine nucleotides are LNA nucleotides). Also, in any of these embodiments, any purine nucleotides present in the siNA are alternatively 2'-methoxyethyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-methoxyethyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-methoxyethyl purine nucleotides). In another embodiment, any modified nucleotides present in the single stranded siNA molecules of the invention comprise modified nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag ed., 1984). As such, chemically modified nucleotides present in the single stranded siNA molecules of the invention are preferably resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the siNA molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence, and wherein one or more pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine

nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the siNA are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the siNA optionally further comprising about 1 to about 4 (e.g., about 1, 2, 3, or 4) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group.

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the siNA molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence, and wherein one or more pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the siNA are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the siNA optionally further comprising about 1 to about 4 (e.g., about 1, 2, 3, or 4) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group.

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the siNA molecule comprises a single stranded polynucleotide having complementarity to a

target nucleic acid sequence, and wherein one or more pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the siNA are locked nucleic acid (LNA) nucleotides (e.g., wherein all purine nucleotides are LNA nucleotides or alternately a plurality of purine nucleotides are LNA nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the siNA optionally further comprising about 1 to about 4 (e.g., about 1, 2, 3, or 4) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group.

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the siNA molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence, and wherein one or more pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the siNA are 2'-methoxyethyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-methoxyethyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-methoxyethyl purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the siNA optionally further comprising about 1 to about 4 (e.g., about 1, 2, 3, or 4) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group.

In another embodiment, any modified nucleotides present in the single stranded siNA molecules of the invention comprise modified nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag ed., 1984). As such, chemically modified nucleotides present in the single stranded siNA molecules of the invention are preferably resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi.

In one embodiment, the invention features a method for modulating the expression of a gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the gene in the cell.

In one embodiment, the invention features a method for modulating the expression of a gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the gene and wherein the sense strand sequence of the siNA comprises a sequence substantially similar to the sequence of the target RNA; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the gene in the cell.

In another embodiment, the invention features a method for modulating the expression of more than one gene within a cell comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the genes; and (b) introducing the siNA molecules into a cell under conditions suitable to modulate the expression of the genes in the cell.

In another embodiment, the invention features a method for modulating the expression of more than one gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the gene and wherein the sense strand sequence of the siNA comprises a sequence substantially similar to the sequence of

the target RNA; and (b) introducing the siNA molecules into a cell under conditions suitable to modulate the expression of the genes in the cell.

In one embodiment, siNA molecules of the invention are used as reagents in ex vivo applications. For example, siNA reagents are intoduced into tissue or cells that are transplanted into a subject for therapeutic effect. The cells and/or tissue can be derived from an organism or subject that later receives the explant, or can be derived from another organism or subject prior to transplantation. The siNA molecules can be used to modulate the expression of one or more genes in the cells or tissue, such that the cells or tissue obtain a desired phenotype or are able to perform a function when transplanted in vivo. In one embodiment, certain target cells from a patient are extracted. These extracted cells are contacted with siNAs targeteing a specific nucleotide sequence within the cells under conditions suitable for uptake of the siNAs by these cells (e.g., using delivery reagents such as cationic lipids, liposomes and the like or using techniques such as electroporation to facilitate the delivery of siNAs into cells). The cells are then reintroduced back into the same patient or other patients. Non-limiting examples of ex vivo applications include use in organ/tissue transplant, tissue grafting, or treatment of pulmonary disease (e.g., restenosis) or prevent neointimal hyperplasia and atherosclerosis in vein grafts. Such ex vivo applications may also used to treat conditions associated with coronary and peripheral bypass graft failure, for example, such methods can be used in conjunction with peripheral vascular bypass graft surgery and coronary artery bypass graft surgery. Additional applications include transplants to treat CNS lesions or injury, including use in treatment of neurodegenerative conditions such as Alzheimer's disease, Parkinson's Disease, Epilepsy, Dementia, Huntington's disease, or amyotrophic lateral sclerosis (ALS).

In one embodiment, the invention features a method of modulating the expression of a gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the gene; and (b) introducing the siNA molecule into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the gene in that organism.

In one embodiment, the invention features a method of modulating the expression of a gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the gene and wherein the sense strand sequence of the siNA comprises a sequence substantially similar to the sequence of the target RNA; and (b) introducing the siNA molecule into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the gene in that organism.

In another embodiment, the invention features a method of modulating the expression of more than one gene in a tissue explant comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the genes; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the genes in that organism.

In one embodiment, the invention features a method of modulating the expression of a gene in an organism comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the gene; and (b) introducing the siNA molecule into the organism under conditions suitable to modulate the expression of the gene in the organism.

In another embodiment, the invention features a method of modulating the expression of more than one gene in an organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the genes; and (b) introducing the siNA molecules into the organism under conditions suitable to modulate the expression of the genes in the organism.

In one embodiment, the invention features a method for modulating the expression of a gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the gene in the cell.

In one embodiment, the invention features a method of modulating the expression of a target gene in an tissue or organ comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the target gene; and (b) introducing the siNA molecule into the tissue or organ under conditions suitable to modulate the expression of the target gene in the organism. In another embodiment, the tissue is ocular tissue and the organ is the eye. In another embodiment, the tissue comprises hepatocytes and/or hepatic tissue and the organ is the liver.

In another embodiment, the invention features a method for modulating the expression of more than one gene within a cell comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the gene; and (b) contacting the siNA molecule with a cell in vitro or in vivo under conditions suitable to modulate the expression of the genes in the cell.

In one embodiment, the invention features a method of modulating the expression of a gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the gene; and (b) contacting the siNA molecule with a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the gene in that organism.

In another embodiment, the invention features a method of modulating the expression of more than one gene in a tissue explant comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA

comprises a single stranded sequence having complementarity to RNA of the gene; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the genes in that organism.

In one embodiment, the invention features a method of modulating the expression of a gene in an organism comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the gene; and (b) introducing the siNA molecule into the organism under conditions suitable to modulate the expression of the gene in the organism.

In another embodiment, the invention features a method of modulating the expression of more than one gene in an organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the gene; and (b) introducing the siNA molecules into the organism under conditions suitable to modulate the expression of the genes in the organism.

In one embodiment, the invention features a method of modulating the expression of a gene in an organism comprising contacting the organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the gene in the organism.

In another embodiment, the invention features a method of modulating the expression of more than one gene in an organism comprising contacting the organism with one or more siNA molecules of the invention under conditions suitable to modulate the expression of the genes in the organism.

The siNA molecules of the invention can be designed to down regulate or inhibit target gene expression through RNAi targeting of a variety of RNA molecules. In one embodiment, the siNA molecules of the invention are used to target various RNAs corresponding to a target gene. Non-limiting examples of such RNAs include messenger RNA (mRNA), alternate RNA splice variants of target gene(s), post-transcriptionally

modified RNA of target gene(s), pre-mRNA of target gene(s), and/or RNA templates. If alternate splicing produces a family of transcripts that are distinguished by usage of appropriate exons, the instant invention can be used to inhibit gene expression through the appropriate exons to specifically inhibit or to distinguish among the functions of gene family members. For example, a protein that contains an alternatively spliced transmembrane domain can be expressed in both membrane bound and secreted forms. Use of the invention to target the exon containing the transmembrane domain can be used to determine the functional consequences of pharmaceutical targeting of membrane bound as opposed to the secreted form of the protein. Non-limiting examples of applications of the invention relating to targeting these RNA molecules include therapeutic pharmaceutical applications, pharmaceutical discovery applications, molecular diagnostic and gene function applications, and gene mapping, for example using single nucleotide polymorphism mapping with siNA molecules of the invention. Such applications can be implemented using known gene sequences or from partial sequences available from an expressed sequence tag (EST).

In another embodiment, the siNA molecules of the invention are used to target conserved sequences corresponding to a gene family or gene families. As such, siNA molecules targeting multiple gene targets can provide increased therapeutic effect. In addition, siNA can be used to characterize pathways of gene function in a variety of applications. For example, the present invention can be used to inhibit the activity of target gene(s) in a pathway to determine the function of uncharacterized gene(s) in gene function analysis, mRNA function analysis, or translational analysis. The invention can be used to determine potential target gene pathways involved in various diseases and conditions toward pharmaceutical development. The invention can be used to understand pathways of gene expression involved in, for example, in development, such as prenatal development and postnatal development, and/or the progression and/or maintenance of cancer, infectious disease, autoimmunity, inflammation, endocrine disorders, renal disease, pulmonary disease, cardiovascular disease, birth defects, ageing, hair growth, any other disease, condition, trait, genotype or phenotype related to gene expression.

In one embodiment, siNA molecule(s) and/or methods of the invention are used to down-regulate or inhibit the expression of gene(s) that encode RNA referred to by Genbank

Accession, for example genes encoding RNA sequence(s) referred to herein by Genbank Accession number.

In one embodiment, the invention features a method comprising: (a) generating a library of siNA constructs having a predetermined complexity; and (b) assaying the siNA constructs of (a) above, under conditions suitable to determine RNAi target sites within the target RNA sequence. In one embodiment, the siNA molecules of (a) have strands of a fixed length, for example, about 23 nucleotides in length. In another embodiment, the siNA molecules of (a) are of differing length, for example having strands of about 19 to about 25 (*e.g.*, about 19, 20, 21, 22, 23, 24, or 25) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. In another embodiment, fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNAse protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by cellular expression in *in vivo* systems.

In one embodiment, the invention features a method comprising: (a) generating a randomized library of siNA constructs having a predetermined complexity, such as of 4^N, where N represents the number of base paired nucleotides in each of the siNA construct strands (*eg.* for a siNA construct having 21 nucleotide sense and antisense strands with 19 base pairs, the complexity would be 4^{19}); and (b) assaying the siNA constructs of (a) above, under conditions suitable to determine RNAi target sites within the target RNA sequence. In another embodiment, the siNA molecules of (a) have strands of a fixed length, for example about 23 nucleotides in length. In yet another embodiment, the siNA molecules of (a) are of differing length, for example having strands of about 19 to about 25 (*e.g.*, about 19, 20, 21, 22, 23, 24, or 25) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described in Example 7 herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. In another embodiment, fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNAse protection assays, to determine the most suitable target site(s) within the target RNA

sequence. In another embodiment, the target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by cellular expression in *in vivo* systems.

In another embodiment, the invention features a method comprising: (a) analyzing the sequence of a RNA target encoded by a target gene; (b) synthesizing one or more sets of siNA molecules having sequence complementary to one or more regions of the RNA of (a); and (c) assaying the siNA molecules of (b) under conditions suitable to determine RNAi targets within the target RNA sequence. In one embodiment, the siNA molecules of (b) have strands of a fixed length, for example about 23 nucleotides in length. In another embodiment, the siNA molecules of (b) are of differing length, for example having strands of about 19 to about 25 (*e.g.*, about 19, 20, 21, 22, 23, 24, or 25) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. Fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNAse protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by expression in *in vivo* systems.

By "target site" is meant a sequence within a target RNA that is "targeted" for cleavage mediated by a siNA construct which contains sequences within its antisense region that are complementary to the target sequence.

By "detectable level of cleavage" is meant cleavage of target RNA (and formation of cleaved product RNAs) to an extent sufficient to discern cleavage products above the background of RNAs produced by random degradation of the target RNA. Production of cleavage products from 1-5% of the target RNA is sufficient to detect above the background for most methods of detection.

In one embodiment, the invention features a composition comprising a siNA molecule of the invention, which can be chemically-modified, in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a pharmaceutical composition comprising siNA molecules of the invention, which can be chemically-modified, targeting one or more genes in a pharmaceutically acceptable carrier

or diluent. In another embodiment, the invention features a method for diagnosing a disease, condition, trait, genotype or phenotype in a subject comprising administering to the subject a composition of the invention under conditions suitable for the diagnosis of the disease, condition, trait, genotype or phenotype in the subject.

In one embodiment, the invention features a method for treating or preventing a disease, condition, trait, genotype or phenotype in a subject, comprising administering to the subject a composition of the invention under conditions suitable for the treatment or prevention of the disease, condition, trait, genotype or phenotype in the subject, alone or in conjunction with one or more other therapeutic compounds. In yet another embodiment, the invention features a method for reducing or preventing tissue rejection in a subject comprising administering to the subject a composition of the invention under conditions suitable for the reduction or prevention of tissue rejection in the subject.

In one embodiment, the invention features a method for alleviating the symptoms of a disease, condition, trait, genotype or phenotype in a subject, comprising administering to the subject a composition of the invention (alone or in combination (simultanesouly or sequentially) with one or more other therapeutic compounds) under conditions suitable for alleviating the symptoms of the disease, condition, trait, genotype or phenotype in the subject.

In another embodiment, the invention features a method for validating a gene target, comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of a target gene; (b) introducing the siNA molecule into a cell, tissue, or organism under conditions suitable for modulating expression of the target gene in the cell, tissue, or organism; and (c) determining the function of the gene by assaying for any phenotypic change in the cell, tissue, or organism.

In another embodiment, the invention features a method for validating a target gene comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of a target gene; (b) introducing the siNA molecule into a biological system under conditions suitable for modulating expression of the target gene in the biological system;

and (c) determining the function of the gene by assaying for any phenotypic change in the biological system.

By "biological system" is meant, material, in a purified or unpurified form, from biological sources, including but not limited to human, animal, plant, insect, bacterial, viral or other sources, wherein the system comprises the components required for RNAi acitivity. The term "biological system" includes, for example, a cell, tissue, or organism, or extract thereof. The term biological system also includes reconstituted RNAi systems that can be used in an *in vitro* setting.

By "phenotypic change" is meant any detectable change to a cell that occurs in response to contact or treatment with a nucleic acid molecule of the invention (e.g., siNA). Such detectable changes include, but are not limited to, changes in shape, size, proliferation, motility, protein expression or RNA expression or other physical or chemical changes as can be assayed by methods known in the art. The detectable change can also include expression of reporter genes/molecules such as Green Florescent Protein (GFP) or various tags that are used to identify an expressed protein or any other cellular component that can be assayed.

In one embodiment, the invention features a kit containing a siNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of a target gene in biological system, including, for example, in a cell, tissue, or organism. In another embodiment, the invention features a kit containing more than one siNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of more than one target gene in a biological system, including, for example, in a cell, tissue, or organism.

In one embodiment, the invention features a kit containing a siNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of a target gene in a biological system. In another embodiment, the invention features a kit containing more than one siNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of more than one target gene in a biological system.

In one embodiment, the invention features a cell containing one or more siNA molecules of the invention, which can be chemically-modified. In another embodiment, the cell containing a siNA molecule of the invention is a mammalian cell. In yet another embodiment, the cell containing a siNA molecule of the invention is a human cell.

In one embodiment, the synthesis of a siNA molecule of the invention, which can be chemically-modified, comprises: (a) synthesis of two complementary strands of the siNA molecule; (b) annealing the two complementary strands together under conditions suitable to obtain a double-stranded siNA molecule. In another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase oligonucleotide synthesis. In yet another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase tandem oligonucleotide synthesis.

In one embodiment, the invention features a method for synthesizing a siNA duplex molecule comprising: (a) synthesizing a first oligonucleotide sequence strand of the siNA molecule, wherein the first oligonucleotide sequence strand comprises a cleavable linker molecule that can be used as a scaffold for the synthesis of the second oligonucleotide sequence strand of the siNA; (b) synthesizing the second oligonucleotide sequence strand of siNA on the scaffold of the first oligonucleotide sequence strand, wherein the second oligonucleotide sequence strand further comprises a chemical moiety than can be used to purify the siNA duplex; (c) cleaving the linker molecule of (a) under conditions suitable for the two siNA oligonucleotide strands to hybridize and form a stable duplex; and (d) purifying the siNA duplex utilizing the chemical moiety of the second oligonucleotide sequence strand. In one embodiment, cleavage of the linker molecule in (c) above takes place during deprotection of the oligonucleotide, for example, under hydrolysis conditions using an alkylamine base such as methylamine. In one embodiment, the method of synthesis comprises solid phase synthesis on a solid support such as controlled pore glass (CPG) or polystyrene, wherein the first sequence of (a) is synthesized on a cleavable linker, such as a succinyl linker, using the solid support as a scaffold. The cleavable linker in (a) used as a scaffold for synthesizing the second strand can comprise similar reactivity as the solid support derivatized linker, such that cleavage of the solid support derivatized linker and the cleavable linker of (a) takes place concomitantly. In another embodiment, the chemical moiety of (b) that can be used to isolate the attached oligonucleotide sequence comprises a trityl group, for example a dimethoxytrityl group, which can be employed in a

trityl-on synthesis strategy as described herein. In yet another embodiment, the chemical moiety, such as a dimethoxytrityl group, is removed during purification, for example, using acidic conditions.

In a further embodiment, the method for siNA synthesis is a solution phase synthesis or hybrid phase synthesis wherein both strands of the siNA duplex are synthesized in tandem using a cleavable linker attached to the first sequence which acts a scaffold for synthesis of the second sequence. Cleavage of the linker under conditions suitable for hybridization of the separate siNA sequence strands results in formation of the double-stranded siNA molecule.

In another embodiment, the invention features a method for synthesizing a siNA duplex molecule comprising: (a) synthesizing one oligonucleotide sequence strand of the siNA molecule, wherein the sequence comprises a cleavable linker molecule that can be used as a scaffold for the synthesis of another oligonucleotide sequence; (b) synthesizing a second oligonucleotide sequence having complementarity to the first sequence strand on the scaffold of (a), wherein the second sequence comprises the other strand of the double-stranded siNA molecule and wherein the second sequence further comprises a chemical moiety than can be used to isolate the attached oligonucleotide sequence; (c) purifying the product of (b) utilizing the chemical moiety of the second oligonucleotide sequence strand under conditions suitable for isolating the full-length sequence comprising both siNA oligonucleotide strands connected by the cleavable linker and under conditions suitable for the two siNA oligonucleotide strands to hybridize and form a stable duplex. In one embodiment, cleavage of the linker molecule in (c) above takes place during deprotection of the oligonucleotide, for example under hydrolysis conditions. In another embodiment, cleavage of the linker molecule in (c) above takes place after deprotection of the oligonucleotide. In another embodiment, the method of synthesis comprises solid phase synthesis on a solid support such as controlled pore glass (CPG) or polystyrene, wherein the first sequence of (a) is synthesized on a cleavable linker, such as a succinyl linker, using the solid support as a scaffold. The cleavable linker in (a) used as a scaffold for synthesizing the second strand can comprise similar reactivity or differing reactivity as the solid support derivatized linker, such that cleavage of the solid support derivatized linker and the cleavable linker of (a) takes place either concomitantly or sequentially. In

one embodiment, the chemical moiety of (b) that can be used to isolate the attached oligonucleotide sequence comprises a trityl group, for example a dimethoxytrityl group.

In another embodiment, the invention features a method for making a double-stranded siNA molecule in a single synthetic process comprising: (a) synthesizing an oligonucleotide having a first and a second sequence, wherein the first sequence is complementary to the second sequence, and the first oligonucleotide sequence is linked to the second sequence via a cleavable linker, and wherein a terminal 5'-protecting group, for example, a 5'-O-dimethoxytrityl group (5'-O-DMT) remains on the oligonucleotide having the second sequence; (b) deprotecting the oligonucleotide whereby the deprotection results in the cleavage of the linker joining the two oligonucleotide sequences; and (c) purifying the product of (b) under conditions suitable for isolating the double-stranded siNA molecule, for example using a trityl-on synthesis strategy as described herein.

In another embodiment, the method of synthesis of siNA molecules of the invention comprises the teachings of Scaringe *et al.*, US Patent Nos. 5,889,136; 6,008,400; and 6,111,086.

In one embodiment, the invention features siNA constructs that mediate RNAi in a cell or reconstituted system, wherein the siNA construct comprises one or more chemical modifications, for example, one or more chemical modifications having any of Formulae I-VII or any combination thereof that increases the nuclease resistance of the siNA construct.

In another embodiment, the invention features a method for generating siNA molecules with increased nuclease resistance comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased nuclease resistance.

In one embodiment, the invention features siNA constructs that mediate RNAi against a target gene, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the sense and antisense strands of the siNA construct.

In one embodiment, the binding affinity between the sense and antisense strands of the siNA construct is modulated to increase the activity of the siNA molecule with regard to the ability of the siNA to mediate RNA interference. In another embodiment the binding affinity between the sense and antisense strands of the siNA construct is decreased. The binding affinity between the sense and antisense strands of the siNA construct can be decreased by introducing one or more chemically modified nucleotides in the siNA sequence that disrupts the duplex stability of the siNA (e.g., lowers the Tm of the duplex). The binding affinity between the sense and antisense strands of the siNA construct can be decreased by introducing one or more nucleotides in the siNA sequence that do not form Watson-Crick base pairs. The binding affinity between the sense and antisense strands of the siNA construct can be decreased by introducing one or more wobble base pairs in the siNA sequence. The binding affinity between the sense and antisense strands of the siNA construct can be decreased by modifying the nucleobase composition of the siNA, such as by altering the G-C content of the siNA sequence (e.g., decreasing the number of G-C base pairs in the siNA sequence). These modifications and alterations in sequence can be introduced selectively at pre-determined positions of the siNA sequence to increase siNA mediated RNAi activity. For example, such modificaitons and sequence alterations can be introduced to disrupt siNA duplex stability between the 5'-end of the antisense strand and the 3'-end of the sense strand, the 3'-end of the antisense strand and the 5'-end of the sense strand, or alternately the middle of the siNA duplex. In another embodiment, siNA molecules are screened for optimized RNAi activity by introducing such modifications and sequence alterations either by rational design based upon observed rules or trends in increasing siNA activity, or randomly via combinatorial selection processes that cover either partial or complete sequence space of the siNA construct.

In another embodiment, the invention features a method for generating siNA molecules with increased binding affinity between the sense and antisense strands of the siNA molecule comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased binding affinity between the sense and antisense strands of the siNA molecule.

In another embodiment, the invention features a method for generating siNA molecules with decreased binding affinity between the sense and antisense strands of the

siNA molecule comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having decreased binding affinity between the sense and antisense strands of the siNA molecule.

In one embodiment, the invention features siNA constructs that mediate RNAi in a cell or reconstituted system, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the antisense strand of the siNA construct and a complementary target RNA sequence within a cell.

In one embodiment, the invention features siNA constructs that mediate RNAi in a cell or reconstituted system, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the antisense strand of the siNA construct and a complementary target DNA sequence within a cell.

In another embodiment, the invention features a method for generating siNA molecules with increased binding affinity between the antisense strand of the siNA molecule and a complementary target RNA sequence comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased binding affinity between the antisense strand of the siNA molecule and a complementary target RNA sequence.

In another embodiment, the invention features a method for generating siNA molecules with increased binding affinity between the antisense strand of the siNA molecule and a complementary target DNA sequence comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased binding affinity between the antisense strand of the siNA molecule and a complementary target DNA sequence.

In another embodiment, the invention features a method for generating siNA molecules with decreased binding affinity between the antisense strand of the siNA molecule and a complementary target RNA sequence comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule,

and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having decreased binding affinity between the antisense strand of the siNA molecule and a complementary target RNA sequence.

In another embodiment, the invention features a method for generating siNA molecules with decreased binding affinity between the antisense strand of the siNA molecule and a complementary target DNA sequence comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having decreased binding affinity between the antisense strand of the siNA molecule and a complementary target DNA sequence.

In one embodiment, the invention features siNA constructs that mediate RNAi in a cell or reconstituted system, wherein the siNA construct comprises one or more chemical modifications described herein that modulate the polymerase activity of a cellular polymerase capable of generating additional endogenous siNA molecules having sequence homology to the chemically-modified siNA construct.

In another embodiment, the invention features a method for generating siNA molecules capable of mediating increased polymerase activity of a cellular polymerase capable of generating additional endogenous siNA molecules having sequence homology to a chemically-modified siNA molecule comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules capable of mediating increased polymerase activity of a cellular polymerase capable of generating additional endogenous siNA molecules having sequence homology to the chemically-modified siNA molecule. In one embodiment, the invention features chemically-modified siNA constructs that mediate RNAi in a cell or reconstituted system, wherein the chemical modifications do not significantly effect the interaction of siNA with a target RNA molecule, DNA molecule and/or proteins or other factors that are essential for RNAi in a manner that would decrease the efficacy of RNAi mediated by such siNA constructs.

In another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity comprising (a) introducing nucleotides having any

of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity.

In yet another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against a target RNA comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity against the target RNA.

In yet another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against a DNA target comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity against the DNA target, such as a gene, chromosome, or portion thereof.

In one embodiment, the invention features siNA constructs that mediate RNAi in a cell or reconstituted system, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the cellular uptake of the siNA construct.

In another embodiment, the invention features a method for generating siNA molecules against a target gene with improved cellular uptake comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved cellular uptake.

In one embodiment, the invention features siNA constructs that mediate RNAi against a target gene, wherein the siNA construct comprises one or more chemical modifications described herein that increases the bioavailability of the siNA construct, for example, by attaching polymeric conjugates such as polyethyleneglycol or equivalent conjugates that improve the pharmacokinetics of the siNA construct, or by attaching conjugates that target specific tissue types or cell types *in vivo*. Non-limiting examples of such conjugates are described in Vargeese *et al.*, U.S. Serial No. 10/201,394.

In one embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability comprising (a) introducing a conjugate into the structure of a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability. Such conjugates can include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; polyamines, such as spermine or spermidine; and others.

In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence is chemically modified in a manner that it can no longer act as a guide sequence for efficiently mediating RNA interference and/or is recognized by cellular proteins that facilitate RNAi.

In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein the second sequence is designed or modified in a manner that prevents its entry into the RNAi pathway as a guide sequence or as a sequence that is complementary to a target nucleic acid (e.g., RNA) sequence. Such design or modifications are expected to enhance the activity of siNA and/or improve the specificity of siNA molecules of the invention. These modifications are also expected to minimize any off-target effects and/or associated toxicity.

In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence is incapable of acting as a guide sequence for mediating RNA interference.

In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary to a target

RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence does not have a terminal 5'-hydroxyl (5'-OH) or 5'-phosphate group.

In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence comprises a terminal cap moiety at the 5'-end of said second sequence. In another embodiment, the terminal cap moiety comprises an inverted abasic, inverted deoxy abasic, inverted nucleotide moiety, a group shown in **Figure 22**, an alkyl or cycloalkyl group, a heterocycle, or any other group that prevents RNAi activity in which the second sequence serves as a guide sequence or template for RNAi.

In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that comprises a first nucleotide sequence complementary to a target RNA sequence or a portion thereof, and a second sequence having complementarity to said first sequence, wherein said second sequence comprises a terminal cap moiety at the 5'-end and 3'-end of said second sequence. In another embodiment, each terminal cap moiety individually comprises an inverted abasic, inverted deoxy abasic, inverted nucleotide moiety, a group shown in **Figure 22**, an alkyl or cycloalkyl group, a heterocycle, or any other group that prevents RNAi activity in which the second sequence serves as a guide sequence or template for RNAi.

In one embodiment, the invention features a method for generating siNA molecules of the invention with improved specificity for down regulating or inhibiting the expression of a target nucleic acid (e.g., a DNA or RNA such as a gene or its corresponding RNA), comprising (a) introducing one or more chemical modifications into the structure of a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved specificity. In another embodiment, the chemical modification used to improve specificity comprises terminal cap modifications at the 5'-end, 3'-end, or both 5' and 3'-ends of the siNA molecule. The terminal cap modifications can comprise, for example, structures shown in **Figure 22** (e.g., inverted deoxyabasic moieties) or any other chemical modification that renders a portion of the

siNA molecule (e.g., the sense strand) incapable of mediating RNA interference against an off target nucleic acid sequence. In a non-limiting example, a siNA molecule is designed such that only the antisense sequence of the siNA molecule can serve as a guide sequence for RISC mediated degradation of a corresponding target RNA sequence. This can be accomplished by rendering the sense sequence of the siNA inactive by introducing chemical modifications to the sense strand that preclude recognition of the sense strand as a guide sequence by RNAi machinery. In one embodiment, such chemical modifications comprise any chemical group at the 5'-end of the sense strand of the siNA, or any other group that serves to render the sense strand inactive as a guide sequence for mediating RNA interference. These modifications, for example, can result in a molecule where the 5'-end of the sense strand no longer has a free 5'-hydroxyl (5'-OH) or a free 5'-phosphate group (e.g., phosphate, diphosphate, triphosphate, cyclic phosphate etc.). Non-limiting examples of such siNA constructs are described herein, such as "Stab 9/10", "Stab 7/8", "Stab 7/19", "Stab 17/22", "Stab 23/24", and "Stab 23/25" chemistries (**Table IV**) and variants thereof wherein the 5'-end and 3'-end of the sense strand of the siNA do not comprise a hydroxyl group or phosphate group.

In one embodiment, the invention features a method for generating siNA molecules of the invention with improved specificity for down regulating or inhibiting the expression of a target nucleic acid (e.g., a DNA or RNA such as a gene or its corresponding RNA), comprising introducing one or more chemical modifications into the structure of a siNA molecule that prevent a strand or portion of the siNA molecule from acting as a template or guide sequence for RNAi acitivity. In one embodiment, the inactive strand or sense region of the siNA molecule is the sense strand or sense region of the siNA molecule, i.e. the strand or region of the siNA that does not have complementarity to the target nucleic acid sequence. In one embodiment, such chemical modifications comprise any chemical group at the 5'-end of the sense strand or region of the siNA that does not comprise a 5'-hydroxyl (5'-OH) or 5'-phosphate group, or any other group that serves to render the sense strand or sense region inactive as a guide sequence for mediating RNA interference. Non-limiting examples of such siNA constructs are described herein, such as "Stab 9/10", "Stab 7/8", "Stab 7/19", "Stab 17/22", "Stab 23/24", and "Stab 23/25" chemistries (**Table IV**) and variants thereof wherein the 5'-end and 3'-end of the sense strand of the siNA do not comprise a hydroxyl group or phosphate group.

In one embodiment, the invention features a method for screening siNA molecules against a target nucleic acid sequence comprising, (a) generating a plurality of unmodified siNA molecules, (b) assaying the siNA molecules of step (a) under conditions suitable for isolating siNA molecules that are active in mediating RNA interference against the target nucleic acid sequence, (c) introducing chemical modifications (e.g., chemical modifications as described herein or as otherwise known in the art) into the active siNA molecules of (b), and (d) optionally re-screening the chemically modified siNA molecules of (c) under conditions suitable for isolating chemically modified siNA molecules that are active in mediating RNA interference against the target nucleic acid sequence.

In one embodiment, the invention features a method for screening siNA molecules against a target nucleic acid sequence comprising, (a) generating a plurality of chemically modified siNA molecules (e.g., siNA molecules as described herein or as otherwise known in the art), and (b) assaying the siNA molecules of step (a) under conditions suitable for isolating chemically modified siNA molecules that are active in mediating RNA interference against the target nucleic acid sequence.

In another embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability comprising (a) introducing an excipient formulation to a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability. Such excipients include polymers such as cyclodextrins, lipids, cationic lipids, polyamines, phospholipids, nanoparticles, receptors, ligands, and others.

In another embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability comprising (a) introducing an excipient formulation to a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability. Such excipients include polymers such as cyclodextrins, lipids, cationic lipids, polyamines, phospholipids, and others.

In another embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability comprising (a) introducing nucleotides having any of Formulae I-VII or any combination thereof into a siNA molecule,

and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability.

In another embodiment, polyethylene glycol (PEG) can be covalently attached to siNA compounds of the present invention. The attached PEG can be any molecular weight, preferably from about 2,000 to about 50,000 daltons (Da).

The present invention can be used alone or as a component of a kit having at least one of the reagents necessary to carry out the *in vitro* or *in vivo* introduction of RNA to test samples and/or subjects. For example, preferred components of the kit include a siNA molecule of the invention and a vehicle that promotes introduction of the siNA into cells of interest as described herein (e.g., using lipids and other methods of transfection known in the art, see for example Beigelman *et al,* US 6,395,713). The kit can be used for target validation, such as in determining gene function and/or activity, or in drug optimization, and in drug discovery (see for example Usman et al., USSN 60/402,996). Such a kit can also include instructions to allow a user of the kit to practice the invention.

The term "short interfering nucleic acid", "siNA", "short interfering RNA", "siRNA", "short interfering nucleic acid molecule", "short interfering oligonucleotide molecule", or "chemically-modified short interfering nucleic acid molecule" as used herein refers to any nucleic acid molecule capable of inhibiting or down regulating gene expression or viral replication, for example by mediating RNA interference "RNAi" or gene silencing in a sequence-specific manner; see for example Zamore *et al.,* 2000, Cell, 101, 25-33; Bass, 2001, *Nature*, 411, 428-429; Elbashir *et al.,* 2001, *Nature*, 411, 494-498; and Kreutzer *et al.,* International PCT Publication No. WO 00/44895; Zernicka-Goetz *et al.,* International PCT Publication No. WO 01/36646; Fire, International PCT Publication No. WO 99/32619; Plaetinck *et al.,* International PCT Publication No. WO 00/01846; Mello and Fire, International PCT Publication No. WO 01/29058; Deschamps-Depaillette, International PCT Publication No. WO 99/07409; and Li *et al.,* International PCT Publication No. WO 00/44914; Allshire, 2002, *Science,* 297, 1818-1819; Volpe *et al.,* 2002, *Science,* 297, 1833-1837; Jenuwein, 2002, *Science,* 297, 2215-2218; and Hall *et al.,* 2002, *Science,* 297, 2232-2237; Hutvagner and Zamore, 2002, *Science*, 297, 2056-60; McManus *et al.,* 2002, *RNA*, 8, 842-850; Reinhart *et al.,* 2002, *Gene & Dev.,* 16, 1616-1626; and Reinhart & Bartel, 2002, *Science*, 297, 1831). Non limiting examples of siNA molecules of the

invention are shown in **Figures 18-20,** and **Table I** herein. For example the siNA can be a double-stranded polynucleotide molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (i.e. each strand comprises nucleotide sequence that is complementary to nucleotide sequence in the other strand; such as where the antisense strand and sense strand form a duplex or double stranded structure, for example wherein the double stranded region is about 19 base pairs); the antisense strand comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense strand comprises nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. Alternatively, the siNA is assembled from a single oligonucleotide, where the self-complementary sense and antisense regions of the siNA are linked by means of a nucleic acid based or non-nucleic acid-based linker(s). The siNA can be a polynucleotide with a duplex, asymmetric duplex, hairpin or asymmetric hairpin secondary structure, having self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a separate target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be a circular single-stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof, and wherein the circular polynucleotide can be processed either *in vivo* or *in vitro* to generate an active siNA molecule capable of mediating RNAi. The siNA can also comprise a single stranded polynucleotide having nucleotide sequence complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof (for example, where such siNA molecule does not require the presence within the siNA molecule of nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof), wherein

the single stranded polynucleotide can further comprise a terminal phosphate group, such as a 5'-phosphate (see for example Martinez *et al.,* 2002, *Cell.,* 110, 563-574 and Schwarz *et al.*, 2002, *Molecular Cell*, 10, 537-568), or 5',3'-diphosphate. In certain embodiment, the siNA molecule of the invention comprises separate sense and antisense sequences or regions, wherein the sense and antisense regions are covalently linked by nucleotide or non-nucleotide linkers molecules as is known in the art, or are alternately non-covalently linked by ionic interactions, hydrogen bonding, van der waals interactions, hydrophobic intercations, and/or stacking interactions. In certain embodiments, the siNA molecules of the invention comprise nucleotide sequence that is complementary to nucleotide sequence of a target gene. In another embodiment, the siNA molecule of the invention interacts with nucleotide sequence of a target gene in a manner that causes inhibition of expression of the target gene. As used herein, siNA molecules need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides. In certain embodiments, the short interfering nucleic acid molecules of the invention lack 2'-hydroxy (2'-OH) containing nucleotides. Applicant describes in certain embodiments short interfering nucleic acids that do not require the presence of nucleotides having a 2'-hydroxy group for mediating RNAi and as such, short interfering nucleic acid molecules of the invention optionally do not include any ribonucleotides (e.g., nucleotides having a 2'-OH group). Such siNA molecules that do not require the presence of ribonucleotides within the siNA molecule to support RNAi can however have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. Optionally, siNA molecules can comprise ribonucleotides at about 5, 10, 20, 30, 40, or 50% of the nucleotide positions. The modified short interfering nucleic acid molecules of the invention can also be referred to as short interfering modified oligonucleotides "siMON." As used herein, the term siNA is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi, for example short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering modified oligonucleotide, chemically-modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), and others. In addition, as used herein, the term RNAi is meant to be equivalent to other terms used to describe sequence specific RNA interference, such as post transcriptional gene silencing, translational inhibition, or epigenetics. For example, siNA

molecules of the invention can be used to epigenetically silence genes at both the post-transcriptional level or the pre-transcriptional level. In a non-limiting example, epigenetic regulation of gene expression by siNA molecules of the invention can result from siNA mediated modification of chromatin structure to alter gene expression (see, for example, Verdel *et al.,* 2004, *Science,* 303, 672-676; Pal-Bhadra *et al.,* 2004, *Science,* 303, 669-672; Allshire, 2002, *Science,* 297, 1818-1819; Volpe *et al.,* 2002, *Science,* 297, 1833-1837; Jenuwein, 2002, *Science,* 297, 2215-2218; and Hall *et al.,* 2002, *Science,* 297, 2232-2237).

In one embodiment, a siNA molecule of the invention is a duplex forming oligonucleotide "DFO", (see for example **Figures 93-94** and Vaish *et al.,* USSN 10/727,780 filed December 3, 2003).

In one embodiment, a siNA molecule of the invention is a multifunctional siNA, or a multi-targeted (see for example **Figures 95-101** and Jadhati *et al.,* USSN 60/543,480 filed February 10, 2004). The multifunctional siNA of the invention can comprise nucleotide sequence totargeting, for example, two regions of a target RNA or nucleotide sequences in two distinct target RNAs (see for example target sequences in **Table I**).

By "asymmetric hairpin" as used herein is meant a linear siNA molecule comprising an antisense region, a loop portion that can comprise nucleotides or non-nucleotides, and a sense region that comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complimentary nucleotides to base pair with the antisense region and form a duplex with loop. For example, an asymmetric hairpin siNA molecule of the invention can comprise an antisense region having length sufficient to mediate RNAi in a cell or in vitro system (e.g., about 19 to about 22 nucleotides) and a loop region comprising about 4 to about 8 nucleotides, and a sense region having about 3 to about 18 nucleotides that are complementary to the antisense region (see for example **Figure 74**). The asymmetric hairpin siNA molecule can also comprise a 5'-terminal phosphate group that can be chemically modified (for example as shown in **Figure 75**). The loop portion of the asymmetric hairpin siNA molecule can comprise nucleotides, non-nucleotides, linker molecules, or conjugate molecules as described herein.

By "asymmetric duplex" as used herein is meant a siNA molecule having two separate strands comprising a sense region and an antisense region, wherein the sense

region comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complimentary nucleotides to base pair with the antisense region and form a duplex. For example, an asymmetric duplex siNA molecule of the invention can comprise an antisense region having length sufficient to mediate RNAi in a cell or in vitro system (e.g., about 19 to about 22 nucleotides) and a sense region having about 3 to about 18 nucleotides that are complementary to the antisense region (see for example **Figure 74**).

By "modulate" is meant that the expression of the gene, or level of RNA molecule or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits is up regulated or down regulated, such that expression, level, or activity is greater than or less than that observed in the absence of the modulator. For example, the term "modulate" can mean "inhibit," but the use of the word "modulate" is not limited to this definition.

By "inhibit", "down-regulate", or "reduce", it is meant that the expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits, is reduced below that observed in the absence of the nucleic acid molecules (e.g., siNA) of the invention. In one embodiment, inhibition, down-regulation or reduction with an siNA molecule is below that level observed in the presence of an inactive or attenuated molecule. In another embodiment, inhibition, down-regulation, or reduction with siNA molecules is below that level observed in the presence of, for example, an siNA molecule with scrambled sequence or with mismatches. In another embodiment, inhibition, down-regulation, or reduction of gene expression with a nucleic acid molecule of the instant invention is greater in the presence of the nucleic acid molecule than in its absence.

By "palindrome" or "repeat" nucleic acid sequence is meant a nucleic acid sequence whose 5'-to-3' sequence is identical to its complementary sequence in a duplex. For example, a palindrome sequence of the invention in a duplex can comprise sequence having the same sequence when one strand of the duplex is read in the 5'-to- 3' direction (left to right) and the sequence other strand based paired to it is read in the 3'- to- 5' direction (right to left). In another example, a repeat sequence of the invention can comprise a sequence having repeated nucleotides so arranged as to provide self complementarity when the sequence self-hybridizes (e.g., 5'-AUAU...-3'; 5'-AAUU...-3';

5'-UAUA...-3'; 5'-UUAA...-3'; 5'-CGCG...-3'; 5'-CCGG...-3', 5'-GGCC...-3'; 5'-CCGG...-3'; or any expanded repeat thereof etc.). The palindrome or repeat sequence can comprise about 2 to about 24 nucleotides in even numbers, (e.g., 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24 nucleotides). All that is required of the palindrome or repeat sequence is that it comprises nucleic acid sequence whose 5'-to-3' sequence is identical when present in a duplex, either alone or as part of a longer nucleic acid sequence. The palindrome or repeat sequence of the invention can comprise chemical modificaitons as described herein that can form, for example, Watson Crick or non-Watson Crick base pairs.

By "gene", or "target gene", is meant, a nucleic acid that encodes an RNA, for example, nucleic acid sequences including, but not limited to, structural genes encoding a polypeptide. A gene or target gene can also encode a functional RNA (fRNA) or non-coding RNA (ncRNA), such as small temporal RNA (stRNA), micro RNA (miRNA), small nuclear RNA (snRNA), short interfering RNA (siRNA), small nucleolar RNA (snRNA), ribosomal RNA (rRNA), transfer RNA (tRNA) and precursor RNAs thereof. Such non-coding RNAs can serve as target nucleic acid molecules for siNA mediated RNA interference in modulating the activity of fRNA or ncRNA involved in functional or regulatory cellular processes. Abberant fRNA or ncRNA activity leading to disease can therefore be modulated by siNA molecules of the invention. siNA molecules targeting fRNA and ncRNA can also be used to manipulate or alter the genotype or phenotype of an organism or cell, by intervening in cellular processes such as genetic imprinting, transcription, translation, or nucleic acid processing (e.g., transamination, methylation etc.). The target gene can be a gene derived from a cell, an endogenous gene, a transgene, or exogenous genes such as genes of a pathogen, for example a virus, which is present in the cell after infection thereof. The cell containing the target gene can be derived from or contained in any organism, for example a plant, animal, protozoan, virus, bacterium, or fungus. Non-limiting examples of plants include monocots, dicots, or gymnosperms. Non-limiting examples of animals include vertebrates or invertebrates. Non-limiting examples of fungi include molds or yeasts.

By "non-canonical base pair" is meant any non-Watson Crick base pair, such as mismatches and/or wobble base pairs, including flipped mismatches, single hydrogen bond mismatches, trans-type mismatches, triple base interactions, and quadruple base interactions. Non-limiting examples of such non-canonical base pairs include, but are not

limited to, AC reverse Hoogsteen, AC wobble, AU reverse Hoogsteen, GU wobble, AA N7 amino, CC 2-carbonyl-amino(H1)-N3-amino(H2), GA sheared, UC 4-carbonyl-amino, UU imino-carbonyl, AC reverse wobble, AU Hoogsteen, AU reverse Watson Crick, CG reverse Watson Crick, GC N3-amino-amino N3, AA N1-amino symmetric, AA N7-amino symmetric, GA N7-N1 amino-carbonyl, GA+ carbonyl-amino N7-N1, GG N1-carbonyl symmetric, GG N3-amino symmetric, CC carbonyl-amino symmetric, CC N3-amino symmetric, UU 2-carbonyl-imino symmetric, UU 4-carbonyl-imino symmetric, AA amino-N3, AA N1-amino, AC amino 2-carbonyl, AC N3-amino, AC N7-amino, AU amino-4-carbonyl, AU N1-imino, AU N3-imino, AU N7-imino, CC carbonyl-amino, GA amino-N1, GA amino-N7, GA carbonyl-amino, GA N3-amino, GC amino-N3, GC carbonyl-amino, GC N3-amino, GC N7-amino, GG amino-N7, GG carbonyl-imino, GG N7-amino, GU amino-2-carbonyl, GU carbonyl-imino, GU imino-2-carbonyl, GU N7-imino, psiU imino-2-carbonyl, UC 4-carbonyl-amino, UC imino-carbonyl, UU imino-4-carbonyl, AC C2-H-N3, GA carbonyl-C2-H, UU imino-4-carbonyl 2 carbonyl-C5-H, AC amino(A) N3(C)-carbonyl, GC imino amino-carbonyl, Gpsi imino-2-carbonyl amino-2- carbonyl, and GU imino amino-2-carbonyl base pairs.

By "homologous sequence" is meant, a nucleotide sequence that is shared by one or more polynucleotide sequences, such as genes, gene transcripts and/or non-coding polynucleotides. For example, a homologous sequence can be a nucleotide sequence that is shared by two or more genes encoding related but different proteins, such as different members of a gene family (e.g., VEGF receptors such as VEGFr1, VEGFr2, and/or VEGFr3), different protein epitopes (e.g., different viral strains), different protein isoforms (e.g., VEGF A, B, C, and/or D) or completely divergent genes, such as a cytokine and its corresponding receptors (e.g., VEGF and VEGF receptors). A homologous sequence can be a nucleotide sequence that is shared by two or more non-coding polynucleotides, such as noncoding DNA or RNA, regulatory sequences, introns, and sites of transcriptional control or regulation. Homologous sequences can also include conserved sequence regions shared by more than one polynucleotide sequence. The homology does not need to be perfect homology (e.g., 100%), as partially homologous sequences are also contemplated by the instant invention (e.g., 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80% etc.).

By "conserved sequence region" is meant, a nucleotide sequence of one or more regions in a polynucleotide that does not vary significantly between generations or from one biological system or organism to another biological system or organism. The polynucleotide can include both coding and non-coding DNA and RNA.

By "cancer" is meant a group of diseases characterized by uncontrolled growth and spread of abnormal cells.

By "sense region" is meant a nucleotide sequence of a siNA molecule having complementarity to an antisense region of the siNA molecule. In addition, the sense region of a siNA molecule can comprise a nucleic acid sequence having homology with a target nucleic acid sequence.

By "antisense region" is meant a nucleotide sequence of a siNA molecule having complementarity to a target nucleic acid sequence. In addition, the antisense region of a siNA molecule can optionally comprise a nucleic acid sequence having complementarity to a sense region of the siNA molecule.

By "target nucleic acid" is meant any nucleic acid sequence whose expression or activity is to be modulated. The target nucleic acid can be DNA "target DNA" or RNA "target RNA", such as endogenous DNA or RNA, viral DNA or viral RNA, or other RNA encoded by a gene, virus, bacteria, fungus, mammal, or plant.

By "complementarity" is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types. In reference to the nucleic molecules of the present invention, the binding free energy for a nucleic acid molecule with its complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., RNAi activity. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner *et al.,* 1987, *CSH Symp. Quant. Biol.* LII pp.123-133; Frier *et al.,* 1986, *Proc. Nat. Acad. Sci.* USA 83:9373-9377; Turner *et al.,* 1987, *J. Am. Chem. Soc.* 109:3783-3785). A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, or 10 nucleotides out of a total of 10 nucleotides in the first oligonuelcotide being based paired to a second nucleic acid sequence having 10 nucleotides

represents 50%, 60%, 70%, 80%, 90%, and 100% complementary respectively). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence.

The siNA molecules of the invention represent a novel therapeutic approach to a broad spectrum of diseases and conditions, including cancer or cancerous disease, infectious disease, cardiovascular disease, neurologic disease, ocular disease, prion disease, inflammatory disease, autoimmune disease, pulmonary disease, renal disease, liver disease, mitochondrial disease, endocrine disease, reproduction related diseases and conditions as are known in the art, and any other indications that can respond to the level of an expressed gene product in a cell or organsim (see for example McSwiggen, International PCT Publication No. WO 03/74654).

By "proliferative disease" or "cancer" as used herein is meant, any disease, condition, trait, genotype or phenotype characterized by unregulated cell growth or replication as is known in the art; including AIDS related cancers such as Kaposi's sarcoma; breast cancers; bone cancers such as Osteosarcoma, Chondrosarcomas, Ewing's sarcoma, Fibrosarcomas, Giant cell tumors, Adamantinomas, and Chordomas; Brain cancers such as Meningiomas, Glioblastomas, Lower-Grade Astrocytomas, Oligodendrocytomas, Pituitary Tumors, Schwannomas, and Metastatic brain cancers; cancers of the head and neck including various lymphomas such as mantle cell lymphoma, non-Hodgkins lymphoma, adenoma, squamous cell carcinoma, laryngeal carcinoma, gallbladder and bile duct cancers, cancers of the retina such as retinoblastoma, cancers of the esophagus, gastric cancers, multiple myeloma, ovarian cancer, uterine cancer, thyroid cancer, testicular cancer, endometrial cancer, melanoma, colorectal cancer, lung cancer, bladder cancer, prostate cancer, lung cancer (including non-small cell lung carcinoma), pancreatic cancer, sarcomas, Wilms' tumor, cervical cancer, head and neck cancer, skin cancers, nasopharyngeal carcinoma, liposarcoma, epithelial carcinoma, renal cell carcinoma, gallbladder adeno carcinoma, parotid adenocarcinoma, endometrial sarcoma, multidrug resistant cancers; and proliferative diseases and conditions, such as neovascularization associated with tumor angiogenesis, macular degeneration (e.g., wet/dry AMD), corneal neovascularization, diabetic retinopathy, neovascular glaucoma, myopic degeneration and other proliferative diseases and conditions such as restenosis and polycystic kidney disease, and any other

cancer or proliferative disease, condition, trait, genotype or phenotype that can respond to the modulation of disease related gene expression in a cell or tissue, alone or in combination with other therapies.

By "inflammatory disease" or "inflammatory condition" as used herein is meant any disease, condition, trait, genotype or phenotype characterized by an inflammatory or allergic process as is known in the art, such as inflammation, acute inflammation, chronic inflammation, atherosclerosis, restenosis, asthma, allergic rhinitis, atopic dermatitis, septic shock, rheumatoid arthritis, inflammatory bowl disease, inflammotory pelvic disease, pain, ocular inflammatory disease, celiac disease, Leigh Syndrome, Glycerol Kinase Deficiency, Familial eosinophilia (FE), autosomal recessive spastic ataxia, laryngeal inflammatory disease; Tuberculosis, Chronic cholecystitis, Bronchiectasis, Silicosis and other pneumoconioses, and any other inflammatory disease, condition, trait, genotype or phenotype that can respond to the modulation of disease related gene expression in a cell or tissue, alone or in combination with other therapies.

By "autoimmune disease" or "autoimmune condition" as used herein is meant, any disease, condition, trait, genotype or phenotype characterized by autoimmunity as is known in the art, such as multiple sclerosis, diabetes mellitus, lupus, celiac disease, Crohn's disease, ulcerative colitis, Guillain-Barre syndrome, scleroderms, Goodpasture's syndrome, Wegener's granulomatosis, autoimmune epilepsy, Rasmussen's encephalitis, Primary biliary sclerosis, Sclerosing cholangitis, Autoimmune hepatitis, Addison's disease, Hashimoto's thyroiditis, Fibromyalgia, Menier's syndrome; transplantation rejection (e.g., prevention of allograft rejection) pernicious anemia, rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, Sjogren's syndrome, lupus erythematosus, multiple sclerosis, myasthenia gravis, Reiter's syndrome, Grave's disease, and any other autoimmune disease, condition, trait, genotype or phenotype that can respond to the modulation of disease related gene expression in a cell or tissue, alone or in combination with other therapies.

By "nuerologic disease" or "neurological disease" is meant any disease, disorder, or condition affecting the central or peripheral nervous system, inlcuding ADHD, AIDS - Neurological Complications, Absence of the Septum Pellucidum, Acquired Epileptiform Aphasia, Acute Disseminated Encephalomyelitis, Adrenoleukodystrophy, Agenesis of the

Corpus Callosum, Agnosia, Aicardi Syndrome, Alexander Disease, Alpers' Disease, Alternating Hemiplegia, Alzheimer's Disease, Amyotrophic Lateral Sclerosis, Anencephaly, Aneurysm, Angelman Syndrome, Angiomatosis, Anoxia, Aphasia, Apraxia, Arachnoid Cysts, Arachnoiditis, Arnold-Chiari Malformation, Arteriovenous Malformation, Aspartame, Asperger Syndrome, Ataxia Telangiectasia, Ataxia, Attention Deficit-Hyperactivity Disorder, Autism, Autonomic Dysfunction, Back Pain, Barth Syndrome, Batten Disease, Behcet's Disease, Bell's Palsy, Benign Essential Blepharospasm, Benign Focal Amyotrophy, Benign Intracranial Hypertension, Bernhardt-Roth Syndrome, Binswanger's Disease, Blepharospasm, Bloch-Sulzberger Syndrome, Brachial Plexus Birth Injuries, Brachial Plexus Injuries, Bradbury-Eggleston Syndrome, Brain Aneurysm, Brain Injury, Brain and Spinal Tumors, Brown-Sequard Syndrome, Bulbospinal Muscular Atrophy, Canavan Disease, Carpal Tunnel Syndrome, Causalgia, Cavernomas, Cavernous Angioma, Cavernous Malformation, Central Cervical Cord Syndrome, Central Cord Syndrome, Central Pain Syndrome, Cephalic Disorders, Cerebellar Degeneration, Cerebellar Hypoplasia, Cerebral Aneurysm, Cerebral Arteriosclerosis, Cerebral Atrophy, Cerebral Beriberi, Cerebral Gigantism, Cerebral Hypoxia, Cerebral Palsy, Cerebro-Oculo-Facio-Skeletal Syndrome, Charcot-Marie-Tooth Disorder, Chiari Malformation, Chorea, Choreoacanthocytosis, Chronic Inflammatory Demyelinating Polyneuropathy (CIDP), Chronic Orthostatic Intolerance, Chronic Pain, Cockayne Syndrome Type II, Coffin Lowry Syndrome, Coma, including Persistent Vegetative State, Complex Regional Pain Syndrome, Congenital Facial Diplegia, Congenital Myasthenia, Congenital Myopathy, Congenital Vascular Cavernous Malformations, Corticobasal Degeneration, Cranial Arteritis, Craniosynostosis, Creutzfeldt-Jakob Disease, Cumulative Trauma Disorders, Cushing's Syndrome, Cytomegalic Inclusion Body Disease (CIBD), Cytomegalovirus Infection, Dancing Eyes-Dancing Feet Syndrome, Dandy-Walker Syndrome, Dawson Disease, De Morsier's Syndrome, Dejerine-Klumpke Palsy, Dementia - Multi-Infarct, Dementia - Subcortical, Dementia With Lewy Bodies, Dermatomyositis, Developmental Dyspraxia, Devic's Syndrome, Diabetic Neuropathy, Diffuse Sclerosis, Dravet's Syndrome, Dysautonomia, Dysgraphia, Dyslexia, Dysphagia, Dyspraxia, Dystonias, Early Infantile Epileptic Encephalopathy, Empty Sella Syndrome, Encephalitis Lethargica, Encephalitis and Meningitis, Encephaloceles, Encephalopathy, Encephalotrigeminal Angiomatosis, Epilepsy, Erb's Palsy, Erb-Duchenne and Dejerine-Klumpke Palsies, Fabry's Disease,

Fahr's Syndrome, Fainting, Familial Dysautonomia, Familial Hemangioma, Familial Idiopathic Basal Ganglia Calcification, Familial Spastic Paralysis, Febrile Seizures (e.g., GEFS and GEFS plus), Fisher Syndrome, Floppy Infant Syndrome, Friedreich's Ataxia, Gaucher's Disease, Gerstmann's Syndrome, Gerstmann-Straussler-Scheinker Disease, Giant Cell Arteritis, Giant Cell Inclusion Disease, Globoid Cell Leukodystrophy, Glossopharyngeal Neuralgia, Guillain-Barre Syndrome, HTLV-1 Associated Myelopathy, Hallervorden-Spatz Disease, Head Injury, Headache, Hemicrania Continua, Hemifacial Spasm, Hemiplegia Alterans, Hereditary Neuropathies, Hereditary Spastic Paraplegia, Heredopathia Atactica Polyneuritiformis, Herpes Zoster Oticus, Herpes Zoster, Hirayama Syndrome, Holoprosencephaly, Huntington's Disease, Hydranencephaly, Hydrocephalus - Normal Pressure, Hydrocephalus, Hydromyelia, Hypercortisolism, Hypersomnia, Hypertonia, Hypotonia, Hypoxia, Immune-Mediated Encephalomyelitis, Inclusion Body Myositis, Incontinentia Pigmenti, Infantile Hypotonia, Infantile Phytanic Acid Storage Disease, Infantile Refsum Disease, Infantile Spasms, Inflammatory Myopathy, Intestinal Lipodystrophy, Intracranial Cysts, Intracranial Hypertension, Isaac's Syndrome, Joubert Syndrome, Kearns-Sayre Syndrome, Kennedy's Disease, Kinsbourne syndrome, Kleine-Levin syndrome, Klippel Feil Syndrome, Klippel-Trenaunay Syndrome (KTS), Klüver-Bucy Syndrome, Korsakoff's Amnesic Syndrome, Krabbe Disease, Kugelberg-Welander Disease, Kuru, Lambert-Eaton Myasthenic Syndrome, Landau-Kleffner Syndrome, Lateral Femoral Cutaneous Nerve Entrapment, Lateral Medullary Syndrome, Learning Disabilities, Leigh's Disease, Lennox-Gastaut Syndrome, Lesch-Nyhan Syndrome, Leukodystrophy, Levine-Critchley Syndrome, Lewy Body Dementia, Lissencephaly, Locked-In Syndrome, Lou Gehrig's Disease, Lupus - Neurological Sequelae, Lyme Disease - Neurological Complications, Machado-Joseph Disease, Macrencephaly, Megalencephaly, Melkersson-Rosenthal Syndrome, Meningitis, Menkes Disease, Meralgia Paresthetica, Metachromatic Leukodystrophy, Microcephaly, Migraine, Miller Fisher Syndrome, Mini-Strokes, Mitochondrial Myopathies, Mobius Syndrome, Monomelic Amyotrophy, Motor Neuron Diseases, Moyamoya Disease, Mucolipidoses, Mucopolysaccharidoses, Multi-Infarct Dementia, Multifocal Motor Neuropathy, Multiple Sclerosis, Multiple System Atrophy with Orthostatic Hypotension, Multiple System Atrophy, Muscular Dystrophy, Myasthenia - Congenital, Myasthenia Gravis, Myelinoclastic Diffuse Sclerosis, Myoclonic Encephalopathy of Infants, Myoclonus, Myopathy - Congenital, Myopathy - Thyrotoxic, Myopathy, Myotonia

Congenita, Myotonia, Narcolepsy, Neuroacanthocytosis, Neurodegeneration with Brain Iron Accumulation, Neurofibromatosis, Neuroleptic Malignant Syndrome, Neurological Complications of AIDS, Neurological Manifestations of Pompe Disease, Neuromyelitis Optica, Neuromyotonia, Neuronal Ceroid Lipofuscinosis, Neuronal Migration Disorders, Neuropathy - Hereditary, Neurosarcoidosis, Neurotoxicity, Nevus Cavernosus, Niemann-Pick Disease, O'Sullivan-McLeod Syndrome, Occipital Neuralgia, Occult Spinal Dysraphism Sequence, Ohtahara Syndrome, Olivopontocerebellar Atrophy, Opsoclonus Myoclonus, Orthostatic Hypotension, Overuse Syndrome, Pain - Chronic, Paraneoplastic Syndromes, Paresthesia, Parkinson's Disease, Parmyotonia Congenita, Paroxysmal Choreoathetosis, Paroxysmal Hemicrania, Parry-Romberg, Pelizaeus-Merzbacher Disease, Pena Shokeir II Syndrome, Perineural Cysts, Periodic Paralyses, Peripheral Neuropathy, Periventricular Leukomalacia, Persistent Vegetative State, Pervasive Developmental Disorders, Phytanic Acid Storage Disease, Pick's Disease, Piriformis Syndrome, Pituitary Tumors, Polymyositis, Pompe Disease, Porencephaly, Post-Polio Syndrome, Postherpetic Neuralgia, Postinfectious Encephalomyelitis, Postural Hypotension, Postural Orthostatic Tachycardia Syndrome, Postural Tachycardia Syndrome, Primary Lateral Sclerosis, Prion Diseases, Progressive Hemifacial Atrophy, Progressive Locomotor Ataxia, Progressive Multifocal Leukoencephalopathy, Progressive Sclerosing Poliodystrophy, Progressive Supranuclear Palsy, Pseudotumor Cerebri, Pyridoxine Dependent and Pyridoxine Responsive Siezure Disorders, Ramsay Hunt Syndrome Type I, Ramsay Hunt Syndrome Type II, Rasmussen's Encephalitis and other autoimmune epilepsies, Reflex Sympathetic Dystrophy Syndrome, Refsum Disease - Infantile, Refsum Disease, Repetitive Motion Disorders, Repetitive Stress Injuries, Restless Legs Syndrome, Retrovirus-Associated Myelopathy, Rett Syndrome, Reye's Syndrome, Riley-Day Syndrome, SUNCT Headache, Sacral Nerve Root Cysts, Saint Vitus Dance, Salivary Gland Disease, Sandhoff Disease, Schilder's Disease, Schizencephaly, Seizure Disorders, Septo-Optic Dysplasia, Severe Myoclonic Epilepsy of Infancy (SMEI), Shaken Baby Syndrome, Shingles, Shy-Drager Syndrome, Sjogren's Syndrome, Sleep Apnea, Sleeping Sickness, Soto's Syndrome, Spasticity, Spina Bifida, Spinal Cord Infarction, Spinal Cord Injury, Spinal Cord Tumors, Spinal Muscular Atrophy, Spinocerebellar Atrophy, Steele-Richardson-Olszewski Syndrome, Stiff-Person Syndrome, Striatonigral Degeneration, Stroke, Sturge-Weber Syndrome, Subacute Sclerosing Panencephalitis, Subcortical Arteriosclerotic Encephalopathy, Swallowing Disorders, Sydenham Chorea, Syncope, Syphilitic Spinal

Sclerosis, Syringohydromyelia, Syringomyelia, Systemic Lupus Erythematosus, Tabes Dorsalis, Tardive Dyskinesia, Tarlov Cysts, Tay-Sachs Disease, Temporal Arteritis, Tethered Spinal Cord Syndrome, Thomsen Disease, Thoracic Outlet Syndrome, Thyrotoxic Myopathy, Tic Douloureux, Todd's Paralysis, Tourette Syndrome, Transient Ischemic Attack, Transmissible Spongiform Encephalopathies, Transverse Myelitis, Traumatic Brain Injury, Tremor, Trigeminal Neuralgia, Tropical Spastic Paraparesis, Tuberous Sclerosis, Vascular Erectile Tumor, Vasculitis including Temporal Arteritis, Von Economo's Disease, Von Hippel-Lindau disease (VHL), Von Recklinghausen's Disease, Wallenberg's Syndrome, Werdnig-Hoffman Disease, Wernicke-Korsakoff Syndrome, West Syndrome, Whipple's Disease, Williams Syndrome, Wilson's Disease, X-Linked Spinal and Bulbar Muscular Atrophy, and Zellweger Syndrome.

By "infectious disease" as used herein is meant any disease, condition, trait, genotype or phenotype associated with an infectious agent, such as a virus, bacteria, fungus, prion, or parasite. Non-limiting examples of various viral genes that can be targeted using siNA molecules of the invention include Hepatitis C Virus (HCV, for example Genbank Accession Nos: D11168, D50483.1, L38318 and S82227), Hepatitis B Virus (HBV, for example GenBank Accession No. AF100308.1), Human Immunodeficiency Virus type 1 (HIV-1, for example GenBank Accession No. U51188), Human Immunodeficiency Virus type 2 (HIV-2, for example GenBank Accession No. X60667), West Nile Virus (WNV for example GenBank accession No. NC_001563), cytomegalovirus (CMV for example GenBank Accession No. NC_001347), respiratory syncytial virus (RSV for example GenBank Accession No. NC_001781), influenza virus (for example example GenBank Accession No. AF037412, rhinovirus (for example, GenBank accession numbers: D00239, X02316, X01087, L24917, M16248, K02121, X01087), papillomavirus (for example GenBank Accession No. NC_001353), Herpes Simplex Virus (HSV for example GenBank Accession No. NC_001345), and other viruses such as HTLV (for example GenBank Accession No. AJ430458). Due to the high sequence variability of many viral genomes, selection of siRNA molecules for broad therapeutic applications would likely involve the conserved regions of the viral genome. Nonlimiting examples of conserved regions of the viral genomes include but are not limited to 5'-Non Coding Regions (NCR), 3'- Non Coding Regions (NCR) and/or internal ribosome entry sites (IRES). siRNA molecules designed against conserved regions of various viral genomes will enable efficient

inhibition of viral replication in diverse patient populations and may ensure the effectiveness of the siRNA molecules against viral quasi species which evolve due to mutations in the non-conserved regions of the viral genome. Non-limiting examples of bacterial infections include Actinomycosis, Anthrax, Aspergillosis, Bacteremia, Bacterial Infections and Mycoses, Bartonella Infections, Botulism, Brucellosis, Burkholderia Infections, Campylobacter Infections, Candidiasis, Cat-Scratch Disease, Chlamydia Infections, Cholera , Clostridium Infections, Coccidioidomycosis, Cross Infection, Cryptococcosis, Dermatomycoses, Dermatomycoses, Diphtheria, Ehrlichiosis, Escherichia coli Infections, Fasciitis, Necrotizing, Fusobacterium Infections, Gas Gangrene, Gram-Negative Bacterial Infections, Gram-Positive Bacterial Infections, Histoplasmosis, Impetigo, Klebsiella Infections, Legionellosis, Leprosy, Leptospirosis, Listeria Infections, Lyme Disease, Maduromycosis, Melioidosis, Mycobacterium Infections, Mycoplasma Infections, Mycoses, Nocardia Infections, Onychomycosis, Ornithosis, Plague, Pneumococcal Infections, Pseudomonas Infections, Q Fever, Rat-Bite Fever, Relapsing Fever, Rheumatic Fever, Rickettsia Infections, Rocky Mountain Spotted Fever, Salmonella Infections, Scarlet Fever, Scrub Typhus, Sepsis, Sexually Transmitted Diseases - Bacterial, Bacterial Skin Diseases, Staphylococcal Infections, Streptococcal Infections, Tetanus, Tick-Borne Diseases, Tuberculosis, Tularemia, Typhoid Fever, Typhus, Epidemic Louse-Borne, Vibrio Infections, Yaws, Yersinia Infections, Zoonoses, and Zygomycosis. Non-limiting examples of fungal infections include Aspergillosis, Blastomycosis, Coccidioidomycosis, Cryptococcosis, Fungal Infections of Fingernails and Toenails, Fungal Sinusitis, Histoplasmosis, Histoplasmosis, Mucormycosis, Nail Fungal Infection, Paracoccidioidomycosis, Sporotrichosis, Valley Fever (Coccidioidomycosis), and Mold Allergy.

By "ocular disease" as used herein is meant, any disease, condition, trait, genotype or phenotype of the eye and related structures, such as Cystoid Macular Edema, Asteroid Hyalosis, Pathological Myopia and Posterior Staphyloma, Toxocariasis (Ocular Larva Migrans), Retinal Vein Occlusion, Posterior Vitreous Detachment, Tractional Retinal Tears, Epiretinal Membrane, Diabetic Retinopathy, Lattice Degeneration, Retinal Vein Occlusion, Retinal Artery Occlusion, Macular Degeneration (e.g., age related macular degeneration such as wet AMD or dry AMD), Toxoplasmosis, Choroidal Melanoma, Acquired Retinoschisis, Hollenhorst Plaque, Idiopathic Central Serous Chorioretinopathy,

Macular Hole, Presumed Ocular Histoplasmosis Syndrome, Retinal Macroaneursym, Retinitis Pigmentosa, Retinal Detachment, Hypertensive Retinopathy, Retinal Pigment Epithelium (RPE) Detachment, Papillophlebitis, Ocular Ischemic Syndrome, Coats' Disease, Leber's Miliary Aneurysm, Conjunctival Neoplasms, Allergic Conjunctivitis, Vernal Conjunctivitis, Acute Bacterial Conjunctivitis, Allergic Conjunctivitis &Vernal Keratoconjunctivitis, Viral Conjunctivitis, Bacterial Conjunctivitis, Chlamydial & Gonococcal Conjunctivitis, Conjunctival Laceration, Episcleritis, Scleritis, Pingueculitis, Pterygium, Superior Limbic Keratoconjunctivitis (SLK of Theodore), Toxic Conjunctivitis, Conjunctivitis with Pseudomembrane, Giant Papillary Conjunctivitis, Terrien's Marginal Degeneration, Acanthamoeba Keratitis, Fungal Keratitis, Filamentary Keratitis, Bacterial Keratitis, Keratitis Sicca/Dry Eye Syndrome, Bacterial Keratitis, Herpes Simplex Keratitis, Sterile Corneal Infiltrates, Phlyctenulosis, Corneal Abrasion & Recurrent Corneal Erosion, Corneal Foreign Body, Chemical Burs, Epithelial Basement Membrane Dystrophy (EBMD), Thygeson's Superficial Punctate Keratopathy, Corneal Laceration, Salzmann's Nodular Degeneration, Fuchs' Endothelial Dystrophy, Crystalline Lens Subluxation, Ciliary-Block Glaucoma, Primary Open-Angle Glaucoma, Pigment Dispersion Syndrome and Pigmentary Glaucoma, Pseudoexfoliation Syndrom and Pseudoexfoliative Glaucoma, Anterior Uveitis, Primary Open Angle Glaucoma, Uveitic Glaucoma & Glaucomatocyclitic Crisis, Pigment Dispersion Syndrome & Pigmentary Glaucoma, Acute Angle Closure Glaucoma, Anterior Uveitis, Hyphema, Angle Recession Glaucoma, Lens Induced Glaucoma, Pseudoexfoliation Syndrome and Pseudoexfoliative Glaucoma, Axenfeld-Rieger Syndrome, Neovascular Glaucoma, Pars Planitis, Choroidal Rupture, Duane's Retraction Syndrome, Toxic/Nutritional Optic Neuropathy, Aberrant Regeneration of Cranial Nerve III, Intracranial Mass Lesions, Carotid-Cavernous Sinus Fistula, Anterior Ischemic Optic Neuropathy, Optic Disc Edema & Papilledema, Cranial Nerve III Palsy, Cranial Nerve IV Palsy, Cranial Nerve VI Palsy, Cranial Nerve VII (Facial Nerve) Palsy, Horner's Syndrome, Internuclear Ophthalmoplegia, Optic Nerve Head Hypoplasia, Optic Pit, Tonic Pupil, Optic Nerve Head Drusen, Demyelinating Optic Neuropathy (Optic Neuritis, Retrobulbar Optic Neuritis), Amaurosis Fugax and Transient Ischemic Attack, Pseudotumor Cerebri, Pituitary Adenoma, Molluscum Contagiosum, Canaliculitis, Verruca and Papilloma, Pediculosis and Pthiriasis, Blepharitis, Hordeolum, Preseptal Cellulitis, Chalazion, Basal Cell Carcinoma, Herpes Zoster Ophthalmicus,

Pediculosis & Phthiriasis, Blow-out Fracture, Chronic Epiphora, Dacryocystitis, Herpes Simplex Blepharitis, Orbital Cellulitis, Senile Entropion, and Squamous Cell Carcinoma.

In one embodiment of the present invention, each sequence of a siNA molecule of the invention is independently about 18 to about 24 nucleotides in length, in specific embodiments about 18, 19, 20, 21, 22, 23, or 24 nucleotides in length. In another embodiment, the siNA duplexes of the invention independently comprise about 17 to about 23 base pairs (*e.g.*, about 17, 18, 19, 20, 21, 22 or 23). In yet another embodiment, siNA molecules of the invention comprising hairpin or circular structures are about 35 to about 55 (*e.g.*, about 35, 40, 45, 50 or 55) nucleotides in length, or about 38 to about 44 (*e.g.*, 38, 39, 40, 41, 42, 43 or 44) nucleotides in length and comprising about 16 to about 22 (*e.g.*, about 16, 17, 18, 19, 20, 21 or 22) base pairs. Exemplary siNA molecules of the invention are shown in **Table I**. and/or **Figures 18-19**.

As used herein "cell" is used in its usual biological sense, and does not refer to an entire multicellular organism, e.g., specifically does not refer to a human. The cell can be present in an organism, e.g., birds, plants and mammals such as humans, cows, sheep, apes, monkeys, swine, dogs, and cats. The cell can be prokaryotic (e.g., bacterial cell) or eukaryotic (e.g., mammalian or plant cell). The cell can be of somatic or germ line origin, totipotent or pluripotent, dividing or non-dividing. The cell can also be derived from or can comprise a gamete or embryo, a stem cell, or a fully differentiated cell.

The siNA molecules of the invention are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through injection, infusion pump or stent, with or without their incorporation in biopolymers. In particular embodiments, the nucleic acid molecules of the invention comprise sequences shown in **Table I** and/or **Figures 18-19**. Examples of such nucleic acid molecules consist essentially of sequences defined in these tables and figures. Furthermore, the chemically modified constructs described in **Table IV** can be applied to any siNA sequence of the invention.

In another aspect, the invention provides mammalian cells containing one or more siNA molecules of this invention. The one or more siNA molecules can independently be targeted to the same or different sites.

By "RNA" is meant a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" is meant a nucleotide with a hydroxyl group at the 2' position of a β-D-ribo-furanose moiety. The terms include double-stranded RNA, single-stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in the RNA molecules of the instant invention can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

By "subject" is meant an organism, which is a donor or recipient of explanted cells or the cells themselves. "Subject" also refers to an organism to which the nucleic acid molecules of the invention can be administered. A subject can be a mammal or mammalian cells, including a human or human cells.

The term "ligand" refers to any compound or molecule, such as a drug, peptide, hormone, or neurotransmitter, that is capable of interacting with another compound, such as a receptor, either directly or indirectly. The receptor that interacts with a ligand can be present on the surface of a cell or can alternately be an intercullular receptor. Interaction of the ligand with the receptor can result in a biochemical reaction, or can simply be a physical interaction or association.

The term "phosphorothioate" as used herein refers to an internucleotide linkage having Formula I, wherein Z and/or W comprise a sulfur atom. Hence, the term phosphorothioate refers to both phosphorothioate and phosphorodithioate internucleotide linkages.

The term "phosphonoacetate" as used herein refers to an internucleotide linkage having Formula I, wherein Z and/or W comprise an acetyl or protected acetyl group.

The term "thiophosphonoacetate" as used herein refers to an internucleotide linkage having Formula I, wherein Z comprises an acetyl or protected acetyl group and W

comprises a sulfur atom or alternately W comprises an acetyl or protected acetyl group and Z comprises a sulfur atom.

The term "universal base" as used herein refers to nucleotide base analogs that form base pairs with each of the natural DNA/RNA bases with little discrimination between them. Non-limiting examples of universal bases include C-phenyl, C-naphthyl and other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole as known in the art (see for example Loakes, 2001, *Nucleic Acids Research,* 29, 2437-2447).

The term "acyclic nucleotide" as used herein refers to any nucleotide having an acyclic ribose sugar, for example where any of the ribose carbons (C1, C2, C3, C4, or C5), are independently or in combination absent from the nucleotide.

The nucleic acid molecules of the instant invention, individually, or in combination or in conjunction with other drugs, can be used to treat diseases or conditions discussed herein (e.g., cancers and othe proliferative conditions, viral infection, inflammatory disease, autoimmunity, pulmonary disease, renal disease, ocular disease, etc.). For example, to treat a particular disease, condition, trait, genotype or phenotype, the siNA molecules can be administered to a subject or can be administered to other appropriate cells evident to those skilled in the art, individually or in combination with one or more drugs under conditions suitable for the treatment.

In one embodiment, the invention features a method for treating or preventing a disease, condition, trait, genotype or phenotype in a subject, wherein the disease, condition, trait, genotype or phenotype is related to angiogenesis or neovascularization, comprising administering to the subject a siNA molecule of the invention under conditions suitable for the treatment or prevention of the disease, condition, trait, genotype or phenotype in the subject, alone or in conjunction with one or more other therapeutic compounds. In another embodiment, the disease, condition, trait, genotype or phenotype comprises tumor angiogenesis and cancerous conditions herein, including but not limited to breast cancer, lung cancer (including non-small cell lung carcinoma), prostate cancer, colorectal cancer, brain cancer, esophageal cancer, bladder cancer, pancreatic cancer, cervical cancer, head and neck cancer, skin cancers, nasopharyngeal carcinoma, liposarcoma, epithelial carcinoma, renal cell carcinoma, gallbladder adeno carcinoma, parotid adenocarcinoma,

ovarian cancer, melanoma, lymphoma, glioma, endometrial sarcoma, multidrug resistant cancers, diabetic retinopathy, macular degeneration, age related macular degeneration, neovascular glaucoma, myopic degeneration, arthritis, psoriasis, endometriosis, female reproduction, verruca vulgaris, angiofibroma of tuberous sclerosis, pot-wine stains, Sturge Weber syndrome, Kippel-Trenaunay-Weber syndrome, Osler-Weber-Rendu syndrome, renal disease such as Autosomal dominant polycystic kidney disease (ADPKD), restenosis, arteriosclerosis, and any other diseases or conditions that are related to gene expression or will respond to RNA interference in a cell or tissue, alone or in combination with other therapies.

In one embodiment, the invention features a method for treating or preventing an ocular disease, condition, trait, genotype or phenotype in a subject, wherein the ocular disease, condition, trait, genotype or phenotype is related to angiogenesis or neovascularization, comprising administering to the subject a siNA molecule of the invention under conditions suitable for the treatment or prevention of the disease, condition, trait, genotype or phenotype in the subject, alone or in conjunction with one or more other therapeutic compounds. In another embodiment, the ocular disease, condition, trait, genotype or phenotype comprises macular degeneration, age related macular degeneration, diabetic retinopathy, neovascular glaucoma, myopic degeneration, trachoma, scarring of the eye, cataract, ocular inflammation and/or ocular infections.

In one embodiment, the invention features a method for treating or preventing tumor angiogenesis in a subject, comprising administering to the subject a siNA molecule of the invention under conditions suitable for the treatment or prevention of tumor angiogenesis in the subject, alone or in conjunction with one or more other therapeutic compounds.

In one embodiment, the invention features a method for treating or preventing viral infection or replication in a subject, comprising administering to the subject a siNA molecule of the invention under conditions suitable for the treatment or prevention of viral infection or replication in the subject, alone or in conjunction with one or more other therapeutic compounds.

In one embodiment, the invention features a method for treating or preventing autoimmune disease in a subject, comprising administering to the subject a siNA molecule of the invention under conditions suitable for the treatment or prevention of autoimmune

disease in the subject, alone or in conjunction with one or more other therapeutic compounds.

In one embodiment, the invention features a method for treating or preventing inflammation in a subject, comprising administering to the subject a siNA molecule of the invention under conditions suitable for the treatment or prevention of inflammation in the subject, alone or in conjunction with one or more other therapeutic compounds.

In a further embodiment, the siNA molecules can be used in combination with other known treatments to treat conditions or diseases discussed above. For example, the described molecules could be used in combination with one or more known therapeutic agents to treat a disease, condition, trait, genotype or phenotype. Non-limiting examples of other therapeutic agents that can be readily combined with a siNA molecule of the invention are enzymatic nucleic acid molecules, allosteric nucleic acid molecules, antisense, decoy, or aptamer nucleic acid molecules, antibodies such as monoclonal antibodies, small molecules, and other organic and/or inorganic compounds including metals, salts and ions.

In one embodiment, the invention features a method for treating or preventing a disease or condition in a subject, wherein the disease or condition is related to angiogenesis or neovascularization, comprising administering to the subject a siNA molecule of the invention under conditions suitable for the treatment or prevention of the disease or condition in the subject, alone or in conjunction with one or more other therapeutic compounds. In another embodiment, the disease or condition resulting from angiogenesis, such as tumor angiogenesis leading to cancer, such as without limitation to breast cancer, lung cancer (including non-small cell lung carcinoma), prostate cancer, colorectal cancer, brain cancer, esophageal cancer, bladder cancer, pancreatic cancer, cervical cancer, head and neck cancer, skin cancers, nasopharyngeal carcinoma, liposarcoma, epithelial carcinoma, renal cell carcinoma, gallbladder adeno carcinoma, parotid adenocarcinoma, ovarian cancer, melanoma, lymphoma, glioma, endometrial sarcoma, and multidrug resistant cancers, diabetic retinopathy, macular degeneration, age related macular degeneration, macular adema, neovascular glaucoma, myopic degeneration, arthritis, psoriasis, endometriosis, female reproduction, verruca vulgaris, angiofibroma of tuberous sclerosis, pot-wine stains, Sturge Weber syndrome, Kippel-Trenaunay-Weber syndrome,

Osler-Weber-Rendu syndrome, renal disease such as Autosomal dominant polycystic kidney disease (ADPKD), restenosis, arteriosclerosis, and any other diseases or conditions that are related to gene expression or will respond to RNA interference in a cell or tissue, alone or in combination with other therapies.

In one embodiment, the invention features a method for treating or preventing an ocular disease or condition in a subject, wherein the ocular disease or condition is related to angiogenesis or neovascularization (such as those involving genes in the vascular endothelial growth factor, VEGF pathway or TGF-beta pathway), comprising administering to the subject a siNA molecule of the invention under conditions suitable for the treatment or prevention of the disease or condition in the subject, alone or in conjunction with one or more other therapeutic compounds. In another embodiment, the ocular disease or condition comprises macular degeneration, age related macular degeneration, diabetic retinopathy, macular adema, neovascular glaucoma, myopic degeneration, trachoma, scarring of the eye, cataract, ocular inflammation and/or ocular infections.

In one embodiment, the invention features a method of locally administering (e.g., by injection, such as intraocular, intratumoral, periocular, intracranial, etc., topical administration, catheter or the like) to a tissue or cell (e.g., ocular or retinal, brain, CNS) a siNA molecule or a vector expressing siNA molecule, comprising nucleotide sequence that is complementary to nucleotide sequence of target RNA, or a portion thereof, (e.g., target RNA encoding VEGF or a VEGF receptor) comprising contacting said tissue of cell with said double stranded RNA under conditions suitable for said local administration.

In one embodiment, the invention features a method of topically administering (e.g. by dermal, transdermal, hair follicle administration etc.,) to a tissue, organ or cell (e.g., skin, hair follicle) a siNA molecule or a vector expressing siNA molecule, comprising nucleotide sequence that is complementary to nucleotide sequence of target RNA, or a portion thereof, expressed in such organ, cell or tissue (e.g., hairless gene, 5-alpha reductase, nude gene, desmoglein 4 gene, TGF-beta, PDGF, BCL-2 and the like) comprising contacting said tissue of cell with said double stranded RNA under conditions suitable for said topical administration. Such topical administration can be used to treat dermatological disease, indication, conditions, trait, genotype or phenotype, or for cosmetic applications such as

acne, psoriasis, melanoma, allopecia, hair removal etc. In one embodiment, the invention features a method of systemically administering (e.g., by injection, such as subcutaneous, intravenous, topical administration, or the like) to a tissue or cell in a subject, a double stranded RNA formed by a siNA molecule or a vector expressing siNA molecule comprising nucleotide sequence that is complementary to nucleotide sequence of target RNA, or a portion thereof, (e.g., target RNA encoding VEGF or a VEGF receptor) comprising contacting said subject with said double stranded RNA under conditions suitable for said systemic administration.

In one embodiment, the invention features a method for treating or preventing tumor angiogenesis in a subject comprising administering to the subject a siNA molecule of the invention under conditions suitable for the treatment or prevention of tumor angiogenesis in the subject, alone or in conjunction with one or more other therapeutic compounds.

In one embodiment, the invention features a method for treating or preventing viral infection or replication in a subject comprising administering to the subject a siNA molecule of the invention under conditions suitable for the treatment or prevention of viral infection or replication in the subject, alone or in conjunction with one or more other therapeutic compounds.

In one embodiment, the invention features a method for treating or preventing autoimmune disease in a subject comprising administering to the subject a siNA molecule of the invention under conditions suitable for the treatment or prevention of autoimmune disease in the subject, alone or in conjunction with one or more other therapeutic compounds.

In one embodiment, the invention features a method for treating or preventing neurologic disease (e.g., Alzheimer's disease, Huntington disease, Parkinson disease, ALS, multiple sclerosis, epilepsy, etc.) in a subject comprising administering to the subject a siNA molecule of the invention under conditions suitable for the treatment or prevention of neurologic disease in the subject, alone or in conjunction with one or more other therapeutic compounds.

In one embodiment, the invention features a method for treating or preventing inflammation in a subject comprising administering to the subject a siNA molecule of the

invention under conditions suitable for the treatment or prevention of inflammation in the subject, alone or in conjunction with one or more other therapeutic compounds.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a non-limiting example of a scheme for the synthesis of siNA molecules. The complementary siNA sequence strands, strand 1 and strand 2, are synthesized in tandem and are connected by a cleavable linkage, such as a nucleotide succinate or abasic succinate, which can be the same or different from the cleavable linker used for solid phase synthesis on a solid support. The synthesis can be either solid phase or solution phase, in the example shown, the synthesis is a solid phase synthesis. The synthesis is performed such that a protecting group, such as a dimethoxytrityl group, remains intact on the terminal nucleotide of the tandem oligonucleotide. Upon cleavage and deprotection of the oligonucleotide, the two siNA strands spontaneously hybridize to form a siNA duplex, which allows the purification of the duplex by utilizing the properties of the terminal protecting group, for example by applying a trityl on purification method wherein only duplexes/oligonucleotides with the terminal protecting group are isolated.

Figure 2 shows a MALDI-TOF mass spectrum of a purified siNA duplex synthesized by a method of the invention. The two peaks shown correspond to the predicted mass of the separate siNA sequence strands. This result demonstrates that the siNA duplex generated from tandem synthesis can be purified as a single entity using a simple trityl-on purification methodology.

Figure 3 shows the results of a stability assay used to determine the serum stability of chemically modified siNA constructs compared to a siNA control consisting of all RNA with 3'-TT termini. T ½ values are shown for duplex stability.

Figure 4 shows the results of an RNAi activity screen of several phosphorothioate modified siNA constructs using a luciferase reporter system.

Figure 5 shows the results of an RNAi activity screen of several phosphorothioate and universal base modified siNA constructs using a luciferase reporter system.

Figure 6 shows the results of an RNAi activity screen of several 2'-O-methyl modified siNA constructs using a luciferase reporter system.

Figure 7 shows the results of an RNAi activity screen of several 2'-O-methyl and 2'-deoxy-2'-fluoro modified siNA constructs using a luciferase reporter system.

Figure 8 shows the results of an RNAi activity screen of a phosphorothioate modified siNA construct using a luciferase reporter system.

Figure 9 shows the results of an RNAi activity screen of an inverted deoxyabasic modified siNA construct generated via tandem synthesis using a luciferase reporter system.

Figure 10 shows the results of an RNAi activity screen of chemically modifed siNA constructs including 3'-glyceryl modified siNA constructs compared to an all RNA control siNA construct using a luciferase reporter system. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by Sirna/RPI number (sense strand/antisense strand). Sequences corresponding to these Sirna/RPI numbers are shown in Table I.

Figure 11 shows the results of an RNAi activity screen of chemically modifed siNA constructs. The screen compared various combinations of sense strand chemical modifications and antisense strand chemical modifications. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by Sirna/RPI number (sense strand/antisense strand). Sequences corresponding to these Sirna/RPI numbers are shown in Table I.

Figure 12 shows the results of an RNAi activity screen of chemically modifed siNA constructs. The screen compared various combinations of sense strand chemical modifications and antisense strand chemical modifications. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by Sirna/RPI number (sense strand/antisense strand). Sequences corresponding to these Sirna/RPI numbers are shown in Table I. In addition, the antisense strand alone (Sirna/RPI 30430) and an inverted control (Sirna/RPI 30227/30229, having matched chemistry to Sirna/RPI (30063/30224) was compared to the siNA duplexes described above.

Figure 13 shows the results of an RNAi activity screen of chemically modifed siNA constructs. The screen compared various combinations of sense strand chemical modifications and antisense strand chemical modifications. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by Sirna/RPI number (sense strand/antisense strand). Sequences corresponding to these Sirna/RPI numbers are shown in Table I. In addition, an inverted control (Sirna/RPI 30226/30229), having matched chemistry to Sirna/RPI (30222/30224) was compared to the siNA duplexes described above.

Figure 14 shows the results of an RNAi activity screen of chemically modifed siNA constructs including various 3'-terminal modified siNA constructs compared to an all RNA control siNA construct using a luciferase reporter system. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by Sirna/RPI number (sense

strand/antisense strand). Sequences corresponding to these Sirna/RPI numbers are shown in Table I.

Figure 15 shows the results of an RNAi activity screen of chemically modifed siNA constructs. The screen compared various combinations of sense strand chemistries compared to a fixed antisense strand chemistry. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by Sirna/RPI number (sense strand/antisense strand). Sequences corresponding to these Sirna/RPI numbers are shown in Table I.

Figure 16 shows the results of a siNA titration study using a luciferase reporter system, wherein the RNAi activity of a phosphorothioate modified siNA construct is compared to that of a siNA construct consisting of all ribonucleotides except for two terminal thymidine residues.

Figure 17 shows a non-limiting proposed mechanistic representation of target RNA degradation involved in RNAi. Double-stranded RNA (dsRNA), which is generated by RNA-dependent RNA polymerase (RdRP) from foreign single-stranded RNA, for example viral, transposon, or other exogenous RNA, activates the DICER enzyme that in turn generates siNA duplexes. Alternately, synthetic or expressed siNA can be introduced directely into a cell by appropriate means. An active siNA complex forms which recognizes a target RNA, resulting in degradation of the target RNA by the RISC endonuclease complex or in the synthesis of additional RNA by RNA-dependent RNA polymerase (RdRP), which can activate DICER and result in additional siNA molecules, thereby amplifying the RNAi response.

Figure 18A-F shows non-limiting examples of chemically-modified siNA constructs of the present invention. In the figure, N stands for any nucleotide (adenosine, guanosine, cytosine, uridine, or optionally thymidine, for example thymidine can be substituted in the overhanging regions designated by parenthesis (N N). Various modifications are shown for the sense and antisense strands of the siNA constructs.

Figure 18A: The sense strand comprises 21 nucleotides wherein the two terminal 3'-nucleotides are optionally base paired and wherein all nucleotides present are ribonucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all nucleotides present are ribonucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the antisense strand.

Figure 18B: The sense strand comprises 21 nucleotides wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the sense and antisense strand.

Figure 18C: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-O-methyl or 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications

described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the antisense strand.

Figure 18D: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein and wherein and all purine nucleotides that may be present are 2'-deoxy nucleotides. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the antisense strand.

Figure 18E: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all pyrimidine nucleotides that may be present are

2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the antisense strand.

Figure 18F: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein and wherein and all purine nucleotides that may be present are 2'-deoxy nucleotides. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and having one 3'-terminal phosphorothioate internucleotide linkage and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-deoxy nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. A modified internucleotide linkage, such as a phosphorothioate, phosphorodithioate or other modified internucleotide linkage as described herein, shown as "s", optionally connects the (N N) nucleotides in the antisense strand. The antisense strand of constructs A-F comprise sequence complementary to any target nucleic acid sequence of the invention. Furthermore, when a glyceryl moiety (L) is present at the 3'-end of the antisense strand for any construct shown in Figure 4 A-F, the modified internucleotide linkage is optional.

Figure 19 shows non-limiting examples of specific chemically modified siNA sequences of the invention. **A-F** applies the chemical modifications described in **Figure 18A-F** to a representative siNA sequence targeting the hepatitis C virus (HCV). However, such chemical modifications can be applied to any target sequence contemplated by the instant invention (see for example target sequences referred to by accession number in McSwiggen et al., International PCT publication No. WO 03/74654.

Figure 20 shows non-limiting examples of different siNA constructs of the invention. The examples shown (constructs 1, 2, and 3) have 19 representative base pairs; however, different embodiments of the invention include any number of base pairs described herein. Bracketed regions represent nucleotide overhangs, for example comprising about 1, 2, 3, or 4 nucleotides in length when present, preferably about 2 nucleotides. Such overhangs can be present or absent (i.e., blunt ends). Such blunt ends can be present on one end or both ends of the siNA molecule, for example where all nucleotides present in a siNA duplex are base paired. Constructs 1 and 2 can be used independently for RNAi activity. Construct 2 can comprise a polynucleotide or non-nucleotide linker, which can optionally be designed as a biodegradable linker. In one embodiment, the loop structure shown in construct 2 can comprise a biodegradable linker that results in the formation of construct 1 *in vivo* and/or *in vitro*. In another example, construct 3 can be used to generate construct 2 under the same principle wherein a linker is used to generate the active siNA construct 2 *in vivo* and/or *in vitro*, which can optionally utilize another biodegradable linker to generate the active siNA construct 1 *in vivo* and/or *in vitro*. As such, the stability and/or activity of the siNA constructs can be modulated based on the design of the siNA construct for use *in vivo* or *in vitro* and/or *in vitro*.

Figure 21 is a diagrammatic representation of a method used to determine target sites for siNA mediated RNAi within a particular target nucleic acid sequence, such as messenger RNA. (A) A pool of siNA oligonucleotides are synthesized wherein the antisense region of the siNA constructs has complementarity to target sites across the target nucleic acid sequence, and wherein the sense region comprises sequence complementary to the antisense region of the siNA. (B) The sequences are transfected into cells. (C) Cells are selected based on phenotypic change that is associated with modulation of the target nucleic acid sequence. (D) The siNA is isolated from the selected cells and is sequenced to identify efficacious target sites within the target nucleic acid sequence.

Figure 22 shows non-limiting examples of different stabilization chemistries (1-10) that can be used, for example, to stabilize the 3'-end of siNA sequences of the invention, including **(1)** [3-3']-inverted deoxyribose; **(2)** deoxyribonucleotide; **(3)** [5'-3']-3'-deoxyribonucleotide; **(4)** [5'-3']-ribonucleotide; **(5)** [5'-3']-3'-O-methyl ribonucleotide; **(6)** 3'-glyceryl; **(7)** [3'-5']-3'-deoxyribonucleotide; **(8)** [3'-3']-deoxyribonucleotide; **(9)** [5'-2']-deoxyribonucleotide; and **(10)**

[5-3']-dideoxyribonucleotide. In addition to modified and unmodified backbone chemistries indicated in the figure, these chemistries can be combined with different backbone modifications as described herein, for example, backbone modifications having Formula I. In addition, the 2'-deoxy nucleotide shown 5' to the terminal modifications shown can be another modified or unmodified nucleotide or non-nucleotide described herein, for example modifications having any of Formulae I-VII or any combination thereof.

Figure 23 shows a non-limiting example of siNA mediated inhibition of VEGF-induced angiogenesis using the rat corneal model of angiogenesis. siNA targeting site 2340 of VEGFR1 RNA (shown as Sirna/RPI No. 29695/29699) were compared to inverted controls (shown as Sirna/RPI No. 29983/29984) at three different concentrations and compared to a VEGF control in which no siNA was administered.

Figure 24 is a non-limiting example of a HBsAg screen of stabilized siNA constructs ("stab 4/5", see **Table IV**) targeting HBV pregenomic RNA in HepG2 cells at 25 nM compared to untreated and matched chemistry inverted sequence controls. The siNA sense and antisense strands are shown by Sirna/RPI number (sense/antisense).

Figure 25 is a non-limiting example of a dose response HBsAg screen of stabilized siNA constructs ("stab 4/5", see **Table IV**) targeting sites 262 and 1580 of the HBV pregenomic RNA in HepG2 cells at 0.5, 5, 10 and 25 nM compared to untreated and matched chemistry inverted sequence controls. The siNA sense and antisense strands are shown by Sirna/RPI number (sense/antisense).

Figure 26 shows a dose response comparison of two different stabilization chemistries ("stab 7/8" and "stab 7/11", see **Table IV**) targeting site 1580 of the HBV pregenomic RNA in HepG2 cells at 5, 10, 25, 50 and 100 nM compared to untreated and matched chemistry inverted sequence controls. The siNA sense and antisense strands are shown by Sirna/RPI number (sense/antisense).

Figure 27 shows a non-limiting example of a strategy used to identify chemically modified siNA constructs of the invention that are nuclease resistance while preserving the ability to mediate RNAi activity. Chemical modifications are introduced into the siNA construct based on educated design parameters (e.g., introducing 2'-modifications, base

modifications, backbone modifications, terminal cap modifications etc). The modified construct in tested in an appropriate system (e.g human serum for nuclease resistance, shown, or an animal model for PK/delivery parameters). In parallel, the siNA construct is tested for RNAi activity, for example in a cell culture system such as a luciferase reporter assay). Lead siNA constructs are then identified which possess a particular characteristic while maintaining RNAi activity, and can be further modified and assayed once again. This same approach can be used to identify siNA-conjugate molecules with improved pharmacokinetic profiles, delivery, and RNAi activity.

Figure 28 shows representative data of a chemically modified siNA construct (Stab 4/5, **Table IV**) targeting HBV site 1580 RNA compared to an unstabilized siRNA construct in a dose response time course HBsAg assay. The constructs were compared at different concentrations (5nM, 10 nM, 25 nM, 50 nM, and 100 nM) over the course of nine days. Activity based on HBsAg levels was determined at day 3, day 6, and day 9.

Figure 29 shows representative data of a chemically modified siNA construct (Stab 7/8, **Table IV**) targeting HBV site 1580 RNA compared to an unstabilized siRNA construct in a dose response time course HBsAg assay. The constructs were compared at different concentrations (5nM, 10 nM, 25 nM, 50 nM, and 100 nM) over the course of nine days. SiNA activity based on HBsAg levels was determined at day 3, day 6, and day 9.

Figure 30 shows representative data of a chemically modified siNA construct (Stab 7/11, **Table IV**) targeting HBV site 1580 RNA compared to an unstabilized siRNA construct in a dose response time course HBsAg assay. The constructs were compared at different concentrations (5nM, 10 nM, 25 nM, 50 nM, and 100 nM) over the course of nine days. SiNA activity based on HBsAg levels was determined at day 3, day 6, and day 9.

Figure 31 shows representative data of a chemically modified siNA construct (Stab 9/10, **Table IV**) targeting HBV site 1580 RNA compared to an unstabilized siRNA construct in a dose response time course HBsAg assay. The constructs were compared at different concentrations (5nM, 10 nM, 25 nM, 50 nM, and 100 nM) over the course of nine days. SiNA activity based on HBsAg levels was determined at day 3, day 6, and day 9.

Figure 32 shows non-limiting examples of inhibition of viral replication of a HCV/poliovirus chimera by siNA constructs targeted to HCV chimera (29579/29586; 29578/29585) compared to control (29593/29600).

Figure 33 shows a non-limiting example of a dose response study demonstrating the inhibition of viral replication of a HCV/poliovirus chimera by siNA construct (29579/29586) at various concentrations (1nM, 5nM, 10nM, and 25nM) compared to control (29593/29600).

Figure 34 shows a non-limiting example demonstrating the inhibition of viral replication of a HCV/poliovirus chimera by a chemically modified siRNA construct (30051/30053) compared to control construct (30052/30054).

Figure 35 shows a non-limiting example demonstrating the inhibition of viral replication of a HCV/poliovirus chimera by a chemically modified siRNA construct (30055/30057) compared to control construct (30056/30058).

Figure 36 shows a non-limiting example of several chemically modified siRNA constructs targeting viral replication of an HCV/poliovirus chimera at 10 nM treatment in comparison to a lipid control and an inverse siNA control construct 29593/ 29600.

Figure 37 shows a non-limiting example of several chemically modified siRNA constructs targeting viral replication of a HCV/poliovirus chimera at 25 nM treatment in comparison to a lipid control and an inverse siNA control construct 29593/ 29600.

Figure 38 shows a non-limiting example of several chemically modified siRNA constructs targeting viral replication of a Huh7 HCV replicon system at 25 nM treatment in comparison to untreated cells ("cells"), cells transfected with lipofectamine ("LFA2K") and inverse siNA control constructs.

Figure 39 shows a non-limiting example of a dose response study using chemically modified siNA molecules (Stab 4/5, see **Table IV**) targeting HCV RNA sites 291, 300, and 303 in a Huh7 HCV replicon system at 5, 10, 25, and 100 nM treatment comparison to untreated cells ("cells"), cells transfected with lipofectamine ("LFA") and inverse siNA control constructs.

Figure 40 shows a non-limiting example of several chemically modified siNA constructs (Stab 7/8, see **Table IV**) targeting viral replication in a Huh7 HCV replicon system at 25 nM treatment in comparison to untreated cells ("cells"), cells transfected with lipofectamine ("Lipid") and inverse siNA control constructs.

Figure 41 shows a non-limiting example of a dose response study using chemically modified siNA molecules (Stab 7/8, see **Table IV**) targeting HCV site 327 in a Huh7 HCV replicon system at 5, 10, 25, 50, and 100 nM treatment in comparison to inverse siNA control constructs.

Figure 42 shows a synthetic scheme for post-synthetic modification of a nucleic acid molecule to produce a folate conjugate.

Figure 43 shows a synthetic scheme for generating an oligonucleotide or nucleic acid-folate conjugate.

Figure 44 shows an alternative synthetic scheme for generating an oligonucleotide or nucleic acid-folate conjugate.

Figure 45 shows an alternative synthetic scheme for post-synthetic modification of a nucleic acid molecule to produce a folate conjugate.

Figure 46 shows a non-limiting example of a synthetic scheme for the synthesis of a N-acetyl-D-galactosamine-2'-aminouridine phosphoramidite conjugate of the invention.

Figure 47 shows a non-limiting example of a synthetic scheme for the synthesis of a N-acetyl-D-galactosamine-D-threoninol phosphoramidite conjugate of the invention.

Figure 48 shows a non-limiting example of a N-acetyl-D-galactosamine siNA nucleic acid conjugate of the invention. **W** shown in the example refers to a biodegradable linker, for example a nucleic acid dimer, trimer, or tetramer comprising ribonucleotides and/or deoxyribonucleotides. The siNA can be conjugated at the 3', 5' or both 3' and 5' ends of the sense strand of a double stranded siNA and/or the 3'-end of the antisense strand of the siNA. A single stranded siNA molecule can be conjugated at the 3'-end of the siNA.

Figure 49 shows a non-limiting example of a synthetic scheme for the synthesis of a dodecanoic acid derived conjugate linker of the invention.

Figure 50 shows a non-limiting example of a synthetic scheme for the synthesis of an oxime linked nucleic acid/peptide conjugate of the invention.

Figure 51 shows non-limiting examples of phospholipid derived siNA conjugates of the invention. **CL** shown in the examples refers to a biodegradable linker, for example a nucleic acid dimer, trimer, or tetramer comprising ribonucleotides and/or deoxyribonucleotides. The siNA can be conjugated at the 3', 5' or both 3' and 5' ends of the sense strand of a double stranded siNA and/or the 3'-end of the antisense strand of the siNA. A single stranded siNA molecule can be conjugated at the 3'-end of the siNA.

Figure 52 shows a non-limiting example of a synthetic scheme for preparing a phospholipid derived siNA conjugates of the invention.

Figure 53 shows a non-limiting example of a synthetic scheme for preparing a poly-N-acetyl-D-galactosamine nucleic acid conjugate of the invention.

Figure 54 shows a non-limiting example of the synthesis of siNA cholesterol conjugates of the invention using a phosphoramidite approach.

Figure 55 shows a non-limiting example of the synthesis of siNA PEG conjugates of the invention using NHS ester coupling.

Figure 56 shows a non-limiting example of the synthesis of siNA cholesterol conjugates of the invention using NHS ester coupling.

Figure 57 shows a non-limiting example of various siNA cholesterol conjugates of the invention.

Figure 58 shows a non-limiting example of various siNA cholesterol conjugates of the invention in which various linker chemistries and/or cleavable linkers can be utilized at different positions of a double stranded siNA molecule.

Figure 59 shows a non-limiting example of various siNA cholesterol conjugates of the invention in which various linker chemistries and/or cleavable linkers can be utilized at different positions of a double stranded siNA molecule.

Figure 60 shows a non-limiting example of various siNA cholesterol conjugates of the invention in which various linker chemistries and/or cleavable linkers can be utilized at different positions of a single stranded siNA molecule.

Figure 61 shows a non-limiting example of various siNA phospholipid conjugates of the invention in which various linker chemistries and/or cleavable linkers can be utilized at different positions of a double stranded siNA molecule.

Figure 62 shows a non-limiting example of various siNA phospholipid conjugates of the invention in which various linker chemistries and/or cleavable linkers can be utilized at different positions of a single stranded siNA molecule.

Figure 63 shows a non-limiting example of various siNA galactosamine conjugates of the invention in which various linker chemistries and/or cleavable linkers can be utilized at different positions of a double stranded siNA molecule.

Figure 64 shows a non-limiting example of various siNA galactosamine conjugates of the invention in which various linker chemistries and/or cleavable linkers can be utilized at different positions of a single stranded siNA molecule.

Figure 65 shows a non-limiting example of various generalized siNA conjugates of the invention in which various linker chemistries and/or cleavable linkers can be utilized at different positions of a double stranded siNA molecule. CONJ in the figure refers to any biologically active compound or any other conjugate compound as described herein and in the Formulae herein.

Figure 66 shows a non-limiting example of various generalized siNA conjugates of the invention in which various linker chemistries and/or cleavable linkers can be utilized at different positions of a single stranded siNA molecule. CONJ in the figure refers to any biologically active compound or any other conjugate compound as described herein and in the Formulae herein.

Figure 67 shows a non-limiting example of the pharmacokinetic distribution of intact siNA in liver after administration of conjugated or unconjugated siNA molecules in mice.

Figure 68 shows a non-limiting example of the activity of conjugated siNA constructs compared to matched chemistry unconjugated siNA constructs in a HBV cell culture system without the use of transfection lipid. As shown in the Figure, siNA conjugates provide efficacy in cell culture without the need for transfection reagent.

Figure 69 shows a non-limiting example of a scheme for the synthesis of a mono-galactosamine phosphoramidite of the invention that can be used to generate galactosamine conjugated nucleic acid molecules.

Figure 70 shows a non-limiting example of a scheme for the synthesis of a tri-galactosamine phosphoramidite of the invention that can be used to generate tri-galactosamine conjugated nucleic acid molecules.

Figure 71 shows a non-limiting example of a scheme for the synthesis of another tri-galactosamine phosphoramidite of the invention that can be used to generate tri-galactosamine conjugated nucleic acid molecules.

Figure 72 shows a non-limiting example of an alternate scheme for the synthesis of a tri-galactosamine phosphoramidite of the invention that can be used to generate tri-galactosamine conjugated nucleic acid molecules.

Figure 73 shows a non-limiting example of a scheme for the synthesis of a cholesterol NHS ester of the invention that can be used to generate cholesterol conjugated nucleic acid molecules.

Figure 74 shows non-limiting exampled of phosphorylated siNA molecules of the invention, including linear and duplex constructs and asymmetric derivatives thereof.

Figure 75 shows non-limiting examples of a chemically modified terminal phosphate groups of the invention.

Figure 76 shows a non-limiting example of inhibition of VEGF induced neovascularization in the rat corneal model. VEGFr1 site 349 active siNA having "Stab 9/10" chemistry (Sirna # 31270/31273) was tested for inhibition of VEGF-induced angiogenesis at three different concentrations (2.0 ug, 1.0 ug, and 0.1 μg dose response) as compared to a matched chemistry inverted control siNA construct (Sirna # 31276/31279) at each concentration and a VEGF control in which no siNA was administered. As shown in the figure, the active siNA construct having "Stab 9/10" chemistry (Sirna # 31270/31273) is highly effective in inhibiting VEGF-induced angiogenesis in the rat corneal model compared to the matched chemistry inverted control siNA at concentrations from 0.1 μg to 2.0 ug.

Figure 77 shows activity of modified siNA constructs having stab 4/5 (Sirna 30355/30366), stab 7/8 (Sirna 30612/30620), and stab 7/11 (Sirna 30612/31175)

chemistries and an all ribo siNA construct (Sirna 30287/30298) in the reduction of HBsAg levels compared to matched inverted controls at **A.** 3 days, **B.** 9 days, and **C.** 21 days post transfection. Also shown is the corresponding percent inhibition as function of time at siNA concentrations of **D.** 100 nM, **E.** 50 nM, and **F.** 25 nM.

Figure 78 shows non-limiting examples of phosphorylated siNA molecules of the invention, including linear and duplex constructs and asymmetric derivatives thereof.

Figure 79 shows non-limiting examples of chemically modified terminal phosphate groups of the invention.

Figure 80 shows a non-limiting example of reduction of serum HBV DNA in mice treated with hydrodynamically administered chemically modified siNA (Stab 7/8 and Stab 9/10) targeting HBV RNA compared to matched chemistry inverted controls and a saline control.

Figure 81 shows a non-limiting example of reduction of serum HBV S antigen (HBsAg) in mice treated with hydrodynamically administered chemically modified siNA (Stab 7/8 and Stab 9/10) targeting HBV RNA compared to matched chemistry inverted controls and a saline control.

Figure 82 shows a non-limiting example of reduction of serum HBV RNA in mice treated with hydrodynamically administered chemically modified siNA (Stab 7/8 and Stab 9/10) targeting HBV RNA compared to matched chemistry inverted controls and a saline control.

Figure 83 shows a non-limiting example of reduction of serum HBV DNA in mice treated with hydrodynamically administered chemically modified siNA (Stab 7/8 and Stab 9/10) targeting HBV RNA at 5 days and 7 days post administration.

Figure 84 shows a non-limiting example of an assay for dose dependent reduction of Luciferase expression utilizing Stab 7/8 chemically modified siNA constructs targeting luciferase RNA sites 80, 237, and 1478 that were selected from a screen using all Stab 7/8 chemically modified siNA constructs.

Figure 85 shows a non-limiting example of an assay for dose dependent reduction of Luciferase expression utilizing Stab 7/8 chemically modified siNA constructs targeting

luciferase RNA sites 1544 and 1607 that were selected from a screen using all Stab 7/8 chemically modified siNA constructs.

Figure 86 shows a non-limiting example of an assay screen of Stab 7/8 siNA constructs targeting various sites of HCV RNA in a replicon system compared to untreated, lipid, and an inverted control. As shown in the figure, several Stab 7/8 constructs were identified with potent anti-HCV activity as shown by reduction in HCV RNA levels.

Figure 87 shows a non-limiting example of an assay screen of Stab 7/8 siNA constructs targeting various sites of HBV RNA in HEpG2 cells compared to untreated cells and an inverted control. As shown in the figure, several Stab 7/8 constructs were identified with potent anti-HBV activity as shown by reduction in HBV S antigen levels.

Figure 88 shows a non-limiting example of an assay screen of various combinations of chemically modified siNA constructs (e.g., Stab 7/8, 7/10, 7/11, 9/8, and 9/10) targeting site 1580 of HBV RNA in HEpG2 cells compared to untreated cells and an matched chemistry inverted controls. As shown in the figure, the combination chemistries tested demonstrated potent anti-HBV activity as shown by reduction in HBV S antigen levels.

Figure 89 shows a non-limiting example of an assay screen of various combinations of chemically modified siNA constructs (e.g., Stab 7/8, 9/10, 6/10, 16/8, 16/10, 18/8, and 18/10) targeting site 1580 of HBV RNA in HEpG2 cells compared to untreated cells and an matched chemistry inverted controls. As shown in the figure, the combination chemistries tested demonstrated potent anti-HBV activity as shown by reduction in HBV S antigen levels.

Figure 90 shows a non-limiting example of an assay screen of various combinations of chemically modified siNA constructs (e.g., Stab 4/8, 4/10, 7/5, 7/10, 9/5, 9/8, and 9/11) targeting site 1580 of HBV RNA in HEpG2 cells compared to untreated cells and an matched chemistry inverted controls. As shown in the figure, the combination chemistries tested demonstrated potent anti-HBV activity as shown by reduction in HBV S antigen levels.

Figure 91 shows a non-limiting example of reduction of serum HBV DNA in mice treated with hydrodynamically administered

polyethylimine-polyethyleneglycol-tri-N-acetylgalactosamine (PEI-PEG-triGAL) formulated Stab 9/10 siNA targeting HBV site 1580 RNA compared to a matched chemistry inverted control.

Figure 92 shows a non-limiting example of reduction of serum HBsAg in mice treated with hydrodynamically administered polyethylimine-polyethyleneglycol-tri-N-acetylgalactosamine (PEI-PEG-triGAL) formulated Stab 9/10 siNA targeting HBV site 1580 RNA compared to a matched chemistry inverted control.

Figure 93 shows a non-limiting example of the general structure of a polyethylimine-polyethyleneglycol-tri-N-acetylgalactosamine (PEI-PEG-triGAL) transfection agent.

Figure 94A shows a non-limiting example of methodology used to design self complementary DFO constructs utilizing palidrome and/or repeat nucleic acid sequences that are identifed in a target nucleic acid sequence. (i) A palindrome or repeat sequence is identified in a nucleic acid target sequence. (ii) A sequence is designed that is complementary to the target nucleic acid sequence and the palindrome sequence. (iii) An inverse repeat sequence of the non-palindrome/repeat portion of the complementary sequence is appended to the 3'-end of the complementary sequence to generate a self complmentary DFO molecule comprising sequence complementary to the nucleic acid target. (iv) The DFO molecule can self-assemble to form a double stranded oligonucleotide. **Figure 94B** shows a non-limiting representative example of a duplex forming oligonucleotide sequence. **Figure 94C** shows a non-limiting example of the self assembly schematic of a representative duplex forming oligonucleotide sequence. **Figure 94D** shows a non-limiting example of the self assembly schematic of a representative duplex forming oligonucleotide sequence followed by interaction with a target nucleic acid sequence resulting in modulation of gene expression.

Figure 95 shows a non-limiting example of the design of self complementary DFO constructs utilizing palidrome and/or repeat nucleic acid sequences that are incorporated into the DFO constructs that have sequence complementary to any target nucleic acid sequence of interest. Incorporation of these palindrome/repeat sequences allow the design of DFO constructs that form duplexes in which each strand is capable of mediating

modulation of target gene expression, for example by RNAi. First, the target sequence is identified. A complementary sequence is then generated in which nucleotide or non-nucleotide modifications (shown as X or Y) are introduced into the complementary sequence that generate an artificial palindrome (shown as XYXYXY in the Figure). An inverse repeat of the non-palindrome/repeat complementary sequence is appended to the 3'-end of the complementary sequence to generate a self complmentary DFO comprising sequence complementary to the nucleic acid target. The DFO can self-assemble to form a double stranded oligonucleotide.

Figure 96 shows non-limiting examples of multifunctional siNA molecules of the invention comprising two separate polynucleotide sequences that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences. **Figure 96A** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a first target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 3'-ends of each polynucleotide sequence in the multifunctional siNA. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. **Figure 96B** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a frist target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 5'-ends of each polynucleotide sequence in the multifunctional siNA. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences.

Figure 97 shows non-limiting examples of multifunctional siNA molecules of the invention comprising a single polynucleotide sequence comprising distinct regions that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences. **Figure 97A** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a frist target nucleic acid sequence

(complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the second complementary region is situated at the 3'-end of the polynucleotide sequence in the multifunctional siNA. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. **Figure 97B** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a frist target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first complementary region is situated at the 5'-end of the polynucleotide sequence in the multifunctional siNA. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. In one embodiment, these multifunctional siNA constructs are processed in vivo or in vitro to generate multifunctional siNA constructs as shown in **Figure 96**.

Figure 98 shows non-limiting examples of multifunctional siNA molecules of the invention comprising two separate polynucleotide sequences that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences and wherein the multifunctional siNA construct further comprises a self complementary, palindrome, or repeat region, thus enabling shorter bifuctional siNA constructs that can mediate RNA interference against differing target nucleic acid sequences. **Figure 98A** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a frist target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 3'-ends of each polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindrome, or repeat region. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. **Figure 98B** shows a non-limiting example of a multifunctional

siNA molecule having a first region that is complementary to a frist target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first and second complementary regions are situated at the 5'-ends of each polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindrome, or repeat region. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences.

Figure 99 shows non-limiting examples of multifunctional siNA molecules of the invention comprising a single polynucleotide sequence comprising distinct regions that are each capable of mediating RNAi directed cleavage of differing target nucleic acid sequences and wherein the multifunctional siNA construct further comprises a self complementary, palindrome, or repeat region, thus enabling shorter bifuctional siNA constructs that can mediate RNA interference against differing target nucleic acid sequences. **Figure 99A** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a frist target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the second complementary region is situated at the 3'-end of the polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindrome, or repeat region. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. **Figure 99B** shows a non-limiting example of a multifunctional siNA molecule having a first region that is complementary to a frist target nucleic acid sequence (complementary region 1) and a second region that is complementary to a second target nucleic acid sequence (complementary region 2), wherein the first complementary region is situated at the 5'-end of the polynucleotide sequence in the multifunctional siNA, and wherein the first and second complementary regions further comprise a self complementary, palindrome, or repeat region. The dashed portions of each polynucleotide sequence of the multifunctional siNA construct have complementarity with regard to

corresponding portions of the siNA duplex, but do not have complementarity to the target nucleic acid sequences. In one embodiment, these multifunctional siNA constructs are processed in vivo or in vitro to generate multifunctional siNA constructs as shown in **Figure 98**.

Figure 100 shows a non-limiting example of how multifunctional siNA molecules of the invention can target two separate target nucleic acid molecules, such as separate RNA molecules encoding differing proteins, for example a cytokine and its corresponding receptor, differing viral strains, a virus and a cellular protein involved in viral infection or replication, or differing proteins involved in a common or divergent biologic pathway that is implicated in the maintenance of progression of disease. Each strand of the multifunctional siNA construct comprises a region having complementarity to separate target nucleic acid molecules. The multifunctional siNA molecule is designed such that each strand of the siNA can be utilized by the RISC complex to initiate RNA interferance mediated cleavage of its corresponding target. These design parameters can include destabilization of each end of the siNA construct (see for example Schwarz *et al.*, 2003, *Cell*, 115, 199-208). Such destabilization can be accomplished for example by using guanosine-cytidine base pairs, alternate base pairs (e.g., wobbles), or destabilizing chemically modified nucleotides at terminal nucleotide positions as is known in the art.

Figure 101 shows a non-limiting example of how multifunctional siNA molecules of the invention can target two separate target nucleic acid seqeunces within the same target nucleic acid molecule, such as alternate coding regions of a RNA, coding and non-coding regions of a RNA, or alternate splice variant regions of a RNA. Each strand of the multifunctional siNA construct comprises a region having complementarity to the separate regions of the target nucleic acid molecule. The multifunctional siNA molecule is designed such that each strand of the siNA can be utilized by the RISC complex to initiate RNA interferance mediated cleavage of its corresponding target region. These design parameters can include destabilization of each end of the siNA construct (see for example Schwarz *et al.*, 2003, *Cell*, 115, 199-208). Such destabilization can be accomplished for example by using guanosine-cytidine base pairs, alternate base pairs (e.g., wobbles), or destabilizing chemically modified nucleotides at terminal nucleotide positions as is known in the art.

Figure 102 shows a non-limiting exmaple of the dose dependent reduction in serum HBV DNA levels following systemic intravenous administration of a Stab 7/8 siNA construct targeting HBV RNA site 263 in mice pre-treated with a HBV expressing vector via hydrodynamic injection. siNA treated groups were compared to inverted control or saline groups. A statistically significant ($P < 0.01$) reduction of 0.93 log was observed in the 30mg/kg group as compared to the saline group. This result demonstrates *in vivo* activity of a systemically administered siNA.

Figure 103 shows activity of a fully stabilized siNA construct compared to a matched chemistry inverted control, an all RNA siNA construct having identical sequence (RNA active), and a corresponding all RNA inverted control (RNA Inv), in a HBV Co-HDI mouse model. A hydrodynamic tail vein injection (HDI) containing 1 ug of the pWTD HBV vector and 0, 0.03, 0.1, 0.3 or 1.0 ug of siNA was performed on C57BL/J6 mice. Active siNA duplexes and inverted sequence controls in both native RNA and stabilized chemistry were tested. The levels of serum HBV DNA and HBsAg were measured 72 hrs post injection. **Figure 103A** shows results for HBV serum DNA levels, **Figure 103B** shows results for serum HBsAg levels, and **Figure 103C** shows results for liver HBV RNA levels in this study.

Figure 104 shows non-limiting examples of the design of self complementary DFO constructs utilizing palidrome and/or repeat nucleic acid sequences that are incorporated into the DFO constructs that have sequence complementary to any target nucleic acid sequence of interest as described in **Figure 95**. The palidrome/repeat sequence comprises chemically modified nucleotides that are able to interact with a portion of the target nucleic acid sequence (e.g., use of modified base analogs that can form Watson Crick base pairs or non-Watson Crick base pairs such as 2-aminopurine or 2-amino-1,6-dihydropurine nucleotides or universal nucleotides). Similarly, other base-modified pyrimidines can be used to make non-natural base pairs with purines or vice versa.

Figure 105 shows non-limiting examples of inhibition of VEGFR1 RNA expression using DFO molecules of the invention. Duplex DFO constructs prepared from compound numbers 32808, 32809, 32810, 32811, and 32812 were assayed along with siNA molecules having known activity against VEGFR1 RNA (compound numbers 32748/32755, 33282/32289, 31270/31273), matched chemistry inverted controls (compound numbers 32772/32779, 32296/32303, 31276/31279), and a transfection agent control (LF2K). As

shown in the Figure, the self complementary DFO sequence 32812 shows potent inhibition of VEGFR1 RNA. Sequences for compound numbers are shown in **Table I**.

Figure 106 shows non-limiting examples of inhibition of HBV RNA expression using DFO molecules of the invention as assayed by HBsAg levels. A duplex DFO construct prepared from compound 32221 and a hairpin formed with the same sequence (32221 fold) was assayed along with a siNA construct having known activity against HBV RNA (compound number 31335/31337), a matched chemistry inverted control (compound number 31336/31338), and untreated cells (Untreated). As shown in the Figure, the self complementary DFO sequence 32221 shows significant inhibition of HBV HBsAg as a duplex. Sequences for compound numbers are shown in **Table I**.

Figure 107 shows a non-limiting example of DFO mediated reduction of TGF-beta receptor-1 target RNA expression. Self complementary DFO palindrome/repeat sequences shown in **Table I** (e.g., compound # 35038, 35041, 35044, and 35045) were designed against TGF-beta receptor-1 RNA targets and were screened in cell culture experiments and irrelevant controls (Control 1, Control 2) and untreated cells along with a

trasfection control (LF2K). NMuMg cells were transfected with 0.5 uL/well of lipid complexed with 25 and 100 nM DFO. Cells were incubated at 37° for 24h in the continued presence of the DFO transfection mixture. At 24h, RNA was prepared from each well of treated cells. The supernatants with the transfection mixtures were first removed and discarded, then the cells were lysed and RNA prepared from each well. Target gene expression following treatment was evaluated by RT-PCR for the TGF-beta receptor mRNA and for a control gene (36B4, an RNA polymerase subunit) for normalization. As shown in the figure, the DFO constructs displayed potent inhibition of TGF-beta receptor-1 RNA expression in this system.

Figure 108 shows a non-limiting example inhibition of HBV RNA using multifunctional siNA constructs targeting HBV and PKC-alpha RNA in HepG2 cells.

Figure 109 shows a non-limiting example inhibition of PKC-alpha RNA using multifunctional siNA constructs targeting HBV and PKC-alpha RNA in HepG2 cells.

Figure 110 (A-H) shows non-limiting examples of tethered multiifunctional siNA constructs of the invention. In the examples shown, a linker (e.g., nucleotide or non-nucleotide linker) connects two siNA regions (e.g., two sense, two antisense, or alternately a sense and an antisense region together. Separate sense (or sense and antisense) sequences corresponding to a first target sequence and second target sequence are hybridized to their corresponding sense and/or antisense sequences in the multifunctional siNA. In addition, various conjugates, ligands, aptamers, polymers or reporter molecules can be attached to the linker region for selective or improved delivery and/or pharmacokinetic properties.

Figure 111 shows a non-limiting example of various dendrimer based multifunctional siNA designs.

Figure 112 shows a non-limiting example of various supramolecular multifunctional siNA designs.

Figure 113 shows a non-limiting example of a dicer enabled multifunctional siNA design using a 30 nucleotide precursor siNA construct. A 30 base pair duplex is cleaved by Dicer into 22 and 8 base pair products from either end (8 b.p. fragments not shown). For

ease of presentation the overhangs generated by dicer are not shown – but can be compensated for. Three targeting sequences are shown. The required sequence identity overlapped is indicated by grey boxes. The N's of the parent 30 b.p. siNA are suggested sites of 2'-OH positions to enable Dicer cleavage if this is tested in stabilized chemistries. Note that processing of a 30mer duplex by Dicer RNase III does not give a precise 22+8 cleavage, but rather produces a series of closely related products (with 22+8 being the primary site). Therefore, processing by Dicer will yield a series of active siNAs.

Figure 114 shows a non-limiting example of a dicer enabled multifunctional siNA design using a 40 nucleotide precursor siNA construct. A 40 base pair duplex is cleaved by Dicer into 20 base pair products from either end. For ease of presentation the overhangs generated by dicer are not shown – but can be compensated for. Four targeting sequences are shown in four colors, blue, light-blue and red and orange. The required sequence identity overlapped is indicated by grey boxes. This design format can be extended to larger RNAs. If chemically stabilized siNAs are bound by Dicer, then strategically located ribonucleotide linkages can enable designer cleavage products that permit our more extensive repertoire of multiifunctional designs. For example cleavage products not limited to the Dicer standard of approximately 22-nucleotides can allow multifunctional siNA constructs with a target sequence identity overlap ranging from, for example, about 3 to about 15 nucleotides.

Figure 115 shows a non-limiting example of inhibition of HBV RNA by dicer enabled multifunctional siNA constructs targeting HBV site 263. When the first 17 nucleotides of a siNA antisense strand (e.g., 21 nucleotide strands in a duplex with 3'-TT overhangs) are complementary to a target RNA, robust silencing was observed at 25 nM. 80% silencing was observed with only 16 nucleotide complementarity in the same format.

Figure 116 shows a non-limiting example of additional multifunctional siNA construct designs of the invention. In one example, a conjugate, ligand, aptamer, label, or other moiety is attached to a region of the multifunctional siNA to enable improved delivery or pharmacokinetic profiling.

Figure 117 shows a non-limiting example of additional multifunctional siNA construct designs of the invention. In one example, a conjugate, ligand, aptamer, label, or

other moiety is attached to a region of the multifunctional siNA to enable improved delivery or pharmacokinetic profiling.

Figure 118 shows a non-limiting example of an experiment designed to determine the effect of absolute based paired sequence length of siNA constructs on RNAi efficacy. A well characterized site for siNA mediated inhibition, HBV RNA site 263 was chosen and siNA molecules ranging in length from 19 to 39 ribonucleotide base pairs in length with 3'-terminal dinucleotide TT overhangs. Transfection of the human hepatocellular carcinoma cell line, Hep G2, with replication-competent HBV DNA results in the expression of HBV proteins and the production of virions. To test the efficacy of differening length siNAs targeted against HBV RNA, several siNA duplexes targeting site 263 within HBV pregenomic RNA were co-transfected with HBV genomic DNA once at 25 nM with lipid at 12.5 ug/ml into Hep G2 cells, and the subsequent levels of HBV RNA analyzed by RT PCR compared to cells treated with an inverted siNA control to site 263 and untreated cells. As shown in the figure, the siNA constructs from 19 to 39 base pairs were all efficacious in inhibiting HBV RNA in this system.

DETAILED DESCRIPTION OF THE INVENTION

Mechanism of action of Nucleic Acid Molecules of the Invention

The discussion that follows discusses the proposed mechanism of RNA interference mediated by short interfering RNA as is presently known, and is not meant to be limiting and is not an admission of prior art. Applicant demonstrates herein that chemically-modified short interfering nucleic acids possess similar or improved capacity to mediate RNAi as do siRNA molecules and are expected to possess improved stability and activity *in vivo*; therefore, this discussion is not meant to be limited to siRNA only and can be applied to siNA as a whole. By "improved capacity to mediate RNAi" or "improved RNAi activity" is meant to include RNAi activity measured *in vitro* and/or *in vivo* where the RNAi activity is a reflection of both the ability of the siNA to mediate RNAi and the stability of the siNAs of the invention. In this invention, the product of these activities can be increased *in vitro* and/or *in vivo* compared to an all RNA siRNA or a siNA containing a plurality of ribonucleotides. In some cases, the activity or stability of the siNA molecule can be decreased (i.e., less than ten-fold), but the overall activity of the siNA molecule is enhanced *in vitro* and/or *in vivo*.

RNA interference refers to the process of sequence specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Zamore *et al.,* 2000, Cell, 101, 25-33; Fire *et al.,* 1998, *Nature,* 391, 806). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes which is commonly shared by diverse flora and phyla (Fire *et al.,* 1999, *Trends Genet.,* 15, 358). Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response though a mechanism that has yet to be fully characterized. This mechanism appears to be different from the interferon response that results from dsRNA-mediated activation of protein kinase PKR and 2', 5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as Dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs) (Berstein *et al.,* 2001, *Nature,* 409, 363). Short interfering RNAs derived from Dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner *et al.,* 2001, *Science,* 293, 834). The RNAi response also features an endonuclease complex containing a siRNA, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence homologous to the siRNA. Cleavage of the target RNA takes place in the middle of the region complementary to the guide sequence of the siRNA duplex (Elbashir *et al.,* 2001, *Genes Dev.,* 15, 188). In addition, RNA interference can also involve small RNA (e.g., micro-RNA or miRNA) mediated gene silencing, presumably though cellular mechanisms that regulate chromatin structure and thereby prevent transcription of target gene sequences (see for example Allshire, 2002, *Science,* 297, 1818-1819; Volpe *et al.,*

2002, *Science,* 297, 1833-1837; Jenuwein, 2002, *Science,* 297, 2215-2218; and Hall *et al.,* 2002, *Science,* 297, 2232-2237). As such, siNA molecules of the invention can be used to mediate gene silencing via interaction with RNA transcripts or alternately by interaction with particular gene sequences, wherein such interaction results in gene silencing either at the transcriptional level or post-transcriptional level.

RNAi has been studied in a variety of systems. Fire *et al.,* 1998, *Nature,* 391, 806, were the first to observe RNAi in *C. elegans*. Wianny and Goetz, 1999, *Nature Cell Biol.,* 2, 70, describe RNAi mediated by dsRNA in mouse embryos. Hammond *et al.,* 2000, *Nature,* 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir *et al.,* 2001, *Nature,* 411, 494, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells. Recent work in Drosophila embryonic lysates has revealed certain requirements for siRNA length, structure, chemical composition, and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21 nucleotide siRNA duplexes are most active when containing two 2-nucleotide 3'-terminal nucleotide overhangs. Furthermore, substitution of one or both siRNA strands with 2'-deoxy or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of 3'-terminal siRNA nucleotides with deoxy nucleotides was shown to be tolerated. Mismatch sequences in the center of the siRNA duplex were also shown to abolish RNAi activity. In addition, these studies also indicate that the position of the cleavage site in the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end (Elbashir *et al.,* 2001, *EMBO J.,* 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA (Nykanen *et al.,* 2001, *Cell,* 107, 309); however, siRNA molecules lacking a 5'-phosphate are active when introduced exogenously, suggesting that 5'-phosphorylation of siRNA constructs may occur *in vivo*.

Duplex Foming Oligonucleotides (DFO) of the Invention

In one embodiment, the invention features siNA molecules comprising duplex forming oligonucleotides (DFO) that can self-assemble into double stranded oligonucleotides. The duplex forming oligonucleotides of the invention can be chemically

synthesized or expressed from transcription units and/or vectors. The DFO molecules of the instant invention provide useful reagents and methods for a variety of therapeutic, diagnostic, agricultural, veterinary, target validation, genomic discovery, genetic engineering and pharmacogenomic applications.

Applicant demonstrates herein that certain oligonucleotides, refered to herein for convenience but not limitation as duplex forming oligonucleotides or DFO molecules, are potent mediators of sequence specific regulation of gene expression. The oligonucleotides of the invention are distinct from other nucleic acid sequences known in the art (e.g., siRNA, miRNA, stRNA, shRNA, antisense oligonucleotides etc.) in that they represent a class of linear polynucleotide sequences that are designed to self-assemble into double stranded oligonucleotides, where each strand in the double stranded oligonucleotides comprises a nucleotide sequence that is complementary to a target nucleic acid molecule. Nucleic acid molecules of the invention can thus self assemble into functional duplexes in which each strand of the duplex comprises the same polynucleotide sequence and each strand comprises a nucleotide sequence that is complementary to a target nucleic acid molecule.

Generally, double stranded oligonucleotides are formed by the assembly of two distinct oligonucleotide sequences where the oligonucleotide sequence of one strand is complementary to the oligonucleotide sequence of the second strand; such double stranded oligonucleotides are assembled from two separate oligonucleotides, or from a single molecule that folds on itself to form a double stranded structure, often referred to in the field as hairpin stem-loop structure (e.g., shRNA or short hairpin RNA). These double stranded oligonucleotides known in the art all have a common feature in that each strand of the duplex has a distict nucleotide sequence.

Distinct from the double stranded nucleic acid molecules known in the art, the applicants have developed a novel, potentially cost effective and simplified method of forming a double stranded nucleic acid molecule starting from a single stranded or linear oligonucleotide. The two strands of the double stranded oligonucleotide formed according to the instant invention have the same nucleotide sequence and are not covalently linked to each other. Such double-stranded oligonucleotides molecules can be readily linked post-synthetically by methods and reagents known in the art and are within the scope of the

invention. In one embodiment, the single stranded oligonucleotide of the invention (the duplex forming oligonucleotide) that forms a double stranded oligonucleotide comprises a first region and a second region, where the second region includes a nucleotide sequence that is an inverted repeat of the nucleotide sequence in the first region, or a portion thereof, such that the single stranded oligonucleotide self assembles to form a duplex oligonucleotide in which the nucleotide sequence of one strand of the duplex is the same as the nucleotide sequence of the second strand. Non-limiting examples of such duplex forming oligonucleotides are illustrated in **Figures 94 and 95**. These duplex forming oligonucleotides (DFOs) can optionally include certain palindrome or repeat sequences where such palindrome or repeat sequences are present in between the first region and the second region of the DFO.

In one embodiment, the invention features a duplex forming oligonucleotide (DFO) molecule, wherein the DFO comprises a duplex forming self complementary nucleic acid sequence that has nucleotide sequence complementary to a target nucleic acid sequence. The DFO molecule can comprise a single self complementary sequence or a duplex resulting from assembly of such self complementary sequences.

In one embodiment, a duplex forming oligonucleotide (DFO) of the invention comprises a first region and a second region, wherein the second region comprises a nucleotide sequence comprising an inverted repeat of nucleotide sequence of the first region such that the DFO molecule can assemble into a double stranded oligonucleotide. Such double stranded oligonucleotides can act as a short interfering nucleic acid (siNA) to modulate gene expression. Each strand of the double stranded oligonucleotide duplex formed by DFO molecules of the invention can comprise a nucleotide sequence region that is complementary to the same nucleotide sequence in a target nucleic acid molecule (e.g., target RNA).

In one embodiment, the invention features a single stranded DFO that can assemble into a double stranded oligonucleotide. The applicant has surprisingly found that a single stranded oligonucleotide with nucleotide regions of self complementarity can readily assemble into duplex oligonucleotide constructs. Such DFOs can assemble into duplexes that can inhibit gene expression in a sequence specific manner. The DFO moleucles of the invention comprise a first region with nucleotide sequence that is complementary to the

nucleotide sequence of a second region and where the sequence of the first region is complementary to a target nucleic acid (e.g., RNA). The DFO can form a double stranded oligonucleotide wherein a portion of each strand of the double stranded oligonucleotide comprises a sequence complementary to a target nucleic acid sequence.

In one embodiment, the invention features a double stranded oligonucleotide, wherein the two strands of the double stranded oligonucleotide are not covalently linked to each other, and wherein each strand of the double stranded oligonucleotide comprises a nucleotide sequence that is complementary to the same nucleotide sequence in a target nucleic acid molecule or a portion thereof. In another embodiment, the two strands of the double stranded oligonucleotide share an identical nucleotide sequence of at least about 15, preferably at least about 16, 17, 18, 19, 20, or 21 nucleotides.

In one embodiment, a DFO molecule of the invention comprises a structure having Formula DFO-I:

5'-p-X Z X'-3'

wherein Z comprises a palindromic or repeat nucleic acid sequence optionally with one or more modified nucleotides (e.g., nucleotide with a modified base, such as 2-amino purine, 2-amino-1,6-dihydro purine or a universal base), for example of length about 2 to about 24 nucleotides in even numbers (e.g., about 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, or 22 or 24 nucleotides), X represents a nucleic acid sequence, for example of length between about 1 to about 21 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleotides), X' comprises a nucleic acid sequence, for example of length about 1 and about 21 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleotides) having nucleotide sequence complementarity to sequence X or a portion thereof, p comprises a terminal phosphate group that can be present or absent, and wherein sequence X and Z, either independently or together, comprise nucleotide sequence that is complementary to a target nucleic acid sequence or a portion thereof and is of length sufficient to interact (e.g., base pair) with the target nucleic acid sequence or a portion thereof. For example, X independently can comprise a sequence from about 12 to about 21 or more (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more) nucleotides in length that is complementary to nucleotide sequence in a target RNA or a portion thereof.

In another non-limiting example, the length of the nucleotide sequence of X and Z together, when X is present, that is complementary to the target RNA or a portion thereof is from about 12 to about 21 or more nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more). In yet another non-limiting example, when X is absent, the length of the nucleotide sequence of Z that is complementary to the target RNA or a portion thereof is from about 12 to about 24 or more nucleotides (e.g., about 12, 14, 16, 18, 20, 22, 24, or more). In one embodiment X, Z and X' are independently oligonucleotides, where X and/or Z comprises a nucleotide sequence of length sufficient to interact (e.g., base pair) with a nucleotide sequence in the target RNA or a portion thereof. In one embodiment, the lengths of oligonucleotides X and X' are identical. In another embodiment, the lengths of oligonucleotides X and X' are not identical. In another embodiment, the lengths of oligonucleotides X and Z, or Z and X', or X, Z and X' are either identical or different.

When a sequence is described in this specification as being of "sufficient" length to interact (*i.e.*, base pair) with another sequence, it is meant that the the length is such that the number of bonds (*e.g.*, hydrogen bonds) formed between the two sequences is enough to enable the two sequence to form a duplex under the conditions of interest. Such conditions can be *in vitro* (*e.g.*, for diagnostic or assay purposes) or *in vivo* (*e.g.*, for therapeutic purposes). It is a simple and routine matter to determine such lengths.

In one embodiment, the invention features a double stranded oligonucleotide construct having Formula DFO-I(a):

5'-p-X Z X'-3'
3'-X' Z X-p-5'

wherein Z comprises a palindromic or repeat nucleic acid sequence or palindromic or repeat-like nucleic acid sequence with one or more modified nucleotides (e.g., nucleotides with a modified base, such as 2-amino purine, 2-amino-1,6-dihydro purine or a universal base), for example of length about 2 to about 24 nucleotides in even numbers (e.g., about 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 or 24 nucleotides), X represents a nucleic acid sequence, for example of length about 1 to about 21 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleotides), X' comprises a nucleic acid sequence, for example of length about 1 to about 21 nucleotides (e.g., about 1, 2, 3, 4, 5, 6,

7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleotides) having nucleotide sequence complementarity to sequence X or a portion thereof, p comprises a terminal phosphate group that can be present or absent, and wherein each X and Z independently comprises a nucleotide sequence that is complementary to a target nucleic acid sequence or a portion thereof and is of length sufficient to interact with the target nucleic acid sequence of a portion thereof. For example, sequence X independently can comprise a sequence from about 12 to about 21 or more nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more) in length that is complementary to a nucleotide sequence in a target RNA or a portion thereof. In another non-limiting example, the length of the nucleotide sequence of X and Z together (when X is present) that is complementary to the target RNA or a portion thereof is from about 12 to about 21 or more nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more). In yet another non-limiting example, when X is absent, the length of the nucleotide sequence of Z that is complementary to the target RNA or a portion thereof is from about 12 to about 24 or more nucleotides (e.g., about 12, 14, 16, 18, 20, 22, 24 or more). In one embodiment X, Z and X' are independently oligonucleotides, where X and/or Z comprises a nucleotide sequence of length sufficient to interact (e.g., base pair) with nucleotide sequence in the target RNA or a portion thereof. In one embodiment, the lengths of oligonucleotides X and X' are identical. In another embodiment, the lengths of oligonucleotides X and X' are not identical. In another embodiment, the lengths of oligonucleotides X and Z or Z and X' or X, Z and X' are either identical or different. In one embodiment, the double stranded oligonucleotide construct of Formula I(a) includes one or more, specifically 1, 2, 3 or 4, mismatches, to the extent such mismatches do not significantly diminish the ability of the double stranded oligonucleotide to inhibit target gene expression.

In one embodiment, a DFO molecule of the invention comprises structure having Formula DFO-II:

5'-p-X X'-3'

wherein each X and X' are independently oligonucleotides of length about 12 nucleotides to about 21 nucleotides, wherein X comprises, for example, a nucleic acid sequence of length about 12 to about 21 nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleotides), X' comprises a nucleic acid sequence, for example of length about 12 to about

21 nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleotides) having nucleotide sequence complementarity to sequence X or a portion thereof, p comprises a terminal phosphate group that can be present or absent, and wherein X comprises a nucleotide sequence that is complementary to a target nucleic acid sequence (e.g., RNA) or a portion thereof and is of length sufficient to interact (e.g., base pair) with the target nucleic acid sequence of a portion thereof. In one embodiment, the length of oligonucleotides X and X' are identical. In another embodiment the length of oligonucleotides X and X' are not identical. In one embodiment, length of the oligonucleotides X and X' are sufficint to form a relatively stable double stranded oligonucleotide.

In one embodiment, the invention features a double stranded oligonucleotide construct having Formula DFO-II(a):

5'-p-X X'-3'
3'-X' X-p-5'

wherein each X and X' are independently oligonucleotides of length about 12 nucleotides to about 21 nucleotides, wherein X comprises a nucleic acid sequence, for example of length about 12 to about 21 nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleotides), X' comprises a nucleic acid sequence, for example of length about 12 to about 21 nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleotides) having nucleotide sequence complementarity to sequence X or a portion thereof, p comprises a terminal phosphate group that can be present or absent, and wherein X comprises nucleotide sequence that is complementary to a target nucleic acid sequence or a portion thereof and is of length sufficient to interact (e.g., base pair) with the target nucleic acid sequence (e.g., RNA) of a portion thereof. In one embodiment, the lengths of oligonucleotides X and X' are identical. In another embodiment, the lengths of oligonucleotides X and X' are not identical. In one embodiment, the lengths of the oligonucleotides X and X' are sufficint to form a relatively stable double stranded oligonucleotide. In one embodiment, the double stranded oligonucleotide construct of Formula II(a) includes one or more, specifically 1, 2, 3 or 4 , mismatches, to the extent such mismatches do not significantly diminish the ability of the double stranded oligonucleotide to inhibit target gene expression.

In one embodiment, the invention features a DFO molecule having Formula DFO-I(b):

5'-p-Z-3'

where Z comprises a palindromic or repeat nucleic acid sequence optionally including one or more non-standard or modified nucleotides (e.g., nucleotide with a modified base, such as 2-amino purine or a universal base) that can facilitate base-pairing with other nucleotides. Z can be, for example, of length sufficient to interact (e.g., base pair) with nucleotide sequence of a target nucleic acid (e.g., RNA) molecule, preferably of length of at least 12 nucleotides, specifically about 12 to about 24 nucleotides (e.g., about 12, 14, 16, 18, 20, 22 or 24 nucleotides). p represents a terminal phosphate group that can be present or absent.

In one embodiment, a DFO molecule having any of Formula DFO-I, DFO-I(a), DFO-I(b), DFO-II(a) or DFO-II can comprise chemical modifications as described herein without limitation, such as, for example, nucleotides having any of Formulae I-VII, stabilization chemistries as described in **Table IV**, or any other combination of modified nucleotides and non-nucleotides as described in the various embodiments herein.

In one embodiment, the palidrome or repeat sequence or modified nucleotide (e.g., nucleotide with a modified base, such as 2-amino purine or a universal base) in Z of DFO constructs having Formula DFO-I, DFO-I(a) and DFO-I(b), comprises chemically modified nucleotides that are able to interact with a portion of the target nucleic acid sequence (e.g., modified base analogs that can form Watson Crick base pairs or non-Watson Crick base pairs).

In one embodiment, a DFO molecule of the invention, for example a DFO having Formula DFO-I or DFO-II, comprises about 15 to about 40 nucleotides (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides). In one embodiment, a DFO molecule of the invention comprises one or more chemical modifications. In a non-limiting example, the introduction of chemically modified nucleotides and/or non-nucleotides into nucleic acid molecules of the invention provides a powerful tool in overcoming potential limitations of *in vivo* stability and bioavailability inherent to unmodified RNA molecules that are delivered exogenously. For

example, the use of chemically modified nucleic acid molecules can enable a lower dose of a particular nucleic acid molecule for a given therapeutic effect since chemically modified nucleic acid molecules tend to have a longer half-life in serum or in cells or tissues. Furthermore, certain chemical modifications can improve the bioavailability and/or potency of nucleic acid molecules by not only enhancing half-life but also facilitating the targeting of nucleic acid molecules to particular organs, cells or tissues and/or improving cellular uptake of the nucleic acid molecules. Therefore, even if the activity of a chemically modified nucleic acid molecule is reduced in vitro as compared to a native/unmodified nucleic acid molecule, for example when compared to an unmodified RNA molecule, the overall activity of the modified nucleic acid molecule can be greater than the native or unmodified nucleic acid molecule due to improved stability, potency, duration of effect, bioavailability and/or delivery of the molecule.

Multifunctional or Multi-targeted siNA molecules of the Invention

In one embodiment, the invention features siNA molecules comprising multifunctional short interfering nucleic acid (multifunctional siNA) molecules that modulate the expression of one or more genes in a biologic system, such as a cell, tissue, or organism. The multifunctional short interfering nucleic acid (multifunctional siNA) molecules of the invention can target more than one region of the target nucleic acid sequence or can target sequences of more than one distinct target nucleic acid molecules. The multifunctional siNA molecules of the invention can be chemically synthesized or expressed from transcription units and/or vectors. The multifunctional siNA molecules of the instant invention provide useful reagents and methods for a variety of human applications, therapeutic, diagnostic, agricultural, veterinary, target validation, genomic discovery, genetic engineering and pharmacogenomic applications.

Applicant demonstrates herein that certain oligonucleotides, refered to herein for convenience but not limitation as multifunctional short interfering nucleic acid or multifunctional siNA molecules, are potent mediators of sequence specific regulation of gene expression. The multifunctional siNA molecules of the invention are distinct from other nucleic acid sequences known in the art (e.g., siRNA, miRNA, stRNA, shRNA, antisense oligonucleotides, *etc*.) in that they represent a class of polynucleotide molecules that are designed such that each strand in the multifunctional siNA construct comprises a

nucleotide sequence that is complementary to a distinct nucleic acid sequence in one or more target nucleic acid molecules. A single multifunctional siNA molecule (generally a double-stranded molecule) of the invention can thus target more than one (e.g., 2, 3, 4, 5, or more) differing target nucleic acid target molecules. Nucleic acid molecules of the invention can also target more than one (e.g., 2, 3, 4, 5, or more) region of the same target nucleic acid sequence. As such multifunctional siNA molecules of the invention are useful in down regulating or inhibiting the expression of one or more target nucleic acid molecules. For example, a multifunctional siNA molecule of the invention can target nucleic acid molecules encoding a cytokine and its corresponding receptor(s), nucleic acid molecules encoding a virus or viral proteins and corresponding cellular proteins required for viral infection and/or replication, or differing strains of a particular virus. By reducing or inhibiting expression of more than one target nucleic acid molecule with one multifunctional siNA construct, multifunctional siNA molecules of the invention represent a class of potent therapeutic agents that can provide simultaneous inhibition of multiple targets within a disease related pathway. Such simultaneous inhibition can provide synergistic therapeutic treatment strategies without the need for separate preclinical and clinical development efforts or complex regulatory approval process.

Use of multifunctional siNA molecules that target more then one region of a target nucleic acid molecule (e.g., messenger RNA) is expected to provide potent inhibition of gene expression. For example, a single multifunctional siNA construct of the invention can target both conserved and variable regions of a target nucleic acid molecule, thereby allowing down regulation or inhibition of different splice variants encoded by a single gene, or allowing for targeting of both coding and non-coding regions of a target nucleic acid molecule.

Generally, double stranded oligonucleotides are formed by the assembly of two distinct oligonucleotides where the oligonucleotide sequence of one strand is complementary to the oligonucleotide sequence of the second strand; such double stranded oligonucleotides are generally assembled from two separate oligonucleotides (e.g., siRNA). Alternately, a duplex can be formed from a single molecule that folds on itself (e.g., shRNA or short hairpin RNA). These double stranded oligonucleotides are known in the art to mediate RNA interference and all have a common feature wherein only one nucleotide sequence region (guide sequence or the antisense sequence) has complementarity to a

target nucleic acid sequence and the other strand (sense sequence) comprises nucleotide sequence that is homologous to the target nucleic acid sequence. Generally, the antisense sequence is retained in the active RISC complex and guides the RISC to the target nucleotide sequence by means of complementary base-pairing of the antisense sequence with the target seqeunce for mediating sequence-specific RNA interference. It is known in the art that in some cell culture systems, certain types of unmodified siRNAs can exhibit "off target" effects. It is hypothesized that this off-target effect involves the participation of the sense sequence instead of the antisense sequence of the siRNA in the RISC complex (see for example Schwarz et al., 2003, Cell, 115, 199-208). In this instance the sense sequence is believed to direct the RISC complex to a sequence (off-target sequence) that is distinct from the intended target sequence, resulting in the inhibition of the off-target sequence. In these double stranded nucleic acid molecules, each strand is complementary to a distinct target nucleic acid sequence. However, the off-targets that are affected by these dsRNAs are not entirely predictable and are non-specific.

Distinct from the double stranded nucleic acid molecules known in the art, the applicants have developed a novel, potentially cost effective and simplified method of down regulating or inhibiting the expression of more than one target nucleic acid sequence using a single multifunctional siNA construct. The multifunctional siNA molecules of the invention are designed to be double-stranded or partially double stranded, such that a portion of each strand or region of the multifunctional siNA is complementary to a target nucleic acid sequence of choice. As such, the multifunctional siNA molecules of the invention are not limited to targeting sequences that are complementary to each other, but rather to any two differing target nucleic acid sequences. Multifunctional siNA molecules of the invention are designed such that each strand or region of the multifunctional siNA molecule, that is complementary to a given target nucleic acid sequence, is of suitable length (*e.g.*, from about 16 to about 28 nucleotides in length, preferably from about 18 to about 28 nucleotides in length) for mediating RNA interference against the target nucleic acid sequence. The complementarity between the target nucleic acid sequence and a strand or region of the multifunctional siNA must be sufficient (at least about 8 base pairs) for cleavage of the target nucleic acid sequence by RNA interference. multifunctional siNA of the invention is expected to minimize off-target effects seen with certain siRNA sequences, such as those described in (Schwarz *et al.*, supra).

It has been reported that dsRNAs of length between 29 base pairs and 36 base pairs (Tuschl *et al.,* International PCT Publication No. WO 02/44321) do not mediate RNAi. One reason these dsRNAs are inactive may be the lack of turnover or dissociation of the strand that interacts with the target RNA sequence, such that the RISC complex is not able to efficiently interact with multiple copies of the target RNA resulting in a significant decrease in the potency and efficiency of the RNAi process. Applicant has surprisingly found that the multifunctional siNAs of the invention can overcome this hurdle and are capable of enhancing the efficiency and potency of RNAi process. As such, in certain embodiments of the invention, multifunctional siNAs of length between about 29 to about 36 base pairs can be designed such that, a portion of each strand of the multifunctional siNA molecule comprises a nucleotide sequence region that is complementary to a target nucleic acid of length sufficient to mediate RNAi efficiently (e.g., about 15 to about 23 base pairs) and a nucleotide sequence region that is not complementary to the target nucleic acid. By having both complementary and non-complementary portions in each strand of the multifunctional siNA, the multifunctional siNA can mediate RNA interference against a target nucleic acid sequence without being prohibitive to turnover or dissociation (*e.g.*, where the length of each strand is too long to mediate RNAi against the respective target nucleic acid sequence). Furthermore, design of multifunctional siNA molecules of the invention with internal overlapping regions allows the multifunctional siNA molecules to be of favorable (decreased) size for mediating RNA interference and of size that is well suited for use as a therapeutic agent (e.g., wherein each strand is independently from about 18 to about 28 nucleotides in length). Non-limiting examples are lillustrated in the enclosed **Figures 96-101 and 112**.

In one embodiment, a multifunctional siNA molecule of the invention comprises a first region and a second region, where the first region of the multifunctional siNA comprises A nucleotide sequence complementary to a nucleic acid sequence of a first target nucleic acid molecule, and the second region of the multifunctional siNA comprises nucleic acid sequence complementary to a nucleic acid sequence of a second target nucleic acid molecule. In one embodiment, a multifunctional siNA molecule of the invention comprises a first region and a second region, where the first region of the multifunctional siNA comprises nucleotide sequence complementary to a nucleic acid sequence of the first region of a target nucleic acid molecule, and the second region of the multifunctional siNA

comprises nucleotide sequence complementary to a nucleic acid sequence of a second region of a the target nucleic acid molecule. In another embodiment, the first region and second region of the multifunctional siNA can comprise separate nucleic acid sequences that share some degree of complementarity (e.g., from about 1 to about 10 complementary nucleotides). In certain embodiments, multifunctional siNA constructs comprising separate nucleic acid seqeunces can be readily linked post-synthetically by methods and reagents known in the art and such linked constructs are within the scope of the invention. Alternately, the first region and second region of the multifunctional siNA can comprise a single nucleic acid sequence having some degree of self complementarity, such as in a hairpin or stem-loop structure. Non-limiting examples of such double stranded and hairpin multifunctional short interfering nucleic acids are illustrated in **Figures 96 and 97** respectively. These multifunctional short interfering nucleic acids (multifunctional siNAs) can optionally include certain overlapping nucleotide sequence where such overlapping nucleotide sequence is present in between the first region and the second region of the multifunctional siNA (see for example **Figures 98 and 99**).

In one embodiment, the invention features a multifunctional short interfering nucleic acid (multifunctional siNA) molecule, wherein each strand of the the multifunctional siNA independently comprises a first region of nucleic acid sequence that is complementary to a distinct target nucleic acid sequence and the second region of nucleotide sequence that is not complementary to the target sequence. The target nucleic acid sequence of each strand is in the same target nucleic acid molecule or different target nucleic acid molecules.

In another embodiment, the multifunctional siNA comprises two strands, where: (a) the first strand comprises a region having sequence complementarity to a target nucleic acid sequence (complementary region 1) and a region having no sequence complementarity to the target nucleotide sequence (non-complementary region 1); (b) the second strand of the multifunction siNA comprises a region having sequence complementarity to a target nucleic acid sequence that is distinct from the target nucleotide sequence complementary to the first strand nucleotide sequence (complementary region 2), and a region having no sequence complementarity to the target nucleotide sequence of complementary region 2 (non-complementary region 2); (c) the complementary region 1 of the first strand comprises a nucleotide sequence that is complementary to a nucleotide sequence in the non-complementary region 2 of the second strand and the complementary region 2 of the

second strand comprises a nucleotide sequence that is complementary to a nucleotide sequence in the non-complementary region 1 of the first strand. The target nucleic acid sequence of complementary region 1 and complementary region 2 is in the same target nucleic acid molecule or different target nucleic acid molecules.

In another embodiment, the multifunctional siNA comprises two strands, where: (a) the first strand comprises a region having sequence complementarity to a target nucleic acid sequence derived from a gene (e.g., mammalian gene, viral gene or genome, bacterial gene or a plant gene) (complementary region 1) and a region having no sequence complementarity to the target nucleotide sequence of complementary region 1 (non-complementary region 1); (b) the second strand of the multifunction siNA comprises a region having sequence complementarity to a target nucleic acid sequence derived from a gene that is distinct from the gene of complementary region 1 (complementary region 2), and a region having no sequence complementarity to the target nucleotide sequence of complementary region 2 (non-complementary region 2); (c) the complementary region 1 of the first strand comprises a nucleotide sequence that is complementary to a nucleotide sequence in the non-complementary region 2 of the second strand and the complementary region 2 of the second strand comprises a nucleotide sequence that is complementary to a nucleotide sequence in the non-complementary region 1 of the first strand.

In another embodiment, the multifunctional siNA comprises two strands, where: (a) the first strand comprises a region having sequence complementarity to a target nucleic acid sequence derived from a gene (e.g., mammalian gene, viral gene or genome, bacterial gene or a plant gene) (complementary region 1) and a region having no sequence complementarity to the target nucleotide sequence of complementary region 1 (non-complementary region 1); (b) the second strand of the multifunction siNA comprises a region having sequence complementarity to a target nucleic acid sequence distinct from the target nucleic acid sequence of complementary region 1(complementary region 2), provided, however, that the target nucleic acid sequence for complementary region 1 and target nucleic acid sequence for complementary region 2 are both derived from the same gene, and a region having no sequence complementarity to the target nucleotide sequence of complementary region 2 (non-complementary region 2); (c) the complementary region 1 of the first strand comprises a nucleotide sequence that is complementary to a nucleotide sequence in the non-complementary region 2 of the second strand and the complementary

region 2 of the second strand comprises a nucleotide sequence that is complementary to nucleotide sequence in the non-complementary region 1 of the first strand.

In one embodiment, the invention features a multifunctional short interfering nucleic acid (multifunctional siNA) molecule, wherein the multifunctional siNA comprises two complementary nucleic acid sequences in which the first sequence comprises a first region having nucleotide sequence complementary to nucleotide sequence within a target nucleic acid molecule, and in which the second seqeunce comprises a first region having nucleotide sequence complementary to a distinct nucleotide sequence within the same target nucleic acid molecule. Preferably, the first region of the first sequence is also complementary to the nucleotide sequence of the second region of the second sequence, and where the first region of the second sequence is complementary to the nucleotide sequence of the second region of the first sequence,

In one embodiment, the invention features a multifunctional short interfering nucleic acid (multifunctional siNA) molecule, wherein the multifunctional siNA comprises two complementary nucleic acid sequences in which the first sequence comprises a first region having a nucleotide sequence complementary to a nucleotide sequence within a first target nucleic acid molecule, and in which the second seqeunce comprises a first region having a nucleotide sequence complementary to a distinct nucleotide sequence within a second target nucleic acid molecule. Preferably, the first region of the first sequence is also complementary to the nucleotide sequence of the second region of the second sequence, and where the first region of the second sequence is complementary to the nucleotide sequence of the second region of the first sequence,

In one embodiment, the invention features a multifunctional siNA molecule comprising a first region and a second region, where the first region comprises a nucleic acid sequence having between about 18 to about 28 nucleotides complementary to a nucleic acid sequence within a first target nucleic acid molecule, and the second region comprises nucleotide sequence having between about 18 to about 28 nucleotides complementary to a distinct nucleic acid sequence within a second target nucleic acid molecule.

In one embodiment, the invention features a multifunctional siNA molecule comprising a first region and a second region, where the first region comprises nucleic acid sequence having between about 18 to about 28 nucleotides complementary to a nucleic acid

sequence within a target nucleic acid molecule, and the second region comprises nucleotide sequence having between about 18 to about 28 nucleotides complementary to a distinct nucleic acid sequence within the same target nucleic acid molecule.

In one embodiment, the invention features a double stranded multifunctional short interfering nucleic acid (multifunctional siNA) molecule, wherein one strand of the multifunctional siNA comprises a first region having nucleotide sequence complementary to a first target nucleic acid sequence, and the second strand comprises a first region having a nucleotide sequence complementary to a second target nucleic acid sequence. The first and second target nucleic acid sequences can be present in separate target nucleic acid molecules or can be different regions within the same target nucleic acid molecule. As such, multifunctional siNA molecules of the invention can be used to target the expression of different genes, splice variants of the same gene, both mutant and conserved regions of one or more gene transcripts, or both coding and non-coding sequences of the same or differeing genes or gene transcripts.

In one embodiment, a target nucleic acid molecule of the invention encodes a single protein. In another embodiment, a target nucleic acid molecule encodes more than one protein (e.g., 1, 2, 3, 4, 5 or more proteins). As such, a multifunctional siNA construct of the invention can be used to down regulate or inhibit the expression of several proteins. For example, a multifunctional siNA molecule comprising a region in one strand having nucleotide sequence complementarity to a first target nucleic acid sequence derived from a gene encoding one protein (e.g., a cytokine, such as vascular endothelial growth factor or VEGF) and the second strand comprising a region with nucleotide sequence complementarity to a second target nucleic acid sequence present in target nucleic acid molecules derived from genes encoding two proteins (e.g., two differing receptors, such as VEGF receptor 1 and VEGF receptor 2, for a single cytokine, such as VEGF) can be used to down regulate, inhibit, or shut down a particular biologic pathway.by targeting, for example, a cytokine and receptors for the cytokine, or a ligand and receptors for the ligand.

In one embodiment the invention takes advantage of conserved nucleotide sequences present in different isoforms of cytokines or ligands and receptors for the cytokines or ligands. By designing multifunctional siNAs in a manner where one strand includes a sequence that is complementary to a target nucleic acid sequence conserved among various

isoforms of a cytokine and the other strand includes sequence that is complementary to a target nucleic acid sequence conserved among the receptors for the cytokine, it is possible to selectively and effectively modulate or inhibit a biological pathway or multiple genes in a biological pathway using a single multifunctional siNA.

In another nonlimiting example, a multifunctional siNA molecule comprising a region in one strand having a nucleotide sequence complementarity to a first target nucleic acid sequence present in target nucleic acid molecules encoding two proteins (e.g., two isoforms of a cytokine such as VEGF, inlcuding for example any of VEGF-A, VEGF-B, VEGF-C, and/or VEGF-D) and the second strand comprising a region with a nucleotide sequence complementarity to a second target nucleic acid sequence present in target nucleotide molecules encoding two additional proteins (e.g., two differing receptors for the cytokine, such as VEGFR1, VEGFR2, and/or VEGFR3) can be used to down regulate, inhibit, or shut down a particular biologic pathway.by targeting different isoforms of a cytokine and receptors for such cytokines.

In another non-limiting example, a multifunctional siNA molecule comprising a region in one strand having a nucleotide sequence complementarity to a first target nucleic acid sequence derived from a target nucleic acid molecule encoding a virus or a viral protein (e.g., HIV) and the second strand comprising a region having a nucleotide sequence complementarity to a second target nucleic acid sequence present in target nucleic acid molecule encoding a cellular protein (e.g., a receptor for the virus, such as CCR5 receptor for HIV) can be used to down regulate, inhibit, or shut down the viral replication and infection by targeting the virus and cellular proteins necessary for viral infection or replication.

In another nonlimiting example, a multifunctional siNA molecule comprising a region in one strand having a nucleotide sequence complementarity to a first target nucleic acid sequence (e.g., conserved sequence) present in a target nucleic acid molecule such as a viral genome (e.g., HIV genomic RNA) and the second strand comprising a region having a nucleotide sequence complementarity to a second target nucleic acid sequence (e.g., conserved sequence) present in target nucleic acid molecule derived from a gene encoding a viral protein (e.g., HIV proteins, such as TAT, REV, ENV or NEF) to down regulate,

inhibit, or shut down the viral replication and infection by targeting the viral genome and viral encoded proteins necessary for viral infection or replication.

In one embodiment the invention takes advantage of conserved nucleotide sequences present in different strains, isotypes or forms of a virus and genes encoded by these different strains, isotypes and forms of the virus. By designing multifunctional siNAs in a manner where one strand includes a sequence that is complementary to target nucleic acid sequence conserved among various strains, isotypes or forms of a virus and the other strand includes sequence that is complementary to target nucleic acid sequence conserved in a protein encoded by the virus, it is possible to selectively and effectively inhibit viral replication or infection using a sinle multifunctional siNA.

In one embodiment, a multifunctional short interfering nucleic acid (multifunctional siNA) of the invention comprises a region in each strand, wherein the region in one strand comprises nucleotide sequence complementary to a cytokine and the region in the second strand comprises nucleotide sequence complementary to a corresponding receptor for the cytokine. Non-limiting examples of cytokines include vascular endothelial growth factors (e.g., VEGF-A, VEGF-B, VEGF-C, VEGF-D), interleukins (e.g., IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13), tumor necrosis factors (e.g., TNF-alpha, TNF-beta), colony stimulating factors (e.g., CSFs), interferons (e.g., IFN-gamma), nerve growth factors (e.g., NGFs), epidermal growth factors (e.g., EGF), platelet derived growth factors (e.g., PDGF), fibroblast growth factors (e.g., FGF), transforming growth factors (e.g., TGF-alpha and TGF-beta), erythropoietins (e.g., Epo), and Insulin like growth factors (e.g., IGF-1, IGF-2) and non-limiting examples of cytokine receptors include receptors for each of the above cytokines.

In one embodiment, a multifunctional short interfering nucleic acid (multifunctional siNA) of the invention comprises a first region and a second region, wherein the first region comprises nucleotide sequence complementary to a viral RNA of a first viral strain and the second region comprises nucleotide sequence complementary to a viral RNA of a second viral strain. In one embodiment, the first and second regions can comprise nucleotide sequence complementary to shared or conserved RNA sequences of differing viral strains or classes or viral strains. Non-limiting examples of viruses include Hepatitis C Virus (HCV), Hepatitis B Virus (HBV), Human Immunodeficiency Virus type 1 (HIV-1),

Human Immunodeficiency Virus type 2 (HIV-2), West Nile Virus (WNV), cytomegalovirus (CMV), respiratory syncytial virus (RSV), influenza virus, rhinovirus, papillomavirus (HPV), Herpes Simplex Virus (HSV), severe acute respiratory virus (SARS), and other viruses such as HTLV.

In one embodiment, a multifunctional short interfering nucleic acid (multifunctional siNA) of the invention comprises a first region and a second region, wherein the first region comprises a nucleotide sequence complementary to a viral RNA encoding one or more viruses (e.g., one or more strains of virus) and the second region comprises a nucleotide sequence complementary to a viral RNA encoding one or more interferon agonist proteins. In one embodiment, the first region can comprise a nucleotide sequence complementary to shared or conserved RNA sequences of differing viral strains or classes or viral strains. Non-limiting examples of viruses include Hepatitis C Virus (HCV), Hepatitis B Virus (HBV), Human Immunodeficiency Virus type 1 (HIV-1), Human Immunodeficiency Virus type 2 (HIV-2), West Nile Virus (WNV), cytomegalovirus (CMV), respiratory syncytial virus (RSV), influenza virus, rhinovirus, papillomavirus (HPV), Herpes Simplex Virus (HSV), severe acute respiratory virus (SARS), and other viruses such as HTLV. Non-limiting example of interferon agonist proteins include any protein that is capable of inhibition or suppressing RNA silencing (e.g., RNA binding proteins such as E3L or NS1 or equivalents thereof, see for example Li *et al.,* 2004, *PNAS*, 101, 1350-1355)

In one embodiment, a multifunctional short interfering nucleic acid (multifunctional siNA) of the invention comprises a first region and a second region, wherein the first region comprises nucleotide sequence complementary to a viral RNA and the second region comprises nucleotide sequence complementary to a cellular RNA that is involved in viral infection and/or replication. Non-limiting examples of viruses include Hepatitis C Virus (HCV), Hepatitis B Virus (HBV), Human Immunodeficiency Virus type 1 (HIV-1), Human Immunodeficiency Virus type 2 (HIV-2), West Nile Virus (WNV), cytomegalovirus (CMV), respiratory syncytial virus (RSV), influenza virus, rhinovirus, papillomavirus (HPV), Herpes Simplex Virus (HSV), severe acute respiratory virus (SARS), and other viruses such as HTLV. Non-limiting examples of cellular RNAs involved in viral infection and/or replication include cellular receptors, cell surface molecules, cellular enzymes, cellular transcription factors, and/or cytokines, second messengers, and cellular accessory molecules including, but not limited to, interferon

agonsit proteins (e.g., E3L or NS1 or equivalents thereof, see for example Li *et al.,* 2004, *PNAS*, 101, 1350-1355), interferon regulatory factors (IRFs); cellular PKR protein kinase (PKR); human eukaryotic initiation factors 2B (eIF2B gamma and/or eIF2gamma); human DEAD Box protein (DDX3); and cellular proteins that bind to the poly(U) tract of the HCV 3'-UTR, such as polypyrimidine tract-binding protein, CD4 receptors, CXCR4 (Fusin; LESTR; NPY3R); CCR5 (CKR-5, CMKRB5); CCR3 (CC-CKR-3, CKR-3, CMKBR3); CCR2 (CCR2b, CMKBR2); CCR1 (CKR1, CMKBR1); CCR4 (CKR-4); CCR8 (ChemR1, TER1, CMKBR8); CCR9 (D6); CXCR2 (IL-8RB); STRL33 (Bonzo; TYMSTR); US28; V28 (CMKBRL1, CX3CR1, GPR13); GPR1; GPR15 (BOB); Apj (AGTRL1); ChemR23 receptors, Heparan Sulfate Proteoglycans, HSPG2; SDC2; SDC4; GPC1; SDC3; SDC1; Galactoceramides; Erythrocyte-expressed Glycolipids; N-myristoyltransferase (NMT, NMT2); Glycosylation Enzymes; gp-160 Processing Enzymes (PCSK5); Ribonucleotide Reductase; Polyamine Biosynthesis enzymes; SP-1; NF-kappa B (NFKB2, RELA, and NFKB1); Tumor Necrosis Factor-alpha (TNF-alpha); Interleukin 1 alpha (IL-1 alpha); Interleukin 6 (IL-6); Phospholipase C (PLC); Protein Kinase C (PKC), Cyclophilins, (PPID, PPIA, PPIE, PPIB, PPIF, PPIG, and PPIC); Mitogen Activated Protein Kinase (MAP-Kinase, MAPK1); and Extracellular Signal-Regulated Kinase (ERK-Kinase), (see for example Schang, 2002, *Journal of Antimicrobial Chemotherapy,* 50, 779–792 and Ludwig *et al.,* 2003, *Trends. Mol. Med.,* 9, 46-52).

In one embodiment, a double stranded multifunctional siNA molecule of the invention comprises a structure having Formula MF-I:

5'-p-X Z X'-3'
3'-Y' Z Y-p-5'

wherein each 5'-p-XZX'-3' and 5'-p-YZY'-3' are independently an oligonucleotide of length between about 20 nucleotides and about 300 nucleotides, preferably between about 20 and about 200 nucleotides, about 20 and about 100 nucleotides, about 20 and about 40 nucleotides, about 20 and about 40 nucleotides, about 24 and about 38 nucleotides, or about 26 and about 38 nucleotides; XZ comprises a nucleic acid sequence that is complementary to a first target nucleic acid sequence; YZ is an oligonucleotide comprising nucleic acid sequence that is complementary to a second target nucleic acid sequence; Z comprises nucleotide sequence of length about 1 to about 24 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7,

8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 nucleotides) that is self complimentary; X comprises nucleotide sequence of length about 1 to about 100 nucleotides, preferably about 1 to about 21 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleotides) that is complementary to nucleotide sequence present in region Y'; Y comprises nucleotide sequence of length about 1 to about 100 nucleotides, prefereably about 1- about 21 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleotides) that is complementary to nucleotide sequence present in region X'; each p comprises a terminal phosphate group that is independently present or absent; each XZ and YZ is independently of length sufficient to stably interact (i.e., base pair) with the first and second target nucleic acid sequence, respectively, or a portion thereof. For example, each sequence X and Y can independently comprise sequence from about 12 to about 21 or more nucleotides in length (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more) that is complementary to a target nucleotide sequence in different target nucleic acid molecules, such as target RNAs or a portion thereof. In another non-limiting example, the length of the nucleotide sequence of X and Z together that is complementary to the first target nucleic acid sequence (e.g., RNA) or a portion thereof is from about 12 to about 21 or more nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more). In another non-limiting example, the length of the nucleotide sequence of Y and Z together, that is complementary to the second target nucleic acid sequence (e.g., RNA) or a portion thereof is from about 12 to about 21 or more nucleotides (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more). In one embodiment, the first target nucleic acid sequence and the second target nucleic acid sequence are present in the same target nucleic acid molecule. In another embodiment, the first target nucleic acid sequence and the second target nucleic acid sequence are present in different target nucleic acid molecules. In one embodiment, Z comprises a palindrome or a repeat sequence. In one embodiment, the lengths of oligonucleotides X and X' are identical. In another embodiment, the lengths of oligonucleotides X and X' are not identical. In one embodiment, the lengths of oligonucleotides Y and Y' are identical. In another embodiment, the lengths of oligonucleotides Y and Y' are not identical. In one embodiment, the double stranded oligonucleotide construct of Formula I(a) includes one or more, specifically 1, 2, 3 or 4, mismatches, to the extent such mismatches do not significantly diminish the ability of the double stranded oligonucleotide to inhibit target gene expression.

In one embodiment, a multifunctional siNA molecule of the invention comprises a structure having Formula MF-II:

5'-p-X X'-3'
3'-Y' Y-p-5'

wherein each 5'-p-XX'-3' and 5'-p-YY'-3' are independently an oligonucleotide of length between about 20 nucleotides and about 300 nucleotides, preferably between about 20 and about 200 nucleotides, about 20 and about 100 nucleotides, about 20 and about 40 nucleotides, about 20 and about 40 nucleotides, about 24 and about 38 nucleotides, or about 26 and about 38 nucleotides; X comprises a nucleic acid sequence that is complementary to a first target nucleic acid sequence; Y is an oligonucleotide comprising nucleic acid sequence that is complementary to a second target nucleic acid sequence; X comprises a nucleotide sequence of length about 1 to about 100 nucleotides, preferably about 1 to about 21 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 nucleotides) that is complementary to nucleotide sequence present in region Y'; Y comprises nucleotide sequence of length about 1 to about 100 nucleotides, prefereably about 1 to about 21 nucleotides (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 nucleotides) that is complementary to nucleotide sequence present in region X'; each p comprises a terminal phosphate group that is independently present or absent; each X and Y independently is of length sufficient to stably interact (i.e., base pair) with the first and second target nucleic acid sequence, respectively, or a portion thereof. For example, each sequence X and Y can independently comprise sequence from about 12 to about 21 or more nucleotides in length (e.g., about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or more) that is complementary to a target nucleotide sequence in different target nucleic acid molecules, such as target RNAs or a portion thereof. In one embodiment, the first target nucleic acid sequence and the second target nucleic acid sequence are present in the same target nucleic acid molecule. In another embodiment, the first target nucleic acid sequence and the second target nucleic acid sequence are present in different target nucleic acid molecules. In one embodiment, Z comprises a palindrome or a repeat sequence. In one embodiment, the lengths of oligonucleotides X and X' are identical. In another embodiment, the lengths of oligonucleotides X and X' are not identical. In one embodiment, the lengths of oligonucleotides Y and Y' are identical. In another embodiment, the lengths of oligonucleotides Y and Y' are not identical. In one

embodiment, the double stranded oligonucleotide construct of Formula I(a) includes one or more, specifically 1, 2, 3 or 4, mismatches, to the extent such mismatches do not significantly diminish the ability of the double stranded oligonucleotide to inhibit target gene expression.

In one embodiment, a multifunctional siNA molecule of the invention comprises a structure having Formula MF-III:

X X'
Y'-W-Y

wherein each X, X', Y, and Y' is independently an oligonucleotide of length between about 15 nucleotides and about 50 nucleotides, preferably between about 18 and about 40 nucleotides, or about 19 and about 23 nucleotides; X comprises nucleotide sequence that is complementary to nucleotide sequence present in region Y'; X' comprises nucleotide sequence that is complementary to nucleotide sequence present in region Y; each X and X' is independently of length sufficient to stably interact (i.e., base pair) with a first and a second target nucleic acid sequence, respectively, or a portion thereof; W represents a nucleotide or non-nucleotide linker that connects sequences Y' and Y; and the multifunctional siNA directs cleavage of the first and second target sequence via RNA interference. In one embodiment, region W connects the 3'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, region W connects the 3'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X'. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y'. In one embodiment, W connects sequences Y and Y' via a biodegradable linker. In one embodiment, W further comprises a conjugate, lable, aptamer, ligand, lipid, or polymer.

In one embodiment, a multifunctional siNA molecule of the invention comprises a structure having Formula MF-IV:

X X'
Y'-W-Y

wherein each X, X', Y, and Y' is independently an oligonucleotide of length between about 15 nucleotides and about 50 nucleotides, preferably between about 18 and about 40 nucleotides, or about 19 and about 23 nucleotides; X comprises nucleotide sequence that is complementary to nucleotide sequence present in region Y'; X' comprises nucleotide sequence that is complementary to nucleotide sequence present in region Y; each Y and Y' is independently of length sufficient to stably interact (i.e., base pair) with a first and a second target nucleic acid sequence, respectively, or a portion thereof; W represents a nucleotide or non-nucleotide linker that connects sequences Y' and Y; and the multifunctional siNA directs cleavage of the first and second target sequence via RNA interference. In one embodiment, region W connects the 3'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, region W connects the 3'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X'. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y'. In one embodiment, W connects sequences Y and Y' via a biodegradable linker. In one embodiment, W further comprises a conjugate, lable, aptamer, ligand, lipid, or polymer.

In one embodiment, a multifunctional siNA molecule of the invention comprises a structure having Formula MF-V:

X X'
Y'-W-Y

wherein each X, X', Y, and Y' is independently an oligonucleotide of length between about 15 nucleotides and about 50 nucleotides, preferably between about 18 and about 40 nucleotides, or about 19 and about 23 nucleotides; X comprises nucleotide sequence that is complementary to nucleotide sequence present in region Y'; X' comprises nucleotide sequence that is complementary to nucleotide sequence present in region Y; each X, X', Y,

or Y' is independently of length sufficient to stably interact (i.e., base pair) with a first, second, third, or fourth target nucleic acid sequence, respectively, or a portion thereof; W represents a nucleotide or non-nucleotide linker that connects sequences Y' and Y; and the multifunctional siNA directs cleavage of the first, second, third, and/or fourth target sequence via RNA interference. In one embodiment, region W connects the 3'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, region W connects the 3'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 5'-end of sequence Y. In one embodiment, region W connects the 5'-end of sequence Y' with the 3'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence X'. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y. In one embodiment, a terminal phosphate group is present at the 5'-end of sequence Y'. In one embodiment, W connects sequences Y and Y' via a biodegradable linker. In one embodiment, W further comprises a conjugate, lable, aptamer, ligand, lipid, or polymer.

In one embodiment, regions X and Y of multifunctional siNA molecule of the invention (e.g., having any of Formula MF-I - MF-V), are complementary to different target nucleic acid sequences that are portions of the same target nucleic acid molecule. In one embodiment, such target nucleic acid sequences are at different locations within the coding region of a RNA transcript. In one embodiment, such target nucleic acid sequences comprise coding and non-coding regions of the same RNA transcript. In one embodiment, such target nucleic acid sequences comprise regions of alternately spliced transcripts or precursors of such alternately spliced transcripts.

In one embodiment, a multifunctional siNA molecule having any of Formula MF-I - MF-V can comprise chemical modifications as described herein without limitation, such as, for example, nucleotides having any of Formulae I-VII described herein, stabilization chemistries as described in **Table IV**, or any other combination of modified nucleotides and non-nucleotides as described in the various embodiments herein.

In one embodiment, the palidrome or repeat sequence or modified nucleotide (e.g., nucleotide with a modified base, such as 2-amino purine or a universal base) in Z of multifunctional siNA constructs having Formula MF-I or MF-II comprises chemically

modified nucleotides that are able to interact with a portion of the target nucleic acid sequence (e.g., modified base analogs that can form Watson Crick base pairs or non-Watson Crick base pairs).

In one embodiment, a multifunctional siNA molecule of the invention, for example each strand of a multifunctional siNA having MF-I – MF-V, independently comprises about 15 to about 40 nucleotides (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides). In one embodiment, a multifunctional siNA molecule of the invention comprises one or more chemical modifications. In a non-limiting example, the introduction of chemically modified nucleotides and/or non-nucleotides into nucleic acid molecules of the invention provides a powerful tool in overcoming potential limitations of *in vivo* stability and bioavailability inherent to unmodified RNA molecules that are delivered exogenously. For example, the use of chemically modified nucleic acid molecules can enable a lower dose of a particular nucleic acid molecule for a given therapeutic effect since chemically modified nucleic acid molecules tend to have a longer half-life in serum or in cells or tissues. Furthermore, certain chemical modifications can improve the bioavailability and/or potency of nucleic acid molecules by not only enhancing half-life but also facilitating the targeting of nucleic acid molecules to particular organs, cells or tissues and/or improving cellular uptake of the nucleic acid molecules. Therefore, even if the activity of a chemically modified nucleic acid molecule is reduced *in vitro* as compared to a native/unmodified nucleic acid molecule, for example when compared to an unmodified RNA molecule, the overall activity of the modified nucleic acid molecule can be greater than the native or unmodified nucleic acid molecule due to improved stability, potency, duration of effect, bioavailability and/or delivery of the molecule.

In another embodiment, the invention features multifunctional siNAs, wherein the multifunctional siNAs are assembled from two separate double-stranded siNAs, with one of the ends of each sense strand is tethered to the end of the sense strand of the other siNA molecule, such that the two antisense siNA strands are annealed to their corresponding sense strand that are tethered to each other at one end (see **Figure 110**). The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 5'-end of one sense strand of the siNA is tethered to the 5'- end of the sense strand of the other siNA molecule, such that the 5'-ends of the two antisense siNA strands, annealed to their corresponding sense strand that are tethered to each other at one end, point away (in the opposite direction) from each other (see **Figure 110 (A)**). The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 3'-end of one sense strand of the siNA is tethered to the 3'- end of the sense strand of the other siNA molecule, such that the 5'-ends of the two antisense siNA strands, annealed to their corresponding sense strand that are tethered to each other at one end, face each other (see **Figure 110 (B)**). The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 5'-end of one sense strand of the siNA is tethered to the 3'- end of the sense strand of the other siNA molecule, such that the 5'-end of the one of the antisense siNA strands annealed to their corresponding sense strand that are tethered to each other at one end, faces the 3'-end of the other antisense strand (see **Figure 110 (C-D)**). The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 5'-end of one antisense strand of the siNA is tethered to the 3'- end of the antisense strand of the other siNA molecule, such that the 5'-end of the one of the sense siNA strands annealed to their corresponding antisense sense strand that are tethered to each other at one end, faces the 3'-end of the other sense strand (see **Figure 110 (G-H)**). In one embodiment, the linkage between the 5'-end of the first antisense strand and the 3'-end of the second antisense strand is designed in such a way as to be readily cleavable (e.g., biodegradable

linker) such that the 5'end of each antisense strand of the multifunctional siNA has a free 5'-end suitable to mediate RNA interefence-based cleavage of the target RNA. The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 5'-end of one antisense strand of the siNA is tethered to the 5'- end of the antisense strand of the other siNA molecule, such that the 3'-end of the one of the sense siNA strands annealed to their corresponding antisense sense strand that are tethered to each other at one end, faces the 3'-end of the other sense strand (see **Figure 110 (E)**). In one embodiment, the linkage between the 5'-end of the first antisense strand and the 5'-end of the second antisense strand is designed in such a way as to be readily cleavable (e.g., biodegradable linker) such that the 5'end of each antisense strand of the multifunctional siNA has a free 5'-end suitable to mediate RNA interefence-based cleavage of the target RNA. The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

In one embodiment, the invention features a multifunctional siNA, wherein the multifunctional siNA is assembled from two separate double-stranded siNAs, with the 3'-end of one antisense strand of the siNA is tethered to the 3'- end of the antisense strand of the other siNA molecule, such that the 5'-end of the one of the sense siNA strands annealed to their corresponding antisense sense strand that are tethered to each other at one end, faces the 3'-end of the other sense strand (see **Figure 110 (F)**). In one embodiment, the linkage between the 5'-end of the first antisense strand and the 5'-end of the second antisense strand is designed in such a way as to be readily cleavable (e.g., biodegradable linker) such that the 5'end of each antisense strand of the multifunctional siNA has a free 5'-end suitable to mediate RNA interefence-based cleavage of the target RNA. The tethers or linkers can be nucleotide-based linkers or non-nucleotide based linkers as generally known in the art and as described herein.

There are several potential advantages and variations to this multifunctional approach. For example, when used in combination with target sites having homology, siNAs that target a sequence present in two genes (e.g. Flt-1 site 3646, which targets

VEGF-R1 and R2), the design can be used to target more than two sites. A single multifunctional siNA can be for example, used to target VEGF R1 RNA and VEGF R2 RNA (using one antisense strand of the multifunctional siNA targeting of conserved sequence between to the two RNAs) and VEGF RNA (using the second antisense strand of the multifunctional siNA targeting VEGF RNA. This approach allows targeting of the cytokines and the two main receptors using a single multifunctional siNA.

Synthesis of Nucleic acid Molecules

Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small nucleic acid motifs ("small" refers to nucleic acid motifs no more than 100 nucleotides in length, preferably no more than 80 nucleotides in length, and most preferably no more than 50 nucleotides in length; *e.g.*, individual siNA oligonucleotide sequences or siNA sequences synthesized in tandem) are preferably used for exogenous delivery. The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of protein and/or RNA structure. Exemplary molecules of the instant invention are chemically synthesized, and others can similarly be synthesized.

Oligonucleotides (*e.g.*, certain modified oligonucleotides or portions of oligonucleotides lacking ribonucleotides) are synthesized using protocols known in the art, for example as described in Caruthers *et al.*, 1992, *Methods in Enzymology* 211, 3-19, Thompson *et al.*, International PCT Publication No. WO 99/54459, Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677-2684, Wincott *et al.*, 1997, *Methods Mol. Bio.*, 74, 59, Brennan *et al.*, 1998, *Biotechnol Bioeng.*, 61, 33-45, and Brennan, U.S. Pat. No. 6,001,311. The synthesis of

oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 µmol scale protocol with a 2.5 min coupling step for 2'-O-methylated nucleotides and a 45 second coupling step for 2'-deoxy nucleotides or 2'-deoxy-2'-fluoro nucleotides. **Table V** outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 µmol scale can be performed on a 96-well plate synthesizer, such as the instrument produced by Protogene

(Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 μL of 0.11 M = 6.6 μmol) of 2'-O-methyl phosphoramidite and a 105-fold excess of S-ethyl tetrazole (60 μL of 0.25 M = 15 μmol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 22-fold excess (40 μL of 0.11 M = 4.4 μmol) of deoxy phosphoramidite and a 70-fold excess of S-ethyl tetrazole (40 μL of 0.25 M = 10 μmol) can be used in each coupling cycle of deoxy residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); and oxidation solution is 16.9 mM I_2, 49 mM pyridine, 9% water in THF (PERSEPTIVE™). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide, 0.05 M in acetonitrile) is used.

Deprotection of the DNA-based oligonucleotides is performed as follows: the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aqueous methylamine (1 mL) at 65 °C for 10 minutes. After cooling to –20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H2O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder.

The method of synthesis used for RNA including certain siNA molecules of the invention follows the procedure as described in Usman *et al.,* 1987, *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.,* 1990, *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.,* 1995, *Nucleic Acids Res.* 23, 2677-2684 Wincott *et al.,* 1997, *Methods Mol. Bio.*, 74, 59, and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μmol scale protocol with a 7.5 min coupling step for alkylsilyl protected nucleotides and a 2.5

min coupling step for 2'-O-methylated nucleotides. **Table V** outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 μmol scale can be done on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 μL of 0.11 M = 6.6 μmol) of 2'-O-methyl phosphoramidite and a 75-fold excess of S-ethyl tetrazole (60 μL of 0.25 M = 15 μmol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 66-fold excess (120 μL of 0.11 M = 13.2 μmol) of alkylsilyl (ribo) protected phosphoramidite and a 150-fold excess of S-ethyl tetrazole (120 μL of 0.25 M = 30 μmol) can be used in each coupling cycle of ribo residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution is 16.9 mM I_2, 49 mM pyridine, 9% water in THF (PERSEPTIVE™). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide0.05 M in acetonitrile) is used.

Deprotection of the RNA is performed using either a two-pot or one-pot protocol. For the two-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 minutes. After cooling to –20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H2O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder. The base deprotected oligoribonucleotide is resuspended in anhydrous TEA/HF/NMP solution (300 μL of a solution of 1.5 mL N-methylpyrrolidinone, 750 μL TEA and 1 mL TEA•3HF to provide a 1.4 M HF concentration) and heated to 65 °C. After 1.5 h, the oligomer is quenched with 1.5 M NH_4HCO_3.

Alternatively, for the one-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 33% ethanolic methylamine/DMSO: 1/1 (0.8 mL) at 65 °C for 15 minutes. The vial is brought to room temperature TEA•3HF (0.1 mL) is added and the vial is heated at 65 °C for 15 minutes. The sample is cooled at –20 °C and then quenched with 1.5 M NH_4HCO_3.

For purification of the trityl-on oligomers, the quenched NH_4HCO_3 solution is loaded onto a C-18 containing cartridge that had been prewashed with acetonitrile followed by 50 mM TEAA. After washing the loaded cartridge with water, the RNA is detritylated with 0.5% TFA for 13 minutes. The cartridge is then washed again with water, salt exchanged with 1 M NaCl and washed with water again. The oligonucleotide is then eluted with 30% acetonitrile.

The average stepwise coupling yields are typically >98% (Wincott *et al.,* 1995 *Nucleic Acids Res.* 23, 2677-2684). Those of ordinary skill in the art will recognize that the scale of synthesis can be adapted to be larger or smaller than the example described above including but not limited to 96-well format.

Alternatively, the nucleic acid molecules of the present invention can be synthesized separately and joined together post-synthetically, for example, by ligation (Moore *et al.,* 1992, *Science* 256, 9923; Draper *et al.,* International PCT publication No. WO 93/23569; Shabarova *et al.,* 1991, *Nucleic Acids Research* 19, 4247; Bellon *et al.,* 1997, *Nucleosides & Nucleotides*, 16, 951; Bellon *et al.,* 1997, *Bioconjugate Chem.* 8, 204), or by hybridization following synthesis and/or deprotection.

The siNA molecules of the invention can also be synthesized via a tandem synthesis methodology as described in Example 1 herein, wherein both siNA strands are synthesized as a single contiguous oligonucleotide fragment or strand separated by a cleavable linker which is subsequently cleaved to provide separate siNA fragments or strands that hybridize and permit purification of the siNA duplex. The linker can be a polynucleotide linker or a non-nucleotide linker. The tandem synthesis of siNA as described herein can be readily adapted to both multiwell/multiplate synthesis platforms such as 96 well or similarly larger multi-well platforms. The tandem synthesis of siNA as described herein can also be readily

adapted to large scale synthesis platforms employing batch reactors, synthesis columns and the like.

A siNA molecule can also be assembled from two distinct nucleic acid strands or fragments wherein one fragment includes the sense region and the second fragment includes the antisense region of the RNA molecule.

The nucleic acid molecules of the present invention can be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-fluoro, 2'-*O*-methyl, 2'-H (for a review see Usman and Cedergren, 1992, *TIBS* 17, 34; Usman *et al.,* 1994, *Nucleic Acids Symp. Ser.* 31, 163). siNA constructs can be purified by gel electrophoresis using general methods or can be purified by high pressure liquid chromatography (HPLC; see Wincott *et al., supra*, the totality of which is hereby incorporated herein by reference) and re-suspended in water.

In another aspect of the invention, siNA molecules of the invention are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. The recombinant vectors capable of expressing the siNA molecules can be delivered as described herein, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of siNA molecules.

Optimizing Activity of the nucleic acid molecule of the invention.

Chemically synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) can prevent their degradation by serum ribonucleases, which can increase their potency (see *e.g.,* Eckstein *et al.,* International Publication No. WO 92/07065; Perrault *et al.,* 1990 *Nature* 344, 565; Pieken *et al.,* 1991, *Science* 253, 314; Usman and Cedergren, 1992, *Trends in Biochem. Sci.* 17, 334; Usman *et al.,* International Publication No. WO 93/15187; and Rossi *et al.,* International Publication No. WO 91/03162; Sproat, U.S. Pat. No. 5,334,711; Gold *et al.,* U.S. Pat. No. 6,300,074; and Burgin *et al., supra;* all of which are incorporated by reference herein). All of the above references describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of the nucleic acid molecules described herein. Modifications that enhance

their efficacy in cells, and removal of bases from nucleic acid molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are desired.

There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-fluoro, 2'-*O*-methyl, 2'-O-allyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992, *TIBS.* 17, 34; Usman *et al.,* 1994, *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.,* 1996, *Biochemistry*, 35, 14090). Sugar modification of nucleic acid molecules have been extensively described in the art (see Eckstein *et al., International Publication* PCT No. WO 92/07065; Perrault *et al. Nature*, 1990, *344*, 565-568; Pieken *et al. Science,* 1991, *253*, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.* , 1992, *17*, 334-339; Usman *et al. International Publication* PCT No. WO 93/15187; Sproat, *U.S. Pat.* No. 5,334,711 and Beigelman *et al.,* 1995, *J. Biol. Chem.,* 270, 25702; Beigelman *et al.,* International PCT publication No. WO 97/26270; Beigelman *et al.,* U.S. Pat. No. 5,716,824; Usman *et al.,* U.S. Pat. No. 5,627,053; Woolf *et al.,* International PCT Publication No. WO 98/13526; Thompson *et al.,* USSN 60/082,404 which was filed on April 20, 1998; Karpeisky *et al.,* 1998, *Tetrahedron Lett.*, 39, 1131; Earnshaw and Gait, 1998, *Biopolymers (Nucleic Acid Sciences),* 48, 39-55; Verma and Eckstein, 1998, *Annu. Rev. Biochem.,* 67, 99-134; and Burlina *et al.,* 1997, *Bioorg. Med. Chem.,* 5, 1999-2010).

Such publications describe general
methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into nucleic acid molecules without modulating catalysis. In view of such teachings, similar
modifications can be used as described herein to modify the siNA nucleic acid molecules of the instant invention so long as the ability of siNA to promote RNAi is cells is not significantly inhibited.

While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorodithioate, and/or 5'-methylphosphonate linkages improves stability, excessive modifications can cause some toxicity or decreased activity. Therefore, when designing nucleic acid molecules, the amount of these internucleotide linkages

should be minimized. The reduction in the concentration of these linkages should lower toxicity, resulting in increased efficacy and higher specificity of these molecules.

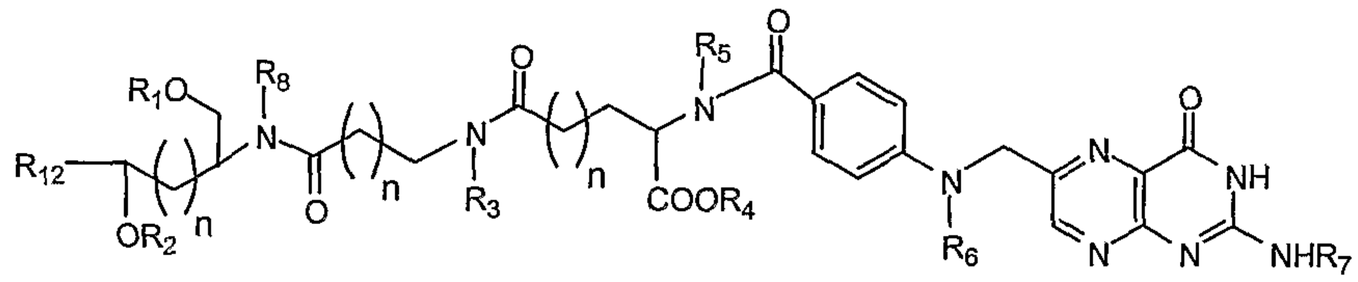
Short interfering nucleic acid (siNA) molecules having chemical modifications that maintain or enhance activity are provided. Such a nucleic acid is also generally more resistant to nucleases than an unmodified nucleic acid. Accordingly, the *in vitro* and/or *in vivo* activity should not be significantly lowered. In cases in which modulation is the goal, therapeutic nucleic acid molecules delivered exogenously should optimally be stable within cells until translation of the target RNA has been modulated long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days depending upon the disease state. Improvements in the chemical synthesis of RNA and DNA (Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677; Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-19) have expanded the ability to
modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability, as described above.

In one embodiment, nucleic acid molecules of the invention include one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) G-clamp nucleotides. A G-clamp nucleotide is a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine within a duplex, see for example Lin and Matteucci, 1998, *J. Am. Chem. Soc.*, 120, 8531-8532. A single G-clamp analog substitution within an oligonucleotide can result in substantially enhanced helical thermal stability and mismatch discrimination when hybridized to complementary oligonucleotides. The inclusion of such nucleotides in nucleic acid molecules of the invention results in both enhanced affinity and specificity to nucleic acid targets, complementary sequences, or template strands. In another embodiment, nucleic acid molecules of the invention include one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) LNA "locked nucleic acid" nucleotides such as a 2', 4'-C methylene bicyclo nucleotide (see for example Wengel *et al.*, International PCT Publication No. WO 00/66604 and WO 99/14226).

In another embodiment, the invention features conjugates and/or complexes of siNA molecules of the invention. Such conjugates and/or complexes can be used to facilitate delivery of siNA molecules into a biological system, such as a cell. The conjugates and

complexes provided by the instant invention can impart therapeutic activity by transferring therapeutic compounds across cellular membranes, altering the pharmacokinetics, and/or modulating the localization of nucleic acid molecules of the invention. The present invention encompasses the design and synthesis of novel conjugates and complexes for the delivery of molecules, including, but not limited to, small molecules, lipids, cholesterol, phospholipids, nucleosides, nucleotides, nucleic acids, antibodies, toxins, negatively charged polymers and other polymers, for example, proteins, peptides, hormones, carbohydrates, polyethylene glycols, or polyamines, across cellular membranes. In general, the transporters described are designed to be used either individually or as part of a multi-component system, with or without degradable linkers. These compounds are expected to improve delivery and/or localization of nucleic acid molecules of the invention into a number of cell types originating from different tissues, in the presence or absence of serum (see Sullenger and Cech, U.S. Pat. No. 5,854,038). Conjugates of the molecules described herein can be attached to biologically active molecules via linkers that are biodegradable, such as biodegradable nucleic acid linker molecules.

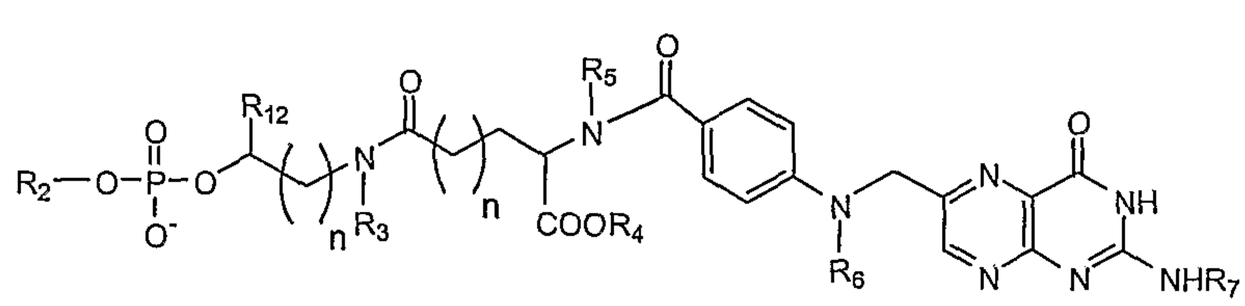
In one embodiment, the invention features a compound having Formula 1:



1

wherein each R_1, R_3, R_4 ,R_5, R_6, R_7 and R_8 is independently hydrogen, alkyl , substituted alkyl, aryl, substituted aryl, or a protecting group, each "n" is independently an integer from 0 to about 200, R_{12} is a straight or branched chain alkyl, substituted alkyl, aryl, or substituted aryl, and R_2 is a siNA molecule or a portion thereof.

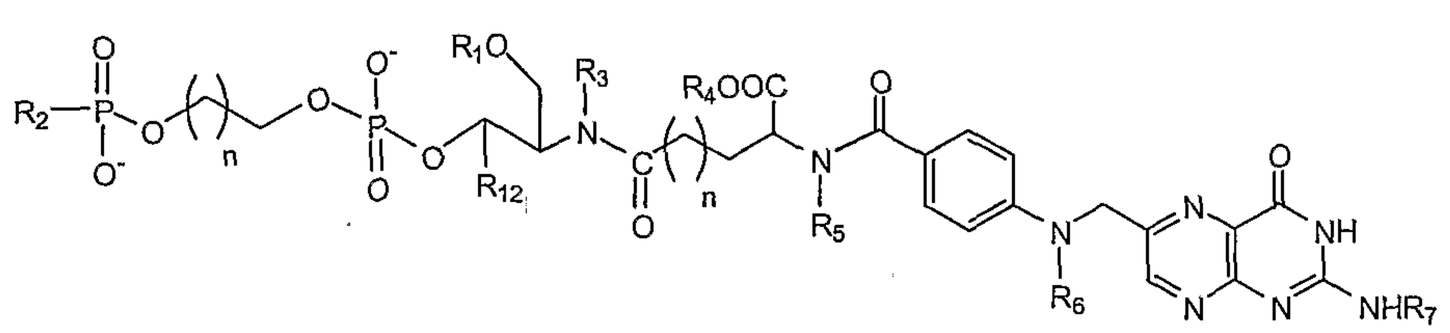
In one embodiment, the invention features a compound having Formula 2:



2

wherein each R_3, R_4 ,R_5, R_6 and R_7 is independently hydrogen, alkyl , substituted alkyl, aryl, substituted aryl, or a protecting group, each "n" is independently an integer from 0 to about 200, R_{12} is a straight or branched chain alkyl, substituted alkyl, aryl, or substituted aryl, and R_2 is a siNA molecule or a portion thereof.

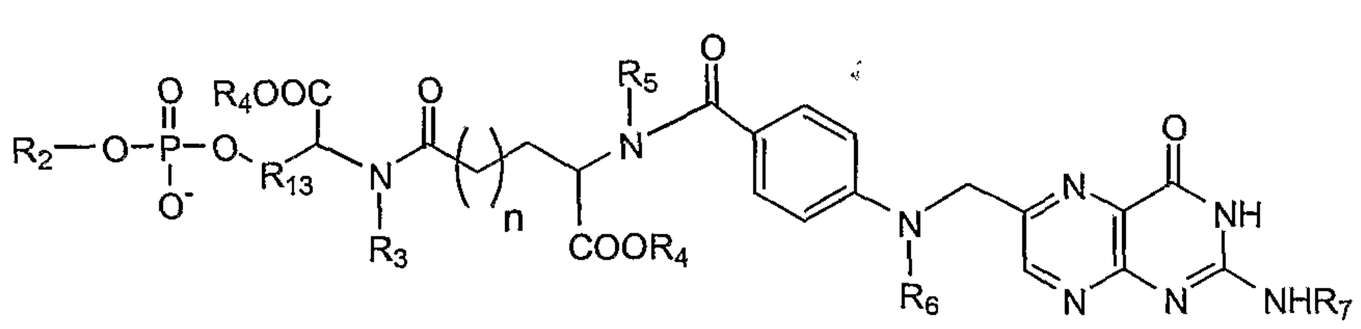
In one embodiment, the invention features a compound having Formula 3:



3

wherein each R_1, R_3, R_4 ,R_5 R_6 and R_7 is independently hydrogen, alkyl , substituted alkyl, aryl, substituted aryl, or a protecting group, each "n" is independently an integer from 0 to about 200, R_{12} is a straight or branched chain alkyl, substituted alkyl, aryl, or substituted aryl, and R_2 is a siNA molecule or a portion thereof,.

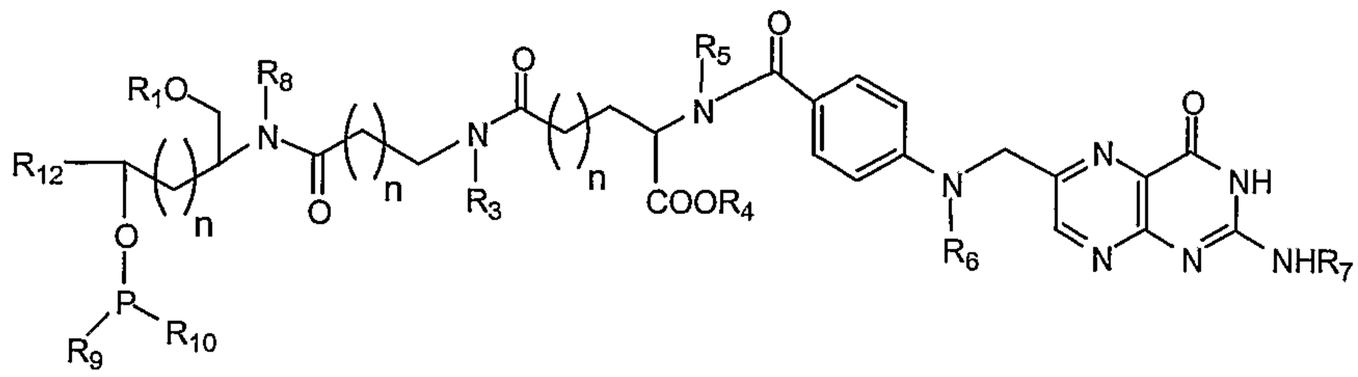
In one embodiment, the invention features a compound having Formula 4:



4

wherein each R_3, R_4 ,R_5, R_6 and R_7 is independently hydrogen, alkyl , substituted alkyl, aryl, substituted aryl, or a protecting group, each "n" is independently an integer from 0 to about 200, R_2 is a siNA molecule or a portion thereof, and R_{13} is an amino acid side chain.

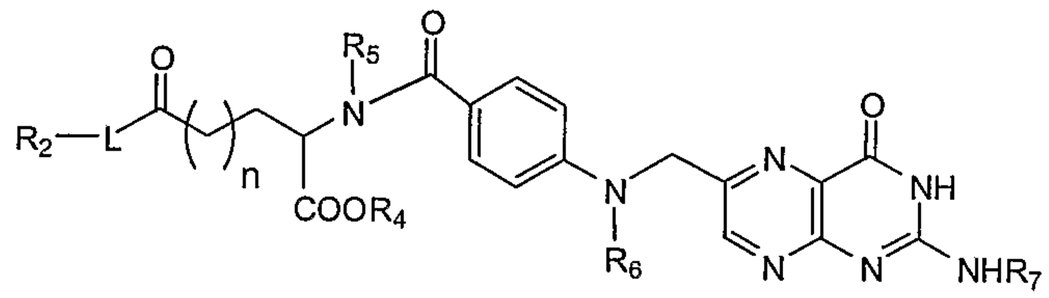
In one embodiment, the invention features a compound having Formula 5:



5

wherein each R_1 and R_4 is independently a protecting group or hydrogen, each R_3, R_5, R_6, R_7 and R_8 is independently hydrogen, alkyl or nitrogen protecting group, each "n" is independently an integer from 0 to about 200, R_{12} is a straight or branched chain alkyl, substituted alkyl, aryl, or substituted aryl, and each R_9 and R_{10} is independently a nitrogen containing group, cyanoalkoxy, alkoxy, aryloxy, or alkyl group.

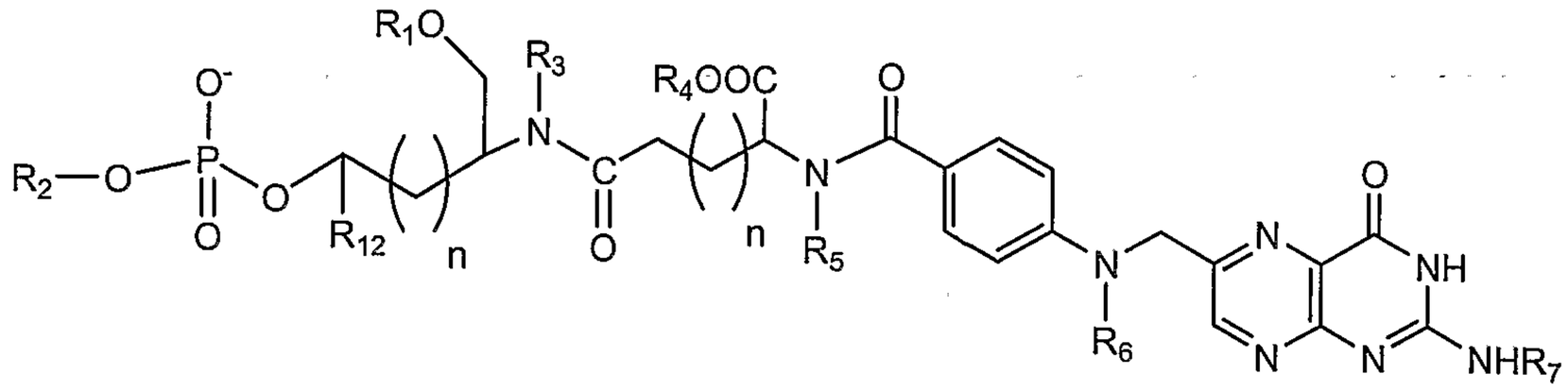
In one embodiment, the invention features a compound having Formula 6:



10

wherein each R_4 ,R_5, R_6 and R_7 is independently hydrogen, alkyl , substituted alkyl, aryl, substituted aryl, or a protecting group, R_2 is a siNA molecule or a portion thereof, each "n" is independently an integer from 0 to about 200, and L is a degradable linker.

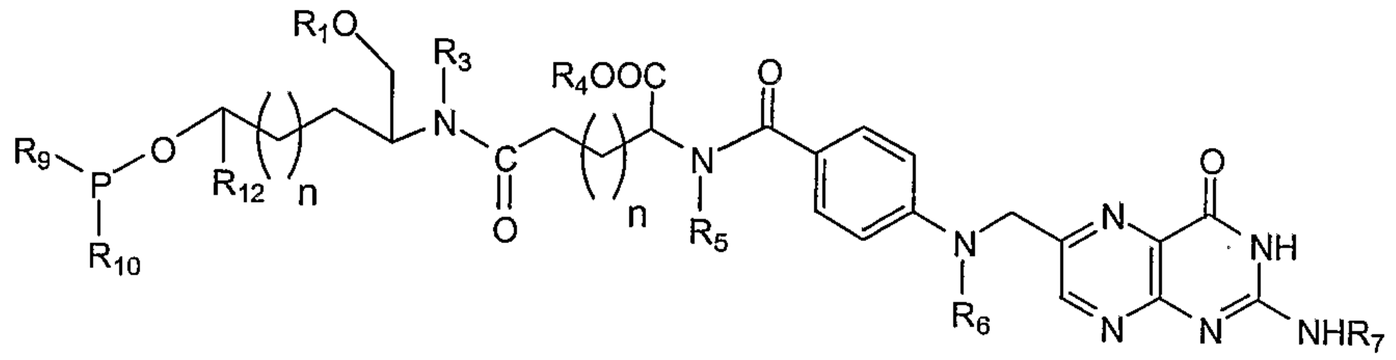
In one embodiment, the invention features a compound having Formula 7:



15

from 0 to about 200, R_{12} is a straight or branched chain alkyl, substituted alkyl, aryl, or substituted aryl, and R_2 is a siNA molecule or a portion thereof.

In one embodiment, the invention features a compound having Formula 8:



wherein each R_1 and R_4 is independently a protecting group or hydrogen, each R_3, R_5, R_6 and R_7 is independently hydrogen, alkyl or nitrogen protecting group, each "n" is independently an integer from 0 to about 200, R_{12} is a straight or branched chain alkyl, substituted alkyl, aryl, or substituted aryl, and each R_9 and R_{10} is independently a nitrogen containing group, cyanoalkoxy, alkoxy, aryloxy, or alkyl group.

In one embodiment, R_{13} of a compound of the invention comprises an alkylamino or an alkoxy group, for example, $-CH_2O-$ or $-CH(CH_2)CH_2O-$.

In another embodiment, R_{12} of a compound of the invention is an alkylhyrdroxyl, for example, $-(CH_2)_nOH$, where n comprises an integer from about 1to about 10.

In another embodiment, L of Formula 6 of the invention comprises serine, threonine, or a photolabile linkage.

In one embodiment, R_9 of a compound of the invention comprises a phosphorus protecting group, for example $-OCH_2CH_2CN$ (oxyethylcyano).

In one embodiment, R_{10} of a compound of the invention comprises a nitrogen containing group, for example, $-N(R_{14})$ wherein R_{14} is a straight or branched chain alkyl having from about 1 to about 10 carbons.

In another embodiment, R_{10} of a compound of the invention comprises a heterocycloalkyl or heterocycloalkenyl ring containing from about 4 to about 7 atoms, and having from about 1 to about 3 heteroatoms comprising oxygen, nitrogen, or sulfur.

In another embodiment, R_1 of a compound of the invention comprises an acid labile protecting group, such as a trityl or substituted trityl group, for example, a dimethoxytrityl or mono-methoxytrityl group.

In another embodiment, R_4 of a compound of the invention comprises a *tert*-butyl, Fm (fluorenyl-methoxy), or allyl group.

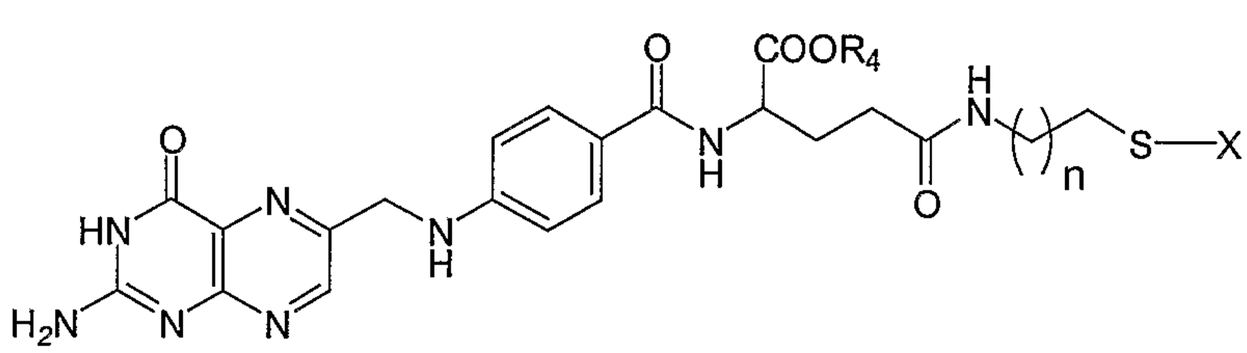
In one embodiment, R_6 of a compound of the invention comprises a TFA (trifluoracetyl) group.

In another embodiment, R_3, R_5 R_7 and R_8 of a compound of the invention are independently hydrogen.

In one embodiment, R_7 of a compound of the invention is independently isobutyryl, dimethylformamide, or hydrogen.

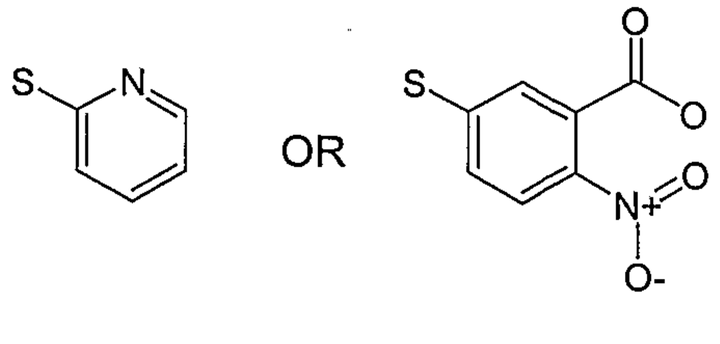
In another embodiment, R_{12} of a compound of the invention comprises a methyl group or ethyl group.

In one embodiment, the invention features a compound having Formula 27:

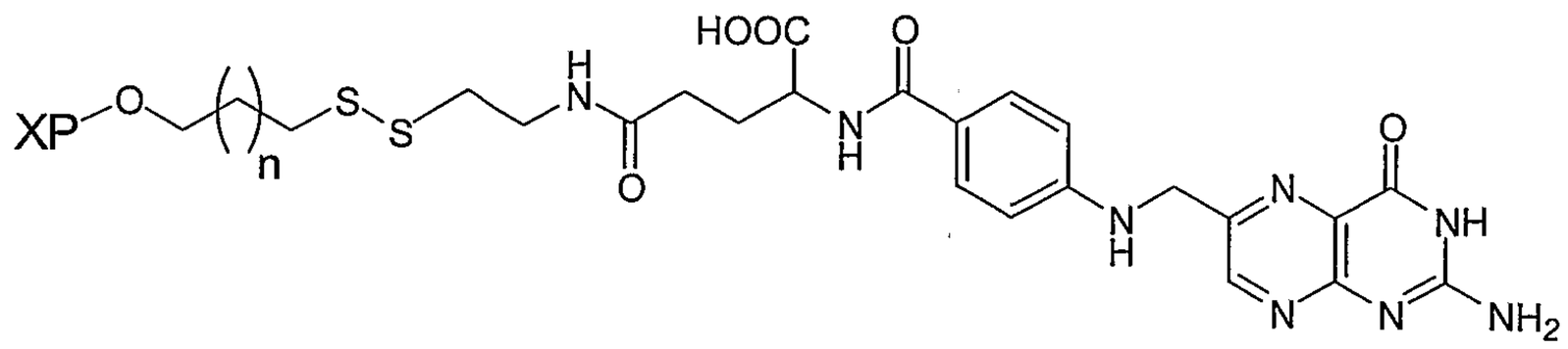


27

wherein "n" is an integer from about 0 to about 20, R_4 is H or a cationic salt, X is a siNA molecule or a portion thereof, and R_{24} is a sulfur containing leaving group, for example a group comprising:



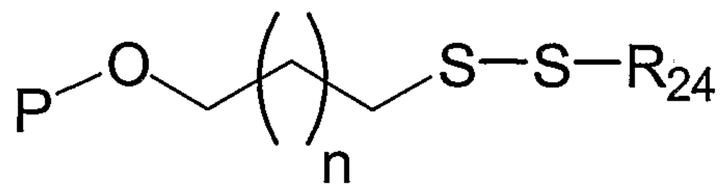
In one embodiment, the invention features a compound having Formula 39:



39

wherein "n" is an integer from about 0 to about 20, X is a siNA molecule or a portion thereof, and P is a phosphorus containing group.

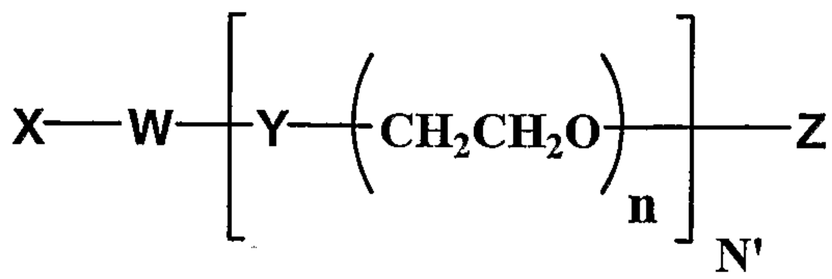
In another embodiment, a thiol containing linker of the invention is a compound having Formula 41:



41

wherein "n" is an integer from about 0 to about 20, P is a phosphorus containing group, for example a phosphine, phosphite, or phosphate, and R_{24} is any alkyl, substituted alkyl, alkoxy, aryl, substituted aryl, alkenyl, substituted alkenyl, alkynyl, or substituted alkynyl group with or without additional protecting groups.

In one embodiment, the invention features a compound having Formula 43:

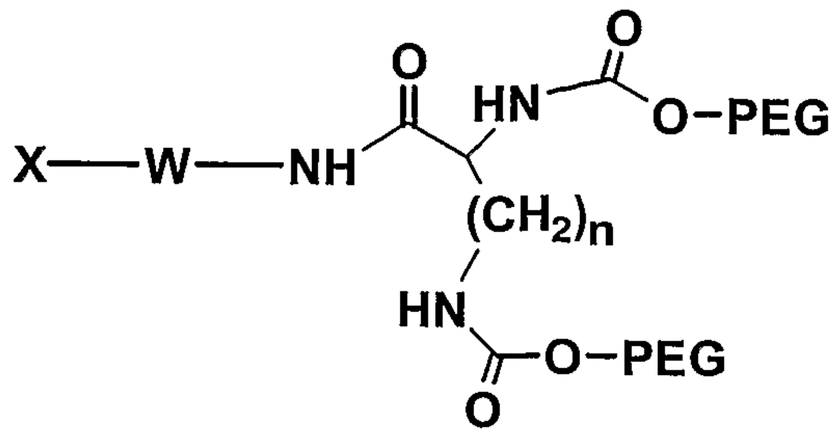


43

wherein X comprises a siNA molecule or portion thereof; W comprises a degradable nucleic acid linker; Y comprises a linker molecule or amino acid that can be present or absent; Z comprises H, OH, O-alkyl, SH, S-alkyl, alkyl, substituted alkyl, aryl, substituted aryl, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, amino acid, peptide, protein, lipid, phospholipid, or label; n is an integer from about 1 to about 100; and N' is an integer from about 1 to about 20. In another

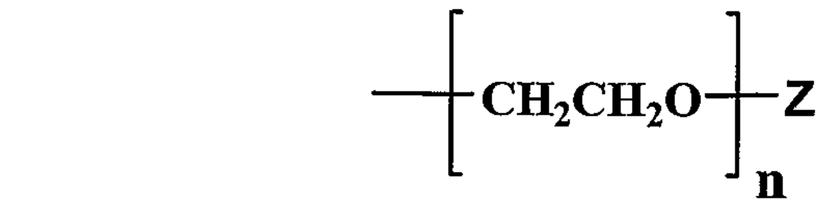
embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

In another embodiment, the invention features a compound having Formula 44:



44

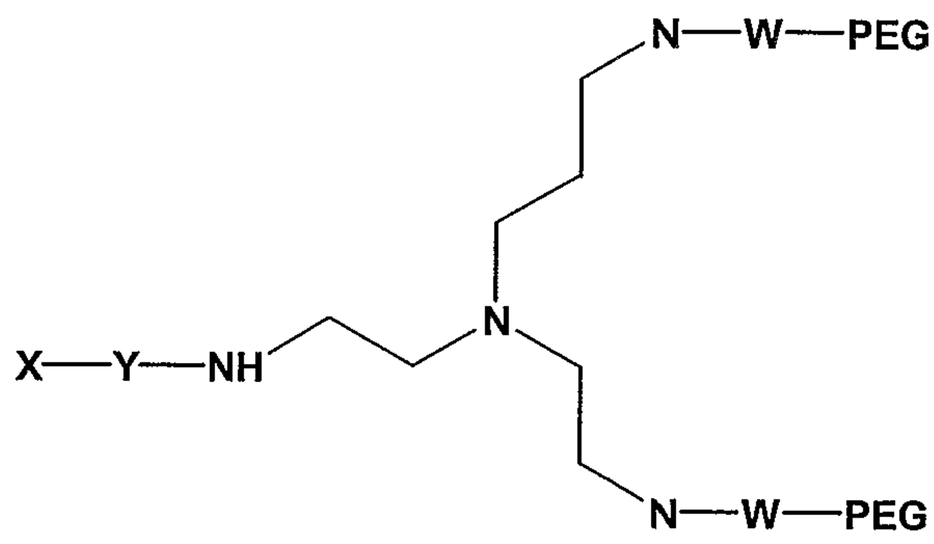
wherein X comprises a siNA molecule or portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent; n is an integer from about 1 to about 50, and PEG represents a compound having Formula 45:



45

wherein Z comprises H, OH, O-alkyl, SH, S-alkyl, alkyl, substituted alkyl, aryl, substituted aryl, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, amino acid, peptide, protein, lipid, phospholipid, or label; and n is an integer from about 1 to about 100. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

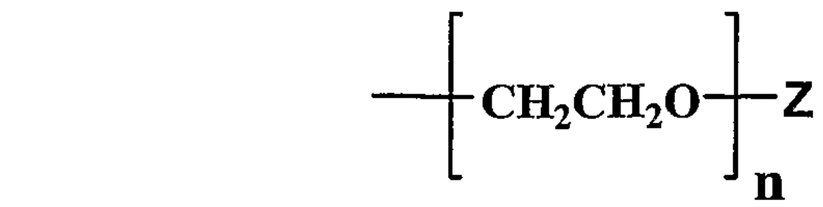
In another embodiment, the invention features a compound having Formula 46:



175

46

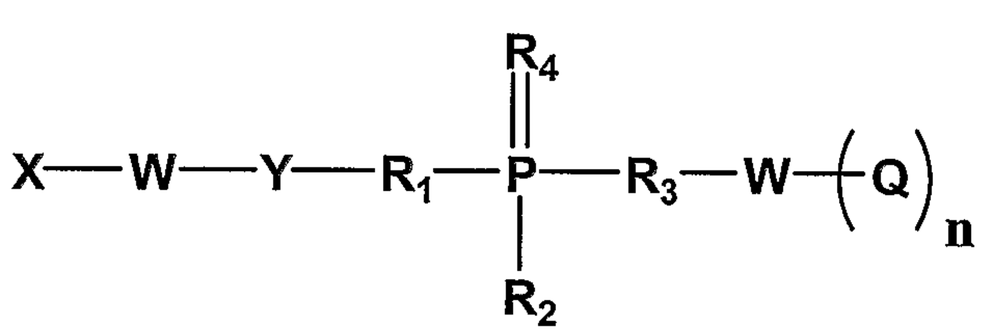
wherein X comprises a siNA molecule or portion thereof; each W independently comprises linker molecule or chemical linkage that can be present or absent, Y comprises a linker molecule or chemical linkage that can be present or absent; and PEG represents a compound having Formula 45:



45

wherein Z comprises H, OH, O-alkyl, SH, S-alkyl, alkyl, substituted alkyl, aryl, substituted aryl, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, amino acid, peptide, protein, lipid, phospholipid, or label; and n is an integer from about 1 to about 100. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

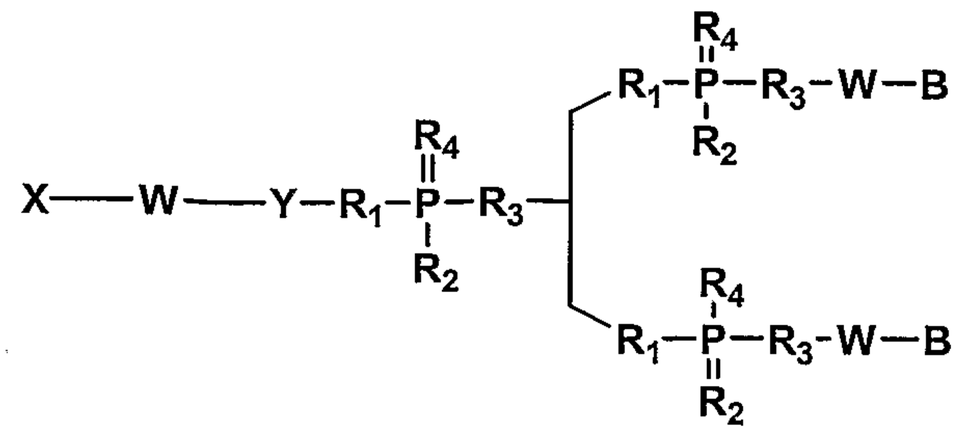
In one embodiment, the invention features a compound having Formula 47:



47

wherein X comprises a siNA molecule or portion thereof; each W independently comprises a linker molecule or chemical linkage that can be the same or different and can be present or absent, Y comprises a linker molecule that can be present or absent; each Q independently comprises a hydrophobic group or phospholipid; each R_1, R_2, R_3, and R_4 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, and n is an integer from about 1 to about 10. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

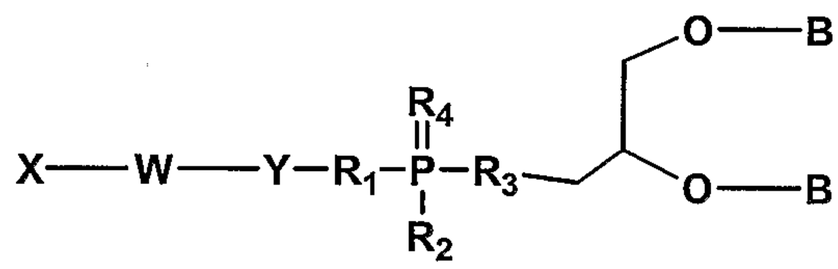
In another embodiment, the invention features a compound having Formula 48:



48

wherein X comprises a siNA molecule or portion thereof; each W independently comprises a linker molecule or chemical linkage that can be present or absent, Y comprises a linker molecule that can be present or absent; each R_1, R_2, R_3, and R_4 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, and B represents a lipophilic group, for example a saturated or unsaturated linear, branched, or cyclic alkyl group, cholesterol, or a derivative thereof. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

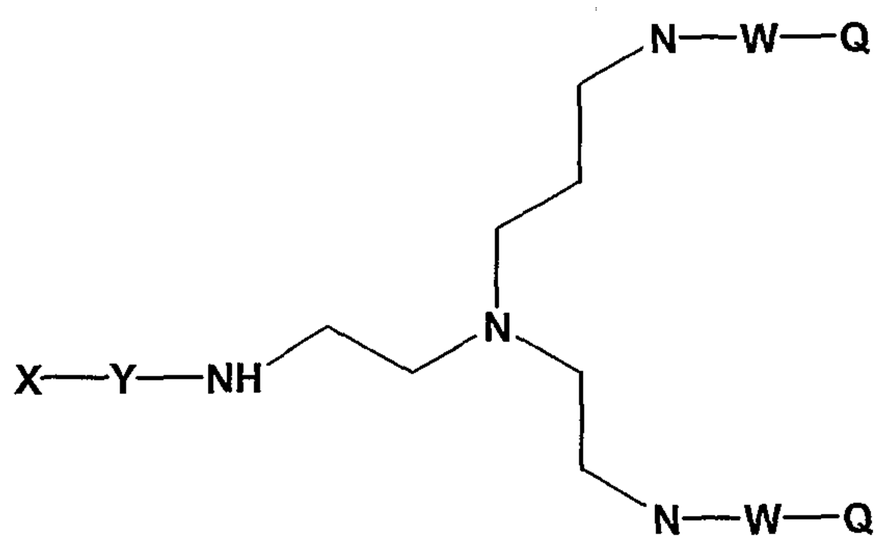
In another embodiment, the invention features a compound having Formula 49:



49

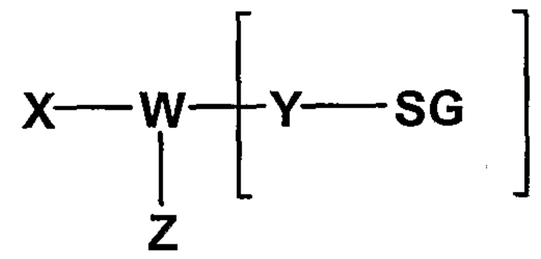
wherein X comprises a siNA molecule or portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent, Y comprises a linker molecule that can be present or absent; each R_1, R_2, R_3, and R_4 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, and B represents a lipophilic group, for example a saturated or unsaturated linear, branched, or cyclic alkyl group, cholesterol, or a derivative thereof. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

In another embodiment, the invention features a compound having Formula 50:



wherein X comprises a siNA molecule or portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent, Y comprises a linker molecule or chemical linkage that can be present or absent; and each Q independently comprises a hydrophobic group or phospholipid. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

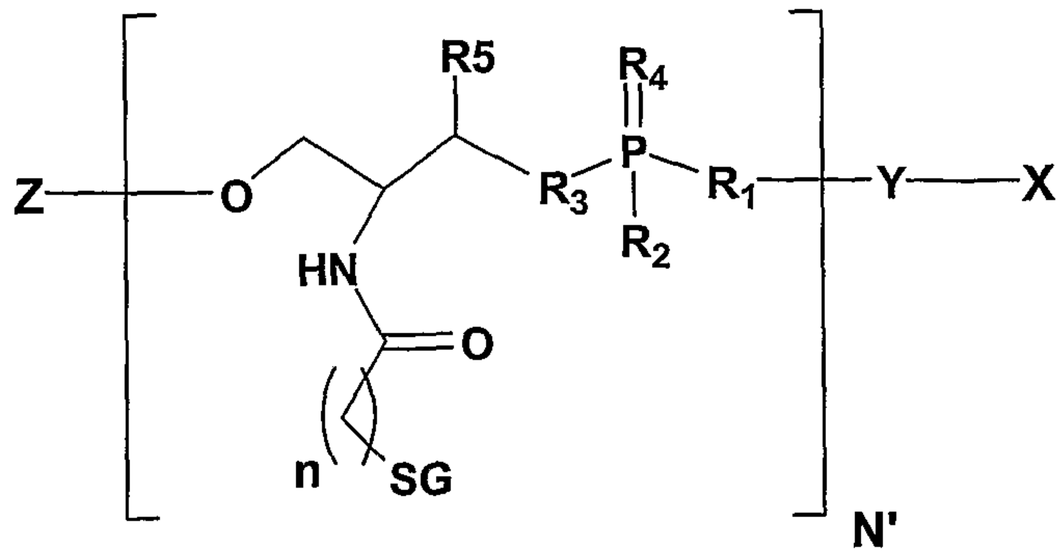
In one embodiment, the invention features a compound having Formula 51:



51

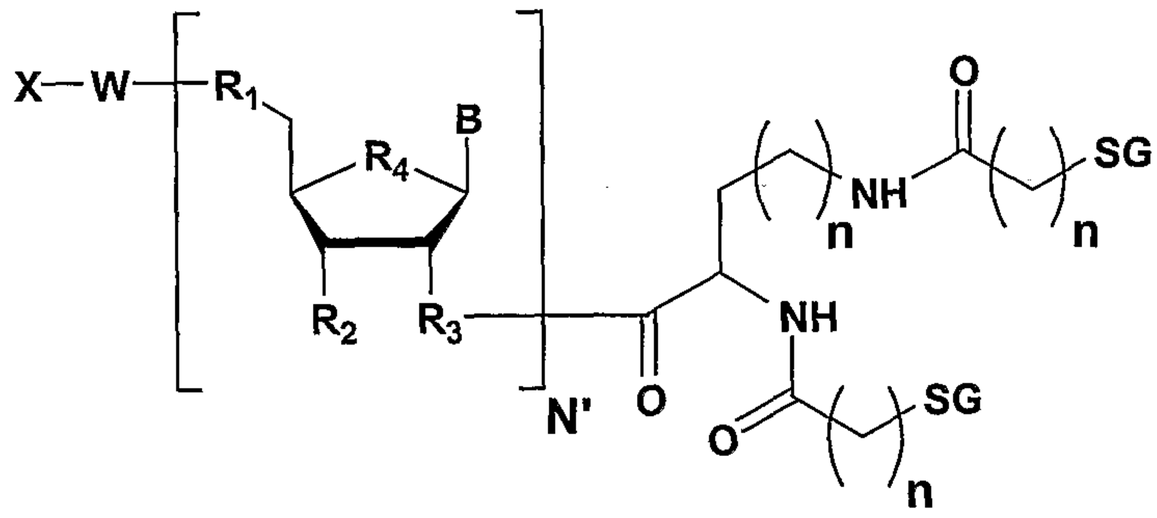
wherein X comprises a siNA molecule or portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent; Y comprises a linker molecule or amino acid that can be present or absent; Z comprises H, OH, O-alkyl, SH, S-alkyl, alkyl, substituted alkyl, aryl, substituted aryl, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, amino acid, peptide, protein, lipid, phospholipid, or label; SG comprises a sugar, for example galactose, galactosamine, N-acetyl-galactosamine, glucose, mannose, fructose, or fucose and the respective D or L, alpha or beta isomers, and n is an integer from about 1 to about 20. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

In another embodiment, the invention features a compound having Formula 52:



wherein X comprises a siNA molecule or portion thereof; Y comprises a linker molecule or chemical linkage that can be present or absent; each R_1, R_2, R_3, R_4, and R_5 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N; Z comprises H, OH, O-alkyl, SH, S-alkyl, alkyl, substituted alkyl, aryl, substituted aryl, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, amino acid, peptide, protein, lipid, phospholipid, or label; SG comprises a sugar, for example galactose, galactosamine, N-acetyl-galactosamine, glucose, mannose, fructose, or fucose and the respective D or L, alpha or beta isomers, n is an integer from about 1 to about 20; and N' is an integer from about 1 to about 20. In another embodiment, X comprises a siNA molecule or a portion thereof. In another embodiment, Y is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

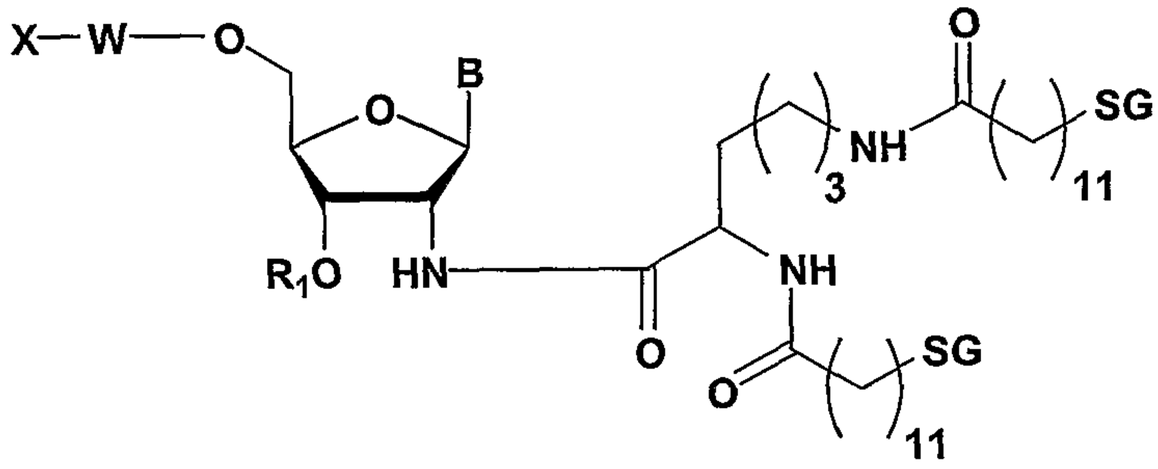
In another embodiment, the invention features a compound having Formula 53:



wherein B comprises H, a nucleoside base, or a non-nucleosidic base with or without protecting groups; each R_1 independently comprises O, N, S, alkyl, or substituted N; each R_2 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylhalo, S, N,

substituted N, or a phosphorus containing group; each R_3 independently comprises N or O-N, each R_4 independently comprises O, CH_2, S, sulfone, or sulfoxy; X comprises H, a removable protecting group, a siNA molecule or a portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent; SG comprises a sugar, for example galactose, galactosamine, N-acetyl-galactosamine, glucose, mannose, fructose, or fucose and the respective D or L, alpha or beta isomers,, each n is independently an integer from about 1 to about 50; and N' is an integer from about 1 to about 10. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

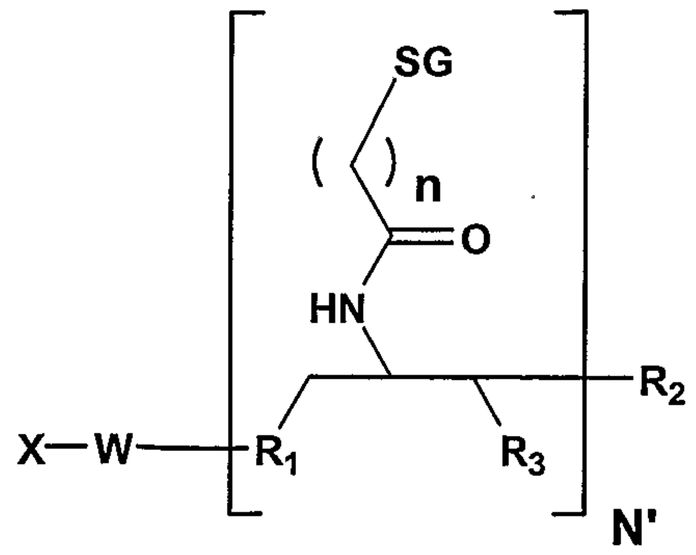
In another embodiment, the invention features a compound having Formula 54:



54

wherein B comprises H, a nucleoside base, or a non-nucleosidic base with or without protecting groups; each R_1 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylhalo, S, N, substituted N, or a phosphorus containing group; X comprises H, a removable protecting group, a siNA molecule or a portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent; and SG comprises a sugar, for example galactose, galactosamine, N-acetyl-galactosamine, glucose, mannose, fructose, or fucose and the respective D or L, alpha or beta isomers. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

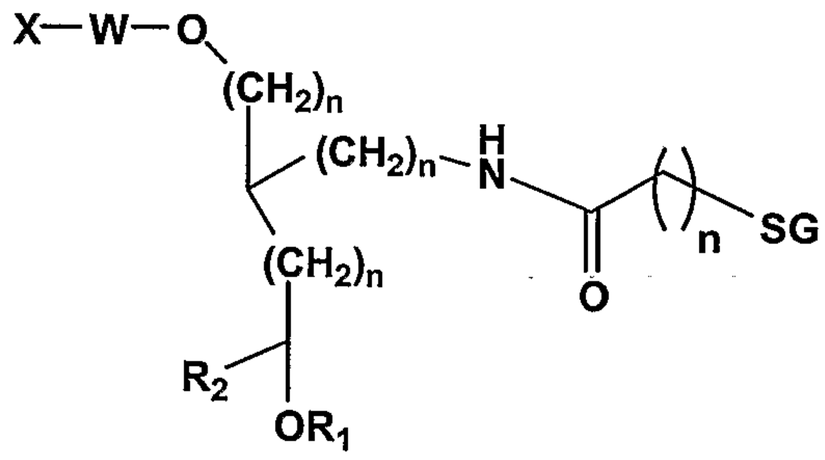
In one embodiment, the invention features a compound having Formula 55:



55

wherein each R_1 independently comprises O, N, S, alkyl, or substituted N; each R_2 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylhalo, S, N, substituted N, or a phosphorus containing group; each R_3 independently comprises H, OH, alkyl, substituted alkyl, or halo; X comprises H, a removable protecting group, a siNA molecule or a portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent; SG comprises a sugar, for example galactose, galactosamine, N-acetyl-galactosamine, glucose, mannose, fructose, or fucose and the respective D or L, alpha or beta isomers, each n is independently an integer from about 1 to about 50; and N' is an integer from about 1 to about 100. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

In another embodiment, the invention features a compound having Formula 56:

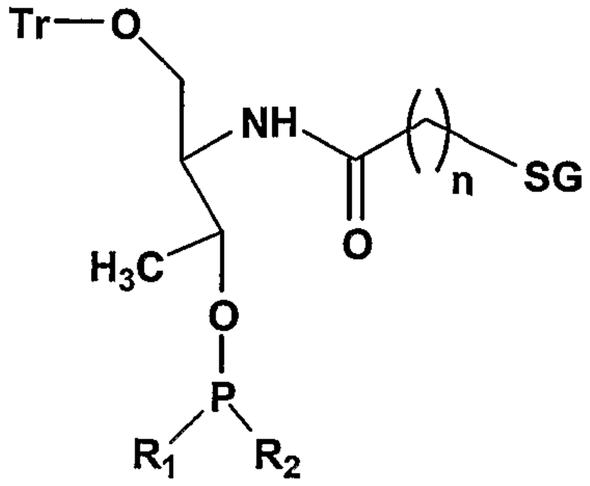


56

wherein R_1 comprises H, alkyl, alkylhalo, N, substituted N, or a phosphorus containing group; R_2 comprises H, O, OH, alkyl, alkylhalo, halo, S, N, substituted N, or a phosphorus containing group; X comprises H, a removable protecting group, a siNA molecule or a portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent; SG comprises a sugar, for example galactose, galactosamine,

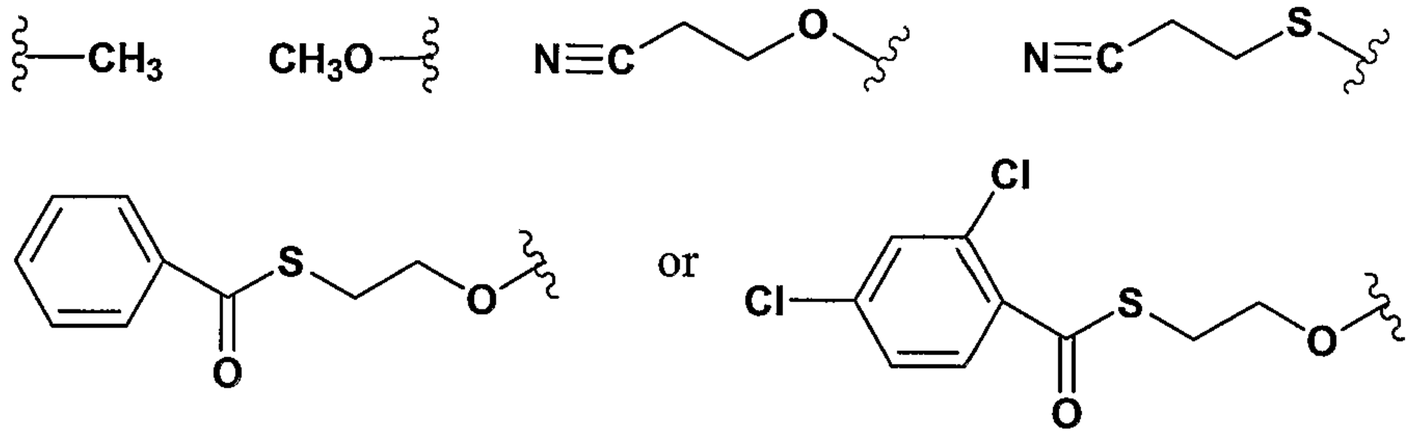
N-acetyl-galactosamine, glucose, mannose, fructose, or fucose and the respective D or L, alpha or beta isomers, and each n is independently an integer from about 0 to about 20. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

In another embodiment, the invention features a compound having Formula 57:

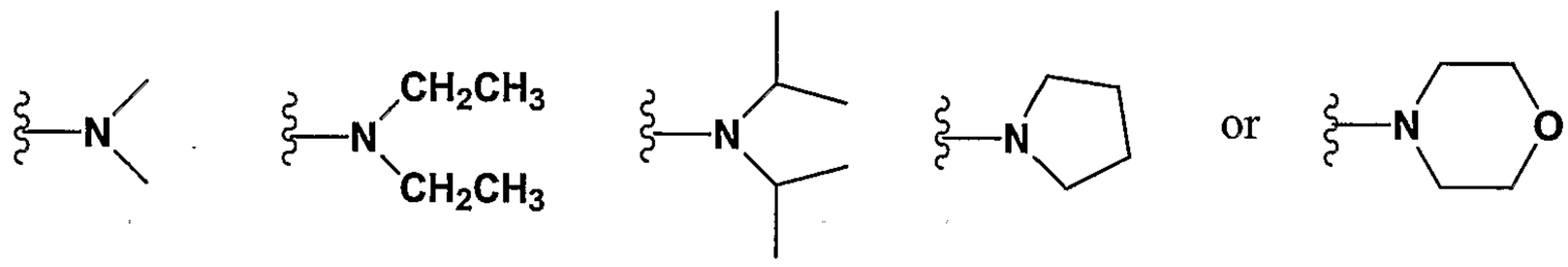


57

wherein R_1 can include the groups:



and wherein R_2 can include the groups:

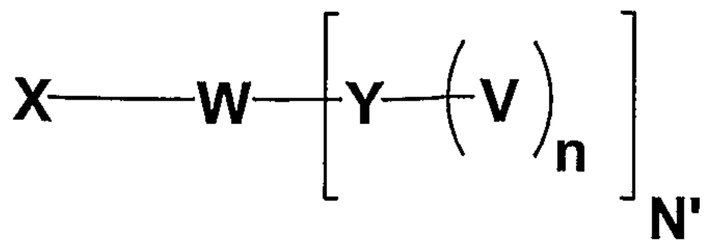


and wherein Tr is a removable protecting group, for example a trityl, monomethoxytrityl, or dimethoxytrityl; SG comprises a sugar, for example galactose, galactosamine, N-acetyl-galactosamine, glucose, mannose, fructose, or fucose and the respective D or L, alpha or beta isomers, and n is an integer from about 1 to about 20.

In one embodiment, compounds having Formula 52, 53, 54, 55, 56, and 57 are featured wherein each nitrogen adjacent to a carbonyl can independently be substituted for

a carbonyl adjacent to a nitrogen or each carbonyl adjacent to a nitrogen can be substituted for a nitrogen adjacent to a carbonyl.

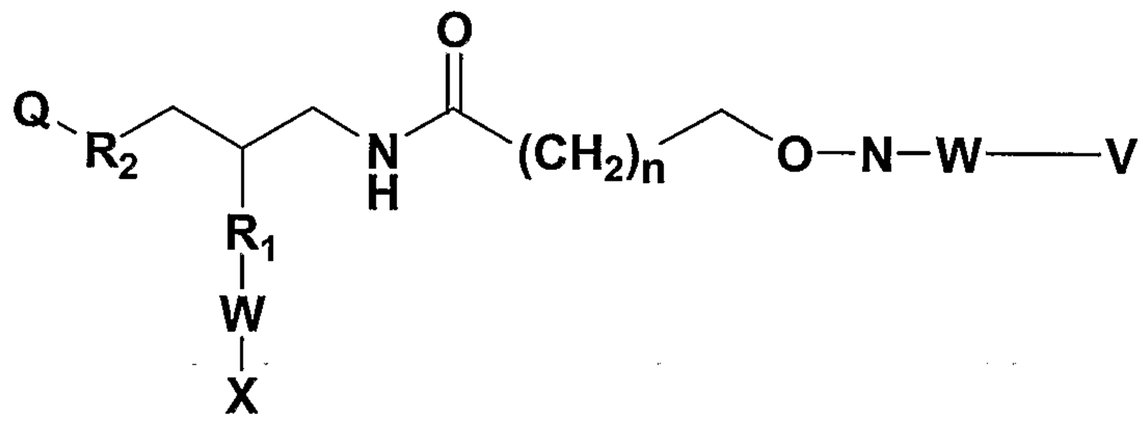
In another embodiment, the invention features a compound having Formula 58:



58

wherein X comprises a siNA molecule or portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent; Y comprises a linker molecule or amino acid that can be present or absent; V comprises a signal protein or peptide, for example Human serum albumin protein, Antennapedia peptide, Kaposi fibroblast growth factor peptide, Caiman crocodylus Ig(5) light chain peptide, HIV envelope glycoprotein gp41 peptide, HIV-1 Tat peptide, Influenza hemagglutinin envelope glycoprotein peptide, or transportan A peptide; each n is independently an integer from about 1 to about 50; and N' is an integer from about 1 to about 100. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

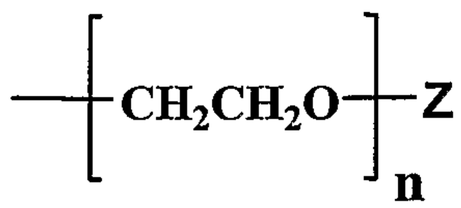
In another embodiment, the invention features a compound having Formula 59:



59

wherein each R_1 independently comprises O, S, N, substituted N, or a phosphorus containing group; each R_2 independently comprises O, S, or N; X comprises H, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, or other biologically active molecule; n is an integer from about 1 to about 50, Q comprises H or a removable protecting group which can be optionally absent, each W independently comprises a linker molecule or chemical linkage that can be present or absent, and V

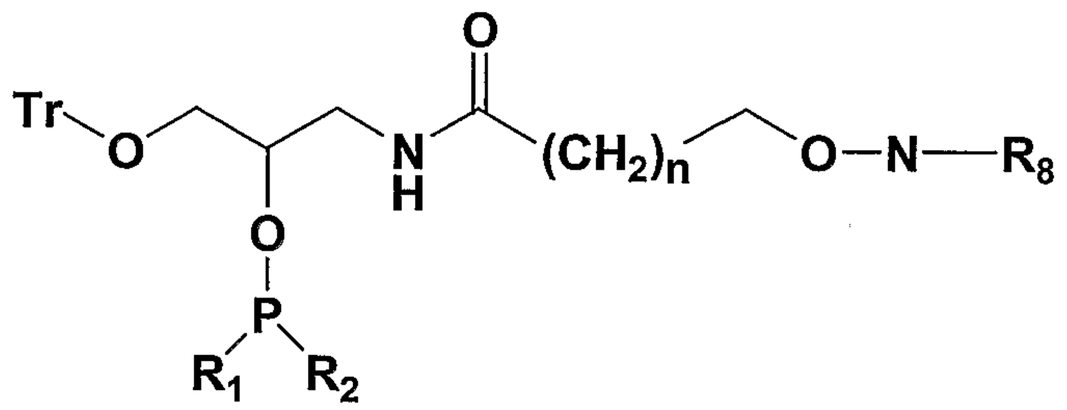
comprises a signal protein or peptide, for example Human serum albumin protein, Antennapedia peptide, Kaposi fibroblast growth factor peptide, Caiman crocodylus Ig(5) light chain peptide, HIV envelope glycoprotein gp41 peptide, HIV-1 Tat peptide, Influenza hemagglutinin envelope glycoprotein peptide, or transportan A peptide, or a compound having Formula 45



45

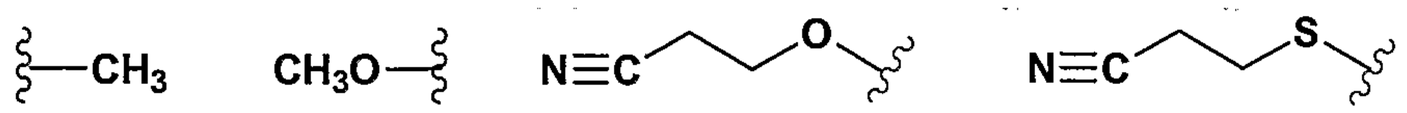
wherein Z comprises H, OH, O-alkyl, SH, S-alkyl, alkyl, substituted alkyl, aryl, substituted aryl, amino, substituted amino, a removable protecting group, a siNA molecule or a portion thereof; and n is an integer from about 1 to about 100. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

In another embodiment, the invention features a compound having Formula 60:

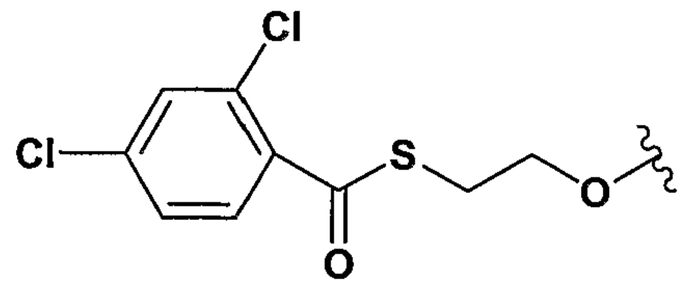


60

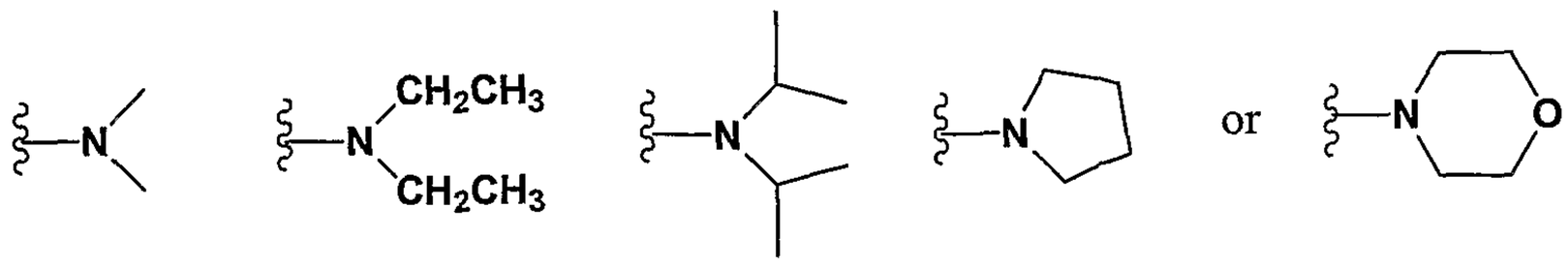
wherein R_1 can include the groups:



or

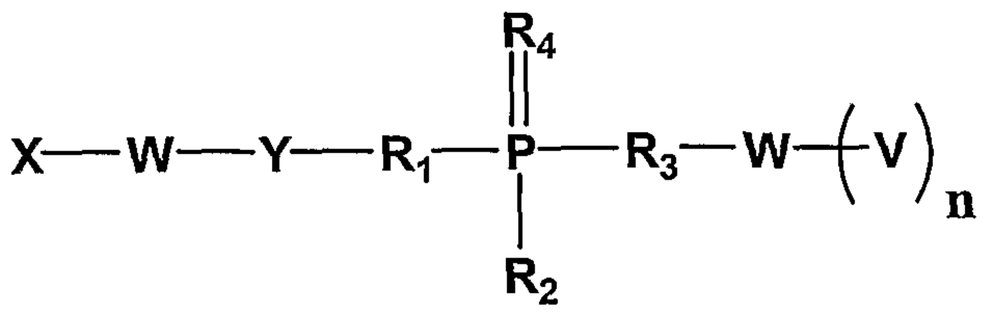


and wherein R_2 can include the groups:



and wherein Tr is a removable protecting group, for example a trityl, monomethoxytrityl, or dimethoxytrityl; n is an integer from about 1 to about 50; and R_8 is a nitrogen protecting group, for example a phthaloyl, trifluoroacetyl, FMOC, or monomethoxytrityl group.

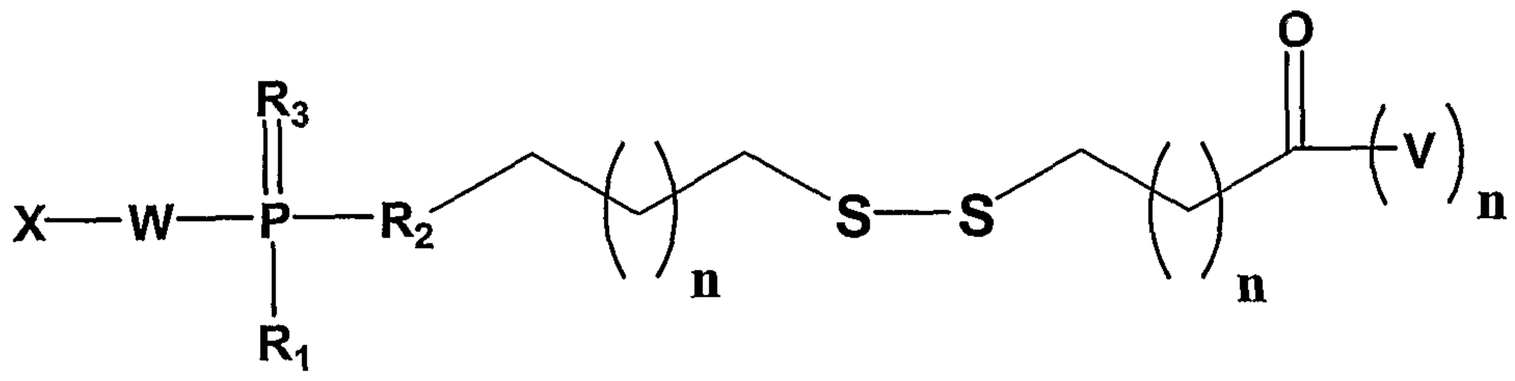
In another embodiment, the invention features a compound having Formula 61:



61

wherein X comprises a siNA molecule or portion thereof; each W independently comprises a linker molecule or chemical linkage that can be the same or different and can be present or absent, Y comprises a linker molecule that can be present or absent; each 5 independently comprises a signal protein or peptide, for example Human serum albumin protein, Antennapedia peptide, Kaposi fibroblast growth factor peptide, Caiman crocodylus Ig(5) light chain peptide, HIV envelope glycoprotein gp41 peptide, HIV-1 Tat peptide, Influenza hemagglutinin envelope glycoprotein peptide, or transportan A peptide;; each R_1, R_2, R_3, and R_4 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, and n is an integer from about 1 to about 10. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

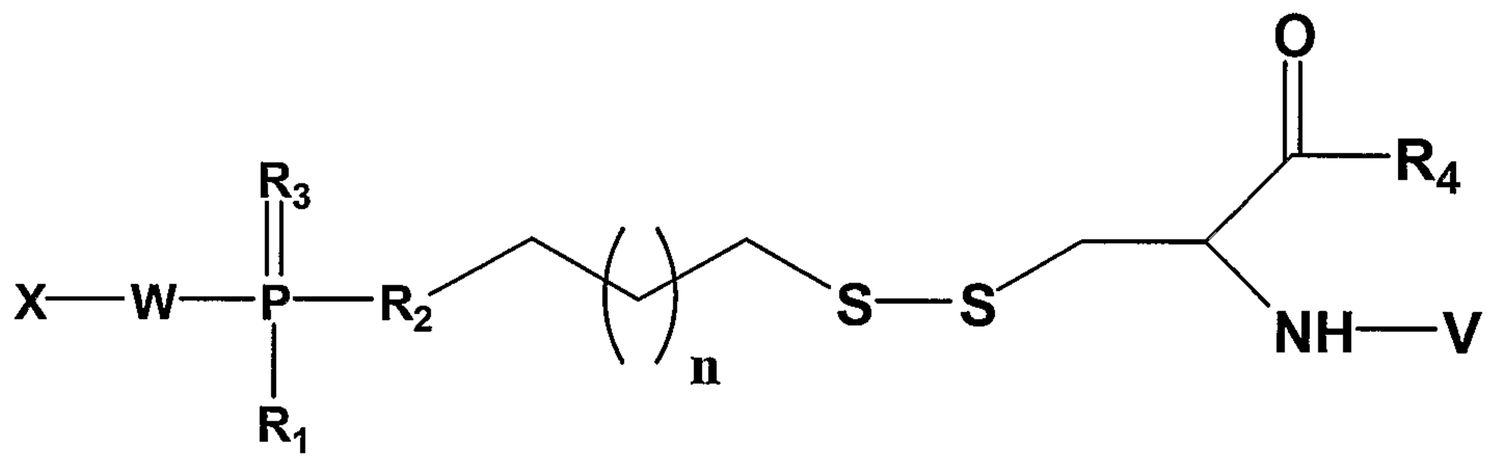
In another embodiment, the invention features a compound having Formula 62:



62

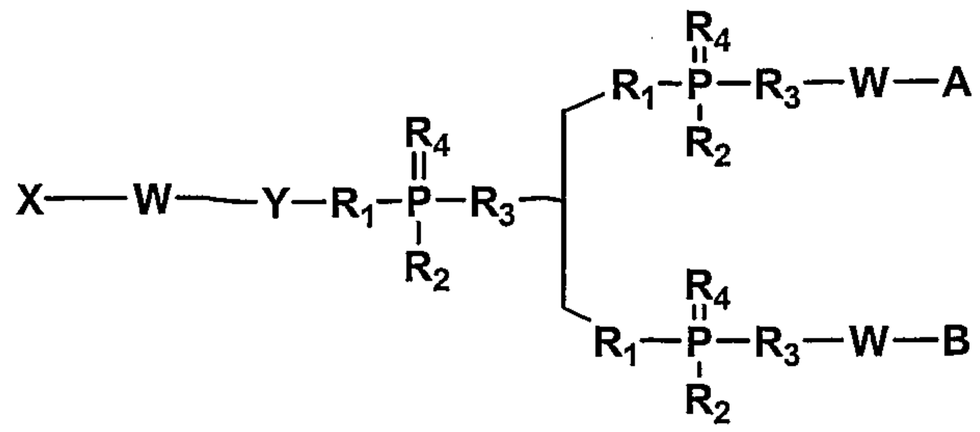
wherein X comprises a siNA molecule or portion thereof; each 5 independently comprises a signal protein or peptide, for example Human serum albumin protein, Antennapedia peptide, Kaposi fibroblast growth factor peptide, Caiman crocodylus Ig(5) light chain peptide, HIV envelope glycoprotein gp41 peptide, HIV-1 Tat peptide, Influenza hemagglutinin envelope glycoprotein peptide, or transportan A peptide; W comprises a linker molecule or chemical linkage that can be present or absent; each R_1, R_2, and R_3 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, and each n is independently an integer from about 1 to about 10. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

In another embodiment, the invention features a compound having Formula 63:



wherein X comprises a siNA molecule or portion thereof; V comprises a signal protein or peptide, for example Human serum albumin protein, Antennapedia peptide, Kaposi fibroblast growth factor peptide, Caiman crocodylus Ig(5) light chain peptide, HIV envelope glycoprotein gp41 peptide, HIV-1 Tat peptide, Influenza hemagglutinin envelope glycoprotein peptide, or transportan A peptide; W comprises a linker molecule or chemical linkage that can be present or absent; each R_1, R_2, R_3 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, R_4 represents an ester, amide, or protecting group, and each n is independently an integer from about 1 to about 10. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

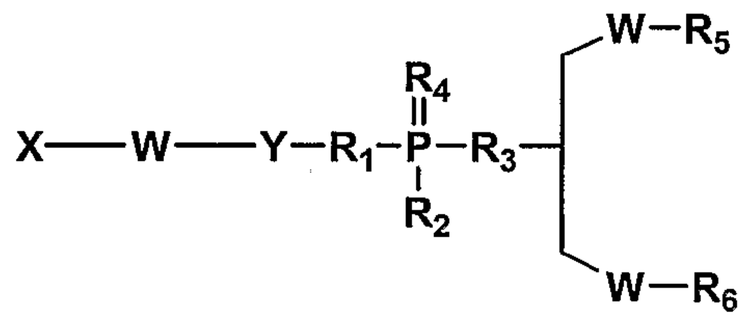
In another embodiment, the invention features a compound having Formula 64:



64

wherein X comprises a siNA molecule or portion thereof; each W independently comprises a linker molecule or chemical linkage that can be present or absent, Y comprises a linker molecule that can be present or absent; each R_1, R_2, R_3, and R_4 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, A comprises a nitrogen containing group, and B comprises a lipophilic group. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

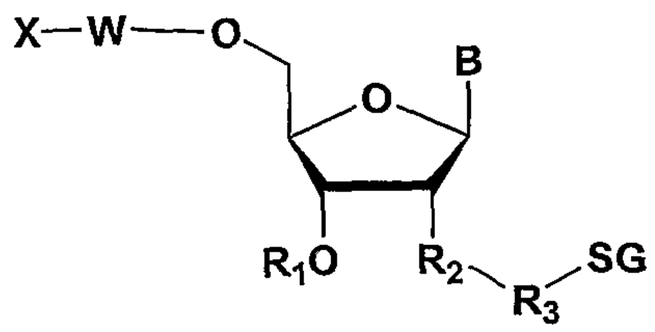
In another embodiment, the invention features a compound having Formula 65:



65

wherein X comprises a siNA molecule or portion thereof; each W independently comprises a linker molecule or chemical linkage that can be present or absent, Y comprises a linker molecule that can be present or absent; each R_1, R_2, R_3, and R_4 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, RV comprises the lipid or phospholipid component of any of Formulae 47-50, and R_6 comprises a nitrogen containing group. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

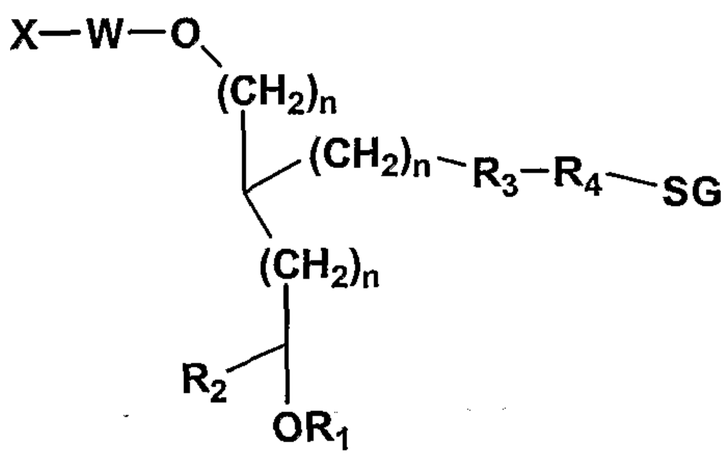
In another embodiment, the invention features a compound having Formula 92:



92

wherein B comprises H, a nucleoside base, or a non-nucleosidic base with or without protecting groups; each R_1 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylhalo, S, N, substituted N, or a phosphorus containing group; X comprises H, a removable protecting group, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, enzymatic nucleic acid, amino acid, peptide, protein, lipid, phospholipid, biologically active molecule or label; W comprises a linker molecule or chemical linkage that can be present or absent; R_2 comprises O, NH, S, CO, COO, ON=C, or alkyl; R_3 comprises alkyl, akloxy, or an aminoacyl side chain; and SG comprises a sugar, for example galactose, galactosamine, N-acetyl-galactosamine, glucose, mannose, fructose, or fucose and the respective D or L, alpha or beta isomers. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

In another embodiment, the invention features a compound having Formula 86:

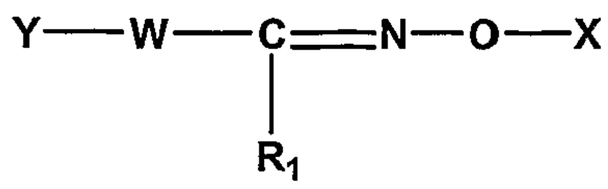


86

wherein R_1 comprises H, alkyl, alkylhalo, N, substituted N, or a phosphorus containing group; R_2 comprises H, O, OH, alkyl, alkylhalo, halo, S, N, substituted N, or a phosphorus containing group; X comprises H, a removable protecting group, a siNA molecule or a portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent; R_3 comprises O, NH, S, CO, COO, ON=C, or alkyl; R_4 comprises alkyl, akloxy, or an aminoacyl side chain; and SG comprises a sugar, for example galactose,

galactosamine, N-acetyl-galactosamine, glucose, mannose, fructose, or fucose and the respective D or L, alpha or beta isomers, and each n is independently an integer from about 0 to about 20. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

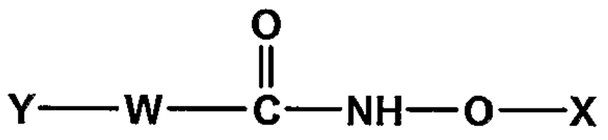
In another embodiment, the invention features a compound having Formula 87:



87

wherein X comprises a protein, peptide, antibody, lipid, phospholipid, oligosaccharide, label, biologically active molecule, for example a vitamin such as folate, vitamin A, E, B6, B12, coenzyme, antibiotic, antiviral, nucleic acid, nucleotide, nucleoside, or oligonucleotide such as an enzymatic nucleic acid, allozyme, antisense nucleic acid, siNA, 2,5-A chimera, decoy, aptamer or triplex forming oligonucleotide, or polymers such as polyethylene glycol; W comprises a linker molecule or chemical linkage that can be present or absent; and Y comprises siNAor a portion thereof; R_1 comprises H, alkyl, or substituted alkyl. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

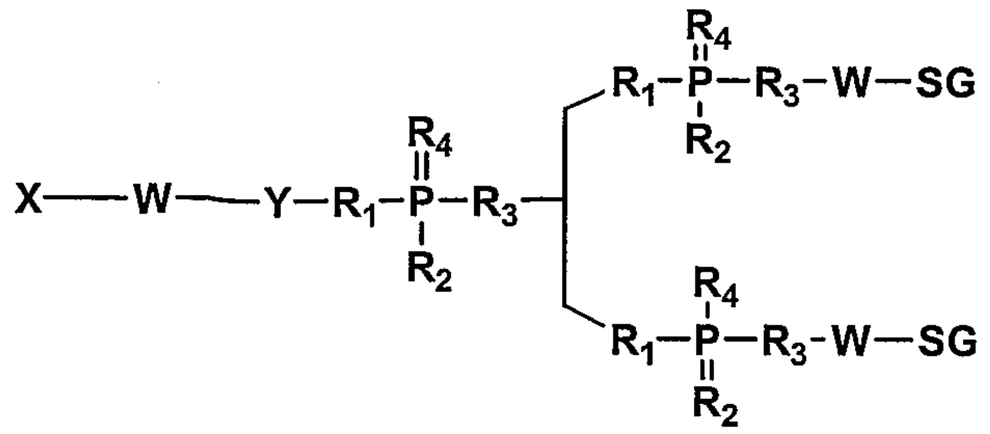
In another embodiment, the invention features a compound having Formula 88:



88

wherein X comprises a protein, peptide, antibody, lipid, phospholipid, oligosaccharide, label, biologically active molecule, for example a vitamin such as folate, vitamin A, E, B6, B12, coenzyme, antibiotic, antiviral, nucleic acid, nucleotide, nucleoside, or oligonucleotide such as an enzymatic nucleic acid, allozyme, antisense nucleic acid, siNA, 2,5-A chimera, decoy, aptamer or triplex forming oligonucleotide, or polymers such as polyethylene glycol; W comprises a linker molecule or chemical linkage that can be present or absent, and Y comprises a siNA or a portion thereof. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

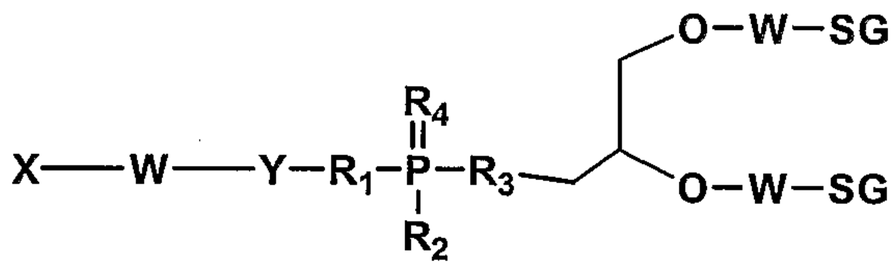
In another embodiment, the invention features a compound having Formula 99:



99

wherein X comprises a siNA molecule or portion thereof; each W independently comprises a linker molecule or chemical linkage that can be present or absent, Y comprises a linker molecule that can be present or absent; each R_1, R_2, R_3, and R_4 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, and SG comprises a sugar, for example galactose, galactosamine, N-acetyl-galactosamine or branched derivative thereof, glucose, mannose, fructose, or fucose and the respective D or L, alpha or beta isomers. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

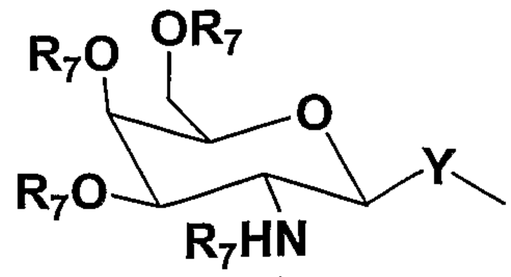
In another embodiment, the invention features a compound having Formula 100:



100

wherein X comprises a siNA molecule or portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent, Y comprises a linker molecule that can be present or absent; each R_1, R_2, R_3, and R_4 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, and SG comprises a sugar, for example galactose, galactosamine, N-acetyl-galactosamine or branched derivative thereof, glucose, mannose, fructose, or fucose and the respective D or L, alpha or beta isomers. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

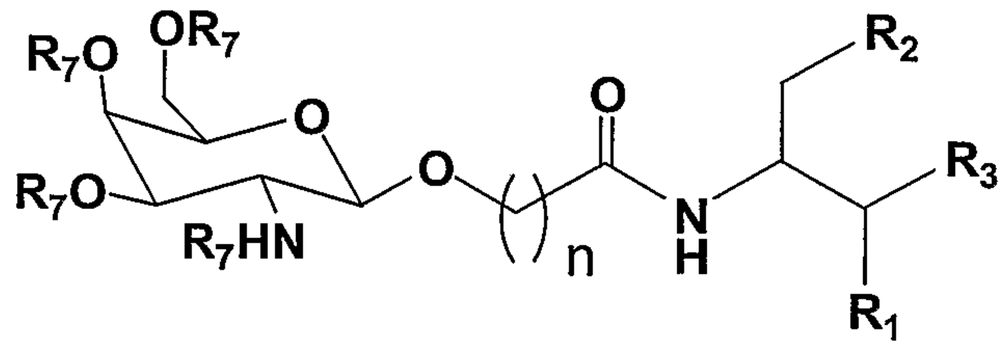
In one embodiment, the SG component of any compound having Formulae 99 or 100 comprises a compound having Formula 101:



101

wherein Y comprises a linker molecule or chemical linkage that can be present or absent and each R_7 independently comprises an acyl group that can be present or absent, for example a acetyl group.

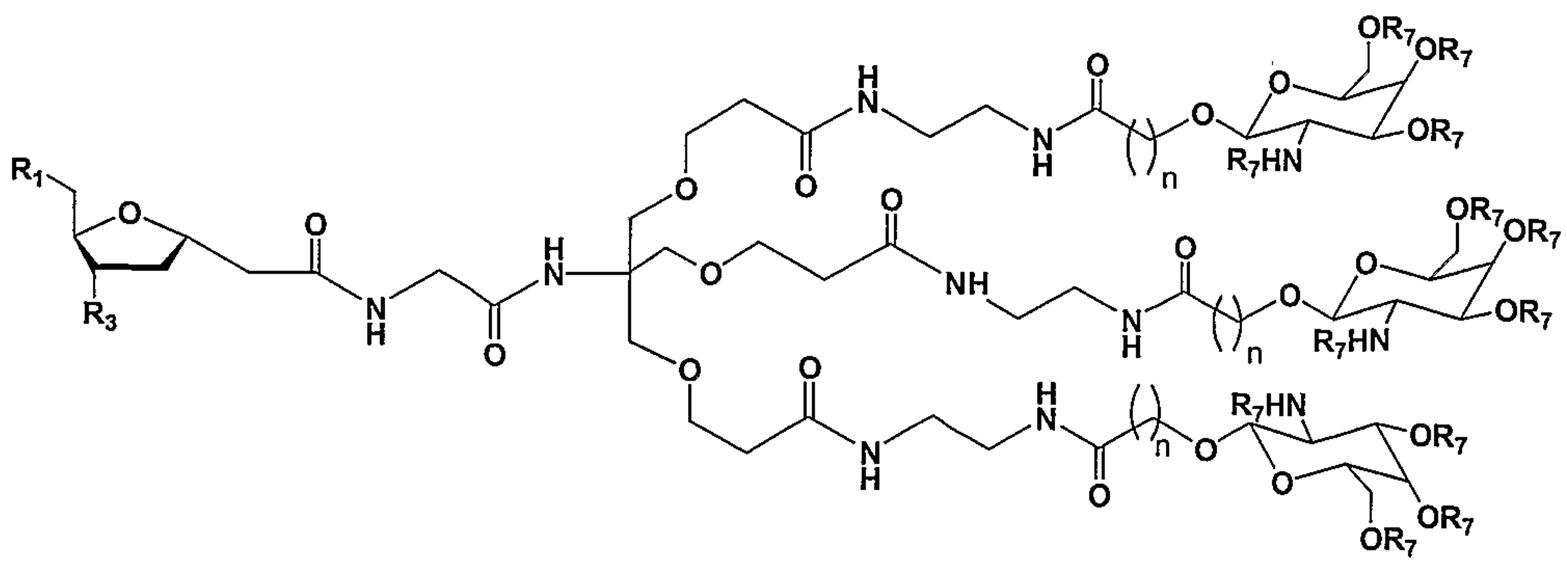
In one embodiment, the W-SG component of a compound having Formulae 99 comprises a compound having Formula 102:



102

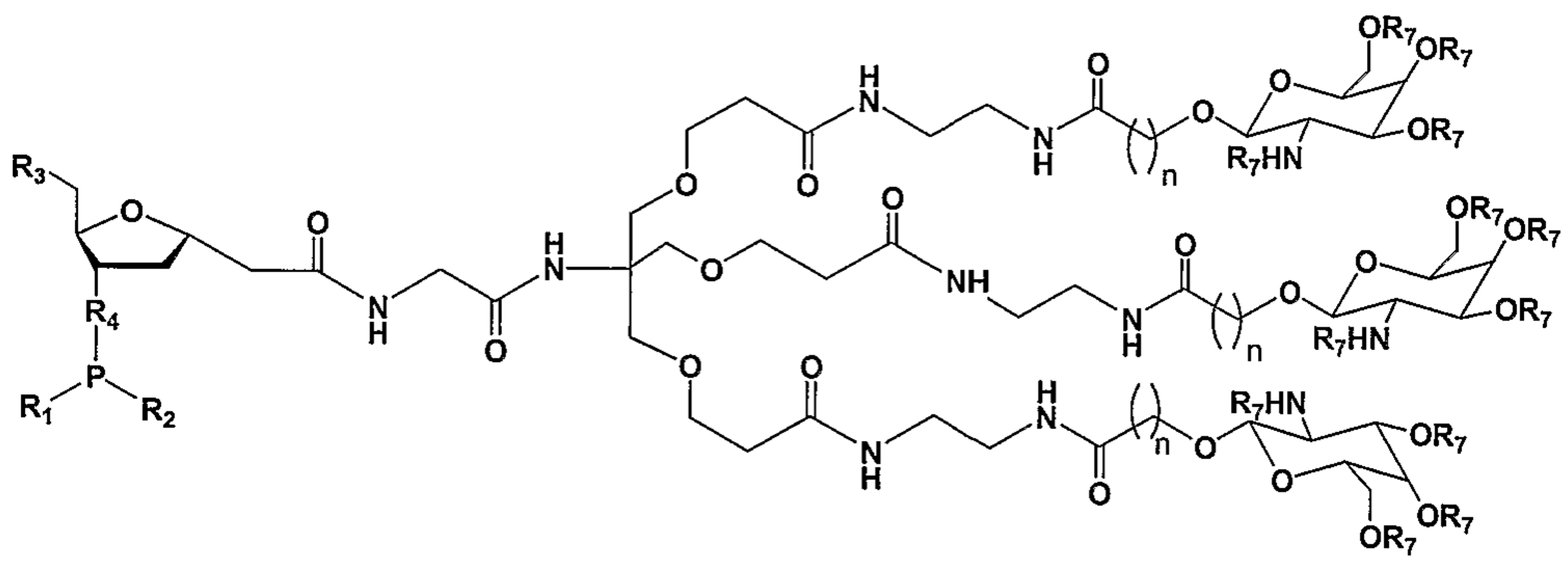
wherein R_2 comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylhalo, S, N, substituted N, a protecting group, or another compound having Formula 102; R_1 independently H, OH, alkyl, substituted alkyl, or halo and each R_7 independently comprises an acyl group that can be present or absent, for example a acetyl group, and R_3 comprises O or R_3 in Formula 99, and n is an integer from about 1 to about 20.

In one embodiment, the W-SG component of a compound having Formulae 99 comprises a compound having Formula 103:



wherein R_1 comprises H, alkyl, alkylhalo, O-alkyl, O-alkylhalo, S, N, substituted N, a protecting group, or another compound having Formula 103; each R_7 independently comprises an acyl group that can be present or absent, for example a acetyl group, and R_3 comprises H or R_3 in Formula 99, and each n is independently an integer from about 1 to about 20.

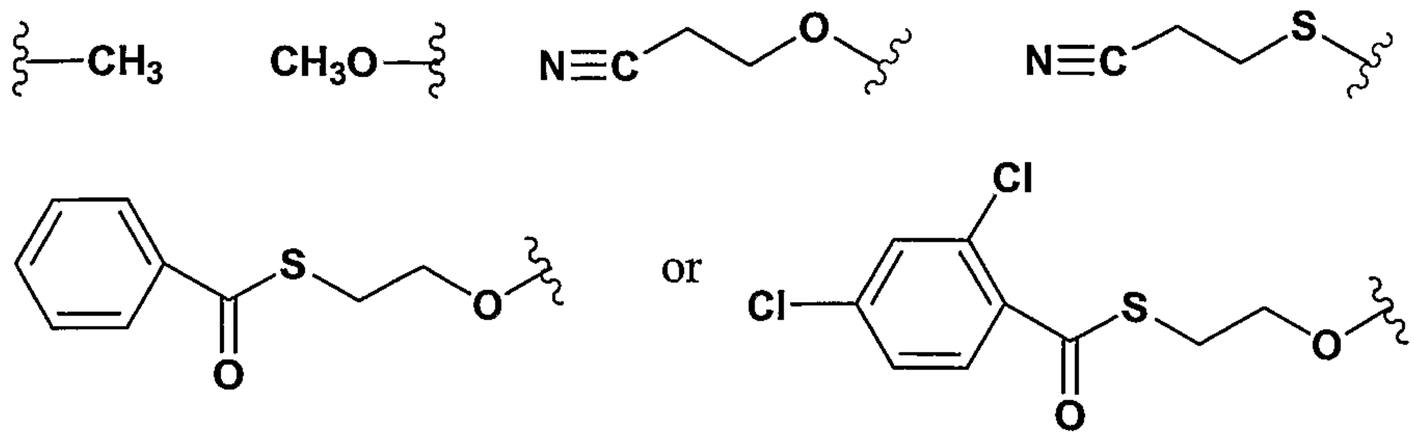
In one embodiment, the invention features a compound having Formula 104:



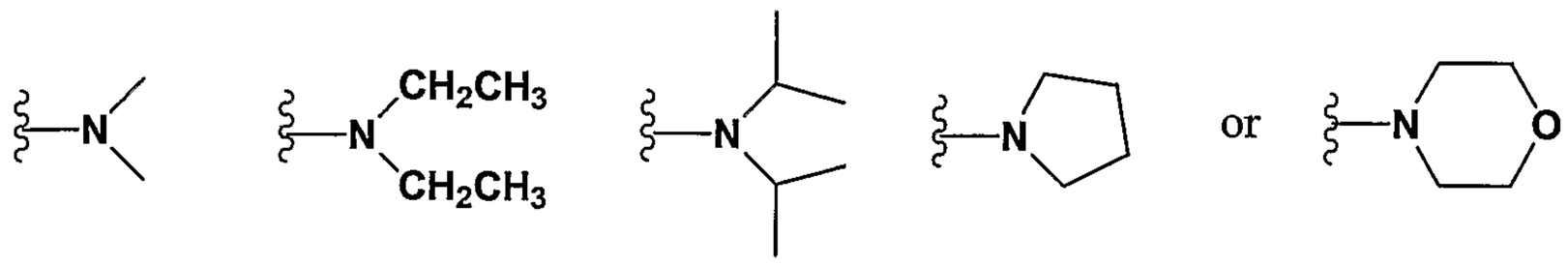
104

wherein R_3 comprises H, OH, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, amino acid, peptide, protein, lipid, phospholipid, label, or a portion thereof, or OR_5 where R_5 a removable protecting group, R_4 comprises O, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, each R_7 independently comprises an acyl group that can be present or absent, for example a acetyl group, and each n is independently an integer from about 1 to about 20, and

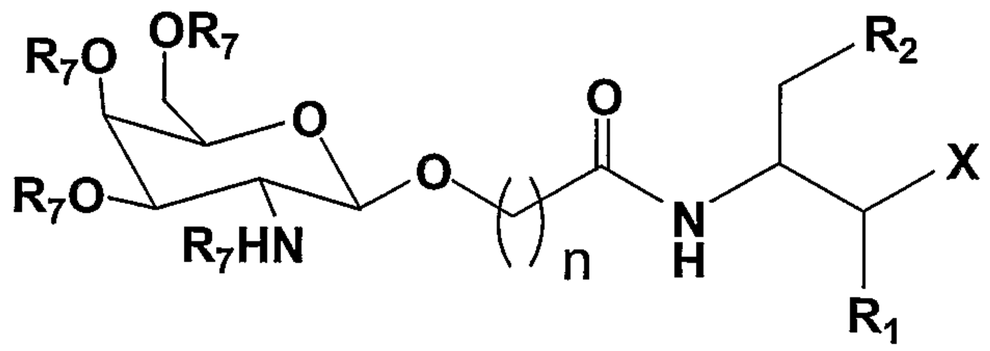
wherein R_1 can include the groups:



and wherein R_2 can include the groups:



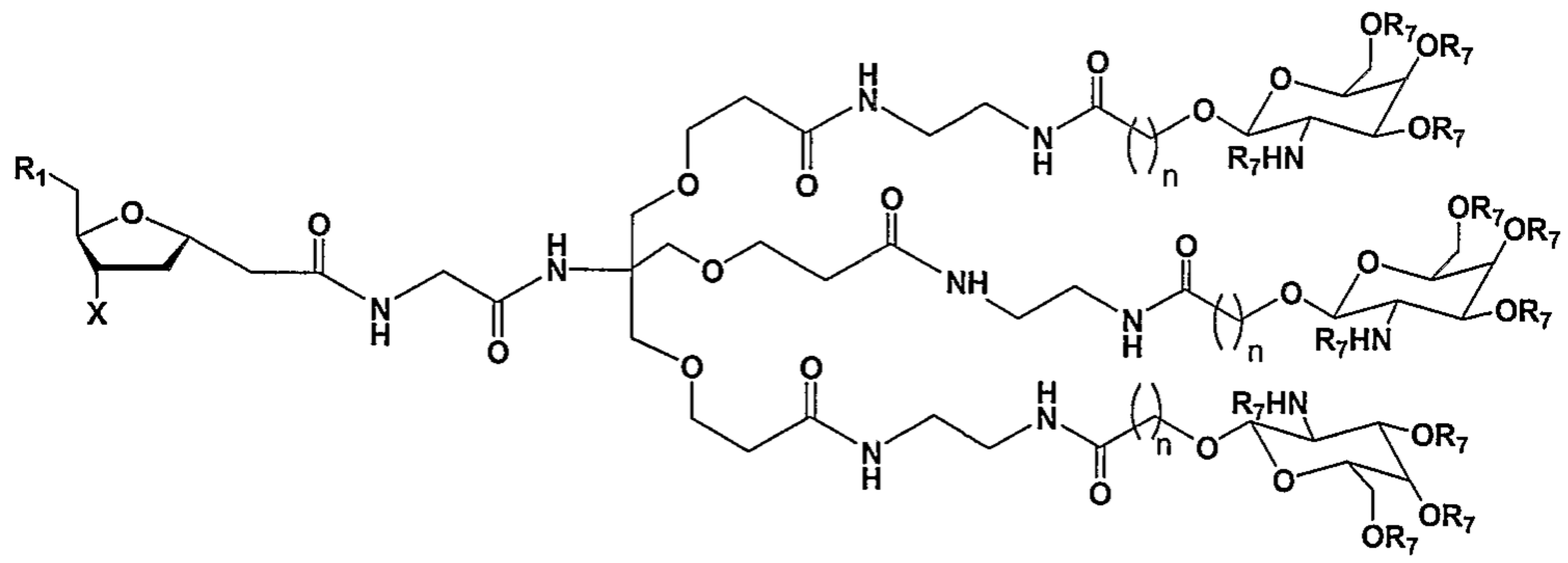
In one embodiment, the invention features a compound having Formula 105:



105

wherein X comprises a siNA molecule or a portion thereof, R_2 comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylhalo, S, N, substituted N, a protecting group, or a nucleotide, polynucleotide, or oligonucleotide or a portion thereof; R_1 independently H, OH, alkyl, substituted alkyl, or halo and each R_7 independently comprises an acyl group that can be present or absent, for example a acetyl group, and n is an integer from about 1 to about 20.

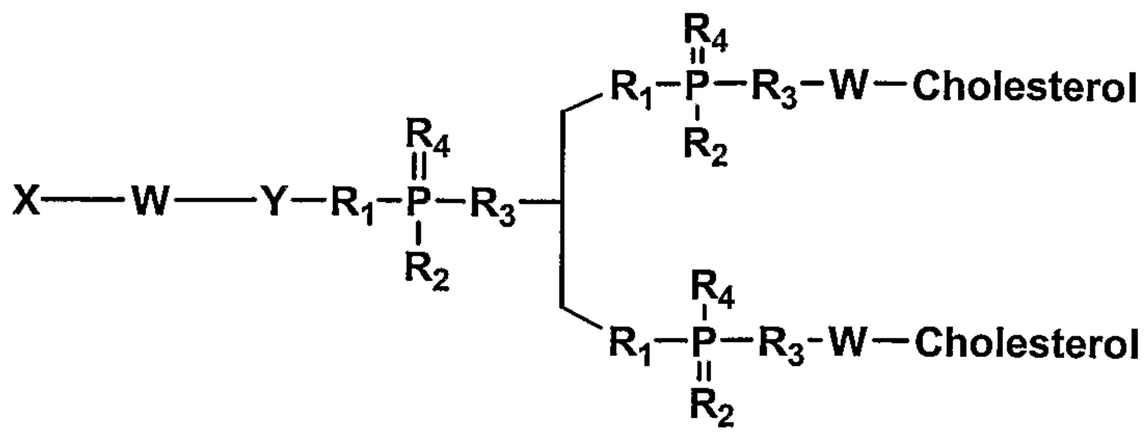
In one embodiment, the invention features a compound having Formula 106:



106

wherein X comprises a siNA molecule or a portion thereof, R_1 comprises H, OH, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, amino acid, peptide, protein, lipid, phospholipid, label, or a portion thereof, or OR_5 where R_5 a removable protecting group, each R_7 independently comprises an acyl group that can be present or absent, for example a acetyl group, and each n is independently an integer from about 1 to about 20

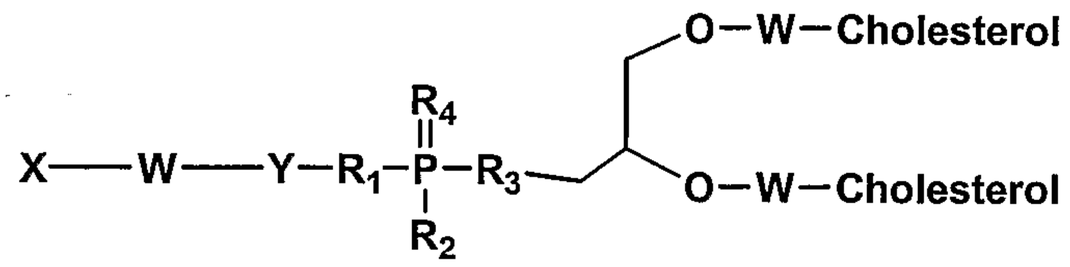
In another embodiment, the invention features a compound having Formula 107:



107

wherein X comprises a siNA molecule or portion thereof; each W independently comprises a linker molecule or chemical linkage that can be present or absent, Y comprises a linker molecule that can be present or absent; each R_1, R_2, R_3, and R_4 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, and Cholesterol comprises cholesterol or an analog, derivative, or metabolite thereof. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

In another embodiment, the invention features a compound having Formula 108:

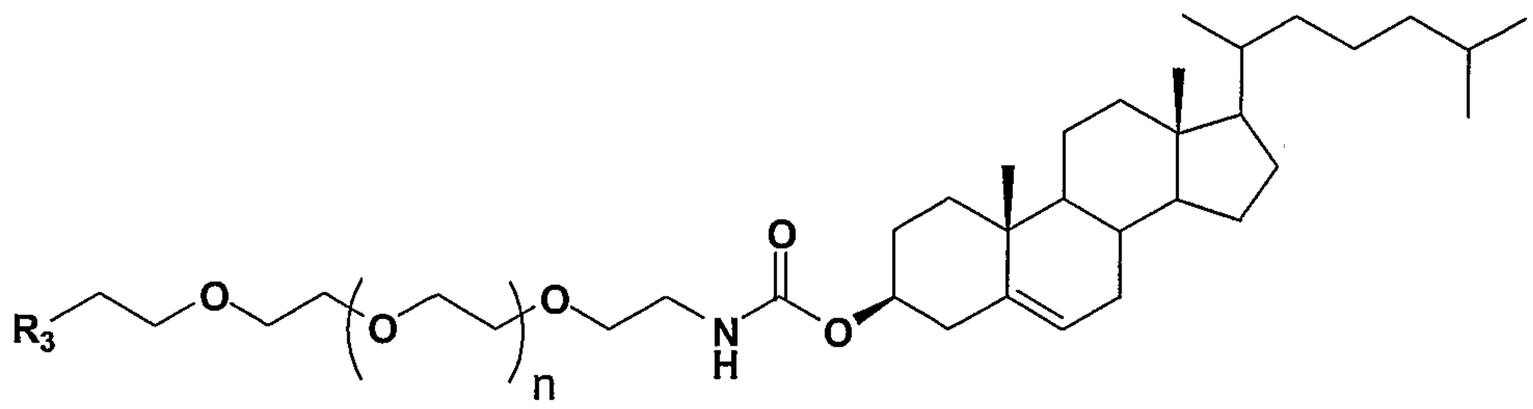


108

wherein X comprises a siNA molecule or portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent, Y comprises a linker molecule that can be present or absent; each R_1, R_2, R_3, and R_4 independently comprises O, OH, H, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, and Cholesterol comprises cholesterol or an analog, derivative, or metabolite thereof. In

another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

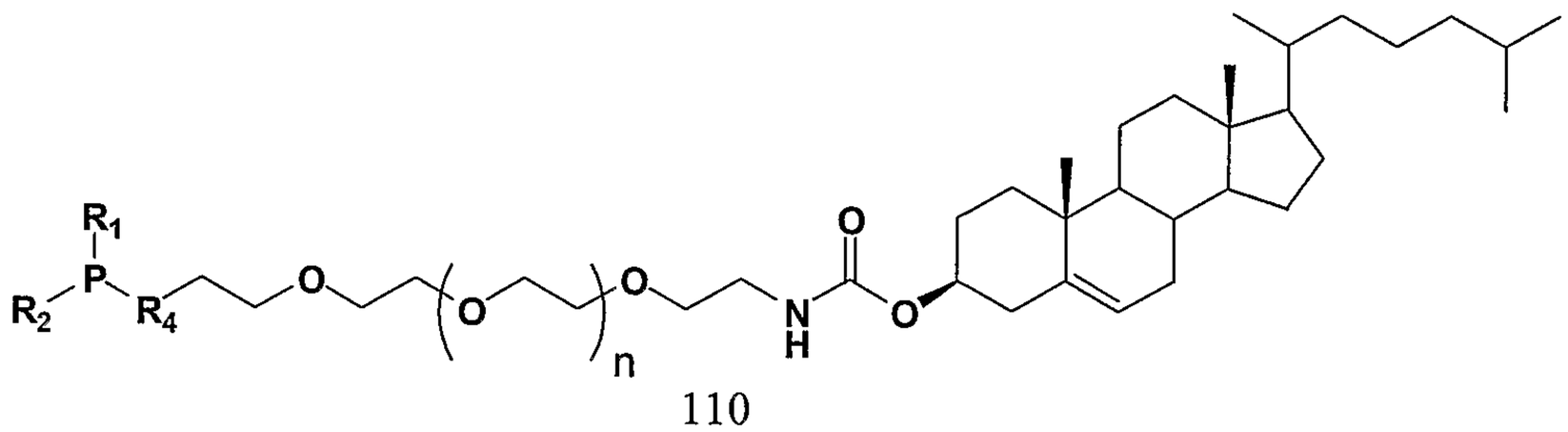
In one embodiment, the W-Cholesterol component of a compound having Formula 107 comprises a compound having Formula 109:



109

wherein R_3 comprises R_3 as described in Formula 107, and n is independently an integer from about 1 to about 20.

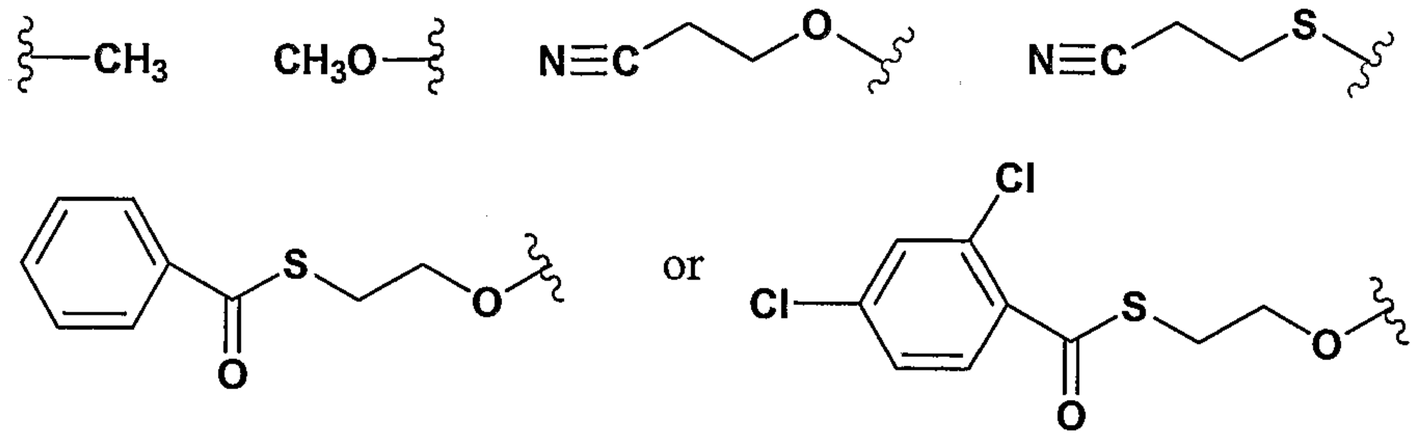
In one embodiment, the invention features a compound having Formula 110:



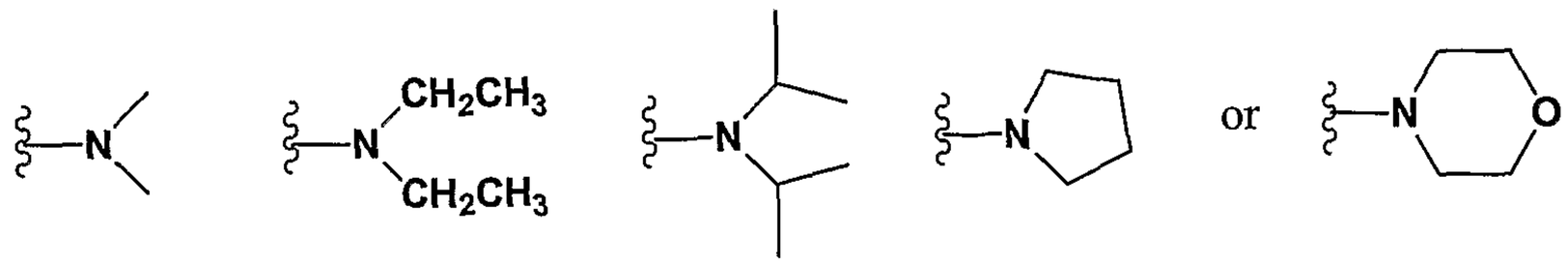
110

wherein R_4 comprises O, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, each n is independently an integer from about 1 to about 20, and

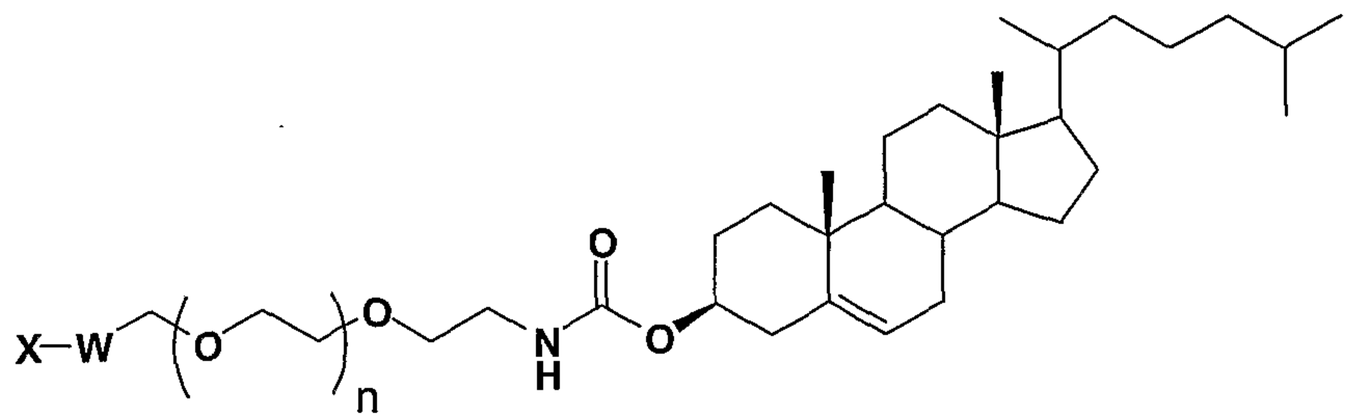
wherein R_1 can include the groups:



and wherein R_2 can include the groups:



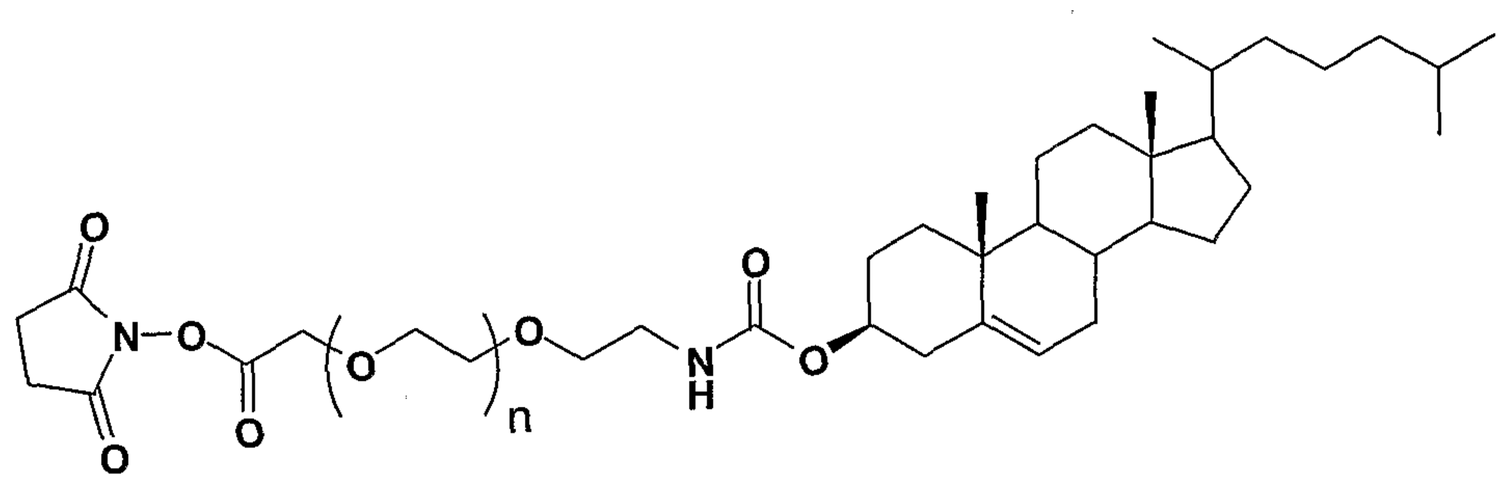
In one embodiment, the invention features a compound having Formula 111:



111

wherein X comprises a siNA molecule or portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent, and n is an integer from about 1 to about 20. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

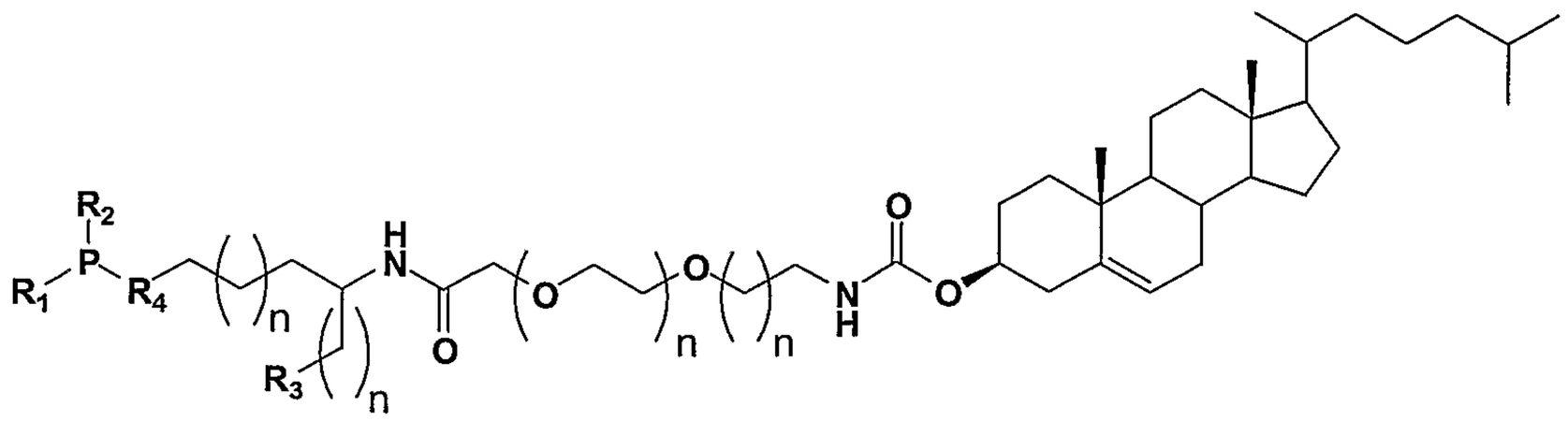
In one embodiment, the invention features a compound having Formula 112:



112

wherein n is an integer from about 1 to about 20. In another embodiment, a compound having Formula 112 is used to generate a compound having Formula 111 via NHS ester mediated coupling with a biologically active molecule, such as a siNA molecule or a portion thereof. In a non-limiting example, the NHS ester coupling can be effectuated via attachment to a free amine present in the siNA molecule, such as an amino linker molecule present on a nucleic acid sugar (e.g., 2'-amino linker) or base (e.g., C5 alkyl amine linker) component of the siNA molecule.

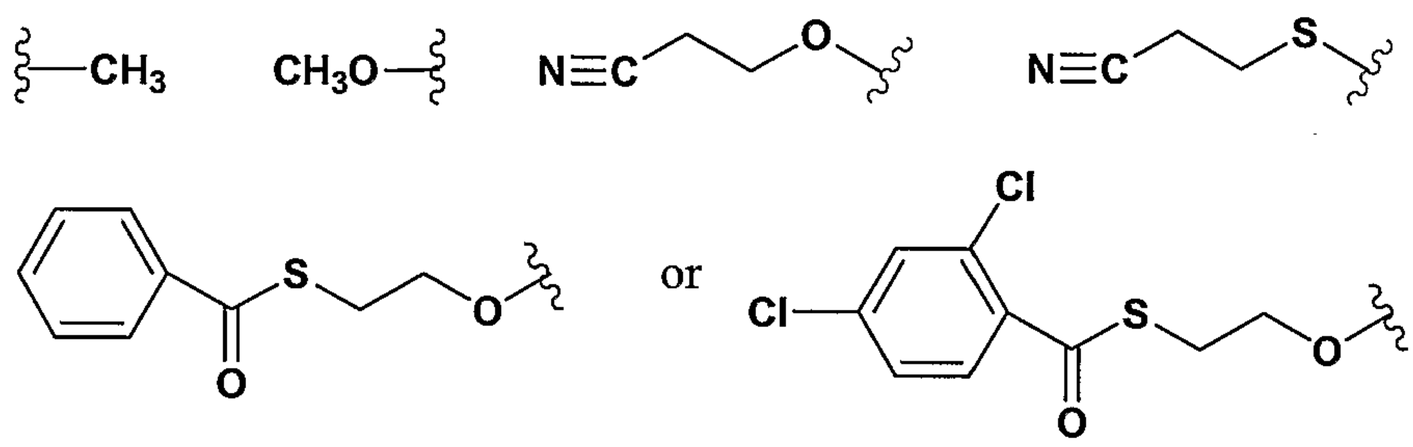
In one embodiment, the invention features a compound having Formula 113:



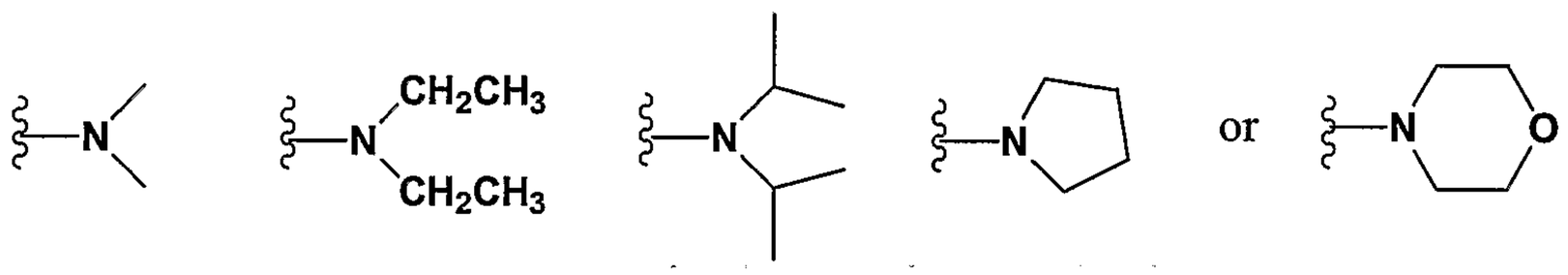
113

wherein R_3 comprises H, OH, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, amino acid, peptide, protein, lipid, phospholipid, label, or a portion thereof, or OR_5 where R_5 a removable protecting group, R_4 comprises O, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, each n is independently an integer from about 1 to about 20, and

wherein R_1 can include the groups:

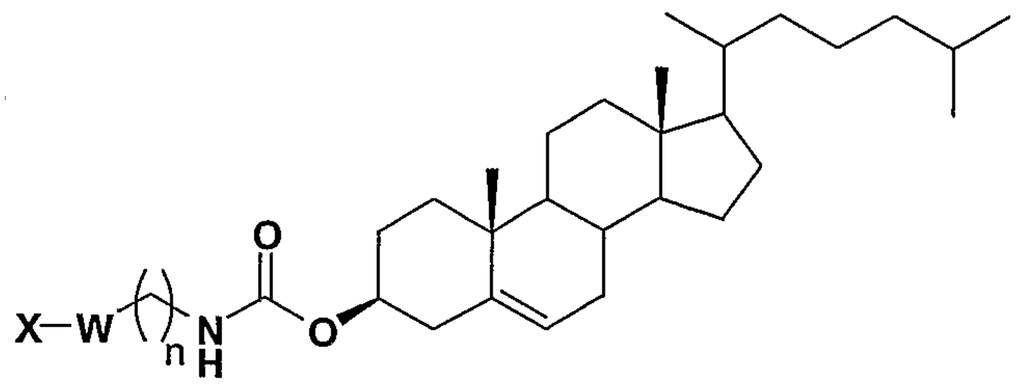


and wherein R_2 can include the groups:



In another embodiment, a compound having Formula 113 is used to generate a compound having Formula 111 via phosphoramidite mediated coupling with a biologically active molecule, such as a siNA molecule or a portion thereof.

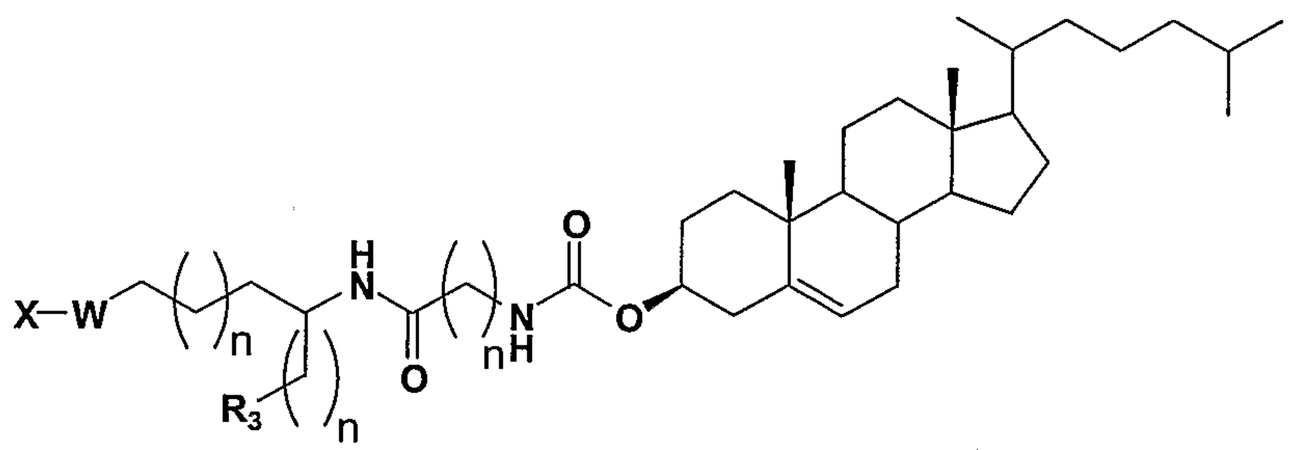
In one embodiment, the invention features a compound having Formula 114:



114

wherein X comprises a siNA molecule or portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent, and n is an integer from about 1 to about 20. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

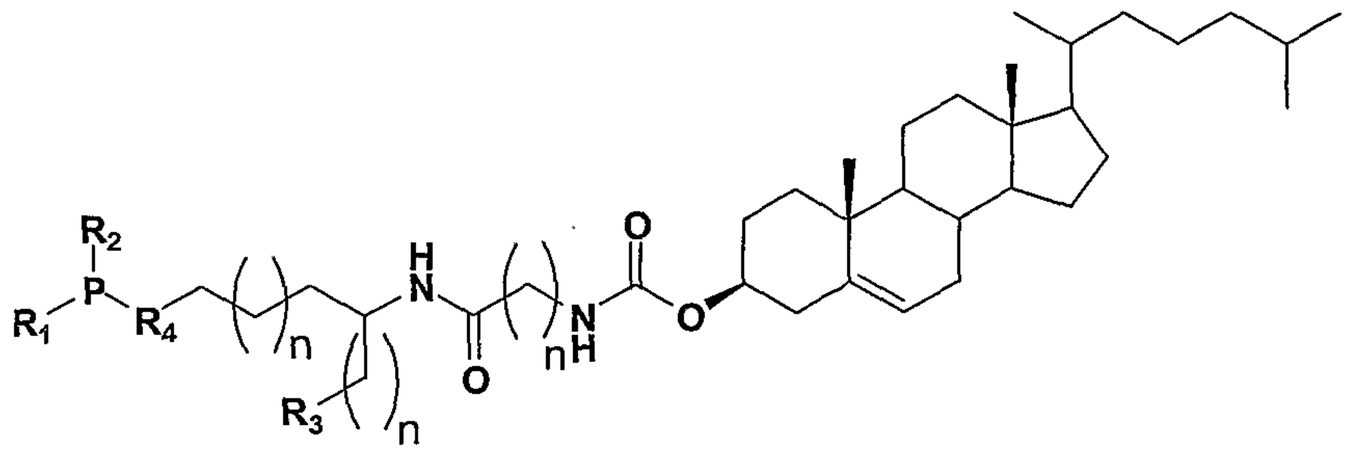
In one embodiment, the invention features a compound having Formula 115:



115

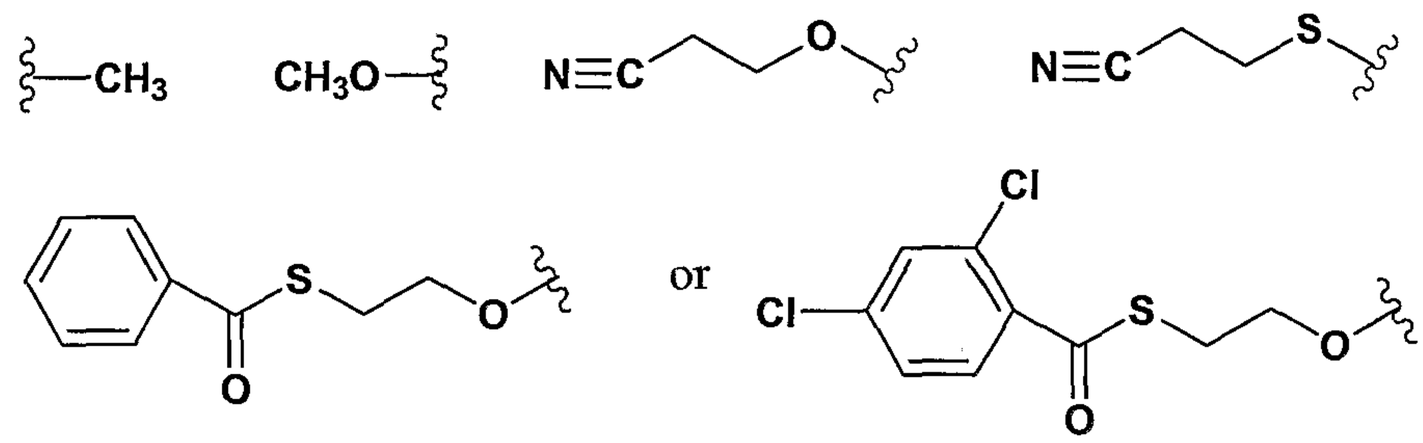
wherein X comprises a siNA molecule or portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent, R_3 comprises H, OH, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, amino acid, peptide, protein, lipid, phospholipid, label, or a portion thereof, or OR_5 where R_5 a removable protecting group, and each n is independently an integer from about 1 to about 20. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

In one embodiment, the invention features a compound having Formula 116:

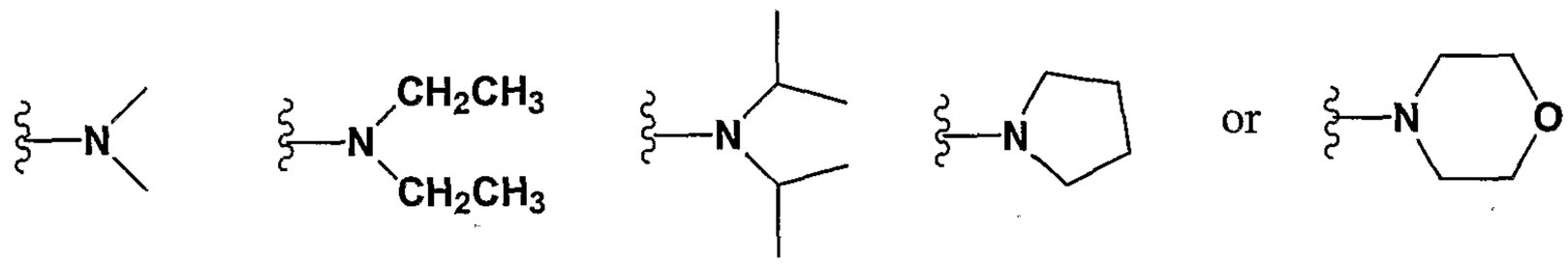


wherein R_3 comprises H, OH, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, amino acid, peptide, protein, lipid, phospholipid, label, or a portion thereof, or OR_5 where R_5 a removable protecting group, R_4 comprises O, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, each n is independently an integer from about 1 to about 20, and

wherein R_1 can include the groups:

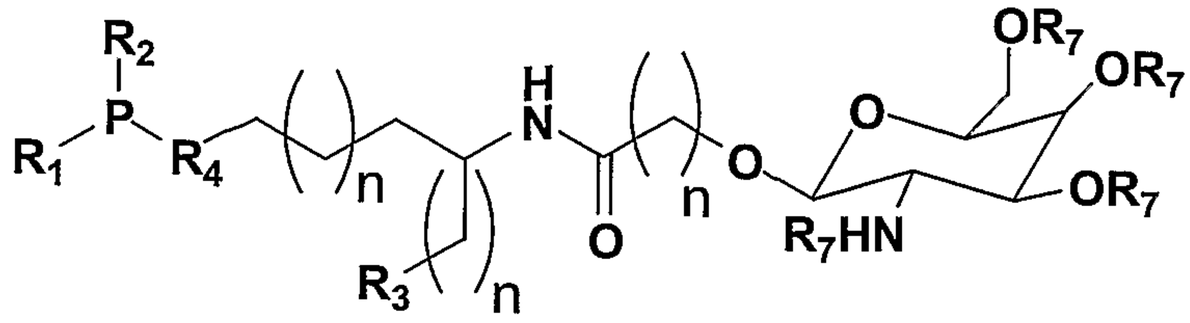


and wherein R_2 can include the groups:



In another embodiment, a compound having Formula 116 is used to generate a compound having Formula 114 or 115 via phosphoramidite mediated coupling with a biologically active molecule, such as a siNA molecule or a portion thereof.

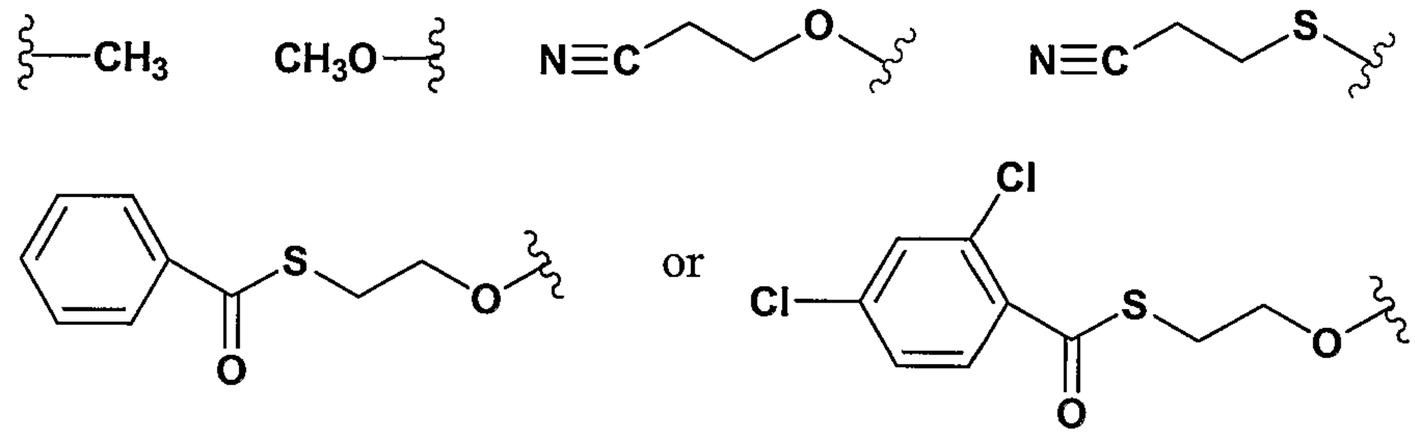
In one embodiment, the invention features a compound having Formula 117:



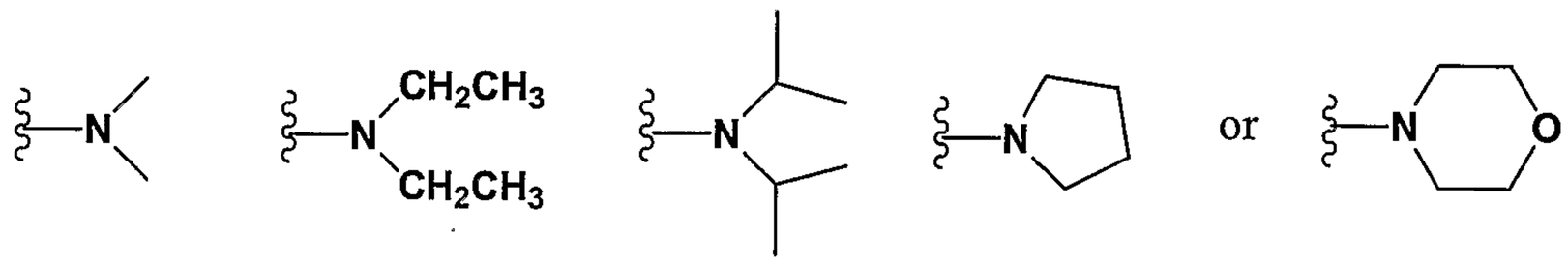
117

wherein R_3 comprises H, OH, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, amino acid, peptide, protein, lipid, phospholipid, label, or a portion thereof, or OR_5 where R_5 a removable protecting group, R_4 comprises O, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, each R_7 independently comprises an acyl group that can be present or absent, for example a acetyl group, each n is independently an integer from about 1 to about 20, and

wherein R_1 can include the groups:

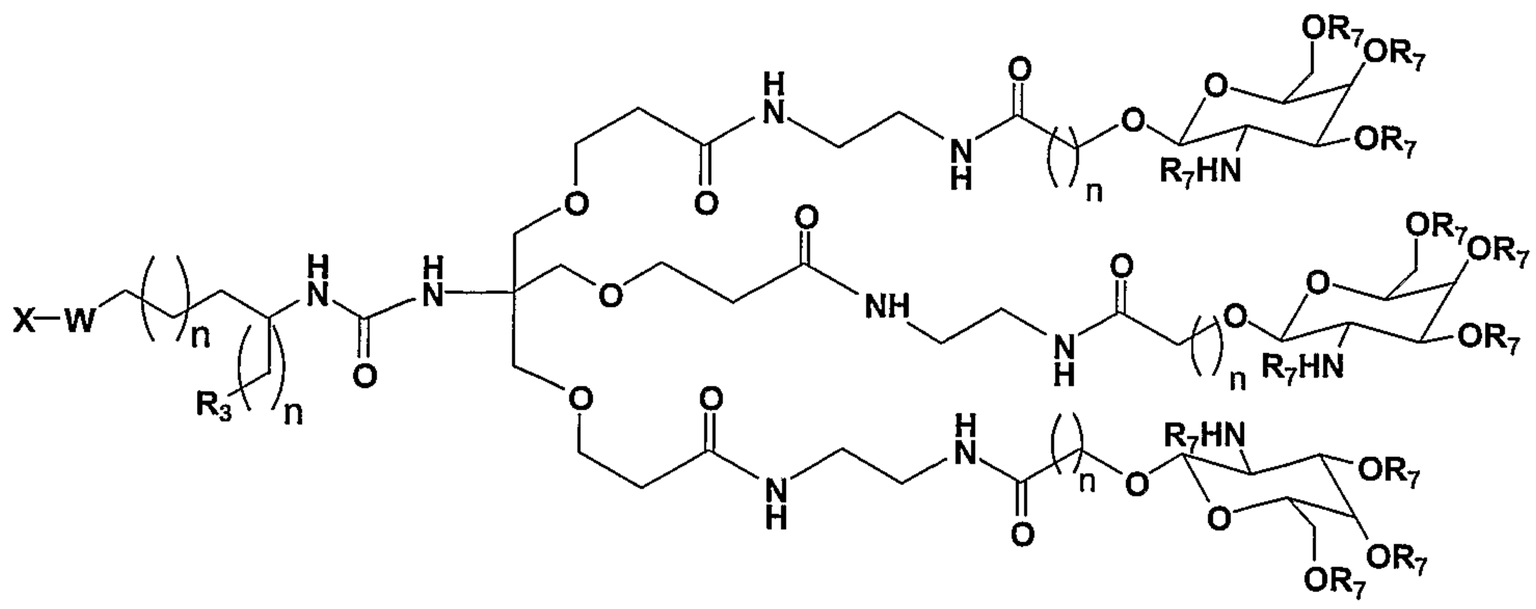


and wherein R_2 can include the groups:



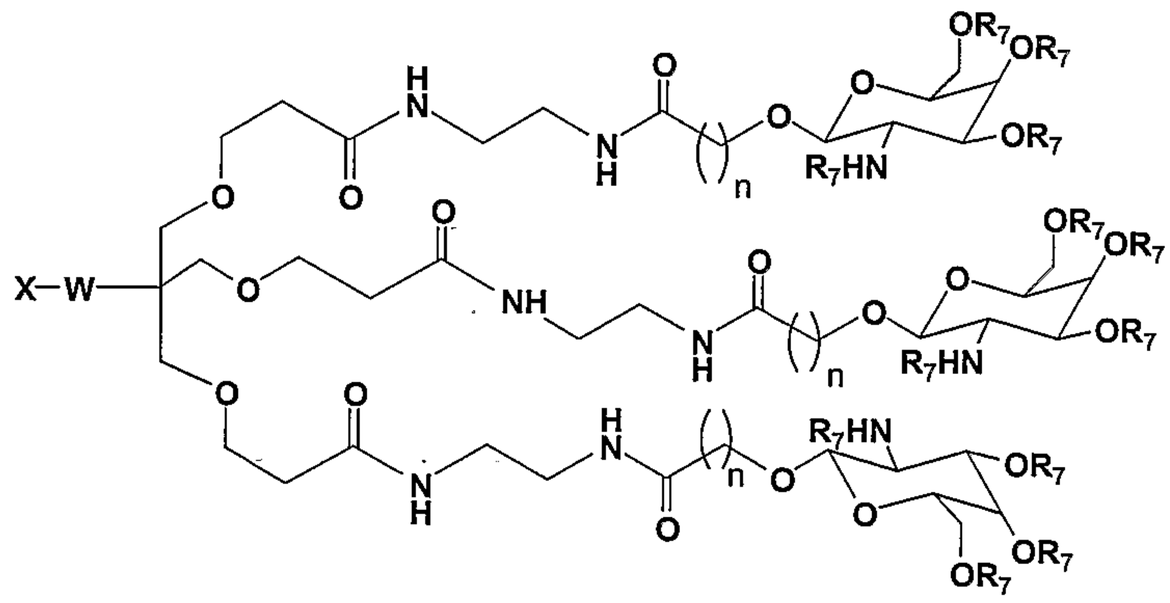
In another embodiment, a compound having Formula 117 is used to generate a compound having Formula 105 via phosphoramidite mediated coupling with a biologically active molecule, such as a siNA molecule or a portion thereof.

In one embodiment, the invention features a compound having Formula 118:



wherein X comprises a siNA molecule or portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent, R_3 comprises H, OH, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, amino acid, peptide, protein, lipid, phospholipid, label, or a portion thereof, or OR_5 where R_5 a removable protecting group, each R_7 independently comprises an acyl group that can be present or absent, for example a acetyl group, and each n is independently an integer from about 1 to about 20. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

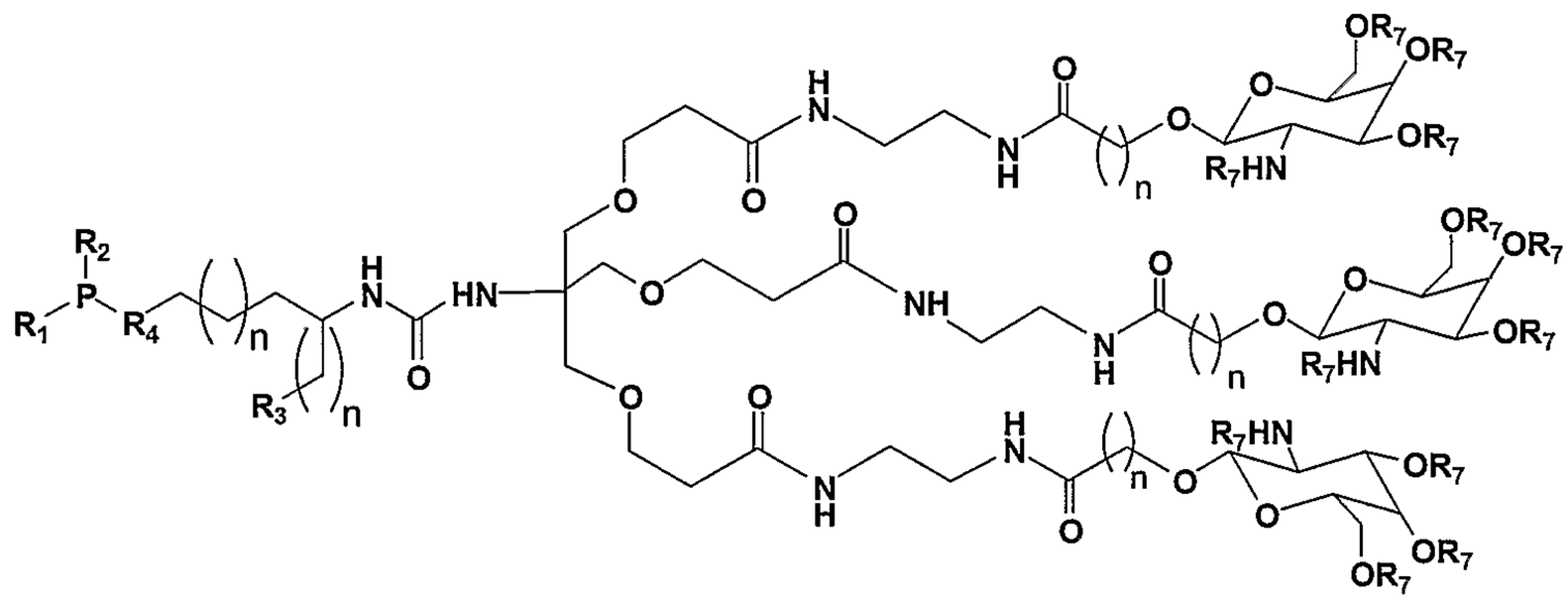
In one embodiment, the invention features a compound having Formula 119:



wherein X comprises a siNA molecule or portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent, each R_7 independently comprises an acyl group that can be present or absent, for example a acetyl group, and each n is independently an integer from about 1 to about 20. In another embodiment, W is

selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

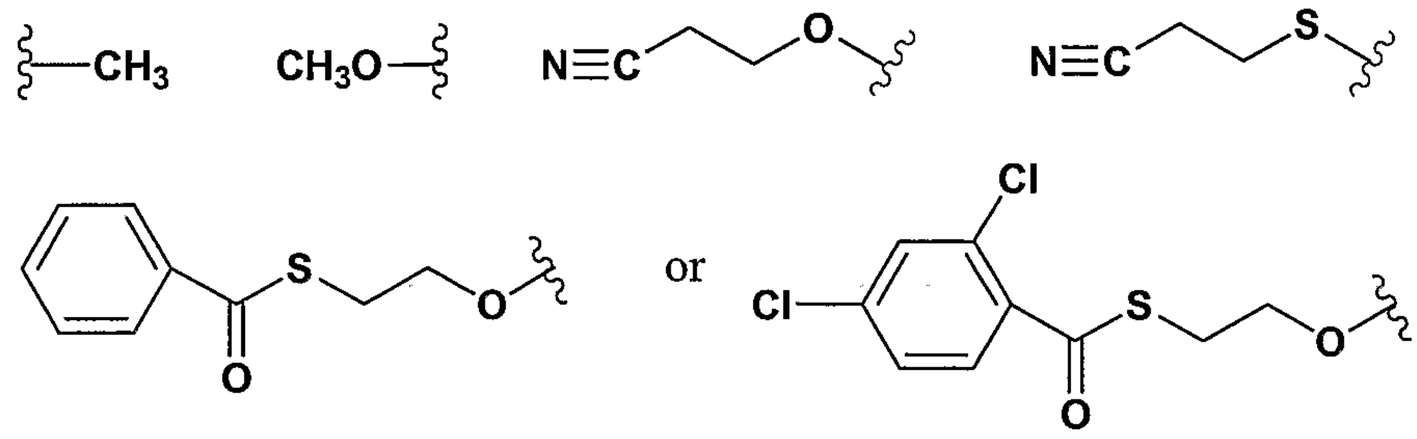
In one embodiment, the invention features a compound having Formula 120:



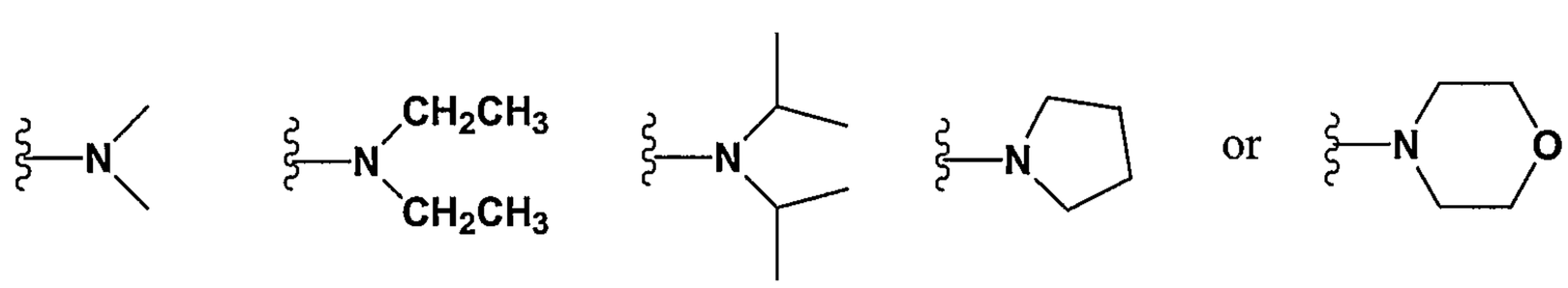
120

wherein R_3 comprises H, OH, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, amino acid, peptide, protein, lipid, phospholipid, label, or a portion thereof, or OR_5 where R_5 a removable protecting group, R_4 comprises O, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, each R_7 independently comprises an acyl group that can be present or absent, for example a acetyl group, each n is independently an integer from about 1 to about 20, and

wherein R_1 can include the groups:

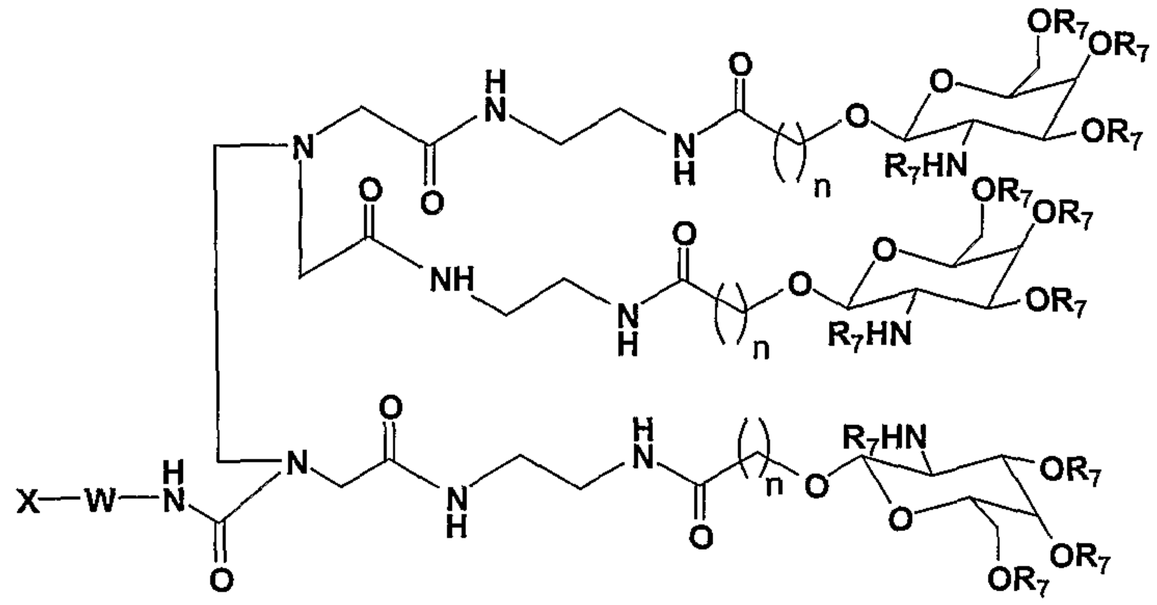


and wherein R_2 can include the groups:



In another embodiment, a compound having Formula 120 is used to generate a compound having Formula 118 or 119 via phosphoramidite mediated coupling with a biologically active molecule, such as a siNA molecule or a portion thereof.

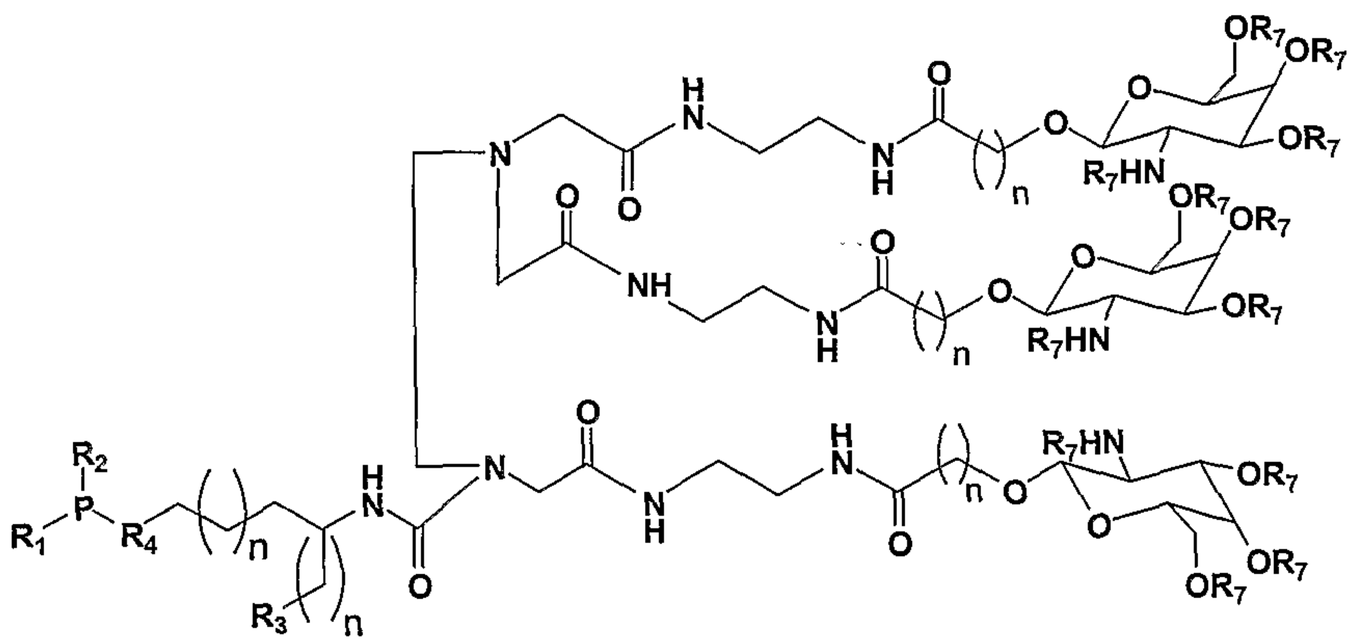
In one embodiment, the invention features a compound having Formula 121:



121

wherein X comprises a siNA molecule or portion thereof; W comprises a linker molecule or chemical linkage that can be present or absent, each R_7 independently comprises an acyl group that can be present or absent, for example a acetyl group, and each n is independently an integer from about 1 to about 20. In another embodiment, W is selected from the group consisting of amide, phosphate, phosphate ester, phosphoramidate, or thiophosphate ester linkage.

In one embodiment, the invention features a compound having Formula 122:

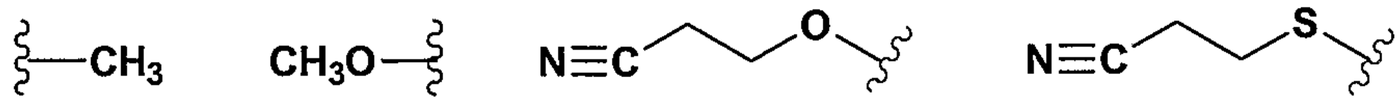


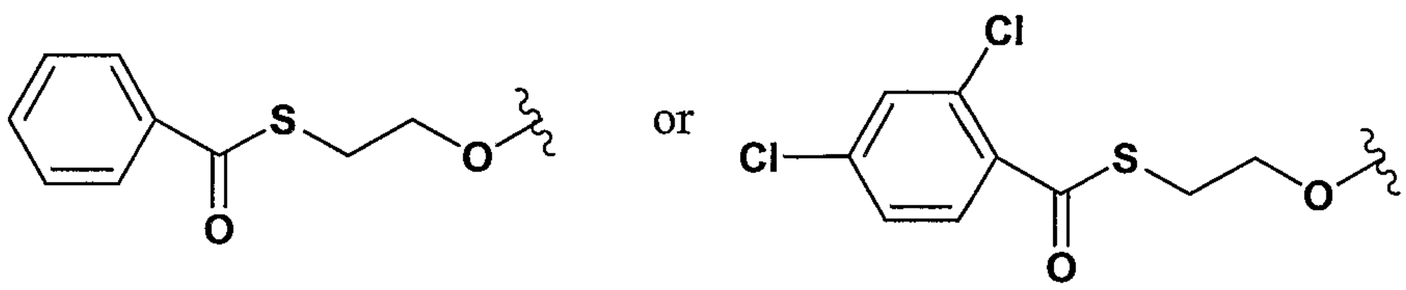
122

wherein R_3 comprises H, OH, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, amino acid, peptide, protein, lipid, phospholipid, label, or a

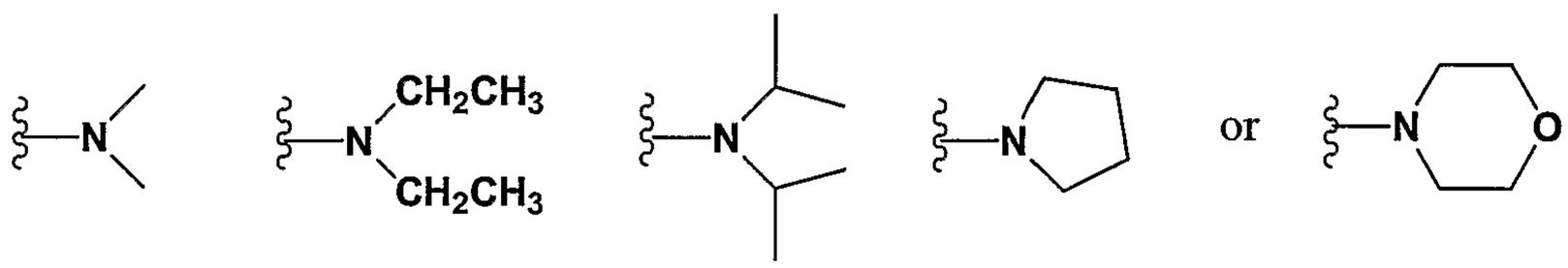
portion thereof, or OR_5 where R_5 a removable protecting group, R_4 comprises O, alkyl, alkylhalo, O-alkyl, O-alkylcyano, S, S-alkyl, S-alkylcyano, N or substituted N, each R_7 independently comprises an acyl group that can be present or absent, for example a acetyl group, each n is independently an integer from about 1 to about 20, and

wherein R_1 can include the groups:



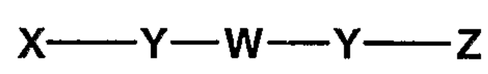


and wherein R_2 can include the groups:



In another embodiment, a compound having Formula 122 is used to generate a compound having Formula 121 via phosphoramidite mediated coupling with a biologically active molecule, such as a siNA molecule or a portion thereof.

In one embodiment, the invention features a compound having Formula 94,



94

wherein X comprises a siNA molecule or a portion thereof; each Y independently comprises a linker or chemical linkage that can be present or absent, W comprises a biodegradable nucleic acid linker molecule, and Z comprises a biologically active molecule, for example an enzymatic nucleic acid, allozyme, antisense nucleic acid, siNA, 2,5-A chimera, decoy, aptamer or triplex forming oligonucleotide, peptide, protein, or antibody.

In another embodiment, W of a compound having Formula 94 of the invention comprises 5'-cytidine-deoxythymidine-3', 5'-deoxythymidine-cytidine-3',

5'-cytidine-deoxyuridine-3', 5'-deoxyuridine-cytidine-3', 5'-uridine-deoxythymidine-3', or 5'-deoxythymidine-uridine-3'.

In yet another embodiment, W of a compound having Formula 94 of the invention comprises 5'-adenosine-deoxythymidine-3', 5'-deoxythymidine-adenosine-3', 5'-adenosine-deoxyuridine-3', or 5'-deoxyuridine-adenosine-3'.

In another embodiment, Y of a compound having Formula 94 of the invention comprises a phosphorus containing linkage, phoshoramidate linkage, phosphodiester linkage, phosphorothioate linkage, amide linkage, ester linkage, carbamate linkage, disulfide linkage, oxime linkage, or morpholino linkage.

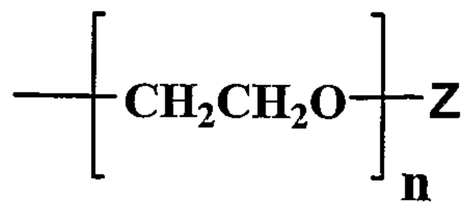
In another embodiment, compounds having Formula 89 and 91 of the invention are synthesized by periodate oxidation of an N-terminal Serine or Threonine residue of a peptide or protein.

In one embodiment, X of compounds having Formulae 43, 44, 46-52, 58, 61-65, 85-88, 92, 94, 95, 99, 100, 105-108, 111, 114, 115, 118, 119, or 121 of the invention comprises a siNA molecule or a portion thereof. In one embodiment, the siNA molecule can be conjugated at the 5' end, 3'-end, or both 5' and 3' ends of the sense strand or region of the siNA. In one embodiment, the siNA molecule can be conjugated at the 3'-end of the antisense strand or region of the siNA with a compound of the invention. In one embodiment, both the sense strand and antisense strands or regions of the siNA molecule are conjugated with a compound of the invention. In one embodiment, only the sense strand or region of the siNA is conjugated with a compound of the invention. In one embodiment, only the antisense strand or region of the siNA is conjugated with a compound of the invention.

In one embodiment, W and/or Y of compounds having Formulae 43, 44, 46-52, 58, 61-65, 85-88, 92, 94, 95, 99, 100, 101, 107, 108, 111, 114, 115, 118, 119, or 121 of the invention comprises a degradable or cleavable linker, for example a nucleic acid sequence comprising ribonucleotides and/or deoxynucleotides, such as a dimer, trimer, or tetramer. A non limiting example of a nucleic acid cleavable linker is an adenosine-deoxythymidine (A-dT) dimer or a cytidine-deoxythymidine (C-dT) dimer. In yet another embodiment, W and/or V of compounds having Formulae 43, 44, 48-51, 58, 63-65, 96, 99, 100, 107, 108, 111, 114, 115, 118, 119, or 121 of the invention comprises a N-hydroxy succinimide

(NHS) ester linkage, oxime linkage, disulfide linkage, phosphoramidate, phosphorothioate, phosphorodithioate, phosphodiester linkage, or NHC(O), $CH_3NC(O)$, CONH, $C(O)NCH_3$, S, SO, SO_2, O, NH, NCH_3 group. In another embodiment, the degradable linker, W and/or Y, of compounds having Formulae Formulae 43, 44, 46-52, 58, 61-65, 85-88, 92, 94, 95, 99, 100, 101, 107, 108, 111, 114, 115, 118, 119, or 121 of the invention comprises a linker that is susceptible to cleavage by carboxypeptidase activity.

In another embodiment, W and/or Y of Formulae Formulae 43, 44, 46-52, 58, 61-65, 85-88, 92, 94, 95, 99, 100, 101, 107, 108, 111, 114, 115, 118, 119, or 121 comprises a polyethylene glycol linker having Formula 45:



45

wherein Z comprises H, OH, O-alkyl, SH, S-alkyl, alkyl, substituted alkyl, aryl, substituted aryl, amino, substituted amino, nucleotide, nucleoside, nucleic acid, oligonucleotide, amino acid, peptide, protein, lipid, phospholipid, or label; and n is an integer from about 1 to about 100.

In one embodiment, the nucleic acid conjugates of the instant invention are assembled by solid phase synthesis, for example on an automated peptide synthesizer, for example a Miligen 9050 synthesizer and/or an automated oligonucleotide synthesizer such as an ABI 394, 390Z, or Pharmacia OligoProcess, OligoPilot, OligoMax, or AKTA synthesizer. In another embodiment, the nucleic acid conjugates of the invention are assembled post synthetically, for example, following solid phase oligonucleotide synthesis (see for example **Figures 45, 50, 53, and 73**).

In another embodiment, V of compounds having Formula 58-63 and 96 comprise peptides having SEQ ID NOS: **1114-1123** (**Table V**).

In one embodiment, the nucleic acid conjugates of the instant invention are assembled post synthetically, for example, following solid phase oligonucleotide synthesis.

The present invention provides compositions and conjugates comprising nucleosidic and non-nucleosidic derivatives. The present invention also provides nucleic acid,

polynucleotide and oligonucleotide derivatives including RNA, DNA, and PNA based conjugates. The attachment of compounds of the invention to nucleosides, nucleotides, non-nucleosides, and nucleic acid molecules is provided at any position within the molecule, for example, at internucleotide linkages, nucleosidic sugar hydroxyl groups such as 5', 3', and 2'-hydroxyls, and/or at nucleobase positions such as amino and carbonyl groups.

The exemplary conjugates of the invention are described as compounds of the formulae herein, however, other peptide, protein, phospholipid, and poly-alkyl glycol derivatives are provided by the invention, including various analogs of the compounds of formulae 1-122, including but not limited to different isomers of the compounds described herein.

The exemplary folate conjugates of the invention are described as compounds shown by formulae herein, however, other folate and antifolate derivatives are provided by the invention, including various folate analogs of the formulae of the invention, including dihydrofloates, tetrahydrofolates, tetrahydorpterins, folinic acid, pteropolyglutamic acid, 1-deza, 3-deaza, 5-deaza, 8-deaza, 10-deaza, 1,5-deaza, 5,10 dideaza, 8,10-dideaza, and 5,8-dideaza folates, antifolates, and pteroic acids. As used herein, the term "folate" is meant to refer to folate and folate derivatives, including pteroic acid derivatives and analogs.

The present invention features compositions and conjugates to facilitate delivery of molecules into a biological system such as cells. The conjugates provided by the instant invention can impart therapeutic activity by transferring therapeutic compounds across cellular membranes. The present invention encompasses the design and synthesis of novel agents for the delivery of molecules, including but not limited to siNA molecules. In general, the transporters described are designed to be used either individually or as part of a multi-component system. The compounds of the invention generally shown in Formulae herein are expected to improve delivery of molecules into a number of cell types originating from different tissues, in the presence or absence of serum.

In another embodiment, the compounds of the invention are provided as a surface component of a lipid aggregate, such as a liposome encapsulated with the predetermined molecule to be delivered. Liposomes, which can be unilamellar or multilamellar, can

introduce encapsulated material into a cell by different mechanisms. For example, the liposome can directly introduce its encapsulated material into the cell cytoplasm by fusing with the cell membrane. Alternatively, the liposome can be compartmentalized into an acidic vacuole (i.e., an endosome) and its contents released from the liposome and out of the acidic vacuole into the cellular cytoplasm.

In one embodiment the invention features a lipid aggregate formulation of the compounds described herein, including phosphatidylcholine (of varying chain length; e.g., egg yolk phosphatidylcholine), cholesterol, a cationic lipid, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-polythyleneglycol-2000 (DSPE-PEG2000). The cationic lipid component of this lipid aggregate can be any cationic lipid known in the art such as dioleoyl 1,2,-diacyl-3-trimethylammonium-propane (DOTAP). In another embodiment this cationic lipid aggregate comprises a covalently bound compound described in any of the Formulae herein.

In another embodiment, polyethylene glycol (PEG) is covalently attached to the compounds of the present invention. The attached PEG can be any molecular weight but is preferably between 2000-50,000 daltons.

The compounds and methods of the present invention are useful for introducing nucleotides, nucleosides, nucleic acid molecules, lipids, peptides, proteins, and/or non-nucleosidic small molecules into a cell. For example, the invention can be used for nucleotide, nucleoside, nucleic acid, lipids, peptides, proteins, and/or non-nucleosidic small molecule delivery where the corresponding target site of action exists intracellularly.

In one embodiment, the compounds of the instant invention provide conjugates of molecules that can interact with cellular receptors, such as high affinity folate receptors and ASGPr receptors, and provide a number of features that allow the efficient delivery and subsequent release of conjugated compounds across biological membranes. The compounds utilize chemical linkages between the receptor ligand and the compound to be delivered of length that can interact preferentially with cellular receptors. Furthermore, the chemical linkages between the ligand and the compound to be delivered can be designed as degradable linkages, for example by utilizing a phosphate linkage that is proximal to a nucleophile, such as a hydroxyl group. Deprotonation of the hydroxyl group or an equivalent group, as a result of pH or interaction with a nuclease, can result in nucleophilic

attack of the phosphate resulting in a cyclic phosphate intermediate that can be hydrolyzed. This cleavage mechanism is analogous RNA cleavage in the presence of a base or RNA nuclease. Alternately, other degradable linkages can be selected that respond to various factors such as UV irradiation, cellular nucleases, pH, temperature etc. The use of degradable linkages allows the delivered compound to be released in a predetermined system, for example in the cytoplasm of a cell, or in a particular cellular organelle.

The present invention also provides ligand derived phosphoramidites that are readily conjugated to compounds and molecules of interest. Phosphoramidite compounds of the invention permit the direct attachment of conjugates to molecules of interest without the need for using nucleic acid phosphoramidite species as scaffolds. As such, the used of phosphoramidite chemistry can be used directly in coupling the compounds of the invention to a compound of interest, without the need for other condensation reactions, such as condensation of the ligand to an amino group on the nucleic acid, for example at the N6 position of adenosine or a 2'-deoxy-2'-amino function. Additionally, compounds of the invention can be used to introduce non-nucleic acid based conjugated linkages into oligonucleotides that can provide more efficient coupling during oligonucleotide synthesis than the use of nucleic acid-based phosphoramidites. This improved coupling can take into account improved steric considerations of abasic or non-nucleosidic scaffolds bearing pendant alkyl linkages.

Compounds of the invention utilizing triphosphate groups can be utilized in the enzymatic incorporation of conjugate molecules into oligonucleotides. Such enzymatic incorporation is useful when conjugates are used in post-synthetic enzymatic conjugation or selection reactions, (see for example Matulic-Adamic *et al.,* 2000, *Bioorg. Med. Chem. Lett.,* 10, 1299-1302; Lee *et al.,* 2001, *NAR.*, 29, 1565-1573; Joyce, 1989, *Gene,* 82, 83-87; Beaudry *et al.,* 1992, *Science* 257, 635-641; Joyce, 1992, *Scientific American* 267, 90-97; Breaker *et al.,* 1994, *TIBTECH* 12, 268; Bartel *et al.,*1993, *Science* 261:1411-1418; Szostak, 1993, *TIBS* 17, 89-93; Kumar *et al.,* 1995, *FASEB J.,* 9, 1183; Breaker, 1996, *Curr. Op. Biotech.*, 7, 442; Santoro *et al.,* 1997, *Proc. Natl. Acad. Sci.,* 94, 4262; Tang *et al.,* 1997, *RNA* 3, 914; Nakamaye & Eckstein, 1994, *supra*; Long & Uhlenbeck, 1994, supra; Ishizaka et al., 1995, *supra*; Vaish *et al.,* 1997, *Biochemistry* 36, 6495; Kuwabara *et al.,* 2000, *Curr. Opin. Chem. Biol.,* 4, 669).

The term "biodegradable linker" as used herein, refers to a nucleic acid or non-nucleic acid linker molecule that is designed as a biodegradable linker to connect one molecule to another molecule, for example, a biologically active molecule to a siNA molecule of the invention or the sense and antisense strands of a siNA molecule of the invention. The biodegradable linker is designed such that its stability can be modulated for a particular purpose, such as delivery to a particular tissue or cell type. The stability of a nucleic acid-based biodegradable linker molecule can be modulated by using various chemistries, for example combinations of ribonucleotides, deoxyribonucleotides, and chemically-modified nucleotides, such as 2'-O-methyl, 2'-fluoro, 2'-amino, 2'-O-amino, 2'-C-allyl, 2'-O-allyl, and other 2'-modified or base modified nucleotides. The biodegradable nucleic acid linker molecule can be a dimer, trimer, tetramer or longer nucleic acid molecule, for example, an oligonucleotide of about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length, or can comprise a single nucleotide with a phosphorus-based linkage, for example, a phosphoramidate or phosphodiester linkage. The biodegradable nucleic acid linker molecule can also comprise nucleic acid backbone, nucleic acid sugar, or nucleic acid base modifications.

The term "biodegradable" as used herein, refers to degradation in a biological system, for example enzymatic degradation or chemical degradation.

The term "biologically active molecule" as used herein, refers to compounds or molecules that are capable of eliciting or modifying a biological response in a system. Non-limiting examples of biologically active siNA molecules either alone or in combination with other molecules contemplated by the instant invention include therapeutically active molecules such as antibodies, cholesterol, hormones, antivirals, peptides, proteins, chemotherapeutics, small molecules, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, 2,5-A chimeras, siNA, dsRNA, allozymes, aptamers, decoys and analogs thereof. Biologically active molecules of the invention also include molecules capable of modulating the pharmacokinetics and/or pharmacodynamics of other biologically active molecules, for example, lipids and polymers such as polyamines, polyamides, polyethylene glycol and other polyethers.

The term "phospholipid" as used herein, refers to a hydrophobic molecule comprising at least one phosphorus group. For example, a phospholipid can comprise a phosphorus-containing group and saturated or unsaturated alkyl group, optionally substituted with OH, COOH, oxo, amine, or substituted or unsubstituted aryl groups.

The term "alkyl" as used herein refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain "isoalkyl", and cyclic alkyl groups. The term "alkyl" also comprises alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, alkenyl, alkynyl, alkoxy, cycloalkenyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, C1-C6 hydrocarbyl, aryl or substituted aryl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably it is a lower alkyl of from about 1 to about 7 carbons, more preferably about 1 to about 4 carbons. The alkyl group can be substituted or unsubstituted. When substituted the substituted group(s) preferably comprise hydroxy, oxy, thio, amino, nitro, cyano, alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, silyl, alkenyl, alkynyl, alkoxy, cycloalkenyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, C1-C6 hydrocarbyl, aryl or substituted aryl groups. The term "alkyl" also includes alkenyl groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has about 2 to about 12 carbons. More preferably it is a lower alkenyl of from about 2 to about 7 carbons, more preferably about 2 to about 4 carbons. The alkenyl group can be substituted or unsubstituted. When substituted the substituted group(s) preferably comprise hydroxy, oxy, thio, amino, nitro, cyano, alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, silyl, alkenyl, alkynyl, alkoxy, cycloalkenyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, C1-C6 hydrocarbyl, aryl or substituted aryl groups. The term "alkyl" also includes alkynyl groups containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has about 2 to about 12 carbons. More preferably it is a lower alkynyl of from about 2 to about 7 carbons, more preferably about 2 to about 4 carbons. The alkynyl group can be substituted or unsubstituted. When substituted the substituted group(s) preferably comprise hydroxy, oxy, thio, amino, nitro, cyano, alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, silyl, alkenyl, alkynyl, alkoxy, cycloalkenyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, C1-C6 hydrocarbyl, aryl or substituted aryl groups. Alkyl groups or moieties of the invention can also include aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and

ester groups. The preferred substituent(s) of aryl groups are halogen, trihalomethyl, hydroxyl, SH, OH, cyano, alkoxy, alkyl, alkenyl, alkynyl, and amino groups. An "alkylaryl" group refers to an alkyl group (as described above) covalently joined to an aryl group (as described above). Carbocyclic aryl groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted. Heterocyclic aryl groups are groups having from about 1 to about 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted. An "amide" refers to an -C(O)-NH-R, where R is either alkyl, aryl, alkylaryl or hydrogen. An "ester" refers to an -C(O)-OR', where R is either alkyl, aryl, alkylaryl or hydrogen.

The term “alkoxyalkyl” as used herein refers to an alkyl-O-alkyl ether, for example, methoxyethyl or ethoxymethyl.

The term “alkyl-thio-alkyl” as used herein refers to an alkyl-S-alkyl thioether, for example, methylthiomethyl or methylthioethyl.

The term “amino” as used herein refers to a nitrogen containing group as is known in the art derived from ammonia by the replacement of one or more hydrogen radicals by organic radicals. For example, the terms “aminoacyl” and “aminoalkyl” refer to specific N-substituted organic radicals with acyl and alkyl substituent groups respectively.

The term “amination” as used herein refers to a process in which an amino group or substituted amine is introduced into an organic molecule.

The term “exocyclic amine protecting moiety” as used herein refers to a nucleobase amino protecting group compatible with oligonucleotide synthesis, for example, an acyl or amide group.

The term “alkenyl” as used herein refers to a straight or branched hydrocarbon of a designed number of carbon atoms containing at least one carbon-carbon double bond. Examples of “alkenyl” include vinyl, allyl, and 2-methyl-3-heptene.

The term “alkoxy” as used herein refers to an alkyl group of indicated number of carbon atoms attached to the parent molecular moiety through an oxygen bridge. Examples of alkoxy groups include, for example, methoxy, ethoxy, propoxy and isopropoxy.

The term "alkynyl" as used herein refers to a straight or branched hydrocarbon of a designed number of carbon atoms containing at least one carbon-carbon triple bond. Examples of “alkynyl” include propargyl, propyne, and 3-hexyne.

The term "aryl" as used herein refers to an aromatic hydrocarbon ring system containing at least one aromatic ring. The aromatic ring can optionally be fused or otherwise attached to other aromatic hydrocarbon rings or non-aromatic hydrocarbon rings. Examples of aryl groups include, for example, phenyl, naphthyl, 1,2,3,4-tetrahydronaphthalene and biphenyl. Preferred examples of aryl groups include phenyl and naphthyl.

The term "cycloalkenyl" as used herein refers to a C3-C8 cyclic hydrocarbon containing at least one carbon-carbon double bond. Examples of cycloalkenyl include cyclopropenyl, cyclobutenyl, cyclopentenyl, cyclopentadiene, cyclohexenyl, 1,3-cyclohexadiene, cycloheptenyl, cycloheptatrienyl, and cyclooctenyl.

The term "cycloalkyl" as used herein refers to a C3-C8 cyclic hydrocarbon. Examples of cycloalkyl include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl.

The term "cycloalkylalkyl," as used herein, refers to a C3-C7 cycloalkyl group attached to the parent molecular moiety through an alkyl group, as defined above. Examples of cycloalkylalkyl groups include cyclopropylmethyl and cyclopentylethyl.

The terms “halogen” or "halo" as used herein refers to indicate fluorine, chlorine, bromine, and iodine.

The term "heterocycloalkyl," as used herein refers to a non-aromatic ring system containing at least one heteroatom selected from nitrogen, oxygen, and sulfur. The heterocycloalkyl ring can be optionally fused to or otherwise attached to other heterocycloalkyl rings and/or non-aromatic hydrocarbon rings. Preferred heterocycloalkyl groups have from 3 to 7 members. Examples of heterocycloalkyl groups include, for

example, piperazine, morpholine, piperidine, tetrahydrofuran, pyrrolidine, and pyrazole. Preferred heterocycloalkyl groups include piperidinyl, piperazinyl, morpholinyl, and pyrolidinyl.

The term "heteroaryl" as used herein refers to an aromatic ring system containing at least one heteroatom selected from nitrogen, oxygen, and sulfur. The heteroaryl ring can be fused or otherwise attached to one or more heteroaryl rings, aromatic or non-aromatic hydrocarbon rings or heterocycloalkyl rings. Examples of heteroaryl groups include, for example, pyridine, furan, thiophene, 5,6,7,8-tetrahydroisoquinoline and pyrimidine. Preferred examples of heteroaryl groups include thienyl, benzothienyl, pyridyl, quinolyl, pyrazinyl, pyrimidyl, imidazolyl, benzimidazolyl, furanyl, benzofuranyl, thiazolyl, benzothiazolyl, isoxazolyl, oxadiazolyl, isothiazolyl, benzisothiazolyl, triazolyl, tetrazolyl, pyrrolyl, indolyl, pyrazolyl, and benzopyrazolyl.

The term "C1-C6 hydrocarbyl" as used herein refers to straight, branched, or cyclic alkyl groups having 1-6 carbon atoms, optionally containing one or more carbon-carbon double or triple bonds. Examples of hydrocarbyl groups include, for example, methyl, ethyl, propyl, isopropyl, n-butyl, sec-butyl, tert-butyl, pentyl, 2-pentyl, isopentyl, neopentyl, hexyl, 2-hexyl, 3-hexyl, 3-methylpentyl, vinyl, 2-pentene, cyclopropylmethyl, cyclopropyl, cyclohexylmethyl, cyclohexyl and propargyl. When reference is made herein to C1-C6 hydrocarbyl containing one or two double or triple bonds it is understood that at least two carbons are present in the alkyl for one double or triple bond, and at least four carbons for two double or triple bonds.

The term "protecting group" as used herein, refers to groups known in the art that are readily introduced and removed from an atom, for example O, N, P, or S. Protecting groups are used to prevent undesirable reactions from taking place that can compete with the formation of a specific compound or intermediate of interest. See also "Protective Groups in Organic Synthesis", 3rd Ed., 1999, Greene, T. W. and related publications.

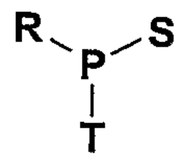
The term "nitrogen protecting group," as used herein, refers to groups known in the art that are readily introduced on to and removed from a nitrogen. Examples of nitrogen protecting groups include Boc, Cbz, benzoyl, and benzyl. See also "Protective Groups in Organic Synthesis", 3rd Ed., 1999, Greene, T. W. and related publications.

The term "hydroxy protecting group," or "hydroxy protection" as used herein, refers to groups known in the art that are readily introduced on to and removed from an oxygen, specifically an –OH group. Examples of hyroxy protecting groups include trityl or substituted trityl goups, such as monomethoxytrityl and dimethoxytrityl, or substituted silyl groups, such as tert-butyldimethyl, trimethylsilyl, or tert-butyldiphenyl silyl groups. See also "Protective Groups in Organic Synthesis", 3rd Ed., 1999, Greene, T. W. and related publications.

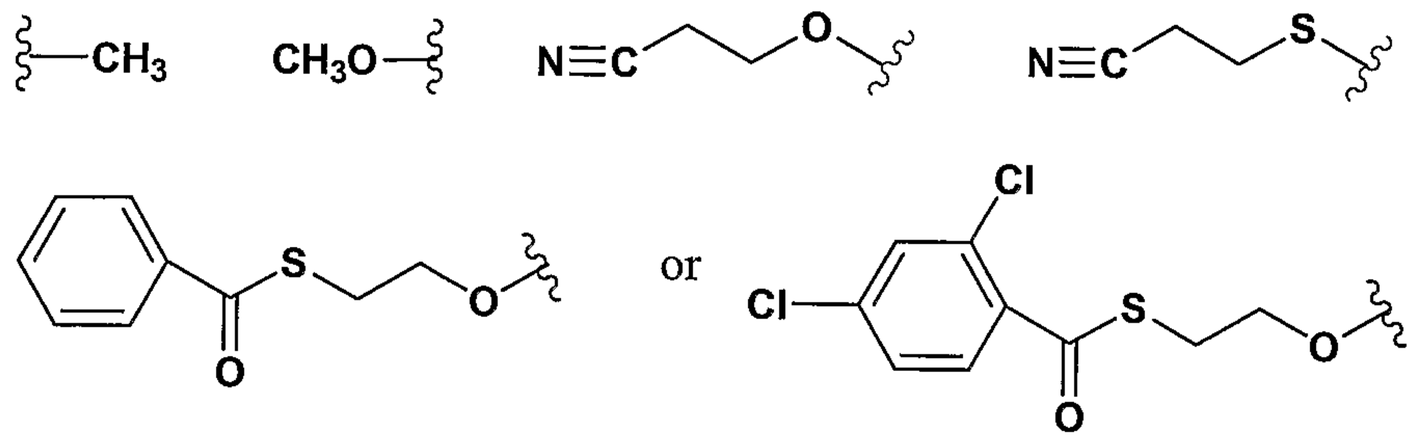
The term "acyl" as used herein refers to -C(O)R groups, wherein R is an alkyl or aryl.

The term "phosphorus containing group" as used herein, refers to a chemical group containing a phosphorus atom. The phosphorus atom can be trivalent or pentavalent, and can be substituted with O, H, N, S, C or halogen atoms. Examples of phosphorus containing groups of the instant invention include but are not limited to phosphorus atoms substituted with O, H, N, S, C or halogen atoms, comprising phosphonate, alkylphosphonate, phosphate, diphosphate, triphosphate, pyrophosphate, phosphorothioate, phosphorodithioate, phosphoramidate, phosphoramidite groups, nucleotides and nucleic acid molecules.

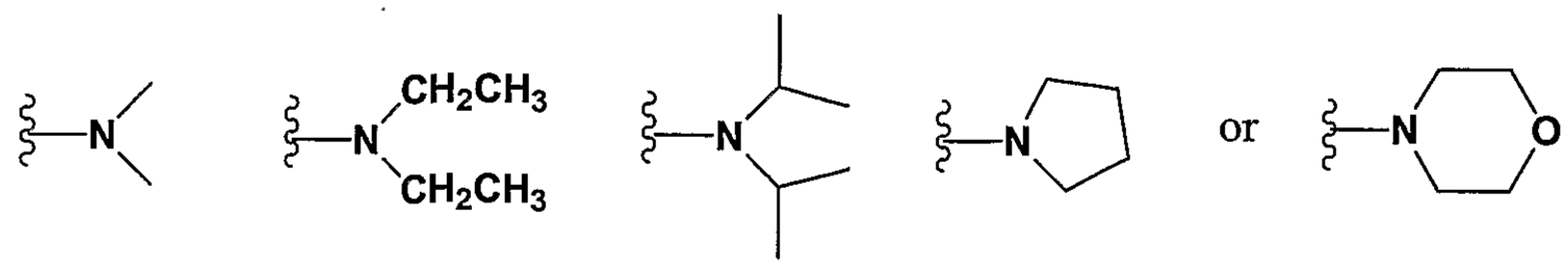
The term "phosphine" or "phosphite" as used herein refers to a trivalent phosphorus species, for example compounds having Formula 97:



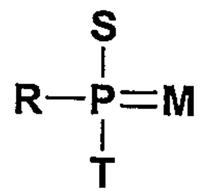
wherein R can include the groups:



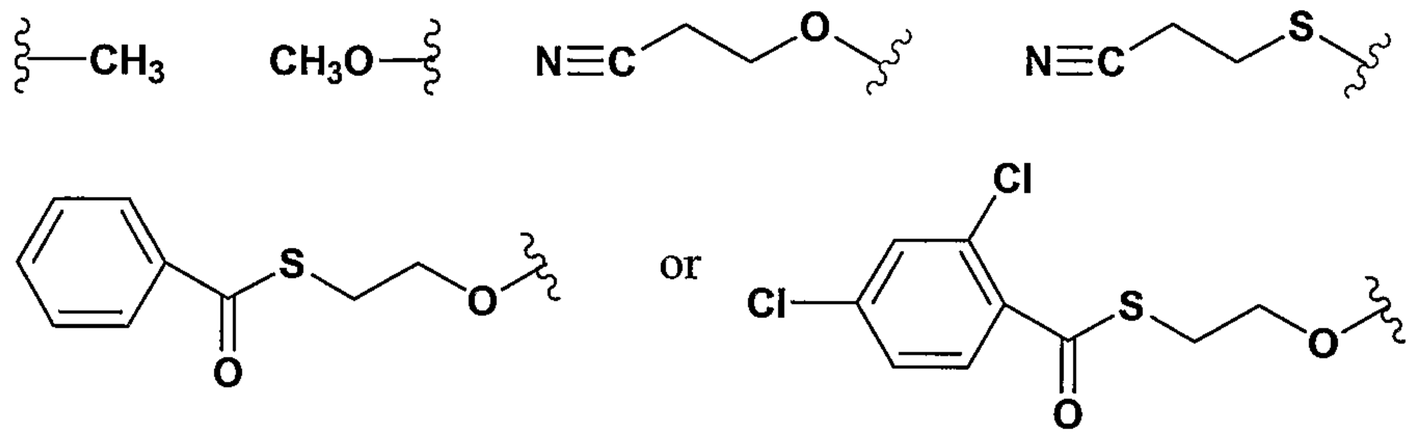
and wherein S and T independently include the groups:



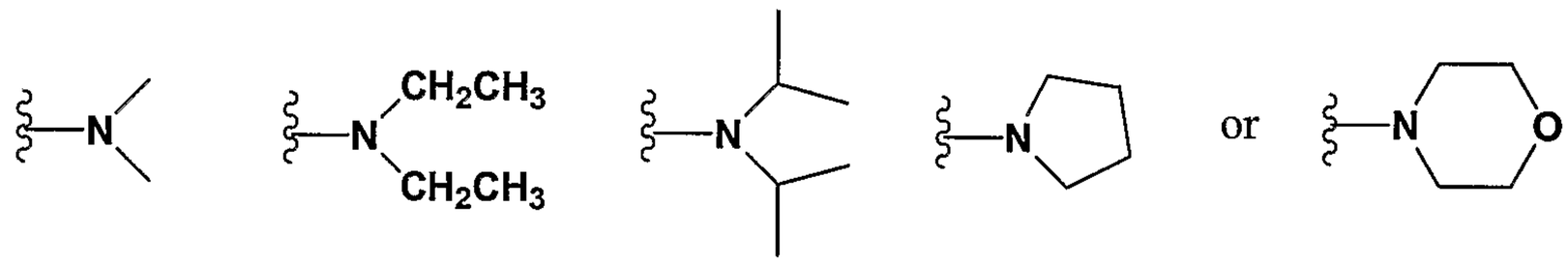
The term "phosphate" as used herein refers to a pentavalent phosphorus species, for example a compound having Formula 98:



wherein R includes the groups:



and wherein S and T each independently can be a sulfur or oxygen atom or a group which can include:



and wherein M comprises a sulfur or oxygen atom. The phosphate of the invention can comprise a nucleotide phosphate, wherein any R, S, or T in Formula 98 comprises a linkage to a nucleic acid or nucleoside.

The term "cationic salt" as used herein refers to any organic or inorganic salt having a net positive charge, for example a triethylammonium (TEA) salt.

The term "degradable linker" as used herein, refers to linker moieties that are capable of cleavage under various conditions. Conditions suitable for cleavage can include but are

not limited to pH, UV irradiation, enzymatic activity, temperature, hydrolysis, elimination, and substitution reactions, and thermodynamic properties of the linkage.

The term "photolabile linker" as used herein, refers to linker moieties as are known in the art, that are selectively cleaved under particular UV wavelengths. Compounds of the invention containing photolabile linkers can be used to deliver compounds to a target cell or tissue of interest, and can be subsequently released in the presence of a UV source.

The term "nucleic acid conjugates" as used herein, refers to nucleoside, nucleotide and oligonucleotide conjugates.

The term "lipid" as used herein, refers to any lipophilic compound. Non-limiting examples of lipid compounds include fatty acids and their derivatives, including straight chain, branched chain, saturated and unsaturated fatty acids, carotenoids, terpenes, bile acids, and steroids, including cholesterol and derivatives or analogs thereof.

The term "folate" as used herein, refers to analogs and derivatives of folic acid, for example antifolates, dihydrofloates, tetrahydrofolates, tetrahydorpterins, folinic acid, pteropolyglutamic acid, 1-deza, 3-deaza, 5-deaza, 8-deaza, 10-deaza, 1,5-deaza, 5,10 dideaza, 8,10-dideaza, and 5,8-dideaza folates, antifolates, and pteroic acid derivatives.

The term "compounds with neutral charge" as used herein, refers to compositions which are neutral or uncharged at neutral or physiological pH. Examples of such compounds are cholesterol and other steroids, cholesteryl hemisuccinate (CHEMS), dioleoyl phosphatidyl choline, distearoylphosphotidyl choline (DSPC), fatty acids such as oleic acid, phosphatidic acid and its derivatives, phosphatidyl serine, polyethylene glycol -conjugated phosphatidylamine, phosphatidylcholine, phosphatidylethanolamine and related variants, prenylated compounds including farnesol, polyprenols, tocopherol, and their modified forms, diacylsuccinyl glycerols, fusogenic or pore forming peptides, dioleoylphosphotidylethanolamine (DOPE), ceramide and the like.

The term "lipid aggregate" as used herein refers to a lipid-containing composition wherein the lipid is in the form of a liposome, micelle (non-lamellar phase) or other aggregates with one or more lipids.

The term "nitrogen containing group" as used herein refers to any chemical group or moiety comprising a nitrogen or substituted nitrogen. Non-limiting examples of nitrogen containing groups include amines, substituted amines, amides, alkylamines, amino acids such as arginine or lysine, polyamines such as spermine or spermidine, cyclic amines such as pyridines, pyrimidines including uracil, thymine, and cytosine, morpholines, phthalimides, and heterocyclic amines such as purines, including guanine and adenine.

Therapeutic nucleic acid molecules (e.g., siNA molecules) delivered exogenously optimally are stable within cells until reverse transcription of the RNA has been modulated long enough to reduce the levels of the RNA transcript. The nucleic acid molecules are resistant to nucleases in order to function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of nucleic acid molecules described in the instant invention and in the art have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability as described above.

In yet another embodiment, siNA molecules having chemical modifications that maintain or enhance enzymatic activity of proteins involved in RNAi are provided. Such nucleic acids are also generally more resistant to nucleases than unmodified nucleic acids. Thus, *in vitro* and/or *in vivo* the activity should not be significantly lowered.

Use of the nucleic acid-based molecules of the invention will lead to better treatment of the disease progression by affording the possibility of combination therapies (*e.g.*, multiple siNA molecules targeted to different genes; nucleic acid molecules coupled with known small molecule modulators; or intermittent treatment with combinations of molecules, including different motifs and/or other chemical or biological molecules). The treatment of subjects with siNA molecules can also include combinations of different types of nucleic acid molecules, such as enzymatic nucleic acid molecules (ribozymes), allozymes, antisense, 2,5-A oligoadenylate, decoys, and aptamers.

In another aspect a siNA molecule of the invention comprises one or more 5' and/or a 3'- cap structure, for example on only the sense siNA strand, the antisense siNA strand, or both siNA strands.

By "cap structure" is meant chemical modifications, which have been incorporated at either terminus of the oligonucleotide (see, for example, Adamic *et al.*, U.S. Pat. No. 5,998,203). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and can help in delivery and/or localization within a cell. The cap can be present at the 5'-terminus (5'-cap) or at the 3'-terminal (3'-cap) or can be present on both termini. Non-limiting examples of the 5'-cap include, but are not limited to, glyceryl, inverted deoxy abasic residue (moiety); 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide, 4'-thio nucleotide; carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety.

Non-limiting examples of the 3'-cap include, but are not limited to, glyceryl, inverted deoxy abasic residue (moiety), 4', 5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate; 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Iyer, 1993, *Tetrahedron* 49, 1925).

By the term "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to

exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine and therefore lacks a base at the 1'-position.

By "nucleotide" as used herein is as recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see, for example, Usman and McSwiggen, *supra*; Eckstein *et al.*, International PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187; Uhlman & Peyman, *supra*). There are several examples of modified nucleic acid bases known in the art as summarized by Limbach *et al.*, 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of base modifications that can be introduced into nucleic acid molecules include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.*, 6-methyluridine), propyne, and others (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents.

In one embodiment, the invention features modified siNA molecules, with phosphate backbone modifications comprising one or more phosphorothioate, phosphonoacetate, and/or thiophosphonoacetate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and/or alkylsilyl, substitutions. For a review of oligonucleotide backbone modifications, see Hunziker and Leumann, 1995, *Nucleic Acid Analogues: Synthesis and Properties,* in *Modern Synthetic Methods,* VCH, 331-417, and Mesmaeker *et al.*, 1994, *Novel Backbone Replacements for Oligonucleotides,* in *Carbohydrate Modifications in Antisense Research,* ACS, 24-39.

By "abasic" is meant sugar moieties lacking a base or having other chemical groups in place of a base at the 1' position, see for example Adamic *et al.*, U.S. Pat. No. 5,998,203.

By "unmodified nucleoside" is meant one of the bases adenine, cytosine, guanine, thymine, or uracil joined to the 1' carbon of β-D-ribo-furanose.

By "modified nucleoside" is meant any nucleotide base which contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate. Non-limiting examples of modified nucleotides are shown by Formulae I-VII and/or other modifications described herein.

In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant 2'-NH_2 or 2'-*O*- NH_2, which can be modified or unmodified. Such modified groups are described, for example, in Eckstein *et al.*, U.S. Pat. No. 5,672,695 and Matulic-Adamic *et al.*, U.S. Pat. No. 6,248,878.

Various modifications to nucleic acid siNA structure can be made to enhance the utility of these molecules. Such modifications will enhance shelf-life, half-life *in vitro*, stability, and ease of introduction of such oligonucleotides to the target site, *e.g.*, to enhance penetration of cellular membranes, and confer the ability to recognize and bind to targeted cells.

Administration of Nucleic Acid Molecules

A siNA molecule of the invention can be adapted for use to treat any disease, infection or condition associated with gene expression, and other indications that can respond to the level of gene product in a cell or tissue, alone or in combination with other therapies. Non-limiting examples of such diseases and conditions include cancer or cancerous disease, infectious disease, cardiovascular disease, neurologic disease, ocular disease, prion disease, inflammatory disease, autoimmune disease, pulmonary disease, renal disease, liver disease, mitochondrial disease, endocrine disease, reproduction related diseases and conditions as are known in the art, and any other indications that can respond to the level of an expressed gene product in a cell or organsim (see for example McSwiggen, International PCT Publication No. WO 03/74654). For example, a siNA molecule can

comprise a delivery vehicle, including liposomes, for administration to a subject, carriers and diluents and their salts, and/or can be present in pharmaceutically acceptable formulations. Methods for the delivery of nucleic acid molecules are described in Akhtar *et al.*, 1992, *Trends Cell Bio.*, 2, 139; *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, ed. Akhtar, 1995, Maurer *et al.*, 1999, *Mol. Membr. Biol.*, 16, 129-140; Hofland and Huang, 1999, *Handb. Exp. Pharmacol.*, 137, 165-192; and Lee *et al.*, 2000, *ACS Symp. Ser.*, 752, 184-192.

Beigelman *et al.*, U.S. Pat. No. 6,395,713 and Sullivan *et al.*, PCT WO 94/02595 further describe the general methods for delivery of nucleic acid molecules. These protocols can be utilized for the delivery of virtually any nucleic acid molecule. Nucleic acid molecules can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as biodegradable polymers, hydrogels, cyclodextrins (see for example Gonzalez *et al.*, 1999, *Bioconjugate Chem.*, 10, 1068-1074; Wang *et al.*, International PCT publication Nos. WO 03/47518 and WO 03/46185), poly(lactic-co-glycolic)acid (PLGA) and PLCA microspheres (see for example US Patent 6,447,796 and US Patent Application Publication No. US 2002130430), biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors (O'Hare and Normand, International PCT Publication No. WO 00/53722). In one embodiment, nucleic acid molecules or the invention are administered via biodegradable implant materials, such as elastic shape memory polymers (see for example Lendelein and Langer, 2002, Science, 296, 1673). In another embodiment, the nucleic acid molecules of the invention can also be formulated or complexed with polyethyleneimine and derivatives thereof, such as polyethyleneimine-polyethyleneglycol-N-acetylgalactosamine (PEI-PEG-GAL) or polyethyleneimine-polyethyleneglycol-tri-N-acetylgalactosamine (PEI-PEG-triGAL) derivatives. Alternatively, the nucleic acid/vehicle combination is locally delivered by direct injection or by use of an infusion pump. Direct injection of the nucleic acid molecules of the invention, whether subcutaneous, intramuscular, or intradermal, can take place using standard needle and syringe methodologies, or by needle-free technologies such as those described in Conry *et al.*, 1999, *Clin. Cancer Res.*, 5, 2330-2337 and Barry *et al.*, International PCT Publication No. WO 99/31262. Many examples in the art describe CNS delivery methods of oligonucleotides by osmotic pump, (see Chun *et al.*, 1998, *Neuroscience Letters*, 257, 135-138, D'Aldin *et al.*, 1998, *Mol. Brain Research*, 55,

151-164, Dryden *et al.*, 1998, *J. Endocrinol.*, 157, 169-175, Ghirnikar *et al.*, 1998, *Neuroscience Letters*, 247, 21-24) or direct infusion (Broaddus *et al.*, 1997, *Neurosurg. Focus*, 3, article 4). Other routes of delivery include, but are not limited to oral (tablet or pill form) and/or intrathecal delivery (Gold, 1997, *Neuroscience*, 76, 1153-1158). More detailed descriptions of nucleic acid delivery and administration are provided in Sullivan *et al.*, supra, Draper *et al.*, PCT WO93/23569, Beigelman *et al.*, PCT WO99/05094, and Klimuk *et al.*, PCT WO99/04819.

The molecules of the instant invention can be used as pharmaceutical agents. Pharmaceutical agents prevent, modulate the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state in a subject.

In addition, the invention features the use of methods to deliver the nucleic acid molecules of the instant invention to the central nervous system and/or peripheral nervous system. Experiments have demonstrated the efficient *in vivo* uptake of nucleic acids by neurons. As an example of local administration of nucleic acids to nerve cells, Sommer *et al.*, 1998, *Antisense Nuc. Acid Drug Dev.*, 8, 75, describe a study in which a 15mer phosphorothioate antisense nucleic acid molecule to c-fos is administered to rats via microinjection into the brain. Antisense molecules labeled with tetramethylrhodamine-isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) were taken up by exclusively by neurons thirty minutes post-injection. A diffuse cytoplasmic staining and nuclear staining was observed in these cells. As an example of systemic administration of nucleic acid to nerve cells, Epa *et al.*, 2000, *Antisense Nuc. Acid Drug Dev.*, 10, 469, describe an *in vivo* mouse study in which beta-cyclodextrin-adamantane-oligonucleotide conjugates were used to target the p75 neurotrophin receptor in neuronally differentiated PC12 cells. Following a two week course of IP administration, pronounced uptake of p75 neurotrophin receptor antisense was observed in dorsal root ganglion (DRG) cells. In addition, a marked and consistent down-regulation of p75 was observed in DRG neurons. Additional approaches to the targeting of nucleic acid to neurons are described in Broaddus *et al.*, 1998, *J. Neurosurg.*, 88(4), 734; Karle *et al.*, 1997, *Eur. J. Pharmocol.*, 340(2/3), 153; Bannai *et al.*, 1998, *Brain Research*, 784(1,2), 304; Rajakumar *et al.*, 1997, *Synapse*, 26(3), 199; Wu-pong *et al.*, 1999, *BioPharm*, 12(1), 32; Bannai *et al.*, 1998, *Brain Res. Protoc.*, 3(1), 83; Simantov *et al.*, 1996, *Neuroscience*, 74(1), 39. Nucleic acid molecules of the invention are therefore

amenable to delivery to and uptake by cells that express repeat expansion allelic variants for modulation of RE gene expression. The delivery of nucleic acid molecules of the invention, targeting RE is provided by a variety of different strategies. Traditional approaches to CNS delivery that can be used include, but are not limited to, intrathecal and intracerebroventricular administration, implantation of catheters and pumps, direct injection or perfusion at the site of injury or lesion, injection into the brain arterial system, or by chemical or osmotic opening of the blood-brain barrier. Other approaches can include the use of various transport and carrier systems, for example though the use of conjugates and biodegradable polymers. Furthermore, gene therapy approaches, for example as described in Kaplitt *et al.,* US 6,180,613 and Davidson, WO 04/013280, can be used to express nucleic acid molecules in the CNS.

In addition, the invention features the use of methods to deliver the nucleic acid molecules of the instant invention to hematopoietic cells, including monocytes and lymphocytes. These methods are described in detail by Hartmann *et al.,* 1998, *J. Phamacol. Exp. Ther.,* 285(2), 920-928; Kronenwett *et al.,* 1998, *Blood,* 91(3), 852-862; Filion and Phillips, 1997, *Biochim. Biophys. Acta.,* 1329(2), 345-356; Ma and Wei, 1996, *Leuk. Res.,* 20(11/12), 925-930; and Bongartz *et al.,* 1994, *Nucleic Acids Research,* 22(22), 4681-8. Such methods, as described above, include the use of free oligonucleitide, cationic lipid formulations, liposome formulations including pH sensitive liposomes and immunoliposomes, and bioconjugates including oligonucleotides conjugated to fusogenic peptides, for the transfection of hematopoietic cells with oligonucleotides.

In one embodiment, a compound, molecule, or composition for the treatment of ocular conditions (e.g., macular degeneration, diabetic retinopathy etc.) is administered to a subject intraocularly or by intraocular means. In another embodiment, a compound, molecule, or composition for the treatment of ocular conditions (e.g., macular degeneration, diabetic retinopathy etc.) is administered to a subject periocularly or by periocular means (see for example Ahlheim et al., International PCT publication No. WO 03/24420). In one embodiment, a siNA molecule and/or formulation or composition thereof is administered to a subject intraocularly or by intraocular means. In another embodiment, a siNA molecule and/or formualtion or composition thereof is administered to a subject periocularly or by periocular means. Periocular administration generally provides a less invasive approach to administering siNA molecules and formualtion or composition

thereof to a subject (see for example Ahlheim et al., International PCT publication No. WO 03/24420). The use of periocular administraction also minimizes the risk of retinal detachment, allows for more frequent dosing or administraction, provides a clinically relevant route of administraction for macular degeneration and other optic conditions, and also provides the possiblilty of using resevoirs (e.g., implants, pumps or other devices) for drug delivery.

In one embodiment, the siNA molecules of the invention and formulations or compositions thereof are administered directly or topically (e.g., locally) to the dermis or follicles as is generally known in the art (see for example Brand, 2001, *Curr. Opin. Mol. Ther.,* 3, 244-8; Regnier *et al.,* 1998, *J. Drug Target,* 5, 275-89; Kanikkannan, 2002, *BioDrugs,* 16, 339-47; Wraight *et al.,* 2001, *Pharmacol. Ther.,* 90, 89-104; and Preat and Dujardin, 2001, STP PharmaSciences, 11, 57-68.

In one embodiment, dermal delivery systems of the invention include, for example, aqueous and nonaqueous gels, creams, multiple emulsions, microemulsions, liposomes, ointments, aqueous and nonaqueous solutions, lotions, aerosols, hydrocarbon bases and powders, and can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic polymers (e.g., polycarbophil and polyvinylpyrolidone). In one embodiment, the pharmaceutically acceptable carrier is a liposome or a transdermal enhancer. Examples of liposomes which can be used in this invention include the following: (1) CellFectin, 1:1.5 (M/M) liposome formulation of the cationic lipid N,NI,NII,NIII-tetramethyl-N,NI,NII,NIII-tetrapalmit-y-spermine and dioleoyl phosphatidylethanolamine (DOPE) (GIBCO BRL); (2) Cytofectin GSV, 2:1 (M/M) liposome formulation of a cationic lipid and DOPE (Glen Research); (3) DOTAP (N-[1-(2,3-dioleoyloxy)-N,N,N-tri-methyl-ammoniummethylsulfate) (Boehringer Manheim); and (4) Lipofectamine, 3:1 (M/M) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE (GIBCO BRL).

In one embodiment, transmucosal delivery systems of the invention include patches, tablets, suppositories, pessaries, gels and creams, and can contain excipients such as solubilizers and enhancers (e.g., propylene glycol, bile salts and amino acids), and other vehicles (e.g., polyethylene glycol, fatty acid esters and derivatives, and hydrophilic polymers such as hydroxypropylmethylcellulose and hyaluronic acid).

In one embodiment, nucleic acid molecules of the invention are administered to the central nervous system (CNS) or peripheral nervous system (PNS). Experiments have demonstrated the efficient *in vivo* uptake of nucleic acids by neurons. As an example of local administration of nucleic acids to nerve cells, Sommer *et al.,* 1998, *Antisense Nuc. Acid Drug Dev.,* 8, 75, describe a study in which a 15mer phosphorothioate antisense nucleic acid molecule to c-fos is administered to rats via microinjection into the brain. Antisense molecules labeled with tetramethylrhodamine-isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) were taken up by exclusively by neurons thirty minutes post-injection. A diffuse cytoplasmic staining and nuclear staining was observed in these cells. As an example of systemic administration of nucleic acid to nerve cells, Epa *et al.,* 2000, *Antisense Nuc. Acid Drug Dev.,* 10, 469, describe an *in vivo* mouse study in which beta-cyclodextrin-adamantane-oligonucleotide conjugates were used to target the p75 neurotrophin receptor in neuronally differentiated PC12 cells. Following a two week course of IP administration, pronounced uptake of p75 neurotrophin receptor antisense was observed in dorsal root ganglion (DRG) cells. In addition, a marked and consistent down-regulation of p75 was observed in DRG neurons. Additional approaches to the targeting of nucleic acid to neurons are described in Broaddus *et al.,* 1998, *J. Neurosurg.,* 88(4), 734; Karle *et al.,* 1997, *Eur. J. Pharmocol.,* 340(2/3), 153; Bannai *et al.,* 1998, *Brain Research,* 784(1,2), 304; Rajakumar *et al.,* 1997, *Synapse,* 26(3), 199; Wu-pong *et al.,* 1999, *BioPharm,* 12(1), 32; Bannai *et al.,* 1998, *Brain Res. Protoc.,* 3(1), 83; Simantov *et al.,* 1996, *Neuroscience,* 74(1), 39. Nucleic acid molecules of the invention are therefore amenable to delivery to and uptake by cells in the CNS and/or PNS.

The delivery of nucleic acid molecules of the invention to the CNS is provided by a variety of different strategies. Traditional approaches to CNS delivery that can be used include, but are not limited to, intrathecal and intracerebroventricular administration, implantation of catheters and pumps, direct injection or perfusion at the site of injury or lesion, injection into the brain arterial system, or by chemical or osmotic opening of the blood-brain barrier. Other approaches can include the use of various transport and carrier systems, for example though the use of conjugates and biodegradable polymers. Furthermore, gene therapy approaches, for example as described in Kaplitt *et al.,* US 6,180,613 and Davidson, WO 04/013280, can be used to express nucleic acid molecules in the CNS.

In one embodiment, the nucleic acid molecules of the invention are administered via pulmonary delivery, such as by inhalation of an aerosol or spray dried formulation administered by an inhalation device or nebulizer, providing rapid local uptake of the nucleic acid molecules into relevant pulmonary tissues. Solid particulate compositions containing respirable dry particles of micronized nucleic acid compositions can be prepared by grinding dried or lyophilized nucleic acid compositions, and then passing the micronized composition through, for example, a 400 mesh screen to break up or separate out large agglomerates. A solid particulate composition comprising the nucleic acid compositions of the invention can optionally contain a dispersant which serves to facilitate the formation of an aerosol as well as other therapeutic compounds. A suitable dispersant is lactose, which can be blended with the nucleic acid compound in any suitable ratio, such as a 1 to 1 ratio by weight.

Aerosols of liquid particles comprising a nucleic acid composition of the invention can be produced by any suitable means, such as with a nebulizer (see for example US 4,501,729). Nebulizers are commercially available devices which transform solutions or suspensions of an active ingredient into a therapeutic aerosol mist either by means of acceleration of a compressed gas, typically air or oxygen, through a narrow venturi orifice or by means of ultrasonic agitation. Suitable formulations for use in nebulizers comprise the active ingredient in a liquid carrier in an amount of up to 40% w/w preferably less than 20% w/w of the formulation. The carrier is typically water or a dilute aqueous alcoholic solution, preferably made isotonic with body fluids by the addition of, for example, sodium chloride or other suitable salts. Optional additives include preservatives if the formulation is not prepared sterile, for example, methyl hydroxybenzoate, anti-oxidants, flavorings, volatile oils, buffering agents and emulsifiers and other formulation surfactants. The aerosols of solid particles comprising the active composition and surfactant can likewise be produced with any solid particulate aerosol generator. Aerosol generators for administering solid particulate therapeutics to a subject produce particles which are respirable, as explained above, and generate a volume of aerosol containing a predetermined metered dose of a therapeutic composition at a rate suitable for human administration. One illustrative type of solid particulate aerosol generator is an insufflator. Suitable formulations for administration by insufflation include finely comminuted powders which can be delivered by means of an insufflator. In the insufflator, the powder,

e.g., a metered dose thereof effective to carry out the treatments described herein, is contained in capsules or cartridges, typically made of gelatin or plastic, which are either pierced or opened in situ and the powder delivered by air drawn through the device upon inhalation or by means of a manually-operated pump. The powder employed in the insufflator consists either solely of the active ingredient or of a powder blend comprising the active ingredient, a suitable powder diluent, such as lactose, and an optional surfactant. The active ingredient typically comprises from 0.1 to 100 w/w of the formulation. A second type of illustrative aerosol generator comprises a metered dose inhaler. Metered dose inhalers are pressurized aerosol dispensers, typically containing a suspension or solution formulation of the active ingredient in a liquified propellant. During use these devices discharge the formulation through a valve adapted to deliver a metered volume to produce a fine particle spray containing the active ingredient. Suitable propellants include certain chlorofluorocarbon compounds, for example, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane and mixtures thereof. The formulation can additionally contain one or more co-solvents, for example, ethanol, emulsifiers and other formulation surfactants, such as oleic acid or sorbitan trioleate, anti-oxidants and suitable flavoring agents. Other methods for pulmonary delivery are described in, for example US Patent Application No. 20040037780, and US Patent Nos. 6,592,904; 6,582,728; 6,565,885.

In one embodiment, a siNA molecule of the invention is complexed with membrane disruptive agents such as those described in U.S. Patent Appliaction Publication No. 20010007666. In another embodiment, the membrane disruptive agent or agents and the siNA molecule are also complexed with a cationic lipid or helper lipid molecule, such as those lipids described in U.S. Patent No. 6,235,310.

In one embodiment, siNA molecules of the invention are formulated or complexed with polyethylenimine (e.g., linear or branched PEI) and/or polyethylenimine derivatives, including for example grafted PEIs such as galactose PEI, cholesterol PEI, antibody derivatized PEI, and polyethylene glycol PEI (PEG-PEI) derivatives thereof (see for example Ogris *et al.*, 2001, *AAPA PharmSci,* 3, 1-11; Furgeson et al., 2003, Bioconjugate Chem., 14, 840-847; Kunath et al., 2002, Phramaceutical Research, 19, 810-817; Choi et al.,

2001, Bull. Korean Chem. Soc., 22, 46-52; Bettinger et al., 1999, Bioconjugate Chem., 10, 558-561; Peterson et al., 2002, Bioconjugate Chem., 13, 845-854; Erbacher et al., 1999, Journal of Gene Medicine Preprint, 1, 1-18; Godbey et al., 1999., PNAS USA, 96, 5177-5181; Godbey et al., 1999, Journal of Controlled Release, 60, 149-160; Diebold et al., 1999, Journal of Biological Chemistry, 274, 19087-19094; Thomas and Klibanov, 2002, PNAS USA, 99, 14640-14645; and Sagara, US 6,586,524.

In one embodiment, a siNA molecule of the invention comprises a bioconjugate, for example a nucleic acid conjugate as described in Vargeese et al., USSN 10/427,160, filed April 30, 2003; US 6,528,631; US 6,335,434; US 6, 235,886; US 6,153,737; US 5,214,136; US 5,138,045.

Thus, the invention features a pharmaceutical composition comprising one or more nucleic acid(s) of the invention in an acceptable carrier, such as a stabilizer, buffer, and the like. The polynucleotides of the invention can be administered (*e.g.*, RNA, DNA or protein) and introduced into a subject by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The compositions of the present invention can also be formulated and used as tablets, capsules or elixirs for oral administration, suppositories for rectal administration, sterile solutions, suspensions for injectable administration, and the other compositions known in the art.

The present invention also includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds, *e.g.*, acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, *e.g.*, systemic administration, into a cell or subject, including for example a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation from reaching a target cell (*i.e.*, a cell to which the negatively charged nucleic acid is desirable for delivery). For example, pharmacological

compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms that prevent the composition or formulation from exerting its effect.

By "systemic administration" is meant *in vivo* systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes that lead to systemic absorption include, without limitation: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes exposes the siNA molecules of the invention to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation that can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach can provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells, such as cancer cells.

By "pharmaceutically acceptable formulation" is meant a composition or formulation that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity. Non-limiting examples of agents suitable for formulation with the nucleic acid molecules of the instant invention include: P-glycoprotein inhibitors (such as Pluronic P85), which can enhance entry of drugs into the CNS (Jolliet-Riant and Tillement, 1999, *Fundam. Clin. Pharmacol.*, 13, 16-26); biodegradable polymers, such as poly (DL-lactide-coglycolide) microspheres for sustained release delivery after intracerebral implantation (Emerich, DF *et al*, 1999, *Cell Transplant*, 8, 47-58) (Alkermes, Inc. Cambridge, MA); and loaded nanoparticles, such as those made of polybutylcyanoacrylate, which can deliver drugs across the blood brain barrier and can alter neuronal uptake mechanisms (*Prog Neuropsychopharmacol Biol Psychiatry*, 23, 941-949, 1999). Other non-limiting examples of delivery strategies for the nucleic acid molecules of the instant invention include material described in Boado *et al.*, 1998, *J. Pharm. Sci.*, 87, 1308-1315; Tyler *et al.*, 1999, *FEBS Lett.*, 421, 280-284; Pardridge *et al.*, 1995, *PNAS USA.*, 92, 5592-5596; Boado, 1995, *Adv. Drug Delivery Rev.*,

15, 73-107; Aldrian-Herrada *et al.*, 1998, *Nucleic Acids Res.*, 26, 4910-4916; and Tyler *et al.*, 1999, *PNAS USA.*, 96, 7053-7058.

The invention also features the use of the composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer a method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic *et al. Chem. Rev.* 1995, 95, 2601-2627; Ishiwata *et al., Chem. Pharm. Bull.* 1995, 43, 1005-1011). Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic *et al., Science* 1995, 267, 1275-1276; Oku *et al.*,1995, *Biochim. Biophys. Acta*, 1238, 86-90). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu *et al., J. Biol. Chem.* 1995, 42, 24864-24870; Choi *et al.*, International PCT Publication No. WO 96/10391; Ansell *et al.*, International PCT Publication No. WO 96/10390; Holland *et al.*, International PCT Publication No. WO 96/10392). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen.

The present invention also includes compositions prepared for storage or administration that include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co. (A.R. Gennaro edit. 1985). For example, preservatives, stabilizers, dyes and
flavoring agents can be provided. These include sodium benzoate, sorbic acid and esters of *p*-hydroxybenzoic acid. In addition, antioxidants and suspending agents can be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state. The pharmaceutically effective dose depends on the type of disease, the

composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors that those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer.

The nucleic acid molecules of the invention and formulations thereof can be administered orally, topically, parenterally, by inhalation or spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and/or vehicles. The term parenteral as used herein includes percutaneous, subcutaneous, intravascular (*e.g.*, intravenous), intramuscular, or intrathecal injection or infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a nucleic acid molecule of the invention and a pharmaceutically acceptable carrier. One or more nucleic acid molecules of the invention can be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants, and if desired other active ingredients. The pharmaceutical compositions containing nucleic acid molecules of the invention can be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

Compositions intended for oral use can be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions can contain one or more such sweetening agents, flavoring agents, coloring agents or preservative agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients that are suitable for the manufacture of tablets. These excipients can be, for example, inert diluents; such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia; and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets can be uncoated or they can be coated by known techniques. In some cases such coatings can be prepared by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For

example, a time delay material such as glyceryl monosterate or glyceryl distearate can be employed.

Formulations for oral use can also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

Aqueous suspensions contain the active materials in a mixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropyl-methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents can be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions can also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.

Oily suspensions can be formulated by suspending the active ingredients in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions can contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents can be added to provide palatable oral preparations. These compositions can be preserved by the addition of an anti-oxidant such as ascorbic acid

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents or suspending agents are exemplified by those already mentioned above.

Additional excipients, for example sweetening, flavoring and coloring agents, can also be present.

Pharmaceutical compositions of the invention can also be in the form of oil-in-water emulsions. The oily phase can be a vegetable oil or a mineral oil or mixtures of these. Suitable emulsifying agents can be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, for example sorbitan monooleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions can also contain sweetening and flavoring agents.

Syrups and elixirs can be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol, glucose or sucrose. Such formulations can also contain a demulcent, a preservative and flavoring and coloring agents. The pharmaceutical compositions can be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents that have been mentioned above. The sterile injectable preparation can also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be employed including synthetic mono-or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The nucleic acid molecules of the invention can also be administered in the form of suppositories, *e.g.*, for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable non-irritating excipient that is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials include cocoa butter and polyethylene glycols.

Nucleic acid molecules of the invention can be administered parenterally in a sterile medium. The drug, depending on the vehicle and concentration used, can either be

suspended or dissolved in the vehicle. Advantageously, adjuvants such as local anesthetics, preservatives and buffering agents can be dissolved in the vehicle.

Dosage levels of the order of from about 0.1 mg to about 140 mg per kilogram of body weight per day are useful in the treatment of the above-indicated conditions (about 0.5 mg to about 7 g per subject per day). The amount of active ingredient that can be combined with the carrier materials to produce a single dosage form varies depending upon the host treated and the particular mode of administration. Dosage unit forms generally contain between from about 1 mg to about 500 mg of an active ingredient.

It is understood that the specific dose level for any particular subject depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

For administration to non-human animals, the composition can also be added to the animal feed or drinking water. It can be convenient to formulate the animal feed and drinking water compositions so that the animal takes in a therapeutically appropriate quantity of the composition along with its diet. It can also be convenient to present the composition as a premix for addition to the feed or drinking water.

The nucleic acid molecules of the present invention can also be administered to a subject in combination with other therapeutic compounds to increase the overall therapeutic effect. The use of multiple compounds to treat an indication can increase the beneficial effects while reducing the presence of side effects.

In one embodiment, the invention comprises compositions suitable for administering nucleic acid molecules of the invention to specific cell types. For example, the asialoglycoprotein receptor (ASGPr) (Wu and Wu, 1987, *J. Biol. Chem.* 262, 4429-4432) is unique to hepatocytes and binds branched galactose-terminal glycoproteins, such as asialoorosomucoid (ASOR). In another example, the folate receptor is overexpressed in many cancer cells. Binding of such glycoproteins, synthetic glycoconjugates, or folates to the receptor takes place with an affinity that strongly depends on the degree of branching of the oligosaccharide chain, for example, triatennary structures are bound with greater affinity than biatenarry or monoatennary chains (Baenziger and Fiete, 1980, *Cell,* 22,

611-620; Connolly *et al.,* 1982, *J. Biol. Chem.,* 257, 939-945). Lee and Lee, 1987, *Glycoconjugate J.*, 4, 317-328, obtained this high specificity through the use of N-acetyl-D-galactosamine as the carbohydrate moiety, which has higher affinity for the receptor, compared to galactose. This "clustering effect" has also been described for the binding and uptake of mannosyl-terminating glycoproteins or glycoconjugates (Ponpipom *et al.,* 1981, *J. Med. Chem.,* 24, 1388-1395). The use of galactose, galactosamine, or folate based conjugates to transport exogenous compounds across cell membranes can provide a targeted delivery approach to, for example, the treatment of liver disease, cancers of the liver, or other cancers. The use of bioconjugates can also provide a reduction in the required dose of therapeutic compounds required for treatment. Furthermore, therapeutic bioavialability, pharmacodynamics, and pharmacokinetic parameters can be modulated through the use of nucleic acid bioconjugates of the invention. Non-limiting examples of such bioconjugates are described in Vargeese *et al.,* USSN 10/201,394, filed August 13, 2001; and Matulic-Adamic *et al.,* USSN 10/151,116, filed May 17, 2002. In one embodiment, nucleic acid molecules of the invention are complexed with or covalently attached to nanoparticles, such as Hepatitis B virus S, M, or L evelope proteins (see for example Yamado *et al.,* 2003, *Nature Biotechnology*, 21, 885). In one embodiment, nucleic acid molecules of the invention are delivered with specificity for human tumor cells, specifically non-apoptotic human tumor cells including for example T-cells, hepatocytes, breast carcinoma cells, ovarian carcinoma cells, melanoma cells, intestinal epithelial cells, prostate cells, testicular cells, non-small cell lung cancers, small cell lung cancers, etc.

In one embodiment, a siNA molecule of the invention is designed or formulated to specifically target endothelial cells or tumor cells. For example, various formulations and conjugates can be utilized to specifically target endothelial cells or tumor cells, including PEI-PEG-folate, PEI-PEG-RGD, PEI-PEG-biotin, PEI-PEG-cholesterol, and other conjugates known in the art that enable specific targeting to endothelial cells and/or tumor cells.

Examples:

The following are non-limiting examples showing the selection, isolation, synthesis and activity of nucleic acids of the instant invention.

Example 1: Tandem synthesis of siNA constructs

Exemplary siNA molecules of the invention are synthesized in tandem using a cleavable linker, for example, a succinyl-based linker. Tandem synthesis as described herein is followed by a one-step purification process that provides RNAi molecules in high yield. This approach is highly amenable to siNA synthesis in support of high throughput RNAi screening, and can be readily adapted to multi-column or multi-well synthesis platforms.

After completing a tandem synthesis of a siNA oligo and its complement in which the 5'-terminal dimethoxytrityl (5'-O-DMT) group remains intact (trityl on synthesis), the oligonucleotides are deprotected as described above. Following deprotection, the siNA sequence strands are allowed to spontaneously hybridize. This hybridization yields a duplex in which one strand has retained the 5'-O-DMT group while the complementary strand comprises a terminal 5'-hydroxyl. The newly formed duplex behaves as a single molecule during routine solid-phase extraction purification (Trityl-On purification) even though only one molecule has a dimethoxytrityl group. Because the strands form a stable duplex, this dimethoxytrityl group (or an equivalent group, such as other trityl groups or other hydrophobic moieties) is all that is required to purify the pair of oligos, for example, by using a C18 cartridge.

Standard phosphoramidite synthesis chemistry is used up to the point of introducing a tandem linker, such as an inverted deoxy abasic succinate or glyceryl succinate linker (see **Figure 1**) or an equivalent cleavable linker. A non-limiting example of linker coupling conditions that can be used includes a hindered base such as diisopropylethylamine (DIPA) and/or DMAP in the presence of an activator reagent such as Bromotripyrrolidinophosphoniumhexaflurorophosphate (PyBrOP). After the linker is coupled, standard synthesis chemistry is utilized to complete synthesis of the second sequence leaving the terminal the 5'-O-DMT intact. Following synthesis, the resulting oligonucleotide is deprotected according to the procedures described herein and quenched with a suitable buffer, for example with 50mM NaOAc or 1.5M $NH_4H_2CO_3$.

Purification of the siNA duplex can be readily accomplished using solid phase extraction, for example using a Waters C18 SepPak 1g cartridge conditioned with 1 column volume (CV) of acetonitrile, 2 CV H_2O, and 2 CV 50mM NaOAc. The sample is loaded

and then washed with 1 CV H_2O or 50mM NaOAc. Failure sequences are eluted with 1 CV 14% ACN (Aqueous with 50mM NaOAc and 50mM NaCl). The column is then washed, for example with 1 CV H_2O followed by on-column detritylation, for example by passing 1 CV of 1% aqueous trifluoroacetic acid (TFA) over the column, then adding a second CV of 1% aqueous TFA to the column and allowing to stand for approximately 10 minutes. The remaining TFA solution is removed and the column washed with H_2O followed by 1 CV 1M NaCl and additional H_2O. The siNA duplex product is then eluted, for example, using 1 CV 20% aqueous CAN.

Figure 2 provides an example of MALDI-TOF mass spectrometry analysis of a purified siNA construct in which each peak corresponds to the calculated mass of an individual siNA strand of the siNA duplex. The same purified siNA provides three peaks when analyzed by capillary gel electrophoresis (CGE), one peak presumably corresponding to the duplex siNA, and two peaks presumably corresponding to the separate siNA sequence strands. Ion exchange HPLC analysis of the same siNA contract only shows a single peak. Testing of the purified siNA construct using a luciferase reporter assay described below demonstrated the same RNAi activity compared to siNA constructs generated from separately synthesized oligonucleotide sequence strands.

Example 2: Serum stability of chemically modified siNA constructs

Chemical modifications were introduced into siNA constructs to determine the stability of these constructs compared to native siNA oligonucleotides (containing two thymidine nucleotide overhangs) in human serum. An investigation of the serum stability of RNA duplexes revealed that siNA constructs consisting of all RNA nucleotides containing two thymidine nucleotide overhangs have a half-life in serum of 15 seconds, whereas chemically modified siNA constructs remained stable in serum for 1 to 3 days depending on the extent of modification (see Figure 3). RNAi stability tests were performed by internally labeling one strand (strand 1) of siNA and duplexing with 1.5 X the concentration of the complementary siNA strand (strand 2) (to insure all labeled material was in duplex form). Duplexed siNA constructs were then tested for stability by incubating at a final concentration of 2μM siNA (strand 2 concentration) in 90% mouse or human serum for time-points of 30sec, 1min, 5min, 30min, 90min, 4hrs 10min, 16hrs 24min, and

49hrs. Time points were run on a 15% denaturing polyacrylamide gels and analyzed on a phosphoimager.

Internal labeling was performed via kinase reactions with polynucleotide kinase (PNK) and ^{32}P-γ-ATP, with addition of radiolabeled phosphate at nucleotide 13 of strand 2, counting in from the 3' side. Ligation of the remaining 8-mer fragments with T4 RNA ligase resulted in the full length, 21-mer, strand 2. Duplexing of RNAi was done by adding appropriate concentrations of the siNA oligonucleotides and heating to 95° C for 5minutes followed by slow cooling to room temperature. Reactions were performed by adding 100% serum to the siNA duplexes and incubating at 37° C, then removing aliquots at desired time-points. Results of this study are summarized in **Figure 3**. As shown in the Figure 3, chemically modified siNA molecules have significantly increased serum stability compared to a siNA construct having all ribonucleotides except a 3'-terminal dithymidine (TT) modification.

Example 3: Identification of potential siNA target sites in any RNA sequence

The sequence of an RNA target of interest, such as a viral or human mRNA transcript, is screened for target sites, for example by using a computer folding algorithm. In a non-limiting example, the sequence of a gene or RNA gene transcript derived from a database, such as Genbank, is used to generate siNA targets having complementarity to the target. Such sequences can be obtained from a database, or can be determined experimentally as known in the art. Target sites that are known, for example, those target sites determined to be effective target sites based on studies with other nucleic acid molecules, for example ribozymes or antisense, or those targets known to be associated with a disease, condition, trait, genotype or phenotype such as those sites containing mutations or deletions, can be used to design siNA molecules targeting those sites. Various parameters can be used to determine which sites are the most suitable target sites within the target RNA sequence. These parameters include but are not limited to secondary or tertiary RNA structure, the nucleotide base composition of the target sequence, the degree of homology between various regions of the target sequence, or the relative position of the target sequence within the RNA transcript. Based on these determinations, any number of target sites within the RNA transcript can be chosen to screen siNA molecules for efficacy, for example by using *in vitro* RNA cleavage assays, cell culture, or animal models. In a

non-limiting example, anywhere from 1 to 1000 target sites are chosen within the transcript based on the size of the siNA construct to be used. High throughput screening assays can be developed for screening siNA molecules using methods known in the art, such as with multi-well or multi-plate assays or combinatorial/siNA library screening assays to determine efficient reduction in target gene expression.

Example 4: Selection of siNA molecule target sites in a RNA

The following non-limiting steps can be used to carry out the selection of siNAs targeting a given gene sequence or transcript.

The target sequence is parsed in silico into a list of all fragments or subsequences of a particular length, for example 23 nucleotide fragments, contained within the target sequence. This step is typically carried out using a custom Perl script, but commercial sequence analysis programs such as Oligo, MacVector, or the GCG Wisconsin Package can be employed as well.

In some instances the siNAs correspond to more than one target sequence; such would be the case for example in targeting different transcripts of the same gene, targeting different transcripts of more than one gene, or for targeting both the human gene and an animal homolog. In this case, a subsequence list of a particular length is generated for each of the targets, and then the lists are compared to find matching sequences in each list. The subsequences are then ranked according to the number of target sequences that contain the given subsequence; the goal is to find subsequences that are present in most or all of the target sequences. Alternately, the ranking can identify subsequences that are unique to a target sequence, such as a mutant target sequence. Such an approach would enable the use of siNA to target specifically the mutant sequence and not effect the expression of the normal sequence.

In some instances the siNA subsequences are absent in one or more sequences while present in the desired target sequence; such would be the case if the siNA targets a gene with a paralogous family member that is to remain untargeted. As in case 2 above, a subsequence list of a particular length is generated for each of the targets, and then the lists are compared to find sequences that are present in the target gene but are absent in the untargeted paralog.

The ranked siNA subsequences can be further analyzed and ranked according to GC content. A preference can be given to sites containing 30-70% GC, with a further preference to sites containing 40-60% GC.

The ranked siNA subsequences can be further analyzed and ranked according to self-folding and internal hairpins. Weaker internal folds are preferred; strong hairpin structures are to be avoided.

The ranked siNA subsequences can be further analyzed and ranked according to whether they have runs of GGG or CCC in the sequence. GGG (or even more Gs) in either strand can make oligonucleotide synthesis problematic and can potentially interfere with RNAi activity, so it is avoided other appropriately suitable sequences are available. CCC is searched in the target strand because that will place GGG in the antisense strand.

The ranked siNA subsequences can be further analyzed and ranked according to whether they have the dinucleotide UU (uridine dinucleotide) on the 3'-end of the sequence, and/or AA on the 5'-end of the sequence (to yield 3' UU on the antisense sequence). These sequences allow one to design siNA molecules with terminal TT thymidine dinucleotides.

Four or five target sites are chosen from the ranked list of subsequences as described above. For example, in subsequences having 23 nucleotides, the right 21 nucleotides of each chosen 23-mer subsequence are then designed and synthesized for the upper (sense) strand of the siNA duplex, while the reverse complement of the left 21 nucleotides of each chosen 23-mer subsequence are then designed and synthesized for the lower (antisense) strand of the siNA duplex (see Tables I). If terminal TT residues are desired for the sequence (as described in paragraph 7), then the two 3' terminal nucleotides of both the sense and antisense strands are replaced by TT prior to synthesizing the oligos.

The siNA molecules are screened in an in vitro, cell culture or animal model system to identify the most active siNA molecule or the most preferred target site within the target RNA sequence.

In an alternate approach, a pool of siNA constructs specific to a target sequence is used to screen for target sites in cells expressing target RNA, such as human HeLa cells. The general strategy used in this approach is shown in **Figure 21**. A non-limiting example

of such a pool is a pool comprising sequences having antisense sequences complementary to the target RNA sequence and sense sequences complementary to the antisense sequences. Cells (e.g., HeLa cells) expressing the target gene are transfected with the pool of siNA constructs and cells that demonstrate a phenotype associated with gene silencing are sorted. The pool of siNA constructs can be chemically modified as described herein and synthesized, for example, in a high throughput manner. The siNA from cells demonstrating a positive phenotypic change (e.g., decreased target mRNA levels or target protein expression), are identified, for example by positional analysis within the assay, and are used to determine the most suitable target site(s) within the target RNA sequence based upon the complementary sequence to the corresponding siNA antisense strand identified in the assay.

Example 5: RNAi activity of chemically modified siNA constructs

Short interfering nucleic acid (siNA) is emerging as a powerful tool for gene regulation. All-ribose siNA duplexes activate the RNAi pathway but have limited utility as therapeutic compounds due to their nuclease sensitivity and short half-life in serum, as shown in Example 2 above. To develop nuclease-resistant siNA constructs for *in vivo* applications, siNAs that target luciferase mRNA and contain stabilizing chemical modifications were tested for activity in HeLa cells. The sequences for the siNA oligonucleotide sequences used in this study are shown in **Table I**. Modifications included phosphorothioate linkages (P=S), 2'–*O*–methyl nucleotides, or 2'–fluoro (F) nucleotides in one or both siNA strands and various 3'-end stabilization chemistries, including 3'-glyceryl, 3'-inverted abasic, 3'-inverted Thymidine, and/or Thymidine. The RNAi activity of chemically stabilized siNA constructs was compared with the RNAi activity of control siNA constructs consisting of all ribonucleotides at every position except the 3'-terminus which comprised two thymidine nucleotide overhangs. Active siNA molecules containing stabilizing modifications such as described herein should prove useful for *in vivo* applications, given their enhanced nuclease-resistance.

A luciferase reporter system was utilized to test RNAi activity of chemically modified siNA constructs compared to siNA constructs consisting of all RNA nucleotides containing two thymidine nucleotide overhangs. Sense and antisense siNA strands (20 uM each) were annealed by incubation in buffer (100 mM potassium acetate, 30 mM

HEPES-KOH, pH 7.4, 2 mM magnesium acetate) for 1 min. at 90°C followed by 1 hour at 37°C. Plasmids encoding firefly luciferase (pGL2) and renilla luciferase (pRLSV40) were purchased from Promega Biotech.

HeLa S3 cells were grown at 37°C in DMEM with 5% FBS and seeded at 15,300 cells in 100 ul media per well of a 96-well plate 24 hours prior to transfection. For transfection, 4 ul Lipofectamine 2000 (Life Technologies) was added to 96 ul OPTI-MEM, vortexed and incubated at room temperature for 5 minutes. The 100 ul diluted lipid was then added to a microtiter tube containing 5 ul pGL2 (200ng/ul), 5 ul pRLSV40 (8 ng/ul) 6 ul siNA (25 nM or 10 nM final), and 84 ul OPTI-MEM, vortexed briefly and incubated at room temperature for 20 minutes. The transfection mix was then mixed briefly and 50 ul was added to each of three wells that contained HeLa S3 cells in 100 ul media. Cells were incubated for 20 hours after transfection and analyzed for luciferase expression using the Dual luciferase assay according to the manufacturer's instructions (Promega Biotech). The results of this study are summarized in **Figures 4-16**. The sequences of the siNA strands used in this study are shown in Table I and are referred to by Sirna/RPI # in the figures. Normalized luciferase activity is reported as the ratio of firefly luciferase activity to renilla luciferase activity in the same sample. Error bars represent standard deviation of triplicate transfections. As shown in **Figures 4-16**, the RNAi activity of chemically modified constructs is often comparable to that of unmodified control siNA constructs, which consist of all ribonucleotides at every position except the 3'-terminus which comprises two thymidine nucleotide overhangs. In some instances, the RNAi activity of the chemically modified constructs is greater than the unmodified control siNA construct consisting of all ribonucleotides..

For example, **Figure 4** shows results obtained from a screen using phosphorothioate modified siNA constructs. The Sirna/RPI 27654/27659 construct contains phosphorothioate substitutions for every pyrimidine nucleotide in both sequences, the Sirna/RPI 27657/27662 construct contains 5 terminal 3'-phosphorothioate substitutions in each strand, the Sirna/RPI 27649/27658 construct contains all phosphorothioate substitutions only in the antisense strand, whereas the Sirna/RPI 27649/27660 and Sirna/RPI 27649/27661 constructs have unmodified sense strands and varying degrees of phosphorothioate substitutions in the antisense strand. All of these constructs show

significant RNAi activity when compared to a scrambled siNA conrol construct (27651/27652).

Figure 5 shows results obtained from a screen using phosphorothioate (Sirna/RPI 28253/28255 and Sirna/RPI 28254/28256) and universal base substitutions (Sirna/RPI 28257/28259 and Sirna/RPI 28258/28260) compared to the same controls described above, these modifications show equivalent or better RNAi activity when compared to the unmodified control siNA construct.

Figure 6 shows results obtained from a screen using 2'-O-methyl modified siNA constructs in which the sense strand contains either 10 (Sirna/RPI 28244/27650) or 5 (Sirna/RPI 28245/27650) 2'-O-methyl substitutions, both with comparable activity to the unmodified control siNA construct.

Figure 7 shows results obtained from a screen using 2'-O-methyl or 2'-deoxy-2'-fluoro modified siNA constructs compared to a control construct consisting of all ribonucleotides at every position except the 3'-terminus which comprises two thymidine nucleotide overhangs.

Figure 8 compares a siNA construct containing six phosphorothioate substitutions in each strand (Sirna/RPI 28460/28461), where 5 phosphorothioates are present at the 3' end and a single phosphorothioate is present at the 5' end of each strand. This motif shows very similar activity to the control siNA construct consisting of all ribonucleotides at every position except the 3'-terminus, which comprises two thymidine nucleotide overhangs.

Figure 9 compares a siNA construct synthesized by the method of the invention described in Example 1, wherein an inverted deoxyabasic succinate linker was used to generate a siNA having a 3'-inverted deoxyabasic cap on the antisense strand of the siNA. This construct shows improved activity compared to the control siNA construct consisting of all ribonucleotides at every position except the 3'-terminus which comprises two thymidine nucleotide overhangs.

Figure 10 shows the results of an RNAi activity screen of chemically modifed siNA constructs including 3'-glyceryl modified siNA constructs compared to an all RNA control siNA construct using a luciferase reporter system. These chemically modified siNAs were

compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by Sirna/RPI number (sense strand/antisense strand). Sequences corresponding to these Sirna/RPI numbers are shown in Table I. As shown in the Figure, the 3'-terminal modified siNA constructs retain significant RNAi activity compared to the unmodified control siNA (siGL2) construct.

Figure 11 shows the results of an RNAi activity screen of chemically modifed siNA constructs. The screen compared various combinations of sense strand chemical modifications and antisense strand chemical modifications. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by Sirna/RPI number (sense strand/antisense strand). Sequences corresponding to these Sirna/RPI numbers are shown in Table I. As shown in the figure, the chemically modified Sirna/RPI 30063/30430, Sirna/RPI 30433/30430, and Sirna/RPI 30063/30224 constructs retain significant RNAi activity compared to the unmodified control siNA construct. It should be noted that Sirna/RPI 30433/30430 is a siNA construct having no ribonucleotides which retains significant RNAi activity compared to the unmodified control siGL2 construct in vitro, therefore, this construct is expected to have both similar RNAi activity and improved stability in vivo compared to siNA constructs having ribonucleotides.

Figure 12 shows the results of an RNAi activity screen of chemically modifed siNA constructs. The screen compared various combinations of sense strand chemical modifications and antisense strand chemical modifications. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by Sirna/RPI number (sense

strand/antisense strand). Sequences corresponding to these Sirna/RPI numbers are shown in Table I. As shown in the figure, the chemically modified Sirna/RPI 30063/30224 and Sirna/RPI 30063/30430 constructs retain significant RNAi activity compared to the control siNA (siGL2) construct. In addition, the antisense strand alone (Sirna/RPI 30430) and an inverted control (Sirna/RPI 30227/30229), having matched chemistry to Sirna/RPI (30063/30224) were compared to the siNA duplexes described above. The antisense strand (Sirna/RPI 30430) alone provides far less inhibition compared to the siNA duplexes using this sequence.

Figure 13 shows the results of an RNAi activity screen of chemically modifed siNA constructs. The screen compared various combinations of sense strand chemical modifications and antisense strand chemical modifications. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by Sirna/RPI number (sense strand/antisense strand). Sequences corresponding to these Sirna/RPI numbers are shown in Table I. In addition, an inverted control (Sirna/RPI 30226/30229, having matched chemistry to Sirna/RPI 30222/30224) was compared to the siNA duplexes described above. As shown in the figure, the chemically modified Sirna/RPI 28251/30430, Sirna/RPI 28251/30224, and Sirna/RPI 30222/30224 constructs retain significant RNAi activity compared to the control siNA construct, and the chemically modified Sirna/RPI 28251/30430 construct demonstrates improved activity compared to the control siNA (siGL2) construct.

Figure 14 shows the results of an RNAi activity screen of chemically modifed siNA constructs including various 3'-terminal modified siNA constructs compared to an all RNA control siNA construct using a luciferase reporter system. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by Sirna/RPI number (sense

strand/antisense strand). Sequences corresponding to these Sirna/RPI numbers are shown in Table I. As shown in the figure, the chemically modified Sirna/RPI 30222/30546, 30222/30224, 30222/30551, 30222/30557 and 30222/30558 constructs retain significant RNAi activity compared to the control siNA construct.

Figure 15 shows the results of an RNAi activity screen of chemically modifed siNA constructs. The screen compared various combinations of sense strand chemistries compared to a fixed antisense strand chemistry. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by Sirna/RPI number (sense strand/antisense strand). Sequences corresponding to these Sirna/RPI numbers are shown in Table I. As shown in the figure, the chemically modified Sirna/RPI 30063/30430, 30434/30430, and 30435/30430 constructs all demonstrate greater activity compared to the control siNA (siGL2) construct.

Example 6: RNAi activity titration

A titration assay was performed to determine the lower range of siNA concentration required for RNAi activity both in a control siNA construct consisting of all RNA nucleotides containing two thymidine nucleotide overhangs and a chemically modified siNA construct comprising five phosphorothioate internucleotide linkages in both the sense and antisense strands. The assay was performed as described above, however, the siNA constructs were diluted to final concentrations between 2.5 nM and 0.025 nM. Results are shown in **Figure 16**. As shown in **Figure 16**, the chemically modified siNA construct shows a very similar concentration dependent RNAi activity profile to the control siNA construct when compared to an inverted siNA sequence control.

Example 7: siNA design

siNA target sites were chosen by analyzing sequences of the target RNA and optionally prioritizing the target sites on the basis of folding (structure of any given sequence analyzed to determine siNA accessibility to the target), by using a library of siNA

molecules as described in Example 4, or alternately by using an *in vitro* siNA system as described in Example 9 herein. siNA molecules were designed that could bind each target and are optionally individually analyzed by computer folding to assess whether the siNA molecule can interact with the target sequence. Varying the length of the siNA molecules can be chosen to optimize activity. Generally, a sufficient number of complementary nucleotide bases are chosen to bind to, or otherwise interact with, the target RNA, but the degree of complementarity can be modulated to accommodate siNA duplexes or varying length or base composition. By using such methodologies, siNA molecules can be designed to target sites within any known RNA sequence, for example those RNA sequences corresponding to the any gene transcript.

Chemically modified siNA constructs are designed to provide nuclease stability for systemic administration in vivo and/or improved pharmacokinetic, localization, and delivery properties while preserving the ability to mediate RNAi activity. Chemical modifications as described herein are introduced synthetically using synthetic methods described herein and those generally known in the art. The synthetic siNA constructs are then assayed for nuclease stability in serum and/or cellular/tissue extracts (e.g., liver extracts). The synthetic siNA constructs are also tested in parallel for RNAi activity using an appropriate assay, such as a luciferase reporter assay as described herein or another suitable assay that can quantity RNAi activity. Synthetic siNA constructs that possess both nuclease stability and RNAi activity can be further modified and re-evaluated in stability and activity assays. The chemical modifications of the stabilized active siNA constructs can then be applied to any siNA sequence targeting any chosen RNA and used, for example, in target screening assays to pick lead siNA compounds for therapeutic development (see for example **Figure 27**).

Example 8: Chemical Synthesis and Purification of siNA

siNA molecules can be designed to interact with various sites in the RNA message, for example, target sequences within the RNA sequences described herein. The sequence of one strand of the siNA molecule(s) is complementary to the target site sequences described above. The siNA molecules can be chemically synthesized using methods described herein. Inactive siNA molecules that are used as control sequences can be synthesized by scrambling the sequence of the siNA molecules such that it is not

DEMANDES OU BREVETS VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS COMPREND PLUS D'UN TOME.

CECI EST LE TOME __1___ DE __3___

NOTE: Pour les tomes additionels, veillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE THAN ONE VOLUME.

THIS IS VOLUME __1___ OF __3___

NOTE: For additional volumes please contact the Canadian Patent Office.

53666-3

CLAIMS:

1. A multifunctional siNA molecule of Formula I:

5'-p-X Z X'-3'
3'-Y'Z Y-p-5'

wherein each 5'-p-XZX'-3' and 5'-p-YZY'-3' independently comprise an oligonucleotide of length between 24 and 38 nucleotides, XZ comprises a nucleic acid sequence that is complementary to a first target nucleic acid sequence, YZ comprises an oligonucleotide comprising nucleic acid sequence that is complementary to a second target nucleic acid sequence, Z comprises nucleotide sequence of length 1 to 24 nucleotides that is complementary between regions XZ and YZ, X comprises nucleotide sequence of length 1 to 21 nucleotides that is complementary to nucleotide sequence present in region Y', Y comprises nucleotide sequence of length 1 to 21 nucleotides that is complementary to nucleotide sequence present in region X', p comprises a terminal phosphate group that can independently be present or absent, and wherein each said XZ and said YZ are independently of length sufficient to stably interact with said first and said second target nucleic acid sequence, respectively, or a portion thereof.

2. The siNA molecule of claim 1, wherein said siNA comprises a 3'-terminal cap moiety.

3. The siNA molecule of claim 2, wherein said terminal cap moiety is an inverted deoxyabasic moiety.

4. The siNA molecule of claim 2, wherein said terminal cap moiety is an inverted deoxynucleotide moiety.

5. The siNA molecule of claim 2, wherein said terminal cap moiety is a dinucleotide moiety.

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6. The siNA molecule of claim 5, wherein said dinucleotide is dithymidine (TT).

7. The siNA molecule of claim 1, wherein said siNA molecule comprises no ribonucleotides.

8. The siNA molecule of claim 1, wherein said siNA molecule comprises ribonucleotides.

9. The siNA molecule of claim 1, wherein any pyrimidine nucleotide in said siNA is a 2'-0-methyl pyrimidine nucleotide.

10. The siNA molecule of claim 1, wherein any purine nucleotide in said siNA is a 2'-deoxy purine nucleotide.

11. The siNA molecule of claim 1, wherein any pyrimidine nucleotide in said siNA is a 2'-deoxy-2'-fluoro pyrimidine nucleotide.

12. The siNA molecule of claim 1, wherein said siNA molecule comprises 3'-nucleotide overhangs.

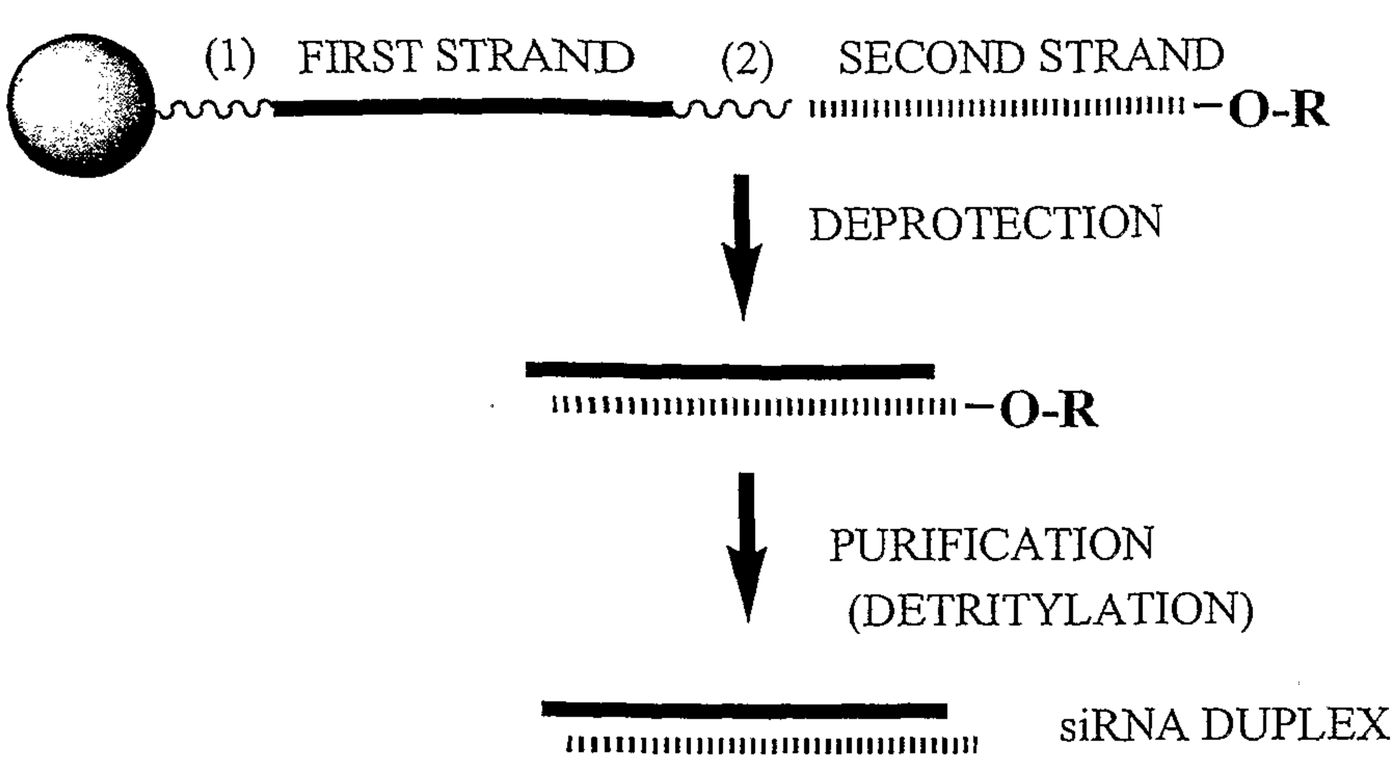
13. The siNA molecule of claim 12, wherein said 3'-overhangs comprise about 1 to about 4 nucleotides.

14. The siNA molecule of claim 13, wherein said nucleotides comprise deoxynucleotides.

15. The siNA molecule of claim 14, wherein said deoxynucleotides are thymidine nucleotides.

16. A composition comprising the siNA molecule of any one of claims 1 to 15 in a pharmaceutically acceptable carrier or diluent.

Figure 1



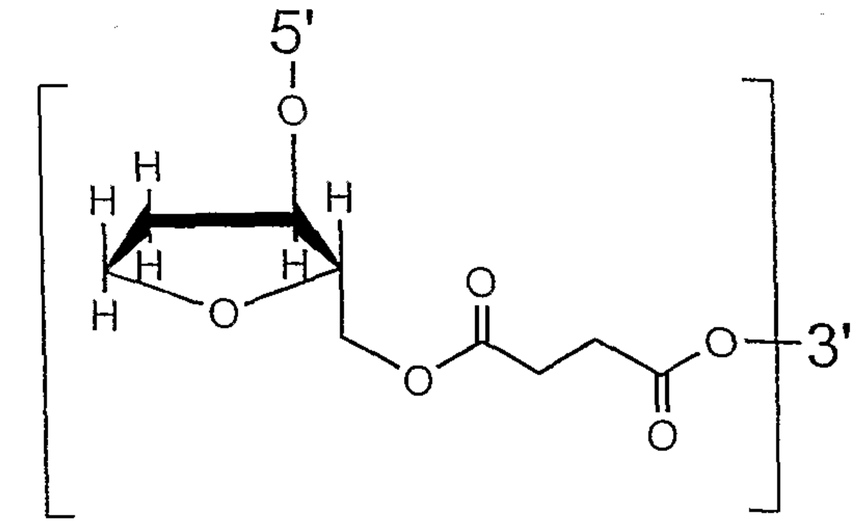
= SOLID SUPPORT

R = TERMINAL PROTECTING GROUP

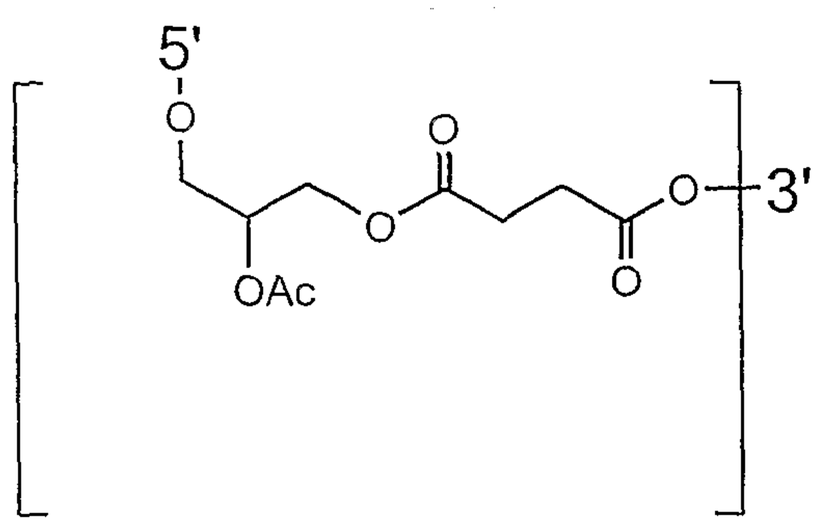
FOR EXAMPLE:
DIMETHOXYTRITYL (DMT)

(1) = CLEAVABLE LINKER
(FOR EXAMPLE: NUCLEOTIDE SUCCINATE OR INVERTED DEOXYABASIC SUCCINATE)

(2) = CLEAVABLE LINKER
(FOR EXAMPLE: NUCLEOTIDE SUCCINATE OR INVERTED DEOXYABASIC SUCCINATE)



INVERTED DEOXYABASIC SUCCINATE LINKAGE



GLYCERYL SUCCINATE LINKAGE

Figure 2

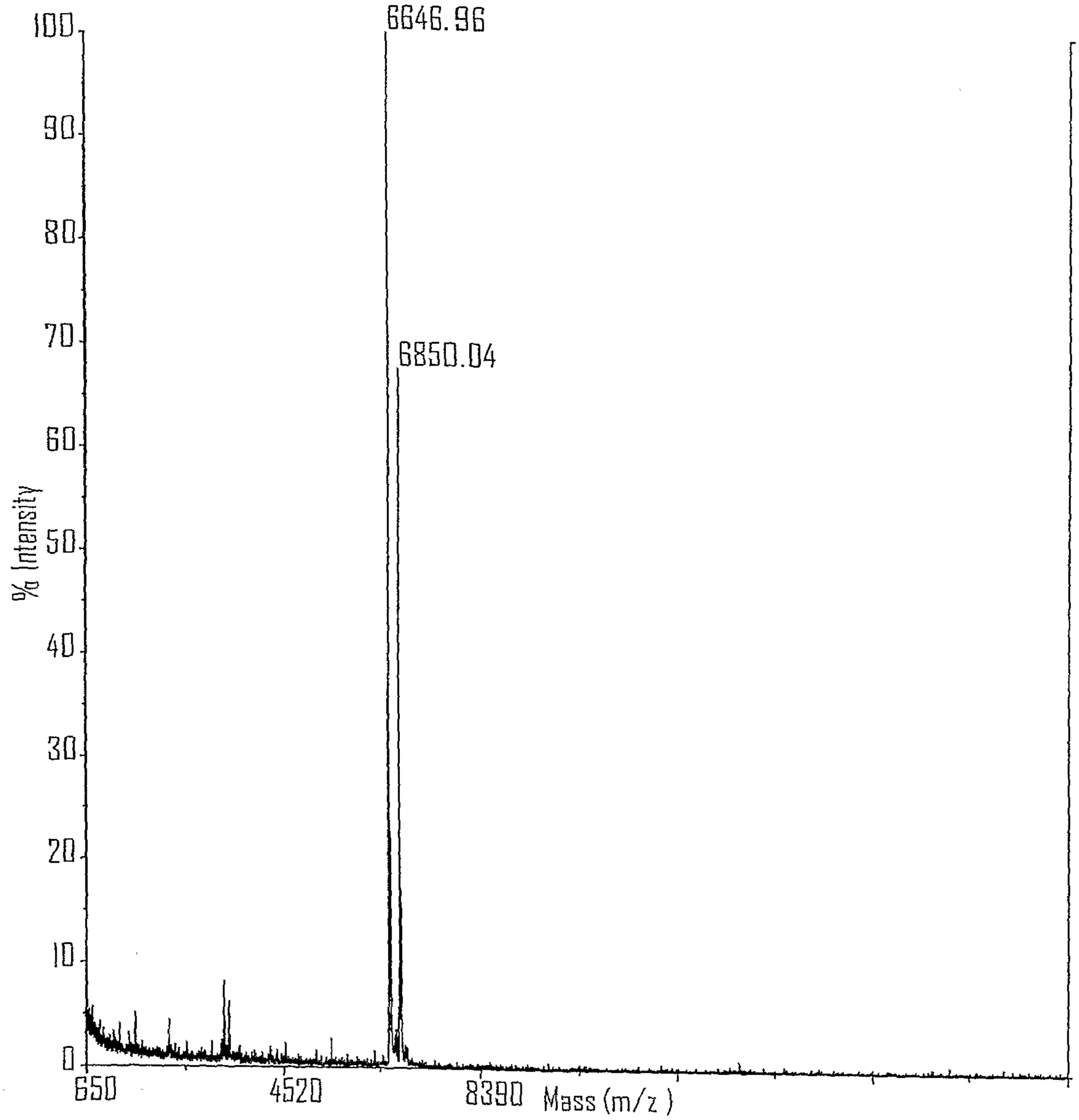

100
90
80
70
60
50
40
30
20
10
0
% Intensity
6646.96
6850.04
650
4520
8390
Mass (m/z)

Figure 3

5'-CGUACGCGGAAUACUUCGATT (SEQ ID NO: 394)
3'-TTGCAUGCGCCUUAUGAAGCU (SEQ ID NO: 395) T ½ = 15 seconds (control)

5'-B cAAccAcAAAAuAcAAcAATT B (SEQ ID NO: 396)
3'-TXGuuGGuGuuuuAuGuuGuu (SEQ ID NO: 397) T ½ = 138 min

5'-B cAAccAcAAAAuAcAAcAATT B (SEQ ID NO: 396)
3'-TDGuuGGuGuuuuAuGuuGuu (SEQ ID NO: 398) T ½ = 3.7 days

5'-B cAAccAcAAAAuAcAAcAATT B (SEQ ID NO: 396)
3'-XTGuuGGuGuuuuAuGuuGuu (SEQ ID NO: 399) T ½ = 72 minutes

5'-B cAAccAcAAAAuAcAAcAATT B (SEQ ID NO: 396)
3'-LTGuuGGuGuuuuAuGuuGuu (SEQ ID NO: 400) T ½ = 40 days

5'-B cAAccAcAAAAuAcAAcAATT B (SEQ ID NO: 396)
3'-tTGuuGGuGuuuuAuGuuGuu (SEQ ID NO: 401) T ½ = 32 days

G, A, U, C = Guanosine, Adenosine, Uridine, Cytidine
T = Thymidine
Lower Case = 2'-deoxy-2'-fluoro
S = phosphorothioate
B = inverted deoxyabasic
D = inverted Thymidine
X = 3'-deoxy Thymidine
t = L-thymidine
L = Glyceryl moiety

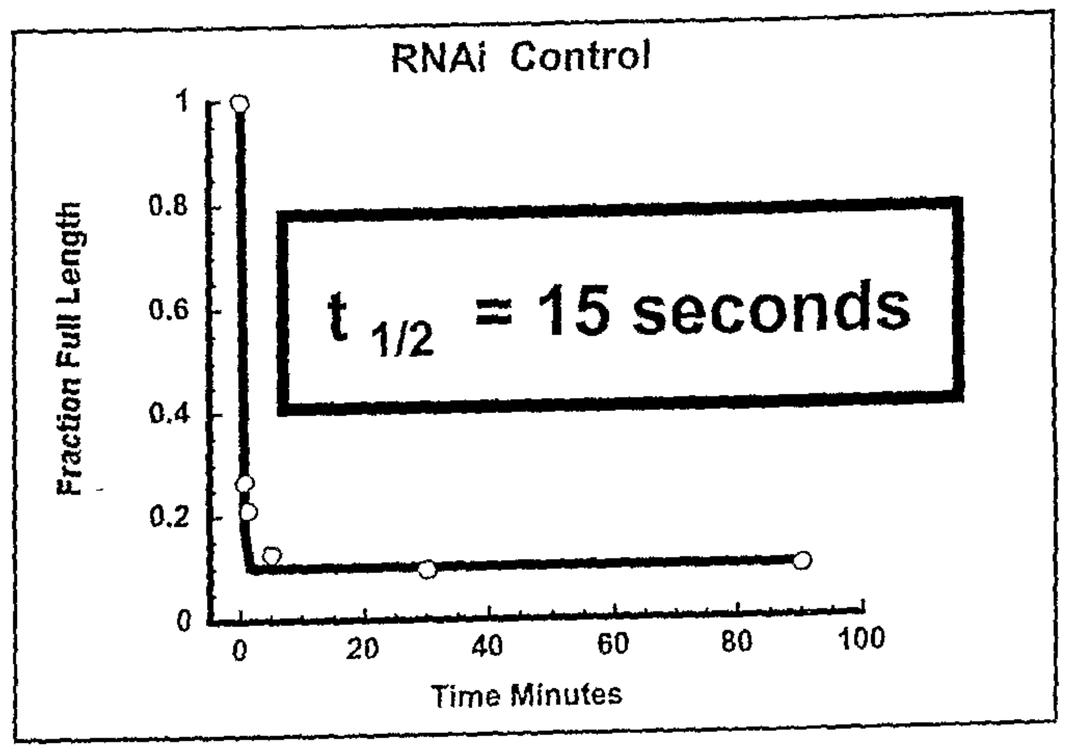
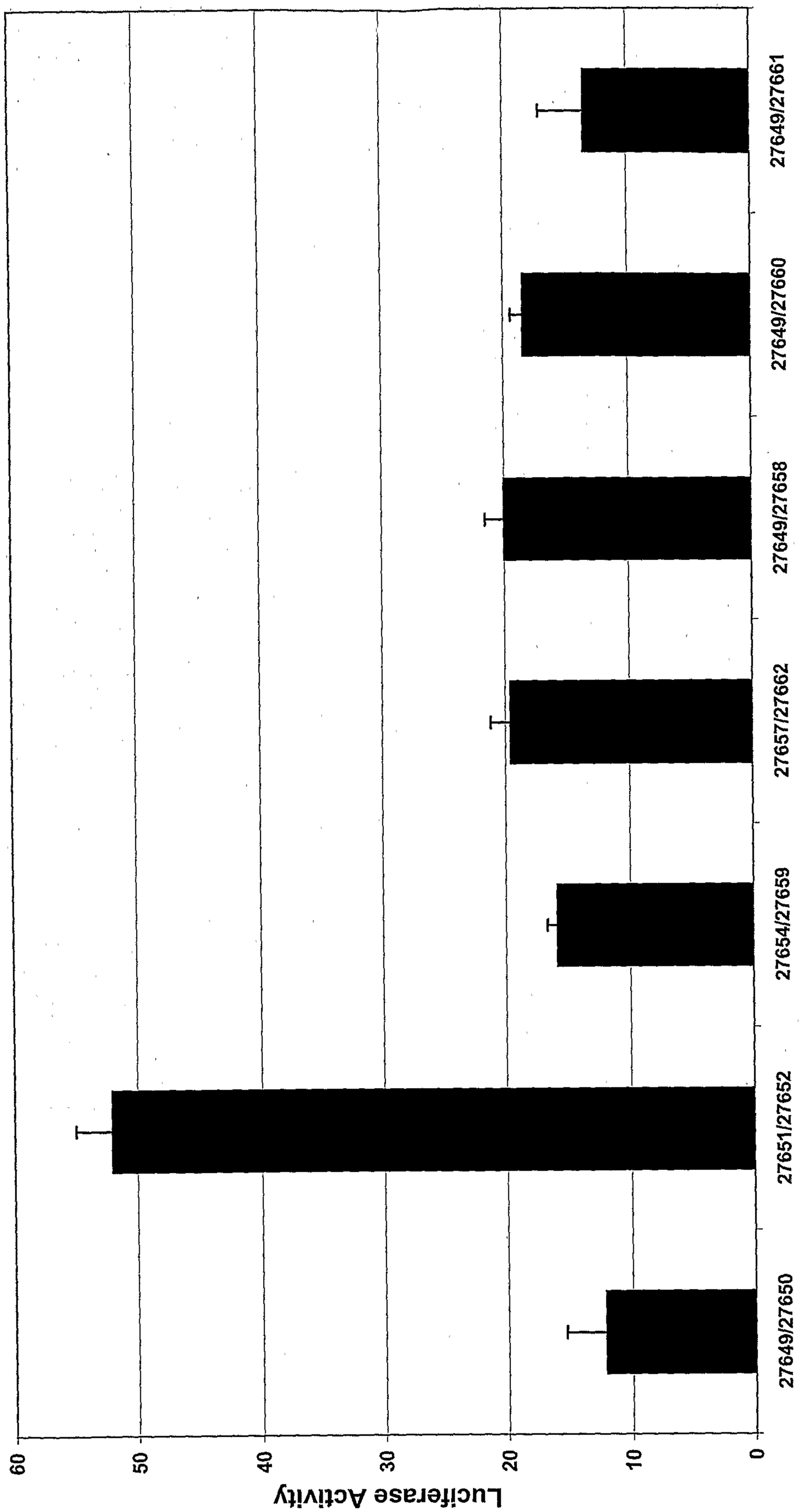


Figure 4


Luciferase Activity
60
50
40
30
20
10
0
27649/27650
27651/27652
27654/27659
27657/27662
27649/27658
27649/27660
27649/27661
RPI # (sense/antisense)

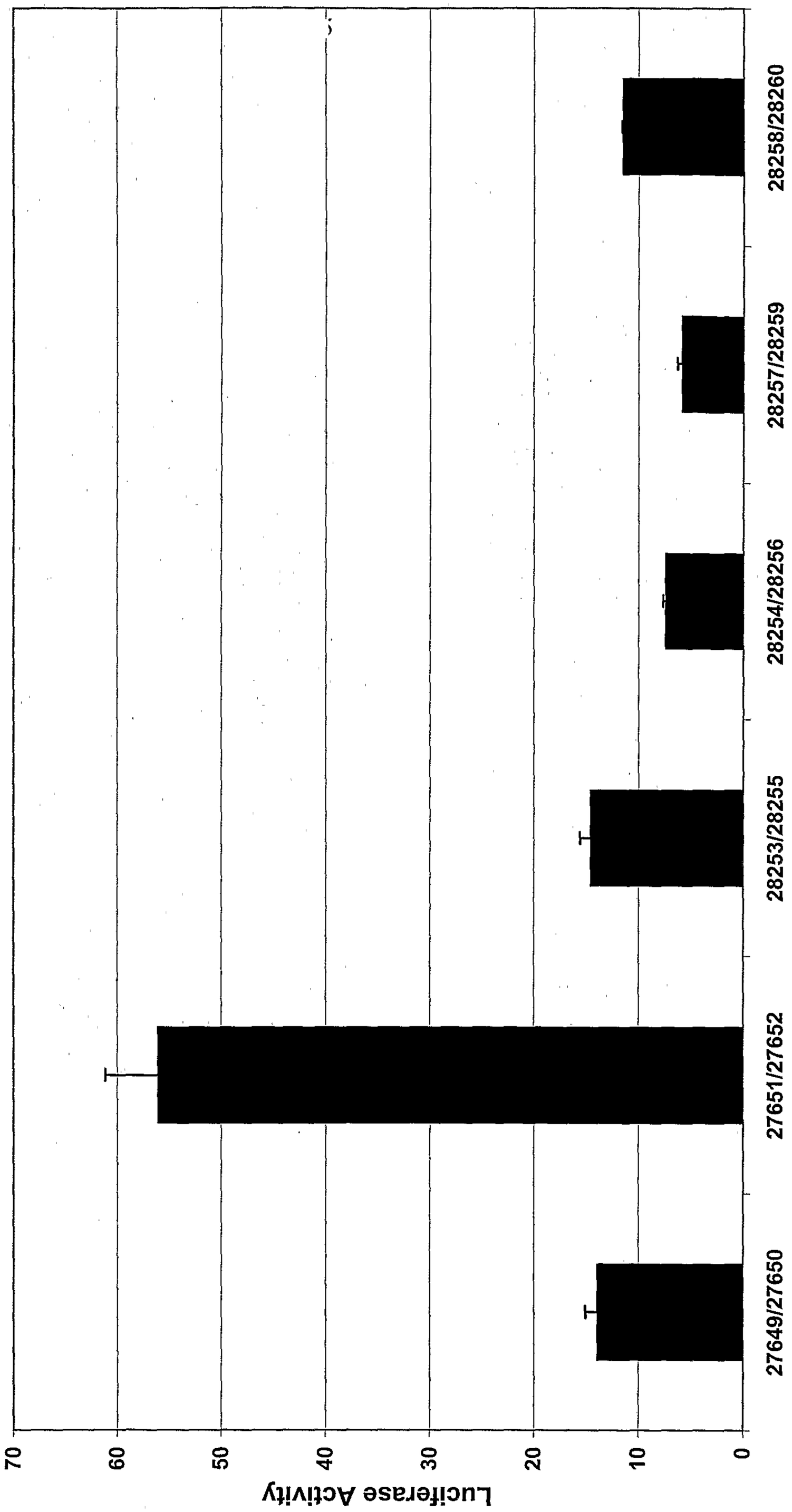

Luciferase Activity
70
60
50
40
30
20
10
0
27649/27650
27651/27652
28253/28255
28254/28256
28257/28259
28258/28260
RPI # (sense/antisense)

Figure 5

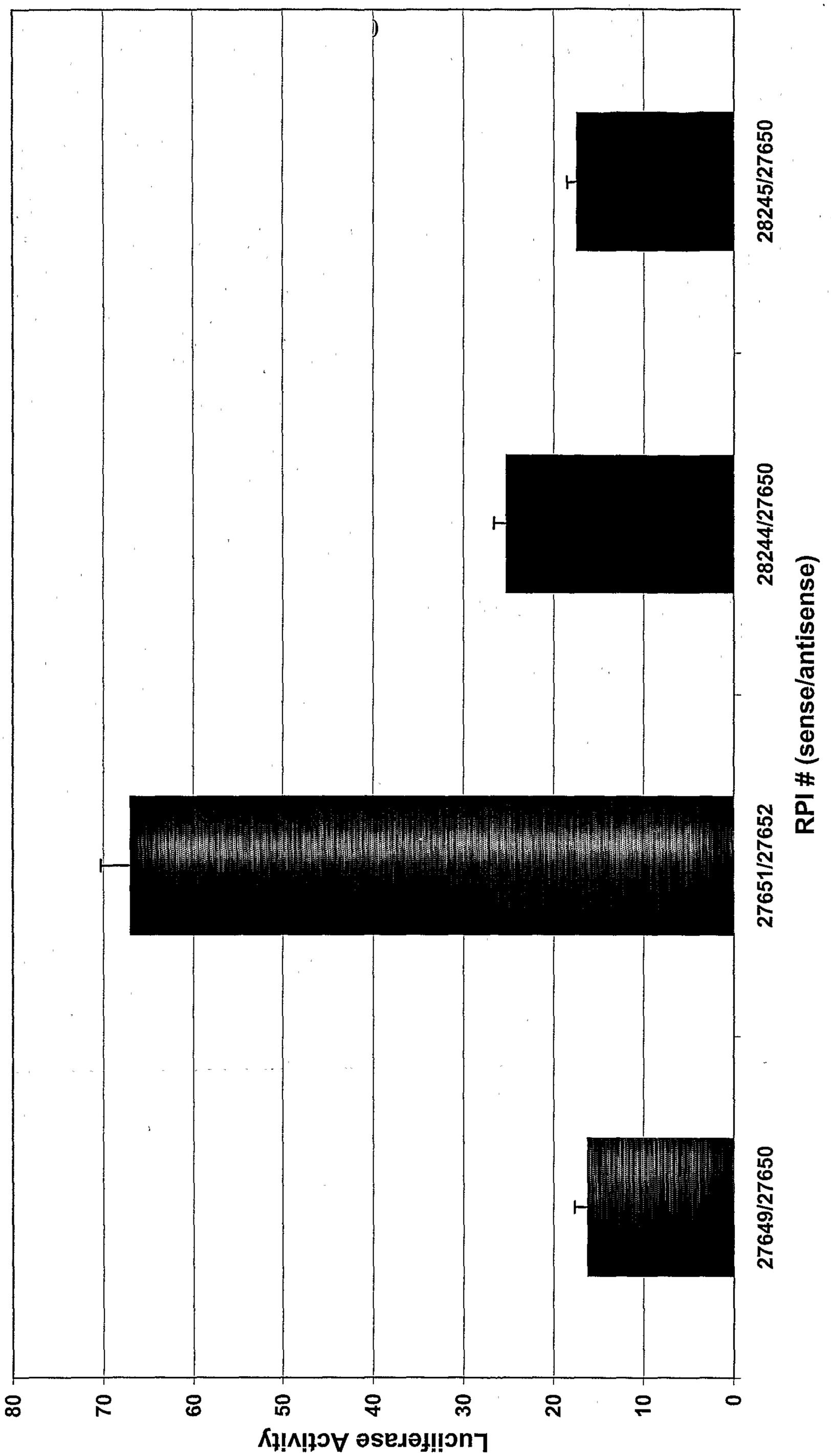

Luciferase Activity
80
70
60
50
40
30
20
10
0
27649/27650
27651/27652
RPI # (sense/antisense)
28244/27650
28245/27650

Figure 6

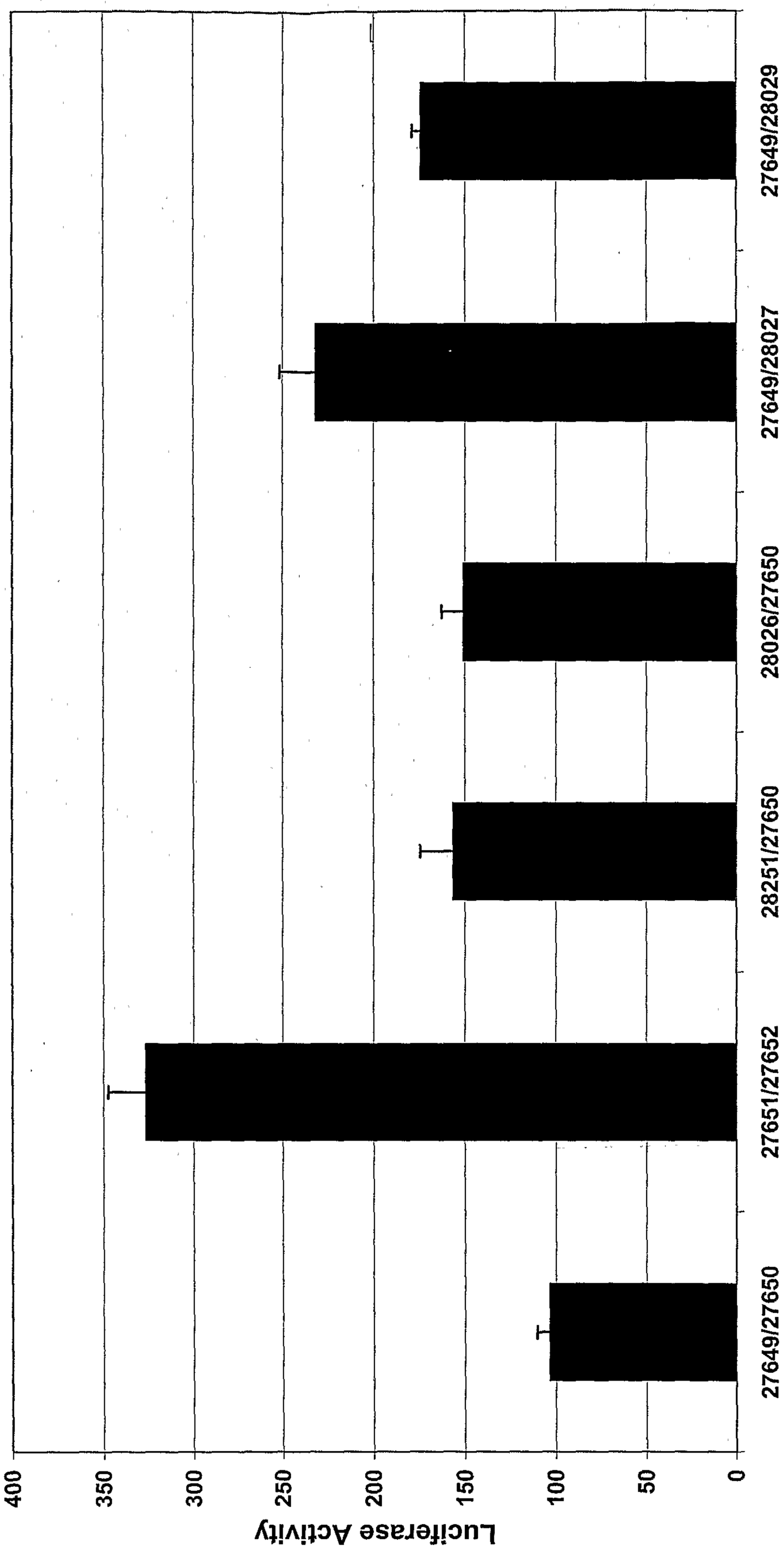

Luciferase Activity
400
350
300
250
200
150
100
50
0
27649/27650
28251/27650
27651/27652
28026/27650
27649/28027
27649/28029
RPI # (sense/antisense)

Figure 7

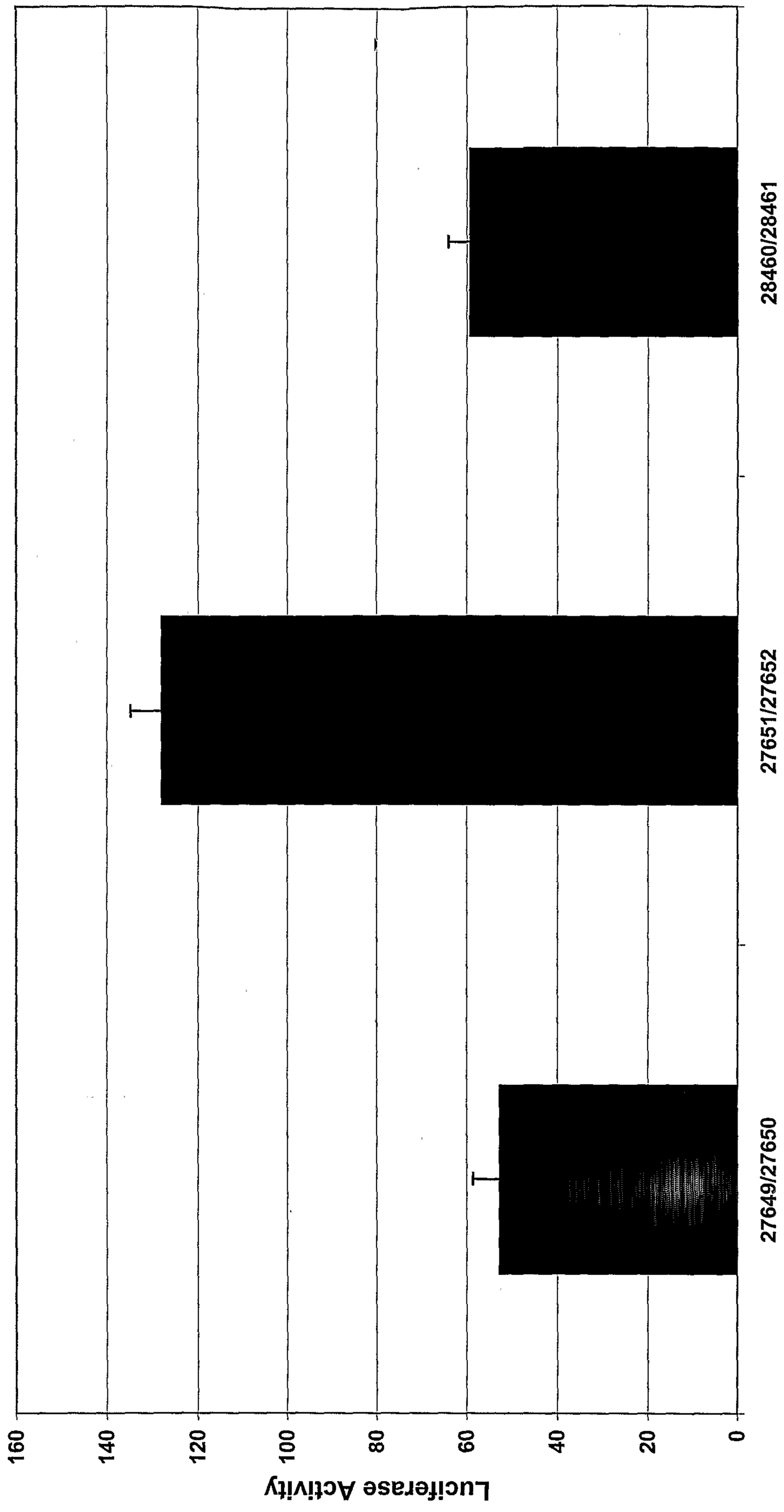
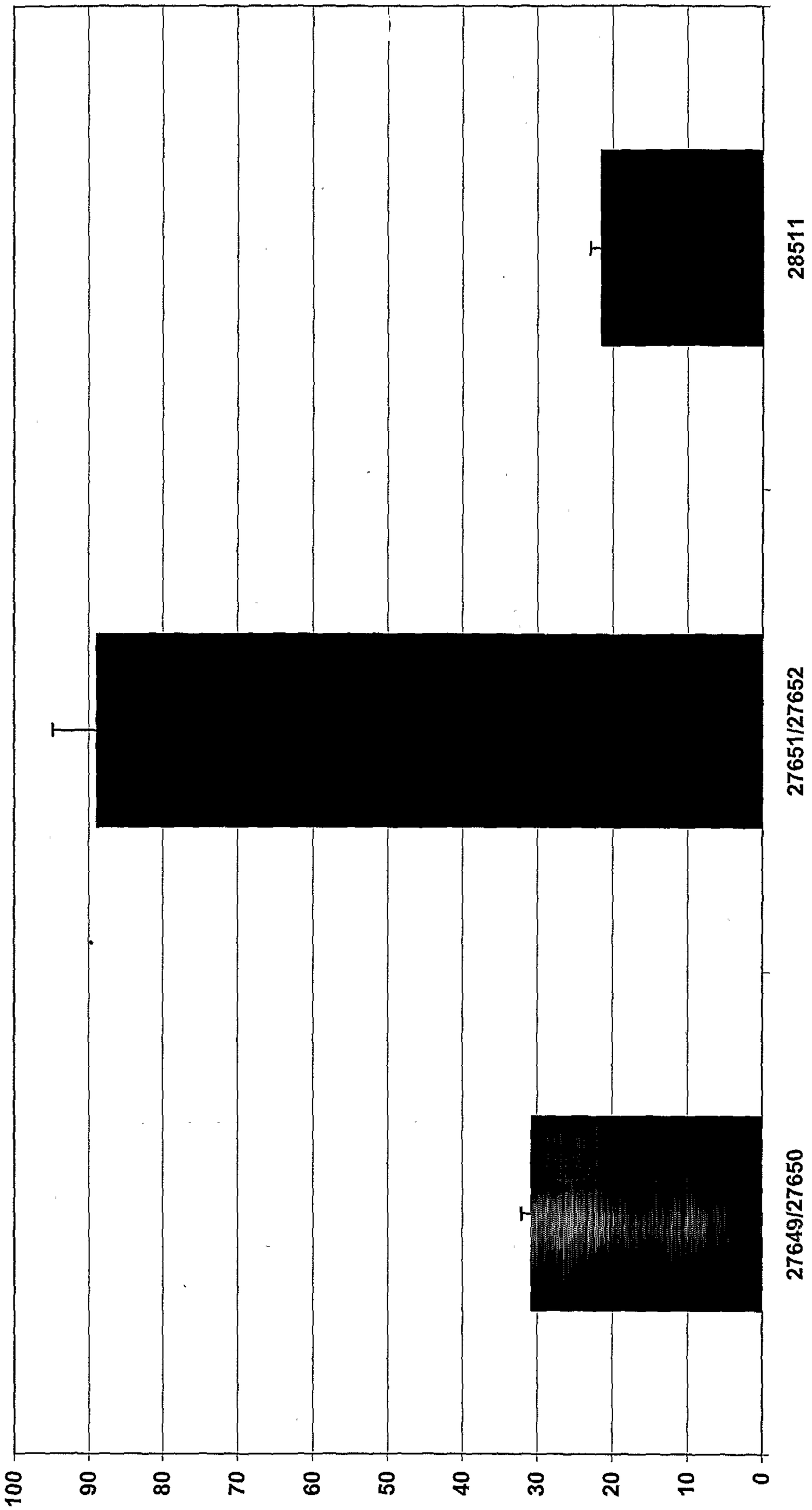

Luciferase Activity
160
140
120
100
80
60
40
20
0
RPI # (sense/antisense)
27649/27650
27651/27652
28460/28461

Figure 8


Luciferase Activity
100
90
80
70
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40
30
20
10
0
27649/27650
27651/27652
28511
RPI # (sense/antisense)

Figure 9

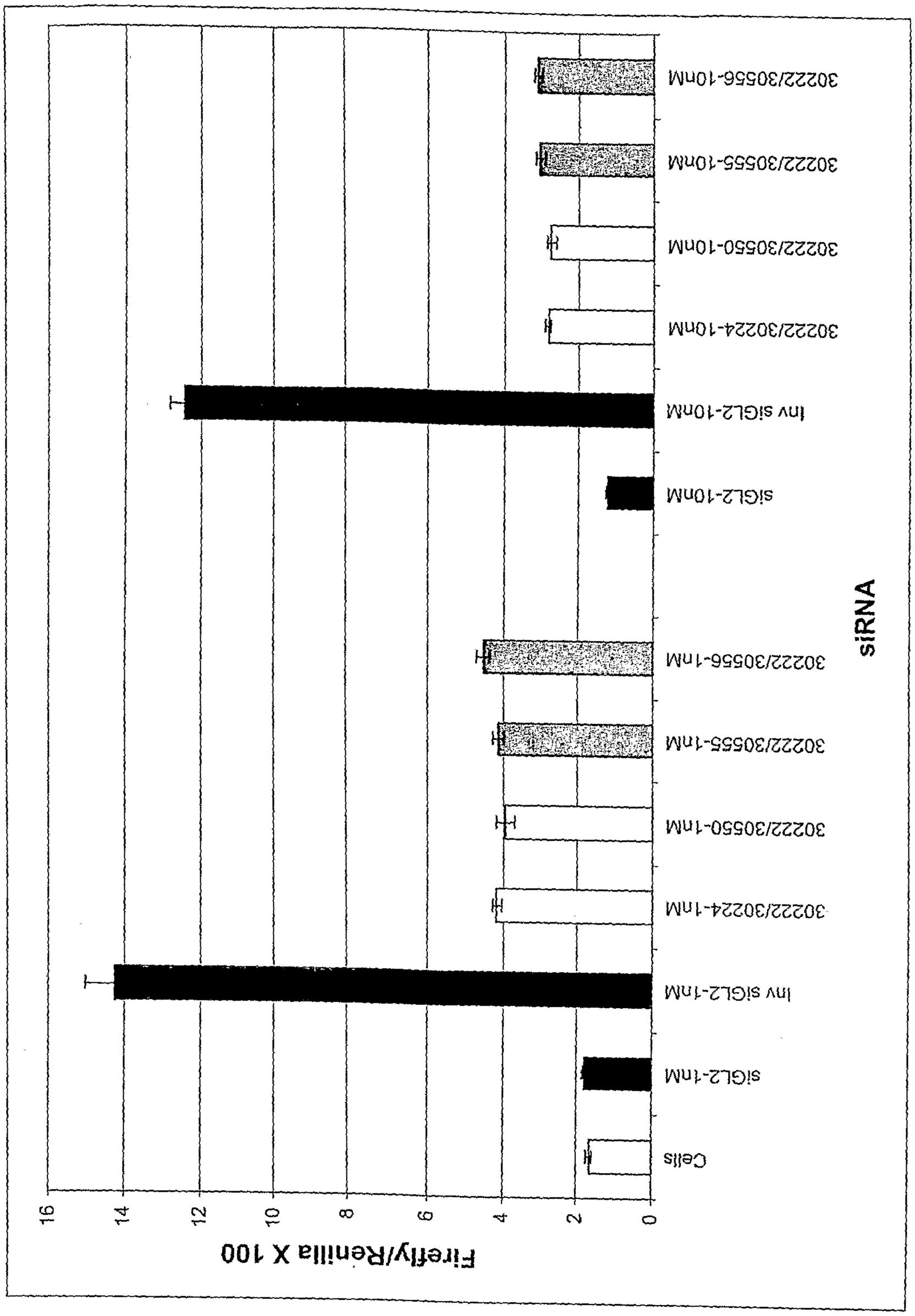
Firefly/Renilla X 100
0
2
4
6
8
10
12
14
16
Cells
siGL2-1nM
Inv siGL2-1nM
30222/30224-1nM
30222/30550-1nM
30222/30555-1nM
30222/30556-1nM
siGL2-10nM
Inv siGL2-10nM
30222/30224-10nM
30222/30550-10nM
30222/30555-10nM
30222/30556-10nM
siRNA

Figure 10

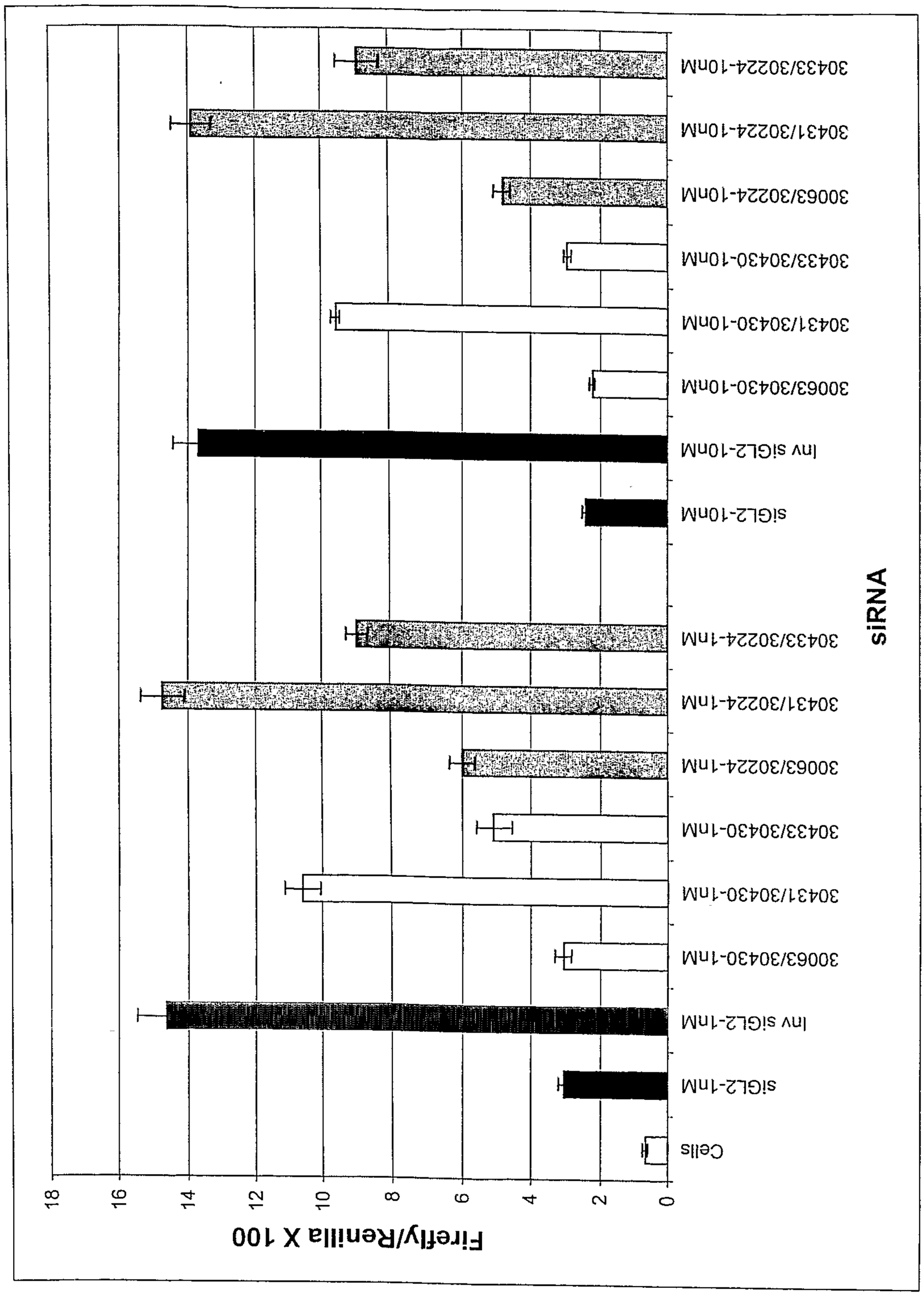
Figure 11
siRNA
Cells
siGL2-10nM
Inv siGL2-10nM
30063/30430-10nM
30431/30430-10nM
30433/30430-10nM
30063/30224-10nM
30431/30224-10nM
30433/30224-10nM
siGL2-1nM
Inv siGL2-1nM
30063/30430-10nM
30431/30430-10nM
30433/30430-10nM
30063/30224-10nM
30431/30224-10nM
30433/30224-10nM
Firefly/Renilla X 100
0
2
4
6
8
10
12
14
16
18

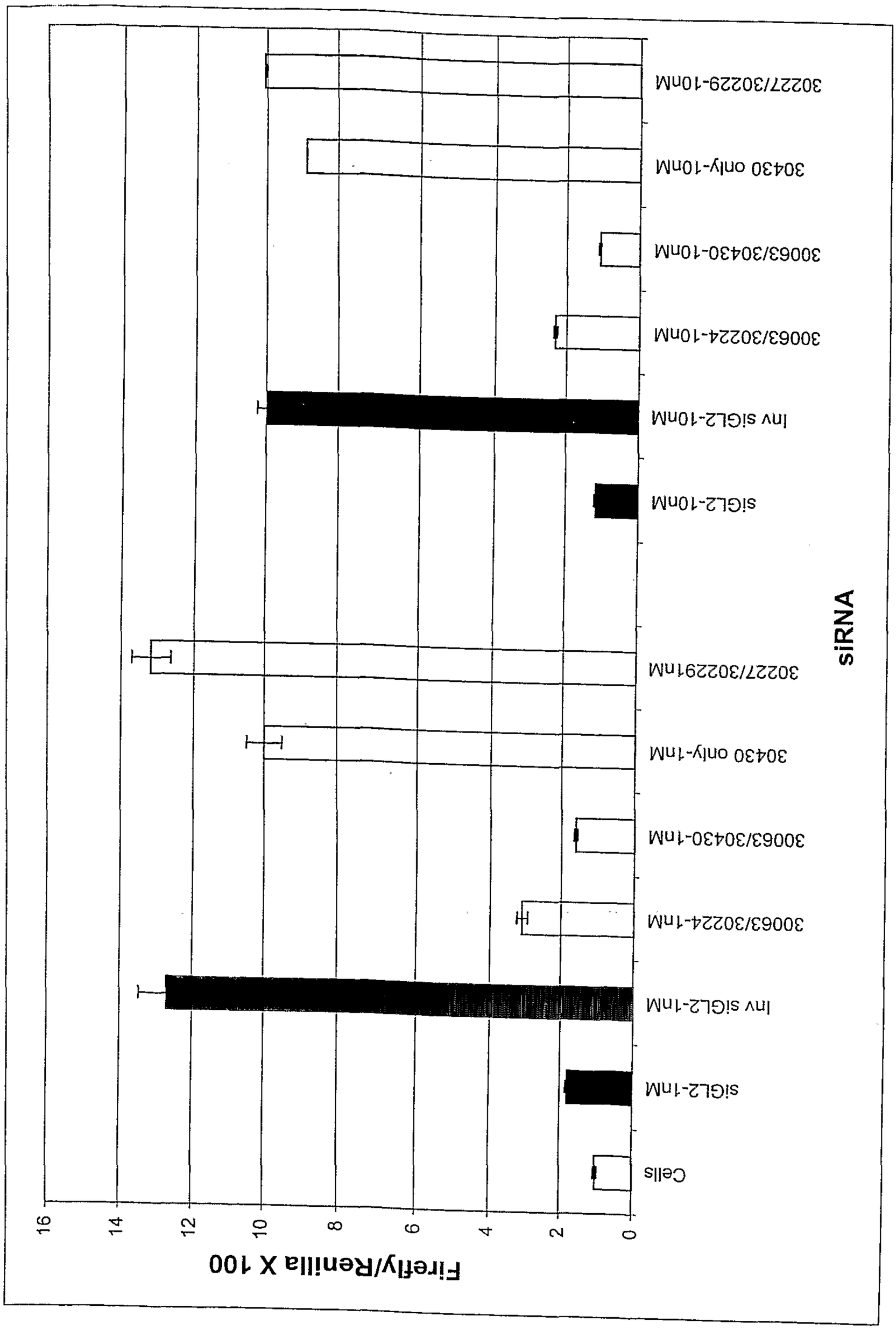
Figure 12
siRNA
Firefly/Renilla X 100
0
2
4
6
8
10
12
14
16
Cells
siGL2-1nM
Inv siGL2-1nM
30063/30224-1nM
30063/30430-1nM
30430 only-1nM
30227/30229-1nM
siGL2-10nM
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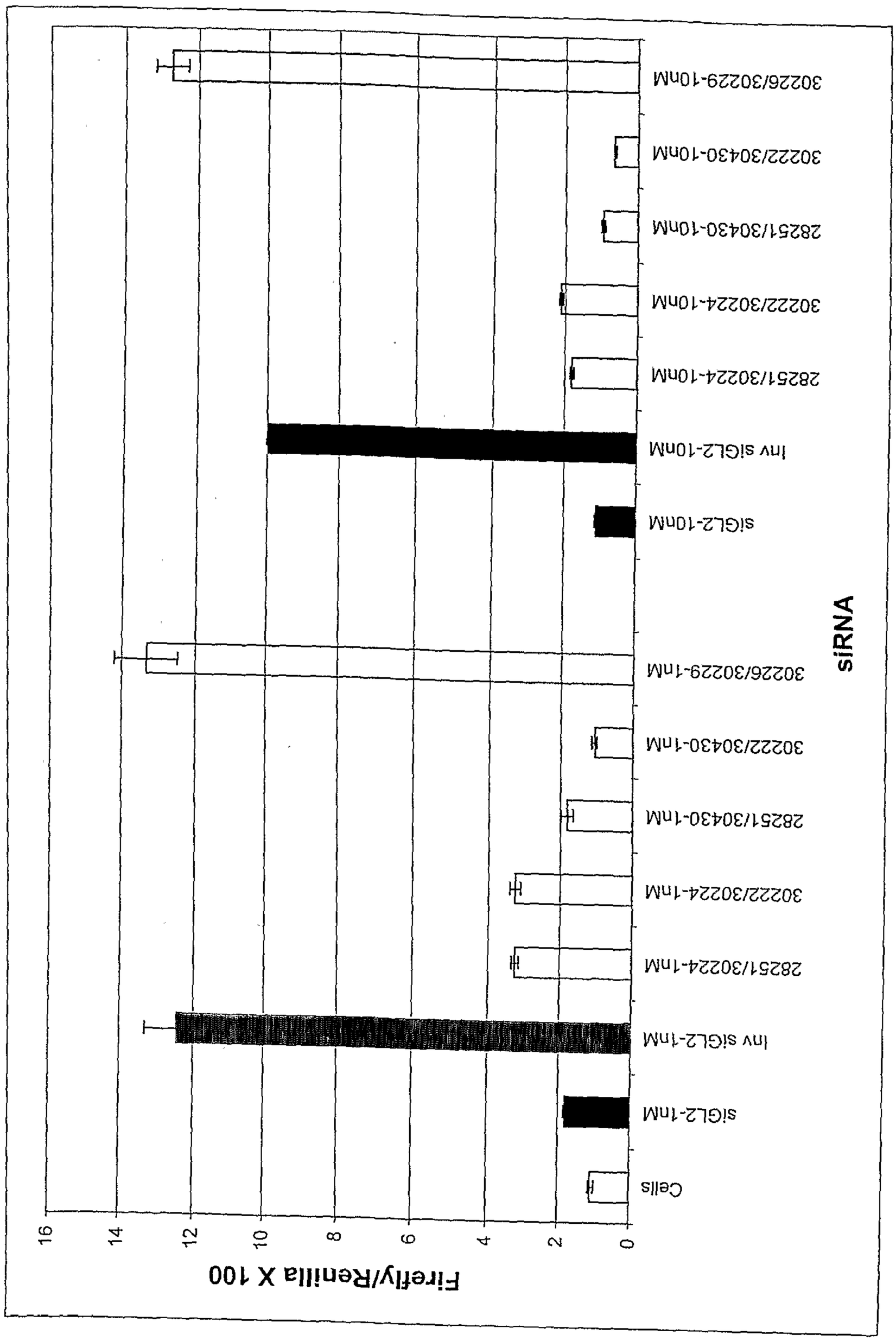
30430 only-10nM
30227/30229-10nM


30226/30229-10nM
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28251/30430-10nM
30222/30224-10nM
28251/30224-10nM
Inv siGL2-10nM
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siRNA
30226/30229-1nM
30222/30430-1nM
28251/30430-1nM
30222/30224-1nM
28251/30224-1nM
Inv siGL2-1nM
siGL2-1nM
Cells
0
2
4
6
8
10
12
14
16
Firefly/Renilla X 100

Figure 13

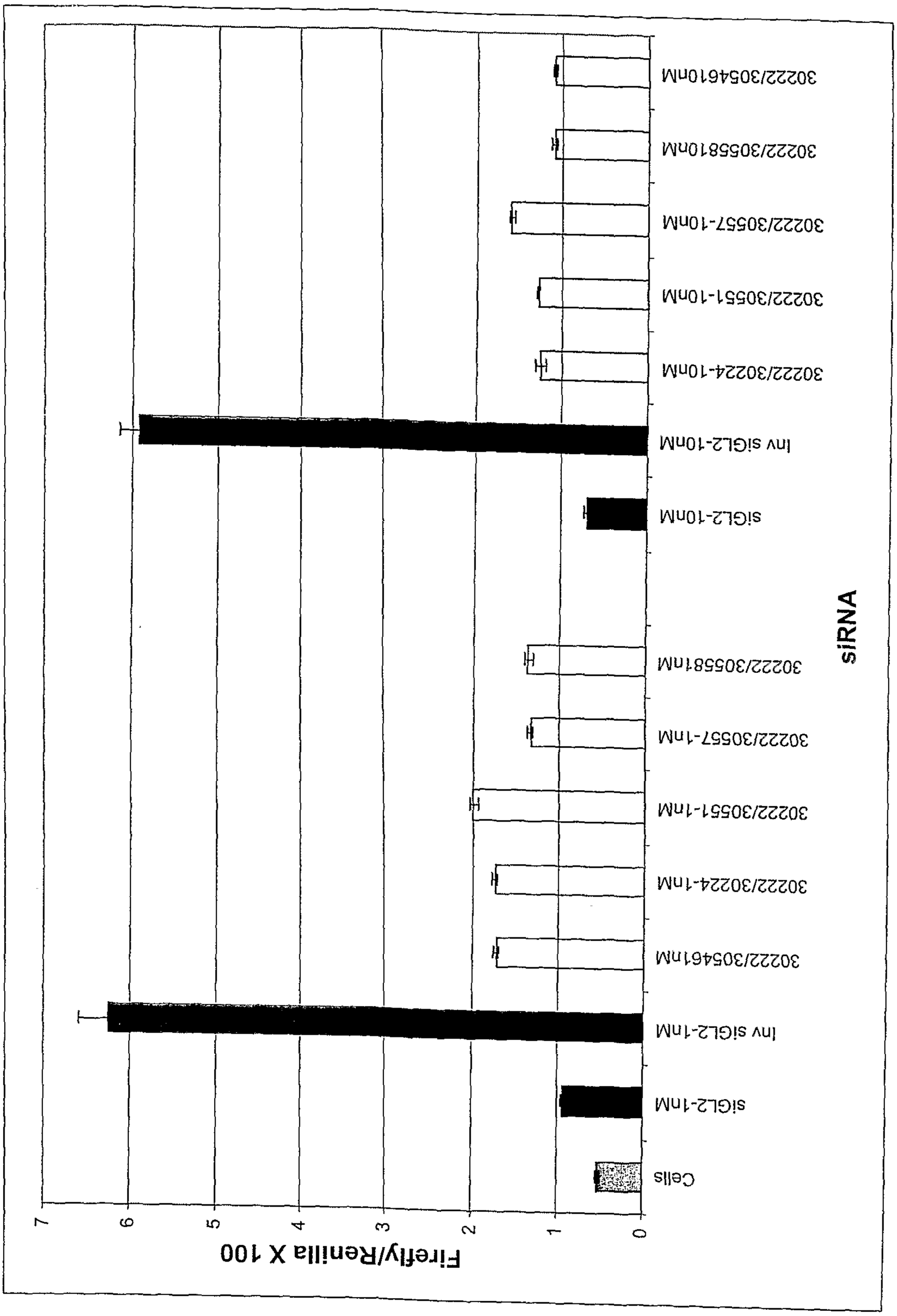


Figure 14
Firefly/Renilla X 100
0
1
2
3
4
5
6
7
Cells
siGL2-1nM
Inv siGL2-1nM
30222/30546 1nM
30222/30558 1nM
30222/30557 1nM
30222/30551 1nM
30222/30224 1nM
30222/30546 10nM
siGL2-10nM
Inv siGL2-10nM
30222/30224-10nM
30222/30551-10nM
30222/30557-10nM
30222/30558 10nM
30222/30546 10nM
siRNA

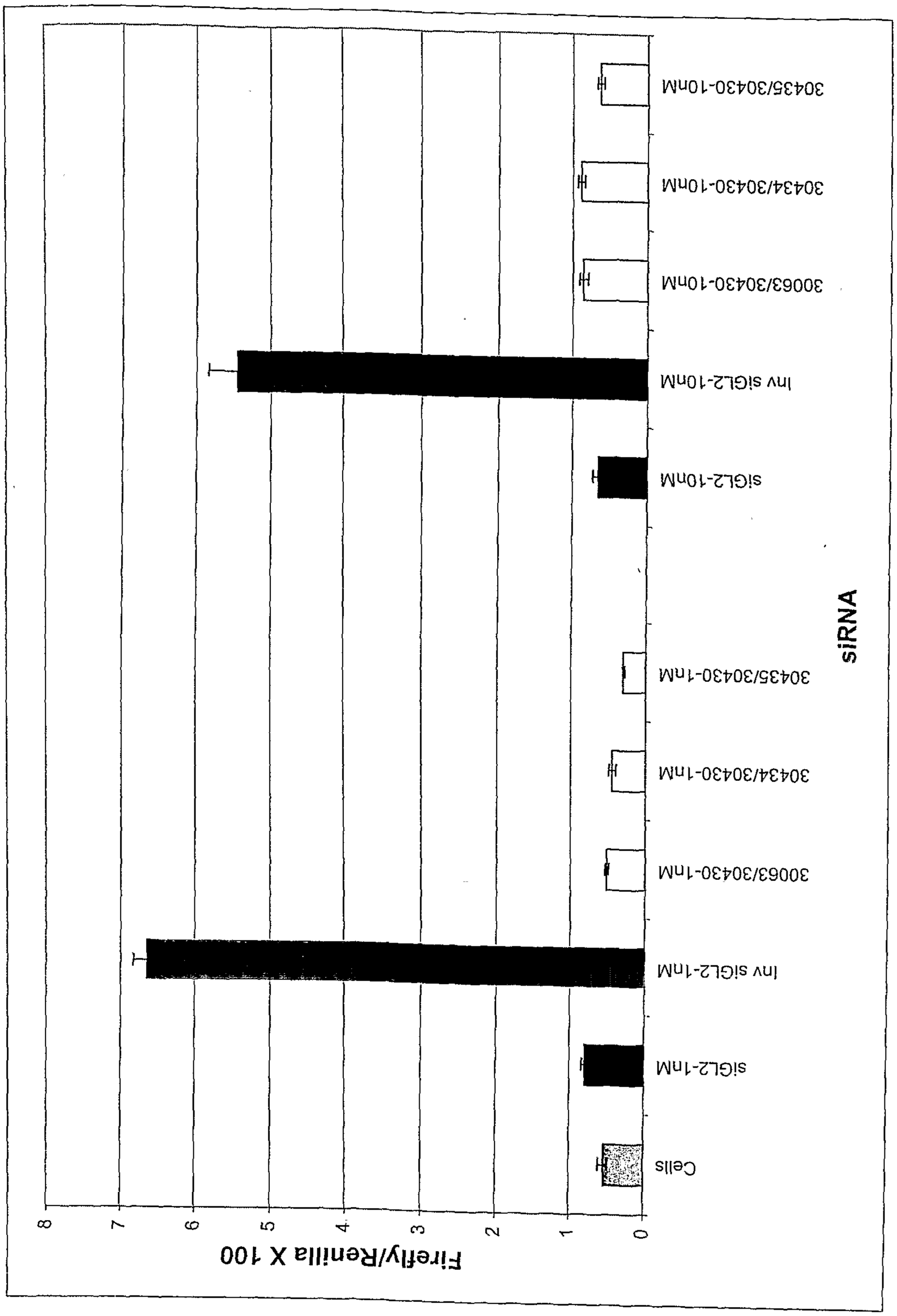

30435/30430-10nM
30434/30430-10nM
30063/30430-10nM
Inv siGL2-10nM
siGL2-10nM
30435/30430-1nM
30434/30430-1nM
30063/30430-1nM
Inv siGL2-1nM
siGL2-1nM
Cells
siRNA
Firefly/Renilla X 100
0
1
2
3
4
5
6
7
8

Figure 15

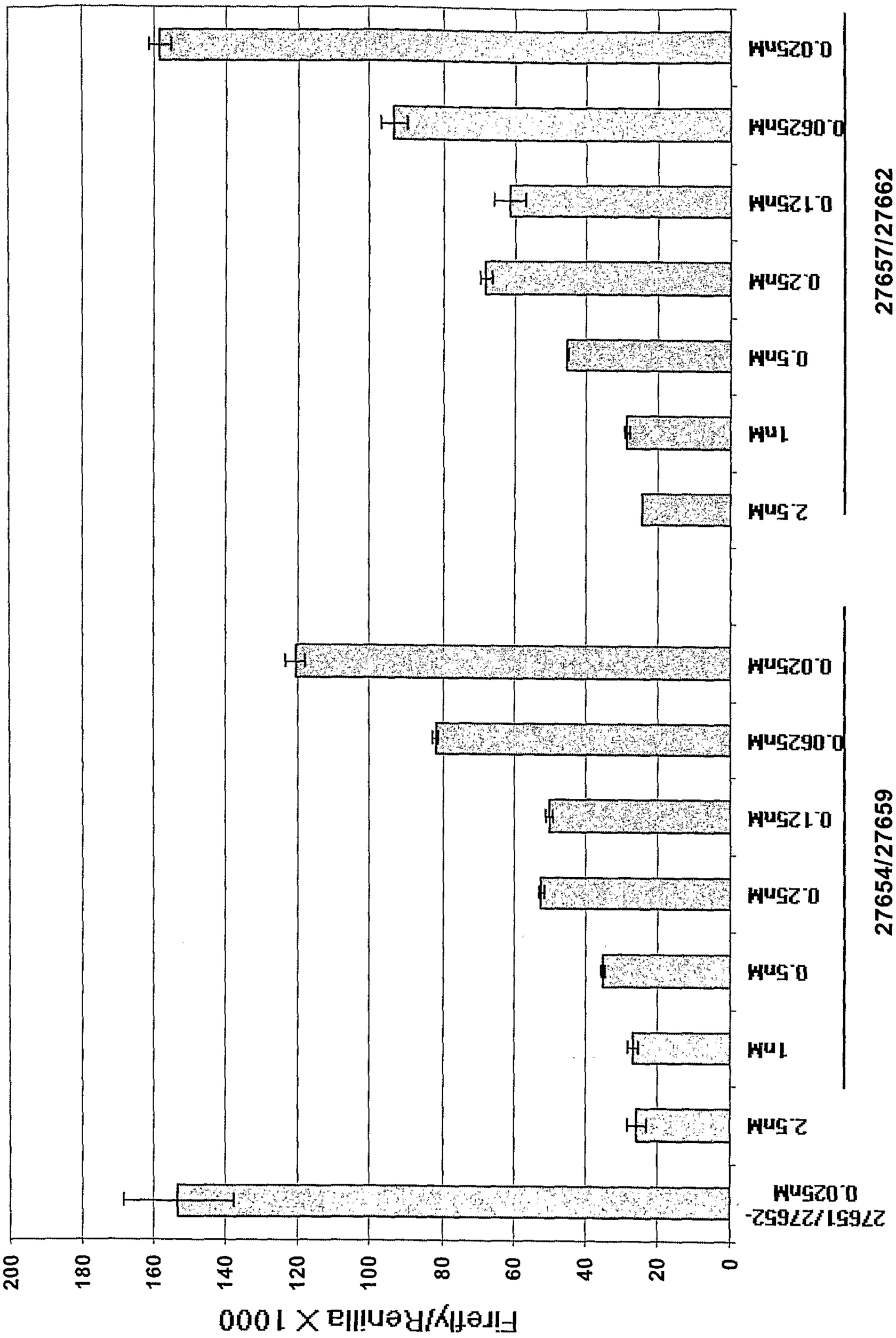

27651/27652-0.025nM
2.5nM
1nM
0.5nM
0.25nM
0.125nM
0.0625nM
0.025nM
27654/27659 siRNA (sense/antisense RPI #) and concentration
0.025nM
0.0625nM
0.125nM
0.25nM
0.5nM
1nM
2.5nM
27657/27662
0.025nM
0.0625nM
0.125nM
0.25nM
0.5nM
1nM
2.5nM
Firefly/Renilla X 1000
0
20
40
60
80
100
120
140
160
180
200

Figure 16

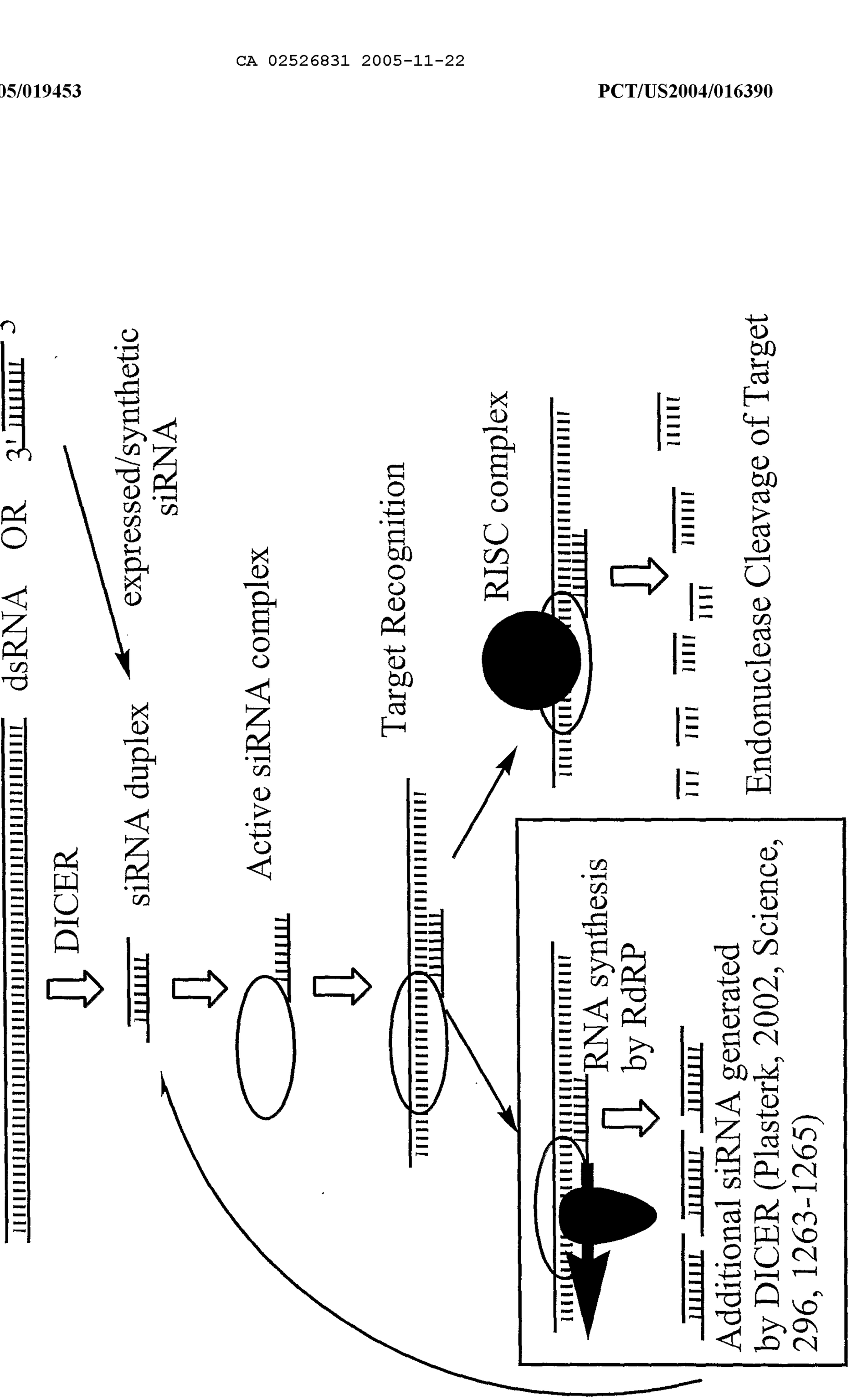

5' 3'
dsRNA OR 3' 5'
expressed/synthetic
siRNA
siRNA duplex
Active siRNA complex
Target Recognition
RISC complex
Endonuclease Cleavage of Target
DICER
siRNA generated
RNA synthesis
by RdRP
Additional siRNA generated
by DICER (Plasterk, 2002, Science,
296, 1263-1265)

Figure 17

Figure 18

A

SENSE STRAND (SEQ ID NO 1134)
ALL PYRIMIDINES = 2'-O-ME OR 2'-FLUORO EXCEPT POSITIONS (N N)

5'- $N_s N_s N_s N_s N N N N N N N N N N N N N N N_s N_s N_s (N_s N)$ -3'

3'- L-$(N_s N)$ N N N N N N N N N N N N N N N N $N_s N_s N_s N_s N$ -5'

ANTISENSE STRAND (SEQ ID NO 1135)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N)

B

SENSE STRAND (SEQ ID NO 1136)
ALL PYRIMIDINES = 2'-O-ME OR 2'-FLUORO EXCEPT POSITIONS (N N)

5'- N N N N N N N N N N N N N N N N N N N N N N $(N_s N)$ -3'

3'- L-(N N) N N N N N N N N N N N N N N N N N N N N N N -5'

ANTISENSE STRAND (SEQ ID NO 1137)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N)

C

SENSE STRAND (SEQ ID NO 1138)
ALL PYRIMIDINES = 2'-O-ME OR 2'-FLUORO EXCEPT POSITIONS (N N)

5'- B-N N N N N N N N N N N N N N N N N N N N N N (N N)-B -3'

3'- L-$(N_s N)$ N N N N N N N N N N N N N N N N N N N N N N -5'

ANTISENSE STRAND (SEQ ID NO 1139)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N)

D

SENSE STRAND (SEQ ID NO 1140)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N) AND ALL PURINES = 2'-DEOXY

5'- B-N N N N N N N N N N N N N N N N N N N N N N (N N)-B -3'

3'- L-$(N_s N)$ N N N N N N N N N N N N N N N N N N N N N N -5'

ANTISENSE STRAND (SEQ ID NO 1141)
ALL PYRIMIDINES = 2'-FLUORO AND ALL PURINES = 2'-O-ME EXCEPT POSITIONS (N N)

E

SENSE STRAND (SEQ ID NO 1142)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N)

5'- B-N N N N N N N N N N N N N N N N N N N N N N (N N)-B -3'

3'- L-$(N_s N)$ N N N N N N N N N N N N N N N N N N N N N N -5'

ANTISENSE STRAND (SEQ ID NO 1141)
ALL PYRIMIDINES = 2'-FLUORO AND ALL PURINES = 2'-O-ME EXCEPT POSITIONS (N N)

F

SENSE STRAND (SEQ ID NO 1140)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N) AND ALL PURINES = 2'-DEOXY

5'- B-N N N N N N N N N N N N N N N N N N N N N N (N N)-B -3'

3'- L-$(N_s N)$ N N N N N N N N N N N N N N N N N N N N N N -5'

ANTISENSE STRAND (SEQ ID NO 1143)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N) AND ALL PURINES = 2'-DEOXY

POSITIONS (NN) CAN COMPRISE ANY NUCLEOTIDE, SUCH AS DEOXYNUCLEOTIDES (eg. THYMIDINE) OR UNIVERSAL BASES

B = ABASIC, INVERTED ABASIC, INVERTED NUCLEOTIDE OR OTHER TERMINAL CAP THAT IS OPTIONALLY PRESENT

L = GLYCERYL or B THAT IS OPTIONALLY PRESENT

S = PHOSPHOROTHIOATE OR PHOSPHORODITHIOATE that is optionally absent

Figure 19

A

SENSE STRAND (SEQ ID NO 1144)

5'- $c_S c_S c_S c_S$G G G A G G u c u c G u $A_S G_S A_S T_S T$ -3'

3'- L-$T_S T$ G G G G *c c c u c c* A G A G $c_S A_S u_S c_S u$ -5'

ANTISENSE STRAND (SEQ ID NO 1145)

B

SENSE STRAND (SEQ ID NO 1146)

5'- c c c c G G G A G G u c u c G u A G A $T_S T$ -3'

3'- L-*T T* G G G G *c c c u c c* A G A G *c* A *u c u* -5'

ANTISENSE STRAND (SEQ ID NO 1147)

C

SENSE STRAND (SEQ ID NO 1148)

5'- iB-c c c c G G G A G G u c u c G u A G A *T T*-iB -3'

3'- L-$T_S T$ G G G G *c c c u c c* A G A G *c* A *u c u* -5'

ANTISENSE STRAND (SEQ ID NO 1149)

D

SENSE STRAND (SEQ ID NO 1150)

5'- iB-*c c c c G G G A G G u c u c G u A G A T T*-iB -3'

3'- L-$T_S T$ g g g g *c c c u c c* a g a g *c* a *u c u* -5'

ANTISENSE STRAND (SEQ ID NO 1151)

E

SENSE STRAND (SEQ ID NO 1152)

5'- iB-*c c c c* G G G A G G *u c u c* G *u* A G A *T T*-iB -3'

3'- L-$T_S T$ g g g g *c c c u c c* a g a g *c* a *u c u* -5'

ANTISENSE STRAND (SEQ ID NO 1151)

F

SENSE STRAND (SEQ ID NO 1150)

5'- iB-*c c c c G G G A G G u c u c G u A G A T T*-iB -3'

3'- L-$T_S T$ *G G G G c c c u c c A G A G c A u c u* -5'

ANTISENSE STRAND (SEQ ID NO 1153)

lower case = 2'-O-Methyl or 2'-deoxy-2'-fluoro

italic lower case = 2'-deoxy-2'-fluoro

underline = 2'-O-methyl

ITALIC UPPER CASE = DEOXY
iB = INVERTED DEOXYABASIC
L = GLYCERYL MOIETY or iB OPTIONALLY PRESENT
S = PHOSPHOROTHIOATE OR
PHOSPHORODITHIOATE OPTIONALLY PRESENT

Figure 20

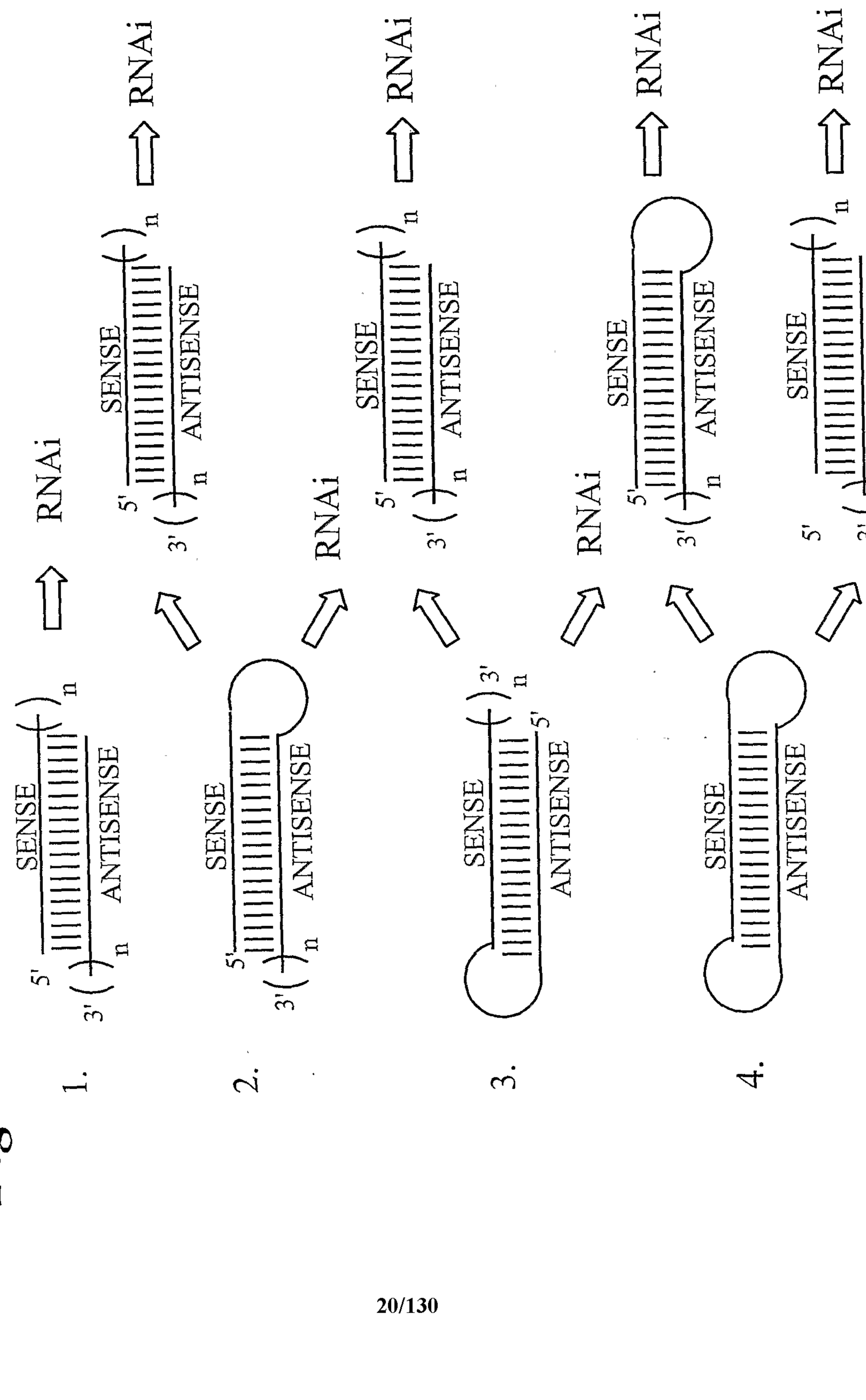


Figure 21: Target site Selection using siRNA

A

siRNA

Synthesize oligos encoding siRNA against Target RNA sequence

B

Transfect Cells

C

Select cells exhibiting desired phenotype

D

Identify efficacious target sites based on siRNA sequence from Positional analysis

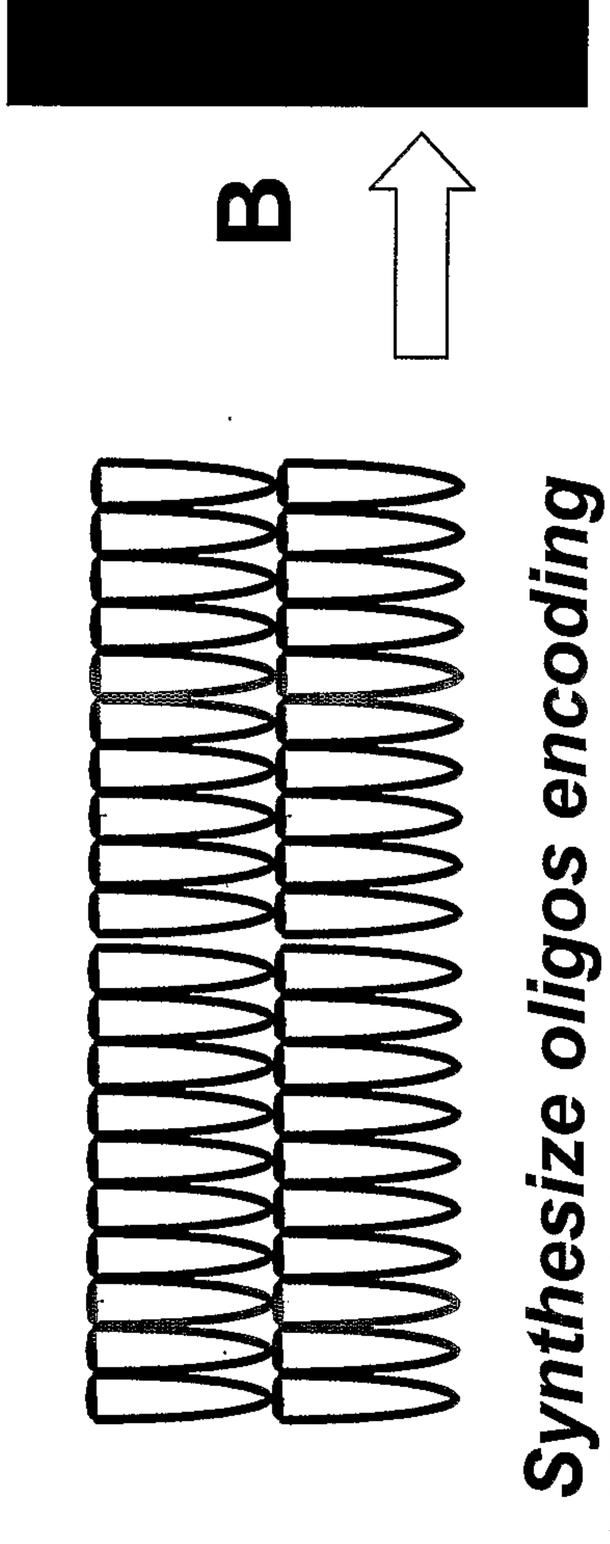
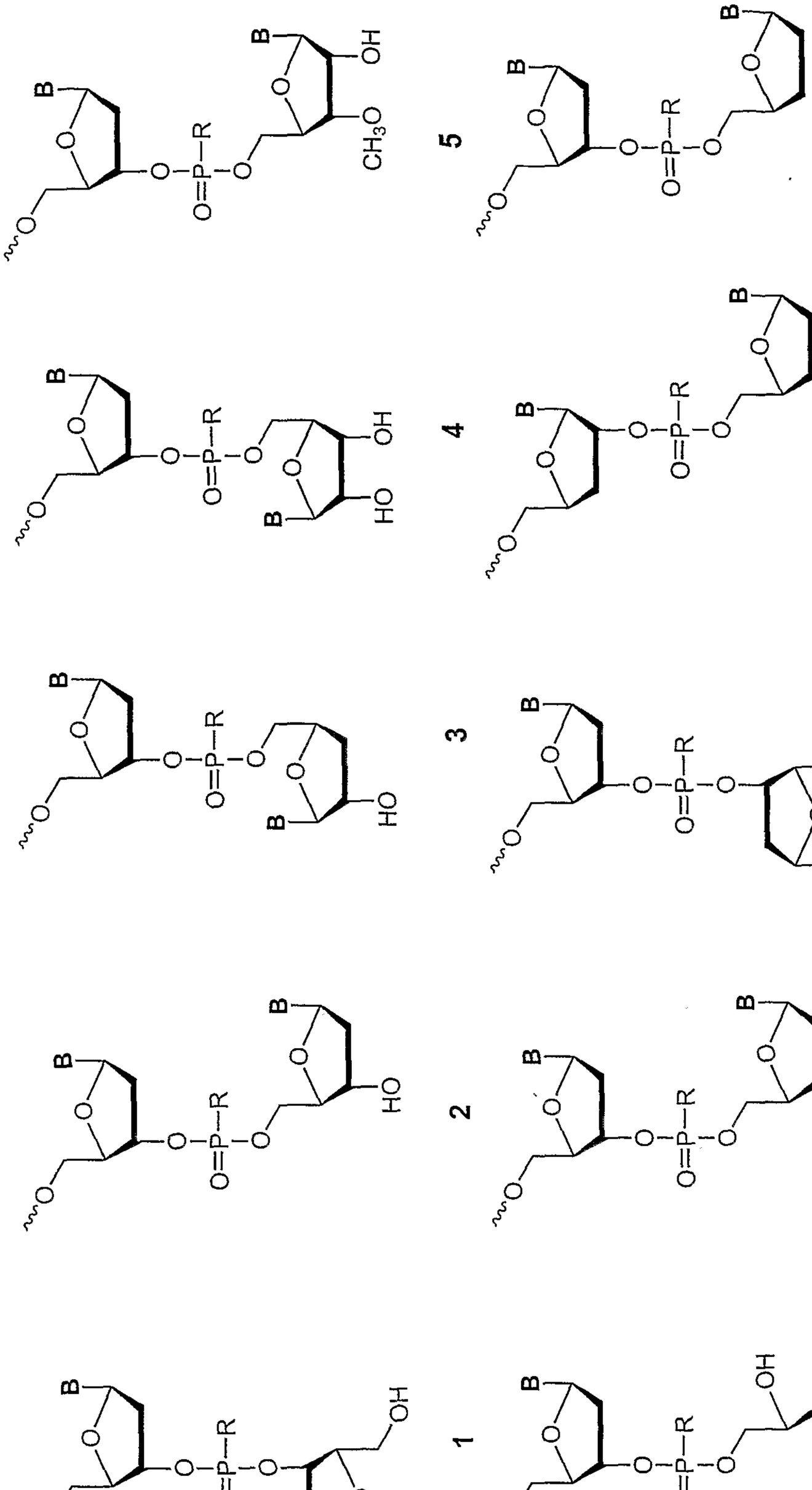


Figure 22



R = O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, or aralkyl
B = Independently any nucleotide base, either naturally occurring or chemically modified, or optionally H (abasic).

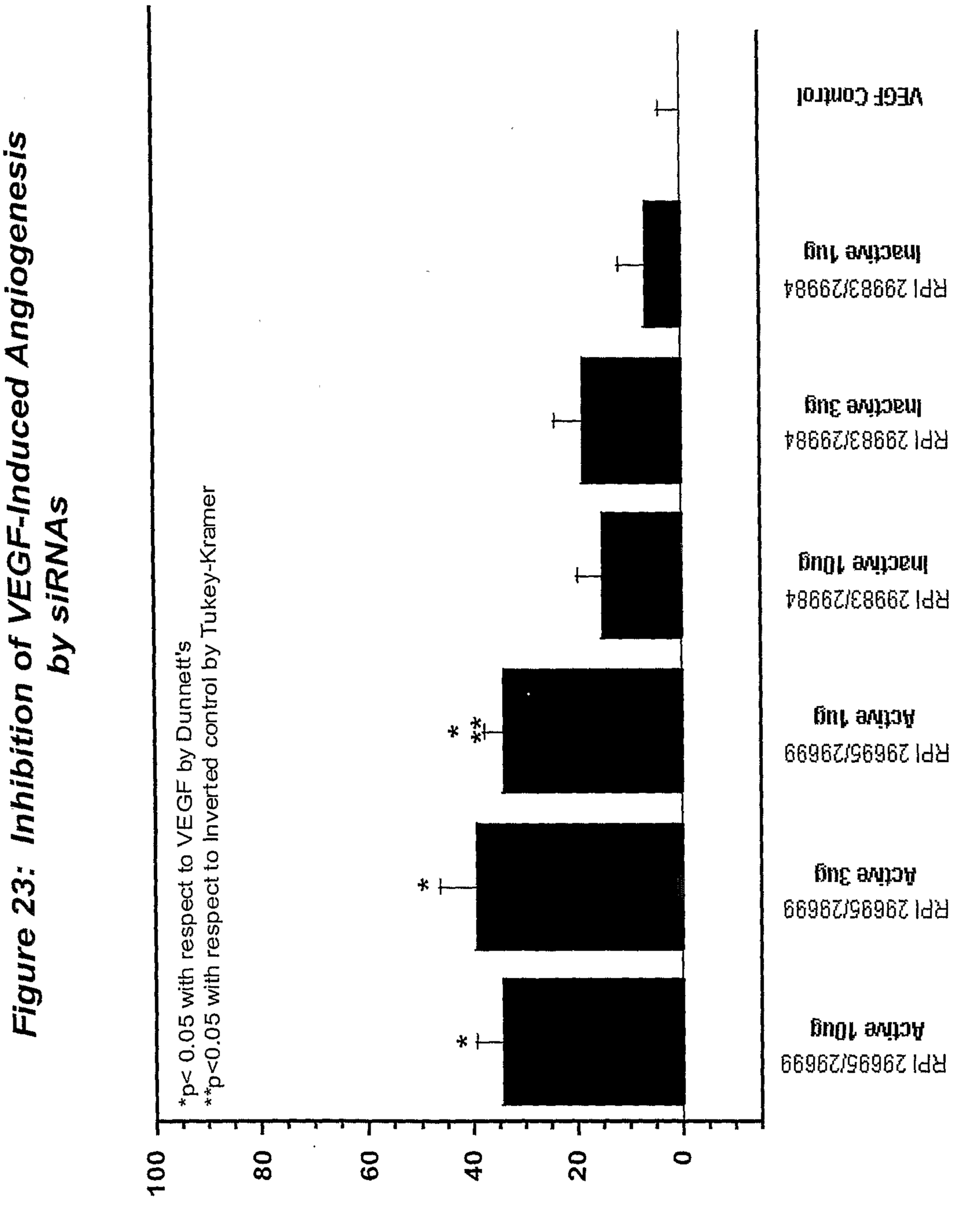


Figure 23: Inhibition of VEGF-Induced Angiogenesis by siRNAs
*p< 0.05 with respect to VEGF by Dunnett's
**p<0.05 with respect to Inverted control by Tukey-Kramer
% Inhibition of VEGF induced Angiogenesis
100
80
60
40
20
0
*
* **
*
*
RPI 29695/29699 Active 3ug
RPI 29695/29699 Active 1ug
RPI 29695/29699 Active 10ug
RPI 29983/29984 Inactive 3ug
RPI 29983/29984 Inactive 1ug
RPI 29983/29984 Inactive 10ug
VEGF Control

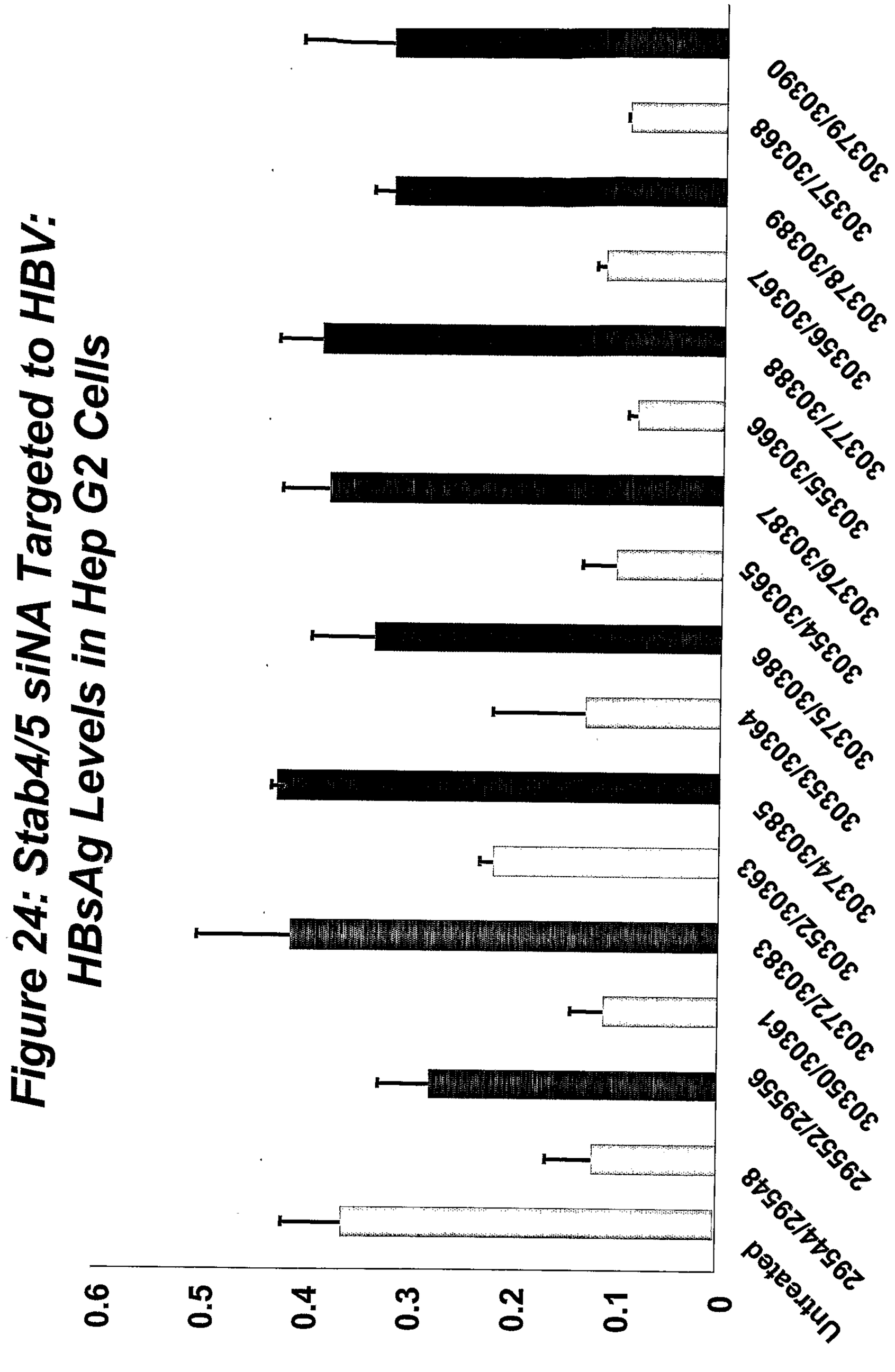


Figure 24: Stabilization/Chemistry Study of Stab4/5 siNA Targeted to HBV: HBsAg Levels in Hep G2 Cells

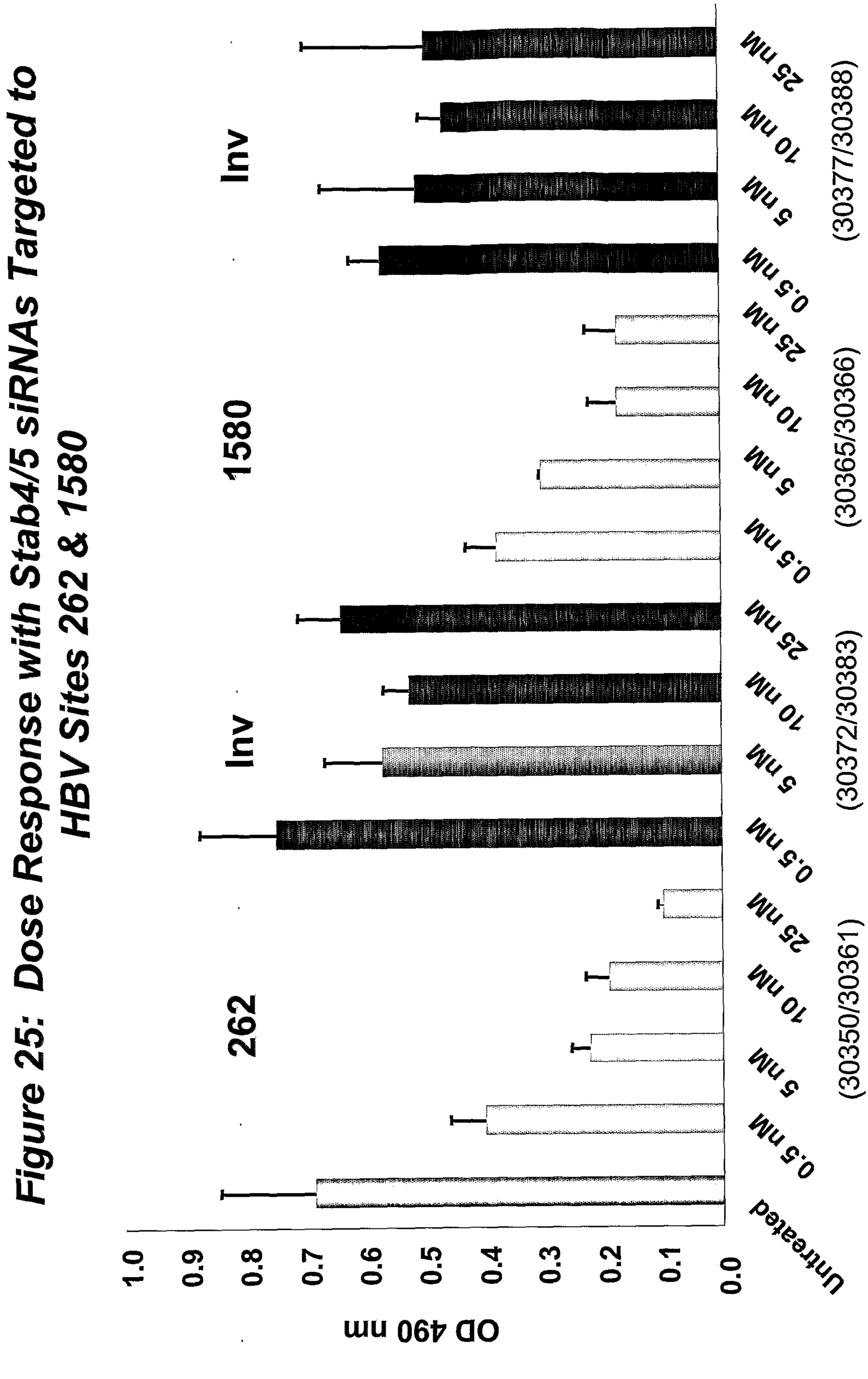


Figure 25: Dose Response with Stab4/5 siRNAs Targeted to HBV Sites 262 & 1580

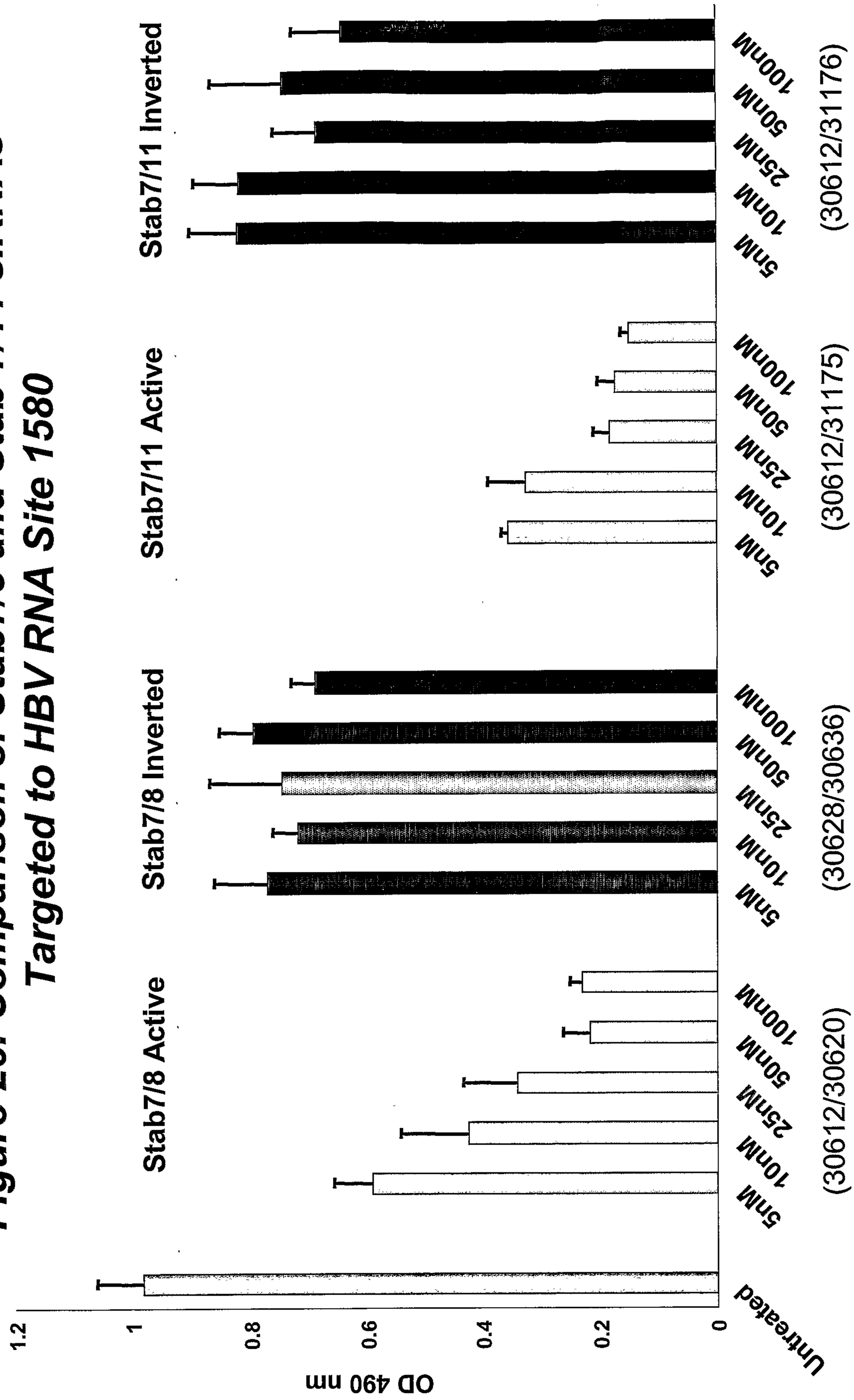


Figure 26: Comparison of Stab7/8 and Stab 7/11 siRNAs Targeted to HBV RNA Site 1580

Figure 27: Modification Strategy

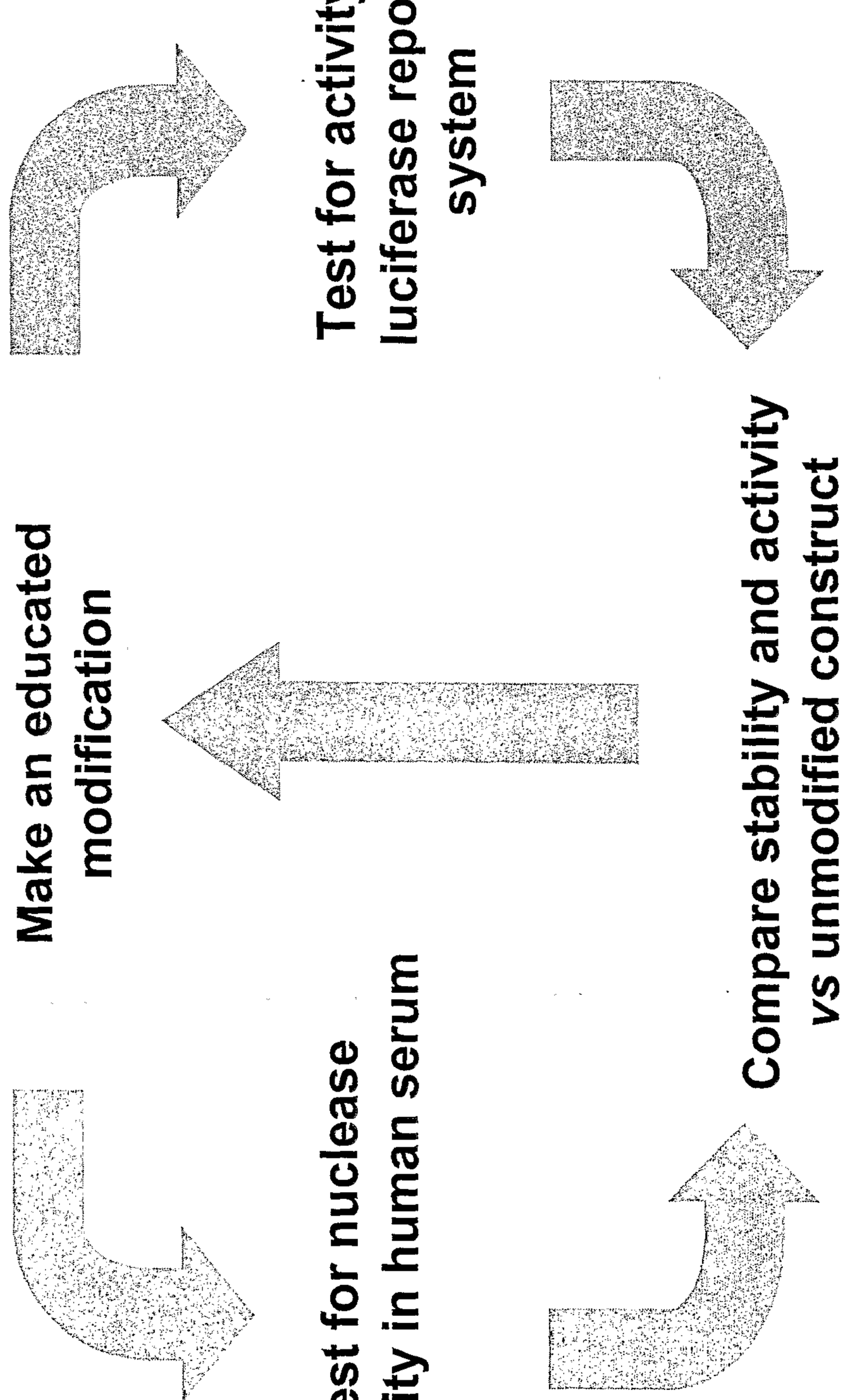
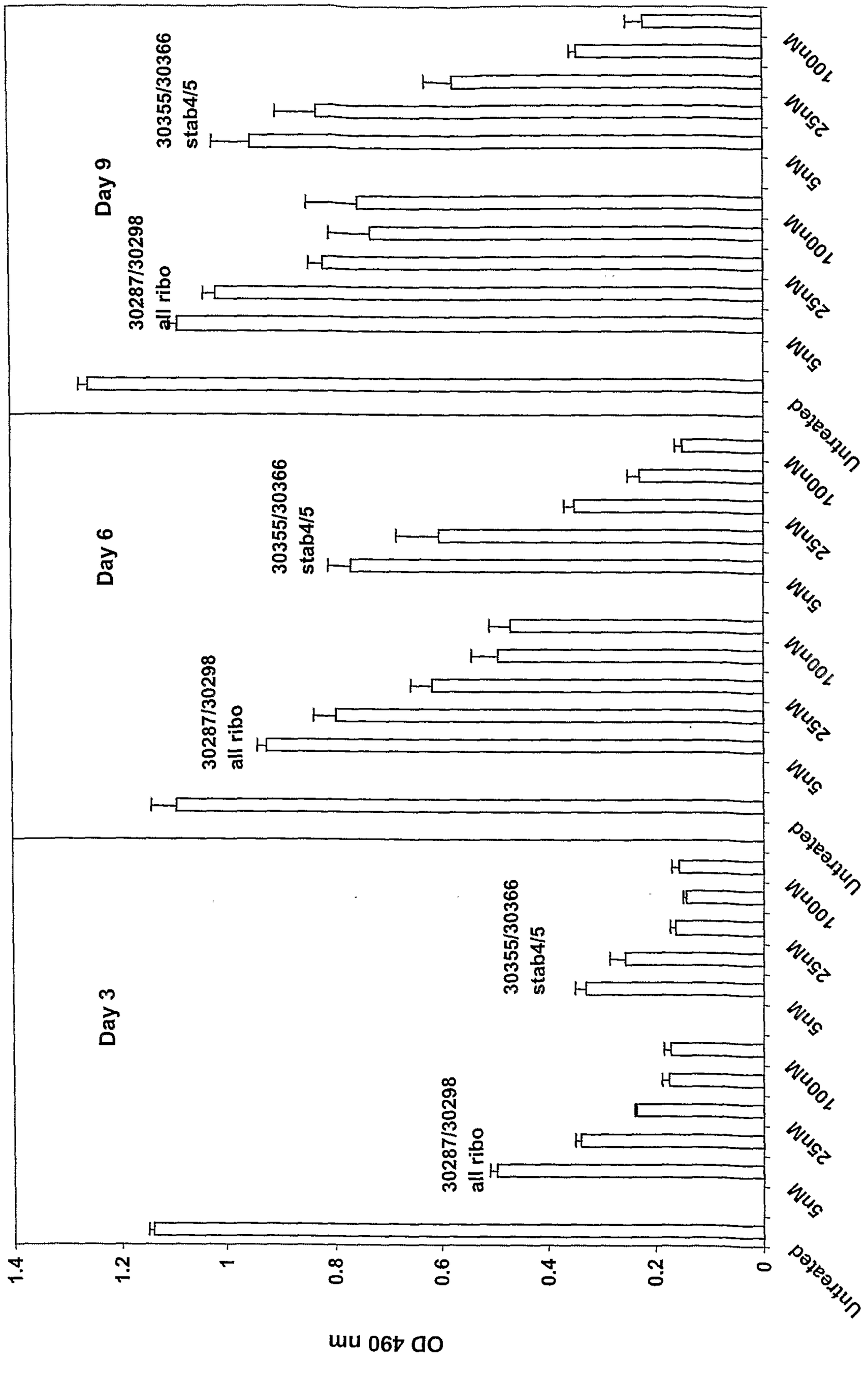


Figure 28: Duration of siRNA Effect All-Ribo vs. Stab4/5 HBV Site 1580: HBsAg Levels



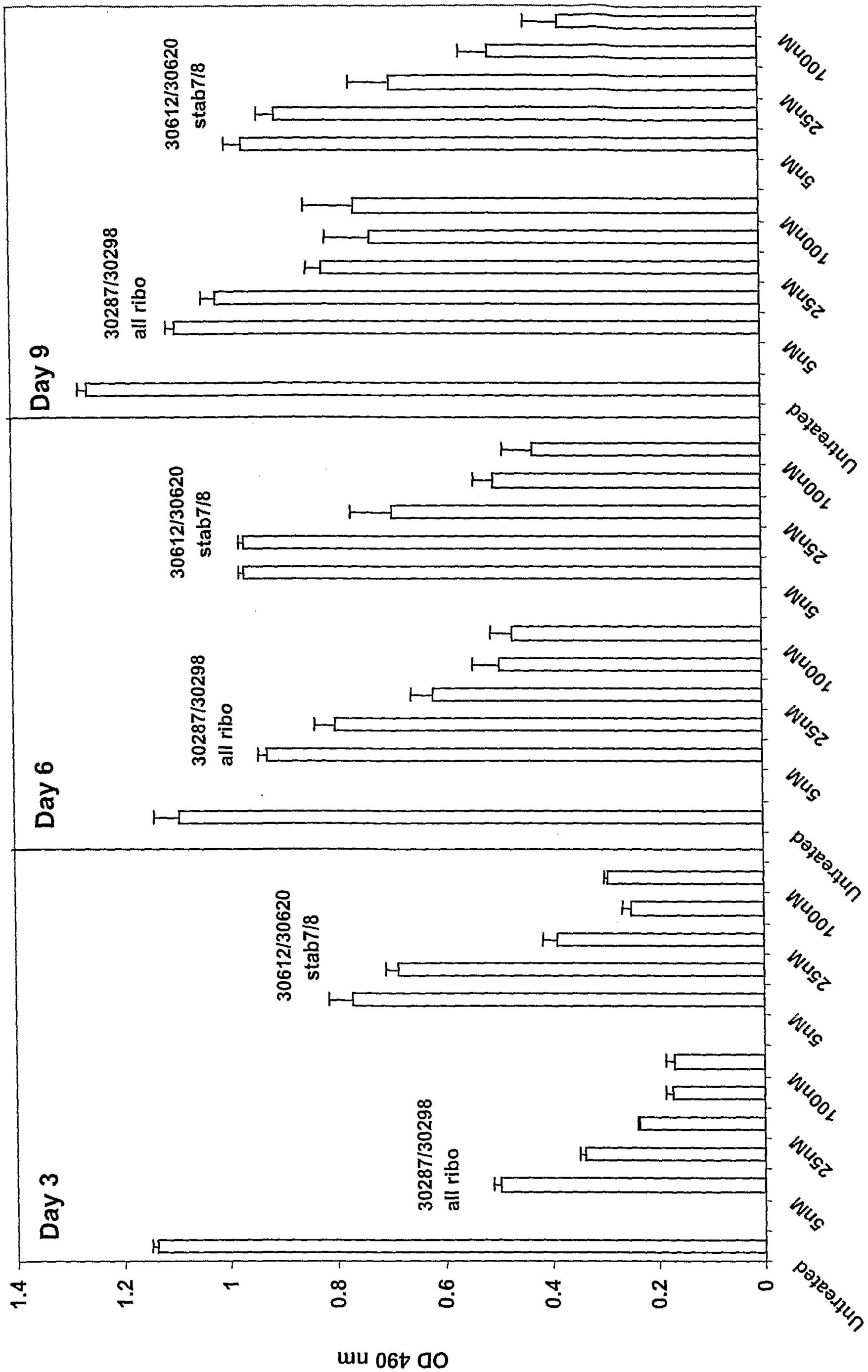


Figure 29: Duration of siRNA Effect All-Ribo vs. Stab7/8 HBV Site 1580: HBsAg Levels

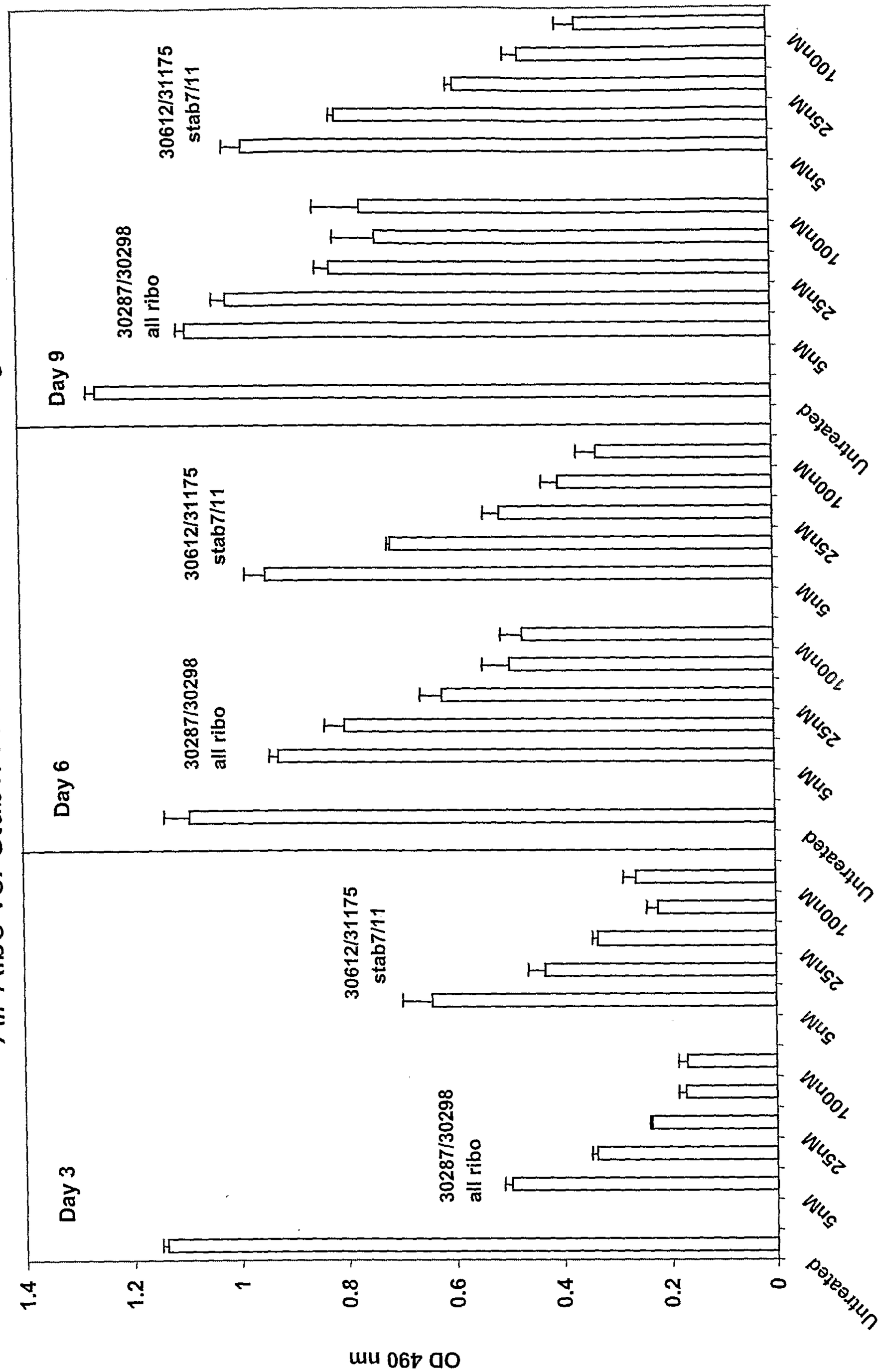


Figure 30: Duration of siRNA Effect
All-Ribo vs. Stab7/11 HBV Site 1580: HBsAg Levels
Day 3
Day 6
Day 9
30287/30298
all ribo
30612/31175
stab7/11
Untreated
5nM
25nM
100nM
OD 490 nm
0
0.2
0.4
0.6
0.8
1
1.2
1.4

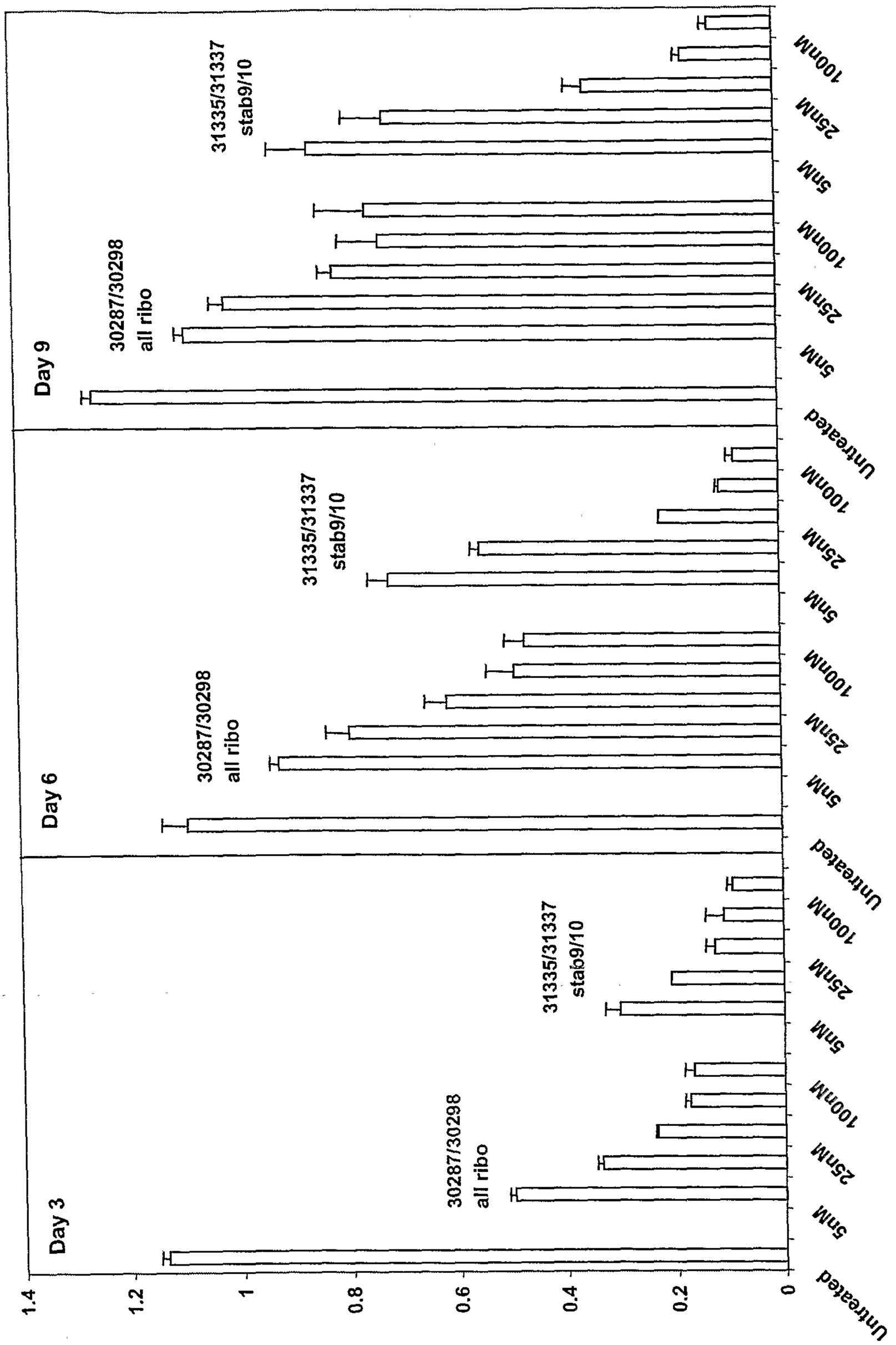
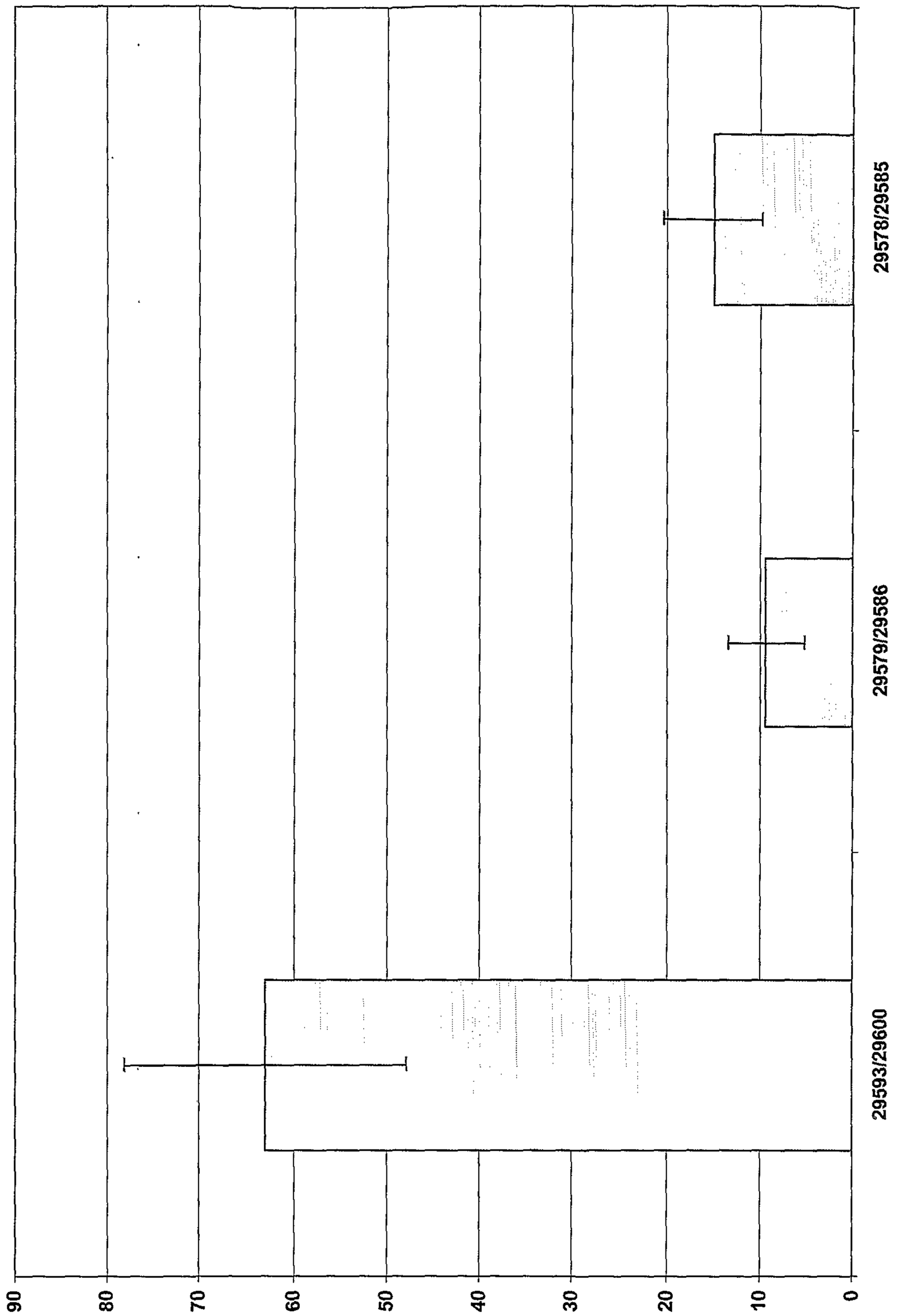


Figure 31: Duration of siRNA Effect All-Ribo vs. Stab9/10 HBV Site 1580: HBsAg Levels

Figure 32 : siRNAs targeting HCV chimera



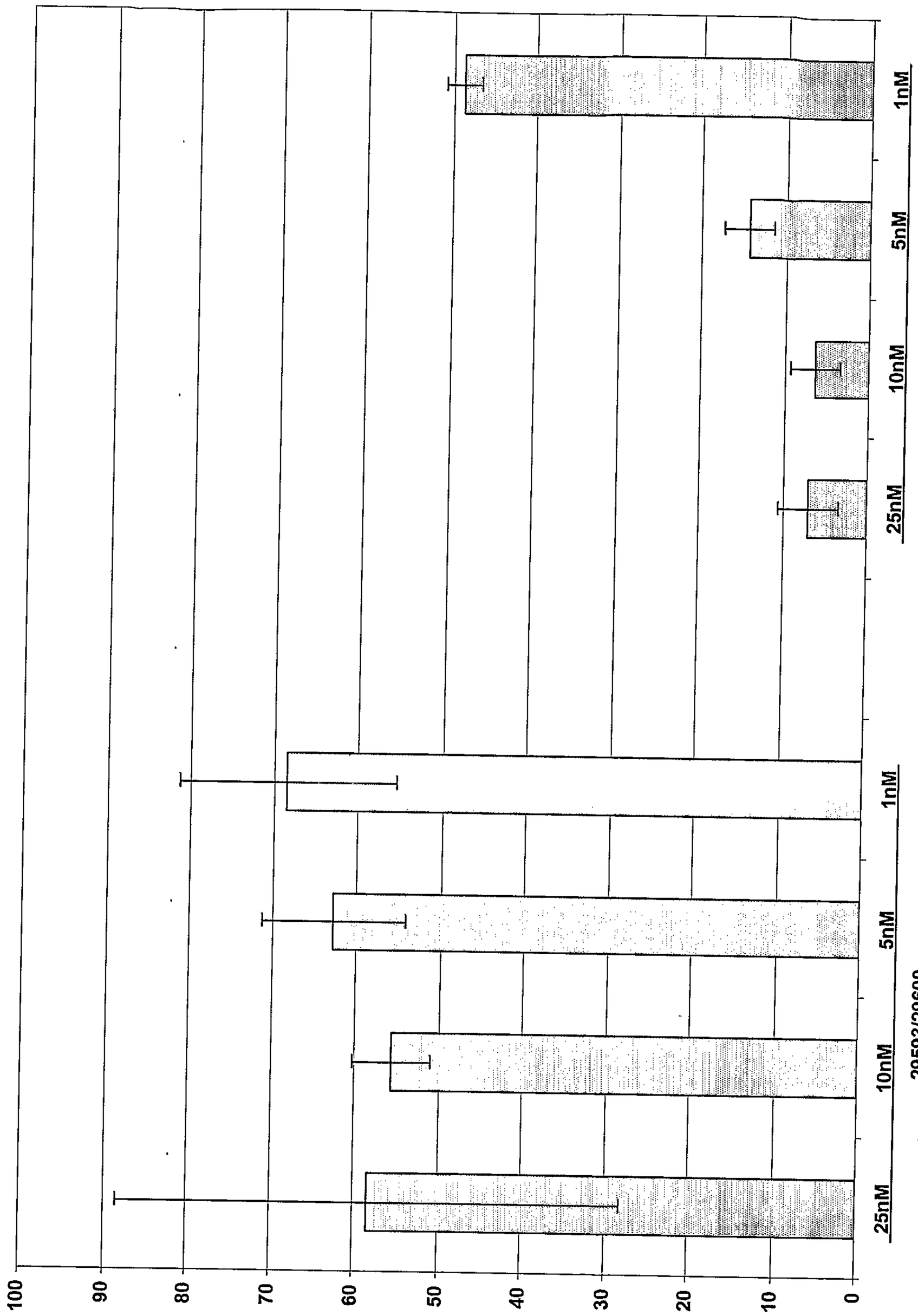
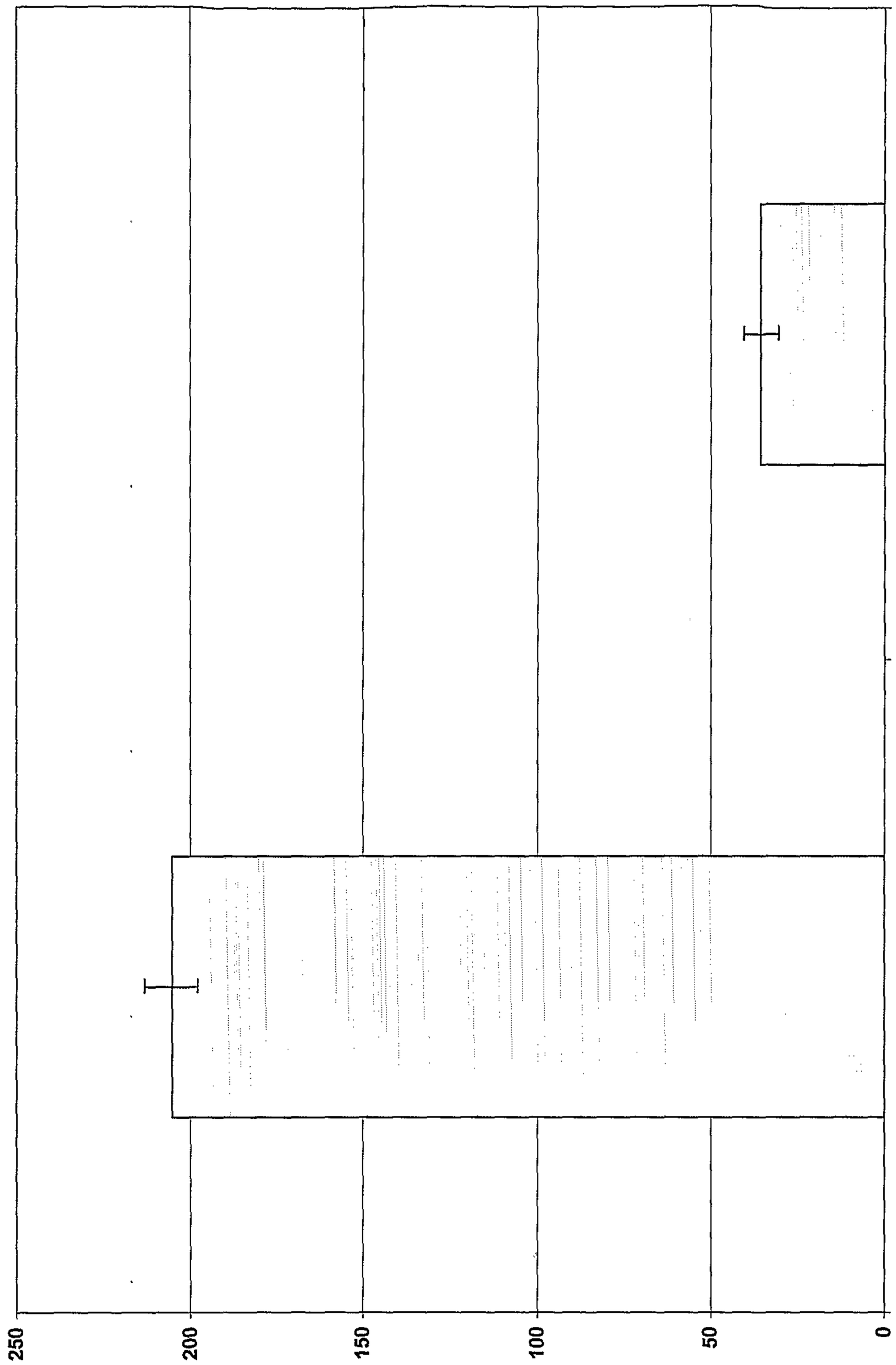


Figure 33: HCV siRNA dose response

Figure 34: Chemically Modified siRNA targeting HCV chimera



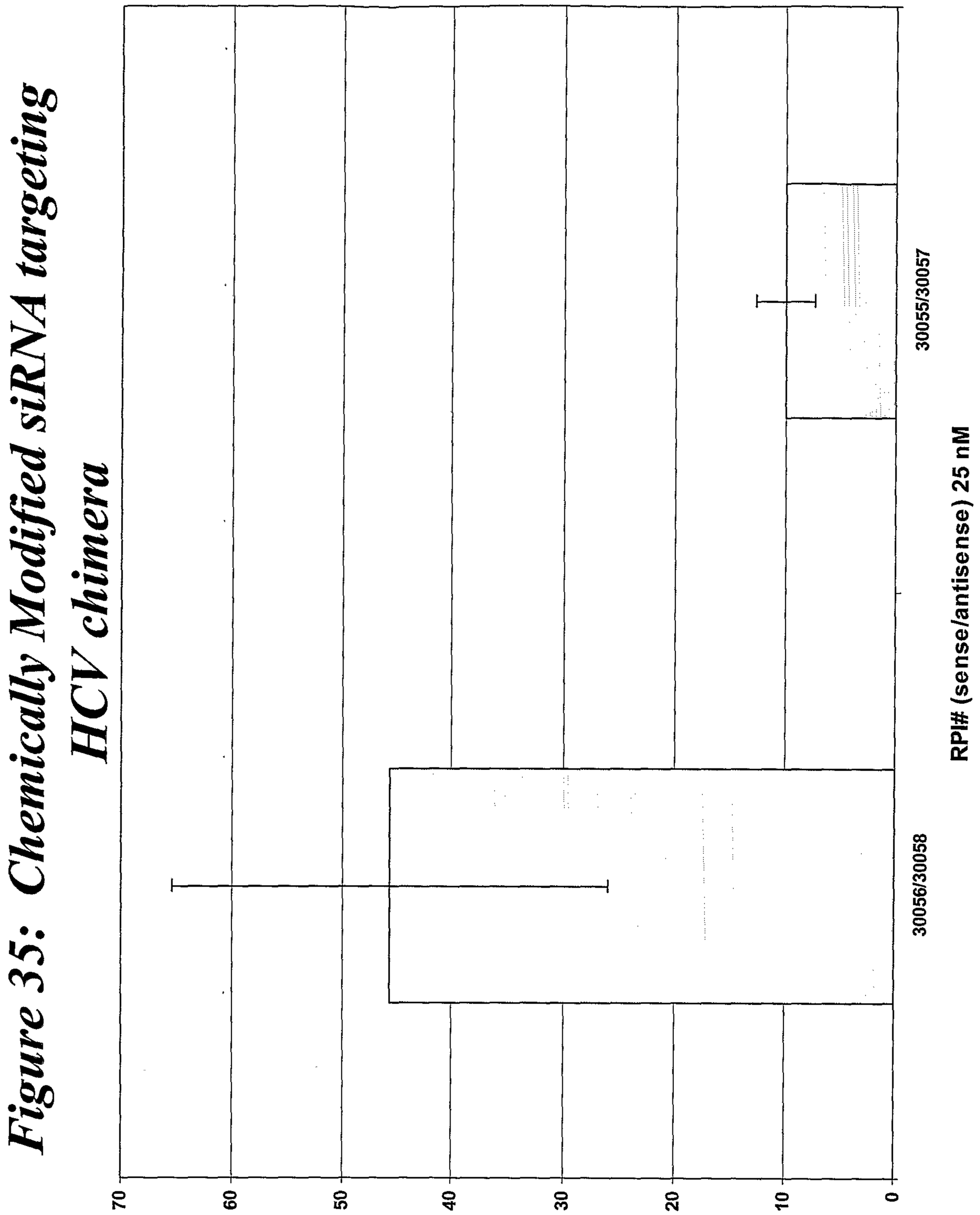


Figure 35: Chemically Modified siRNA targeting HCV chimera

Figure 36: Chemically Modified siRNA targeting HCV chimera

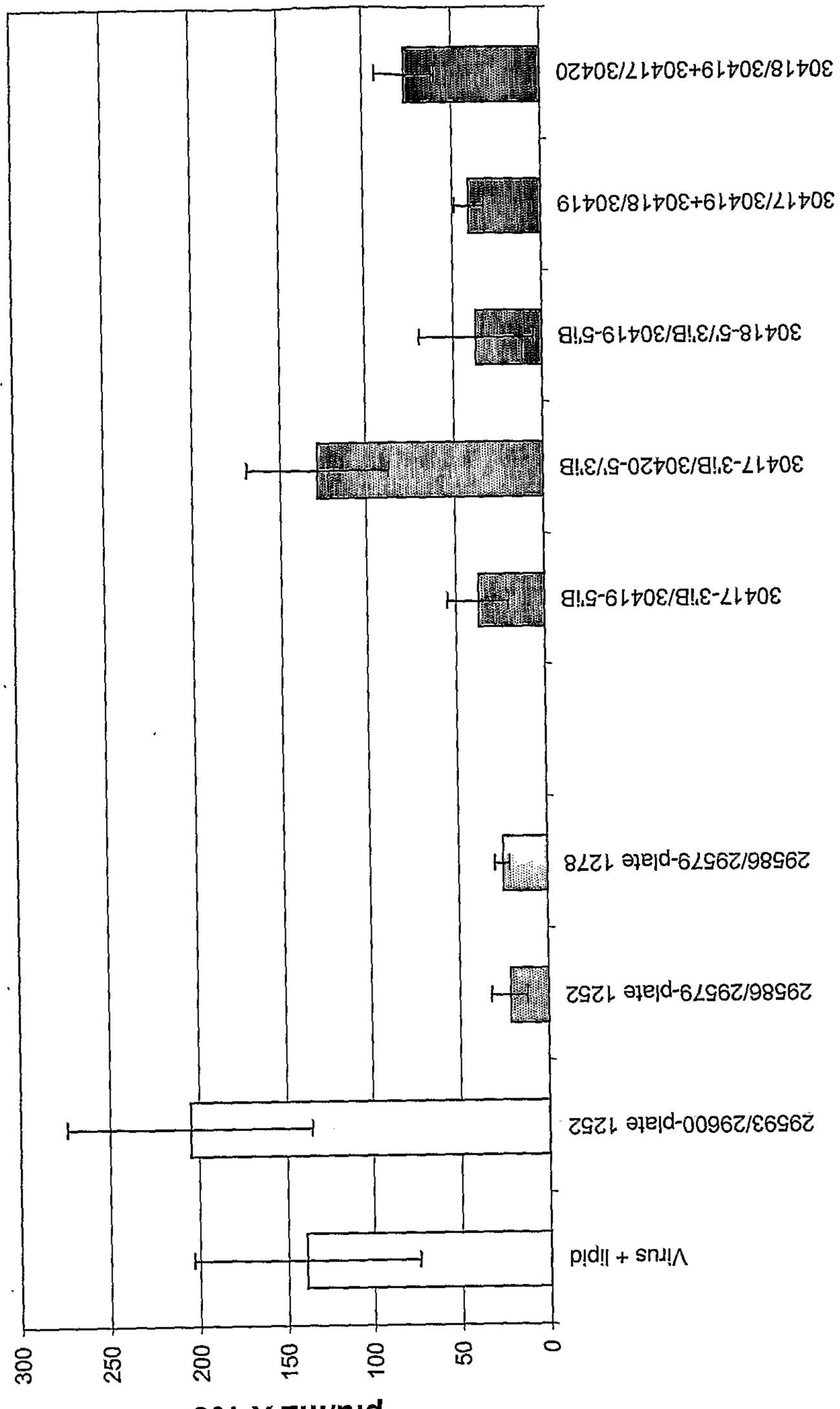
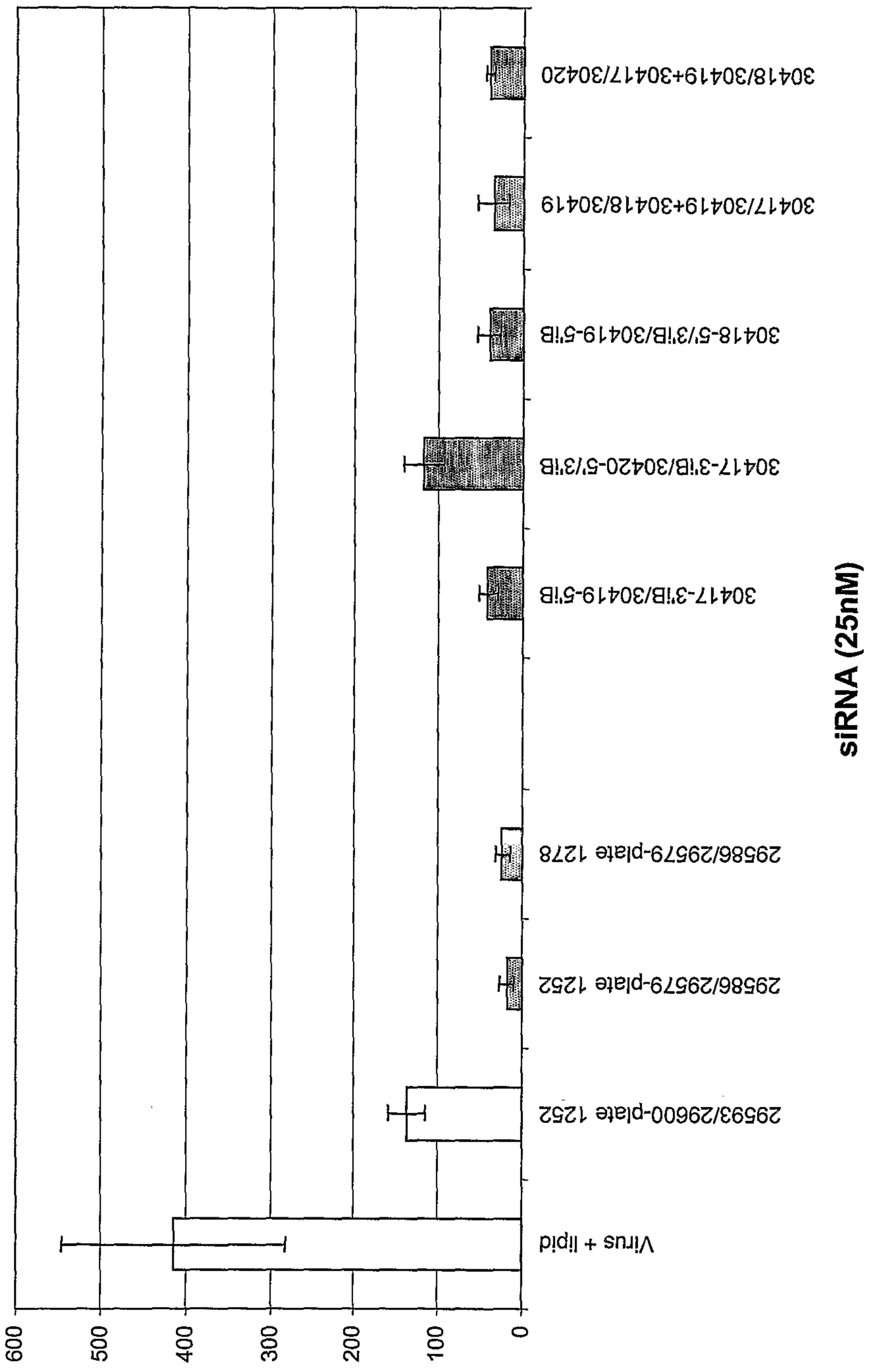


Figure 37: Chemically Modified siRNA targeting HCV chimera



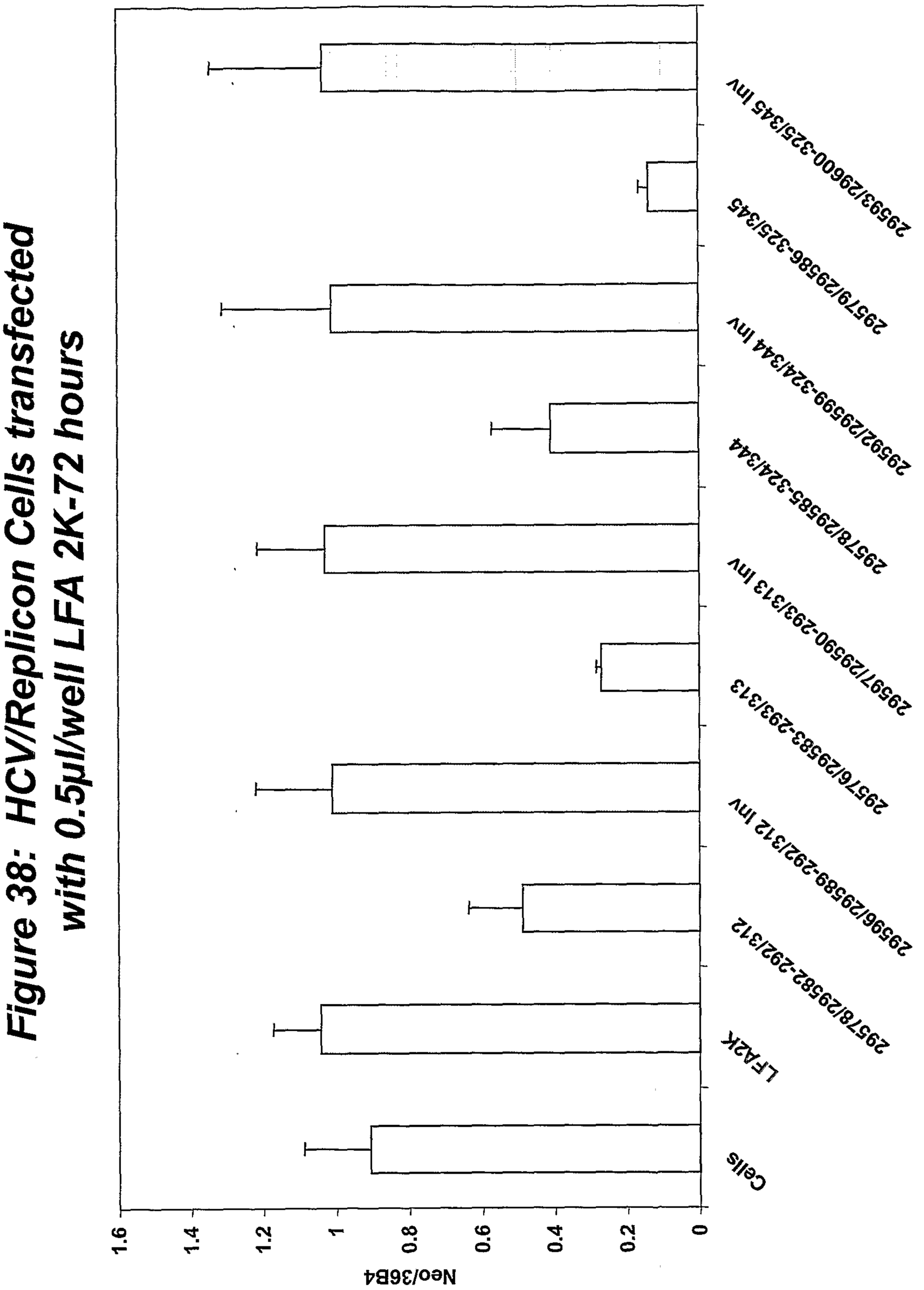


Figure 38: HCV/Replicon Cells transfected with 0.5µl/well LFA 2K-72 hours

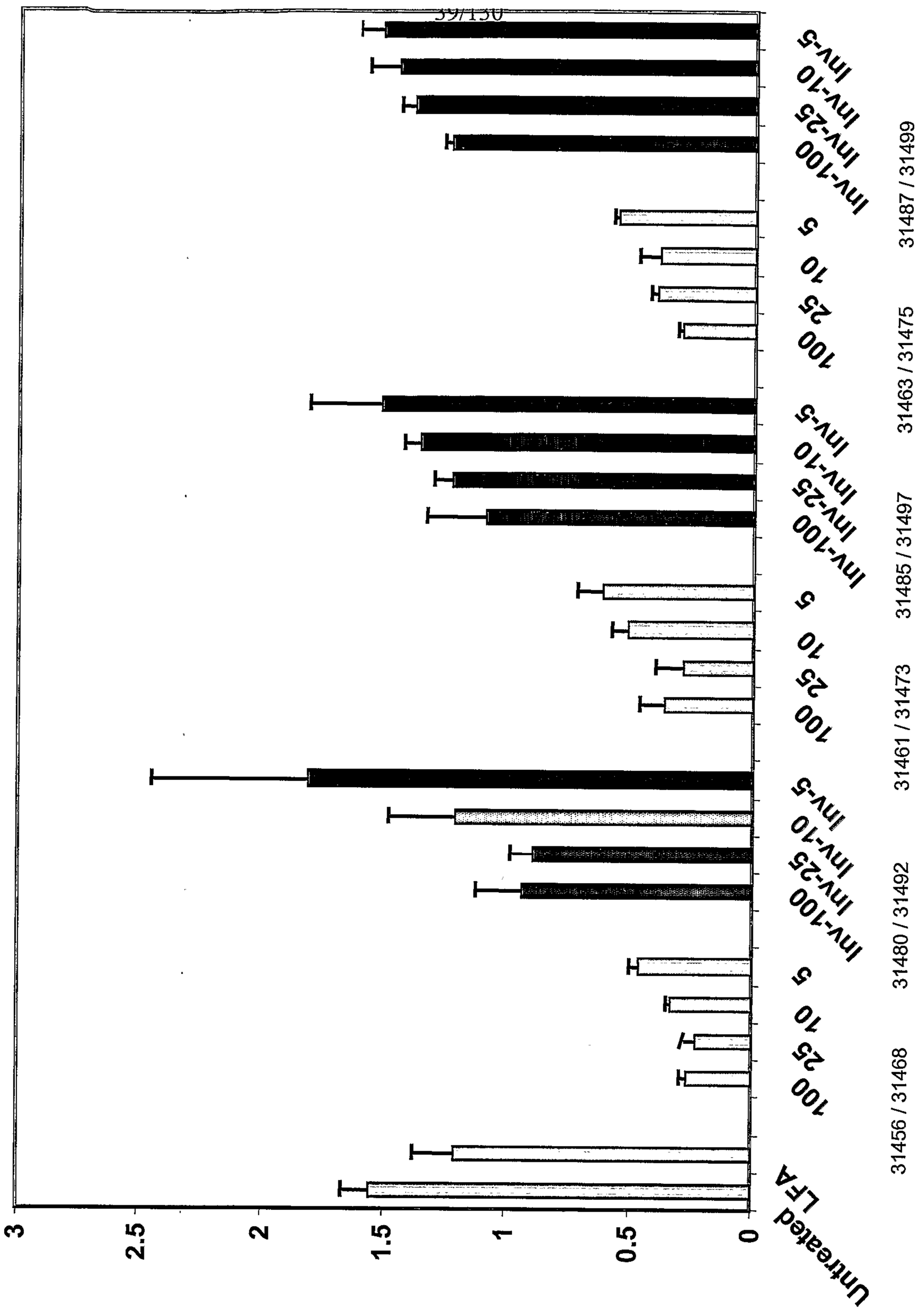


Figure 39: Dose Response with Stab4/5 siNA Leads in HCV Subgenomic Replicon

Figure 40: Activity of Stab 7/8 siNA Leads in Human Subgenomic HCV Replicon

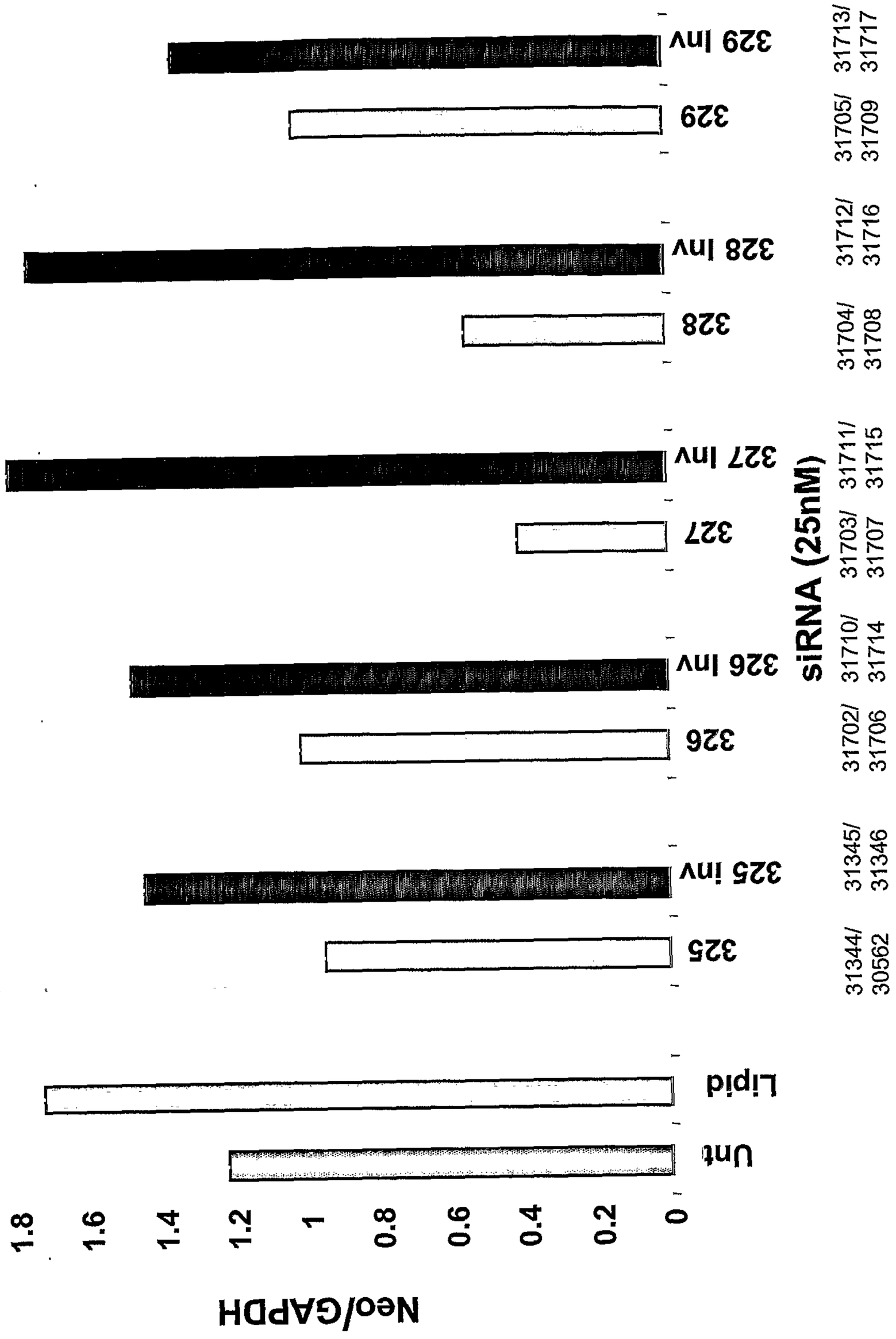


Figure 41: Dose Response with Fully Modified HCV Site 327 siNA

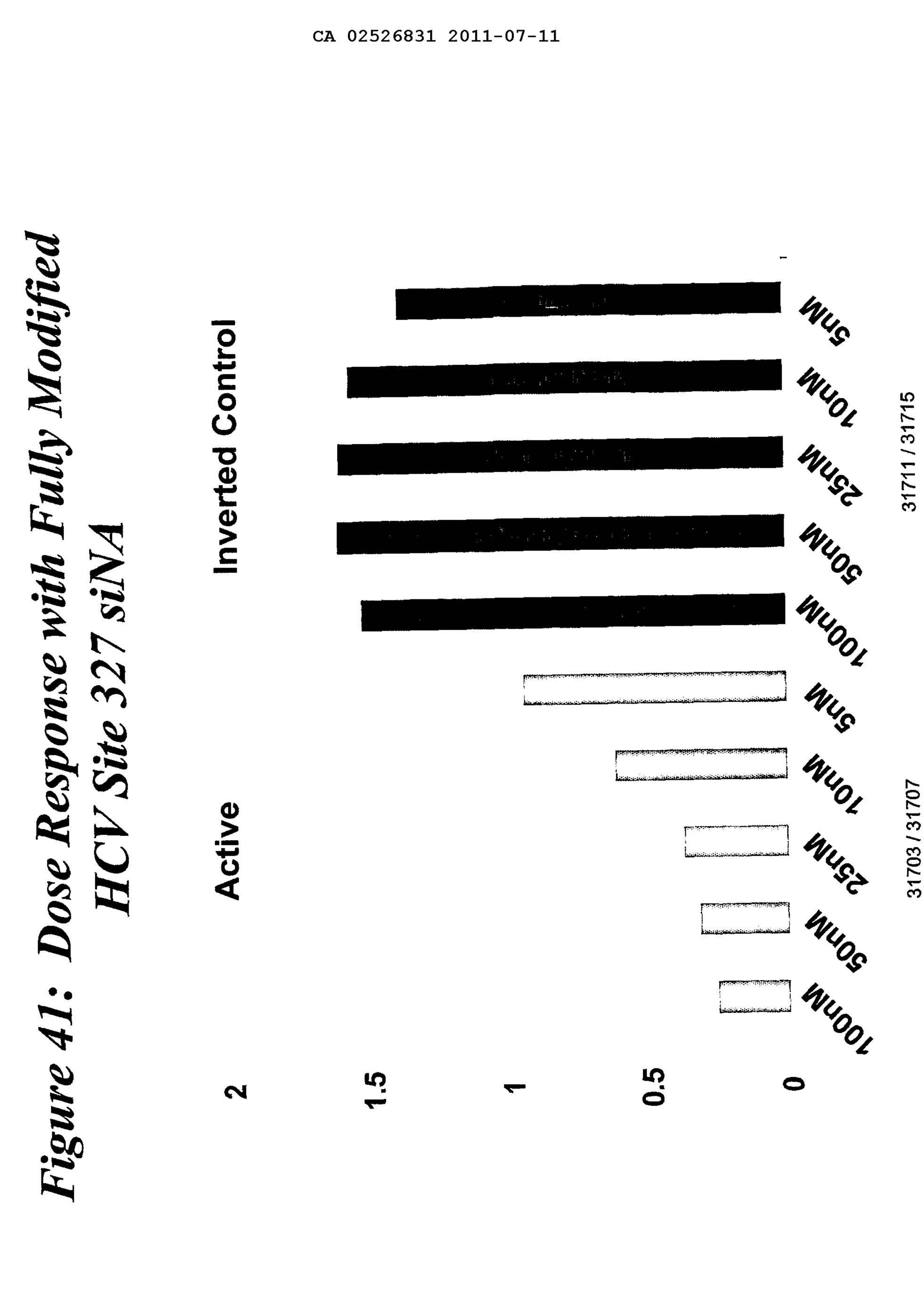
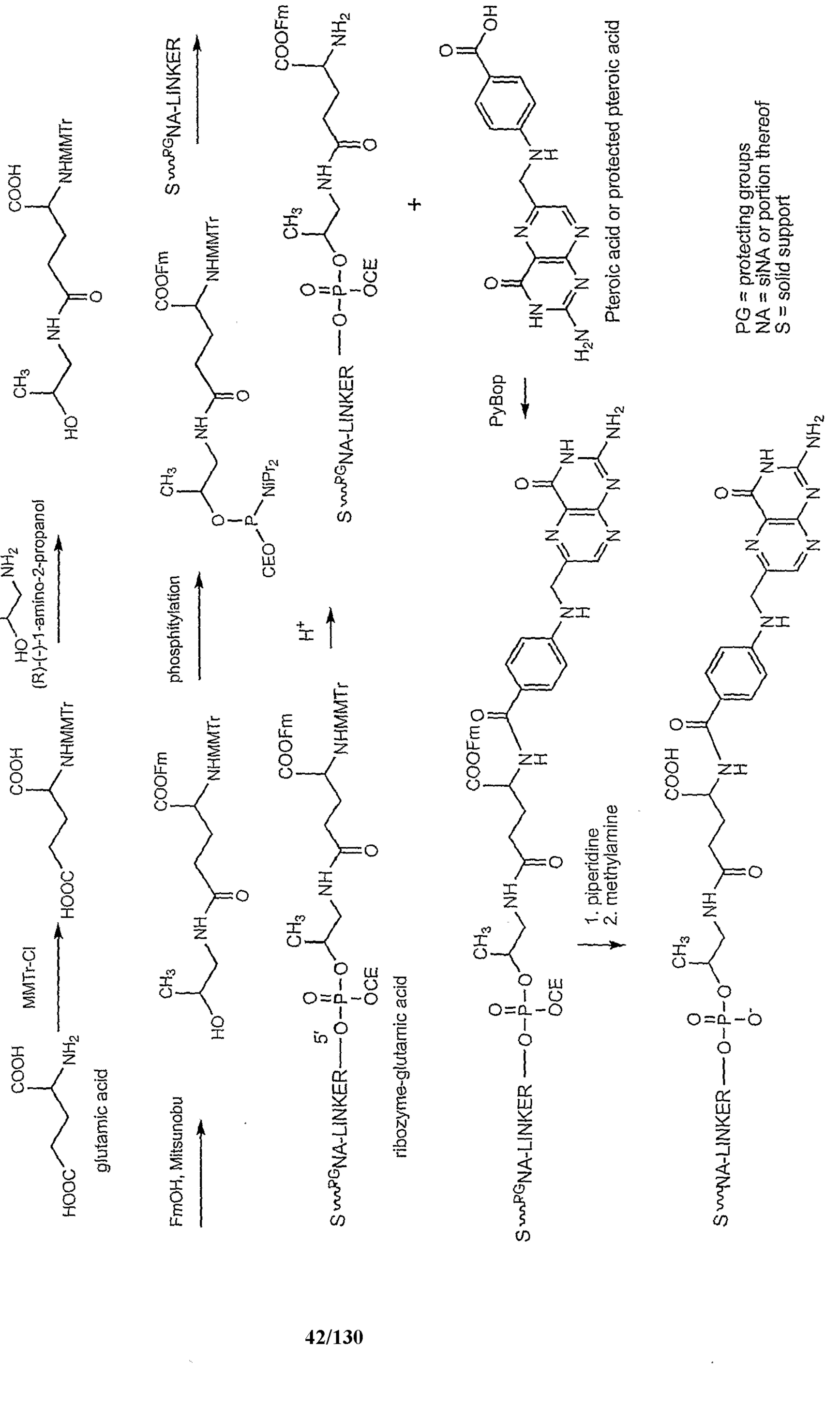


Figure 42: Solid Phase Post-synthetic conjugation of pteroic acid



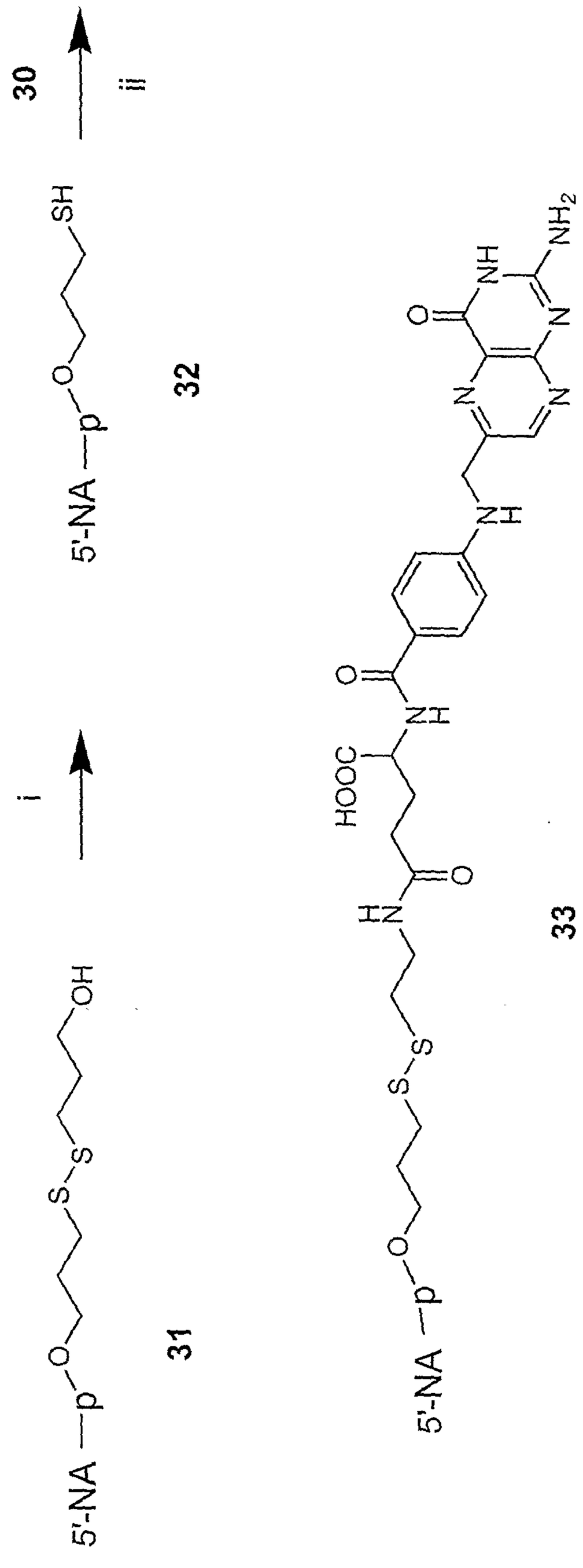

30
i
5'-NA —p— O— SH
32
i
5'-NA —p— O— S— S— OH
31
5'-NA —p— O— S— S—
HOOC
O
HN
NH
O
NH
NH
N
N
N
N
O
NH2
33
NA = siNA or a portion thereof
p = phosphorous moiety

Figure 43

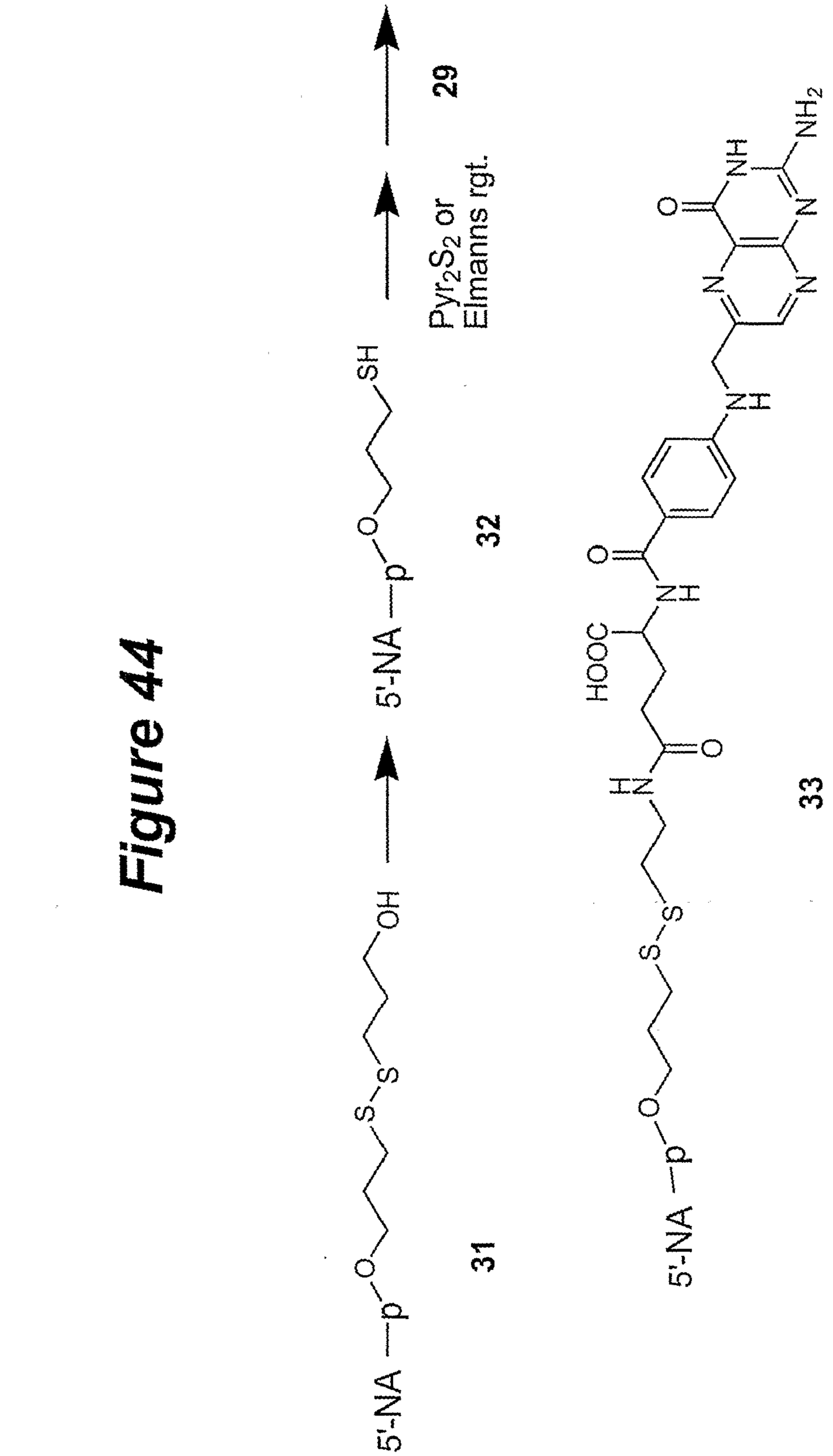

5'-NA —p
O
S
S
OH
31
Pyr2S2 or
Elmanns rgt.
32
5'-NA —p
O
S
S
SH
33
NA = siNA or a portion thereof
p = phosphorous moiety

Figure 44

Figure 45: Solid Phase Post-synthetic conjugation of pteroic acid

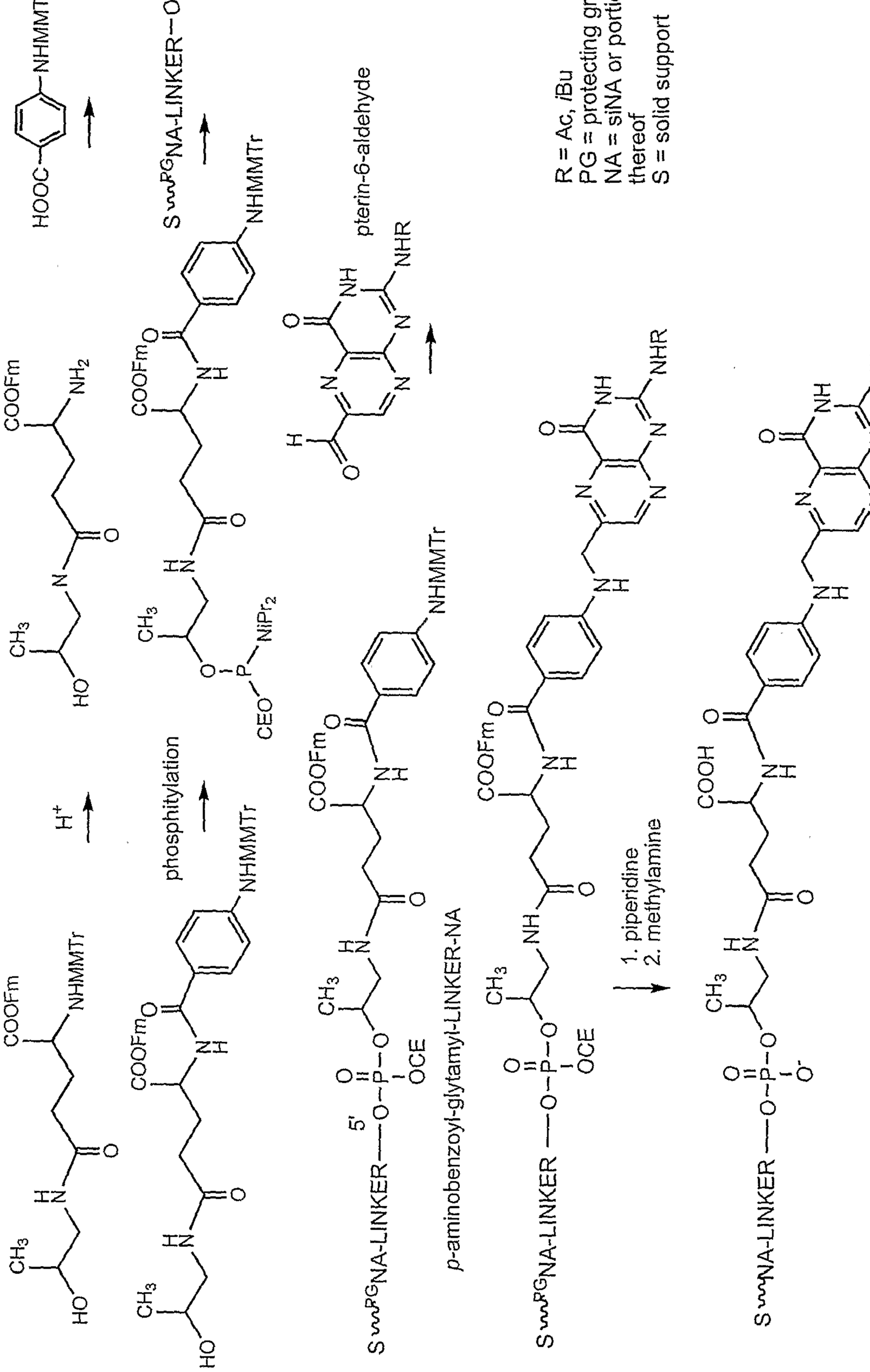
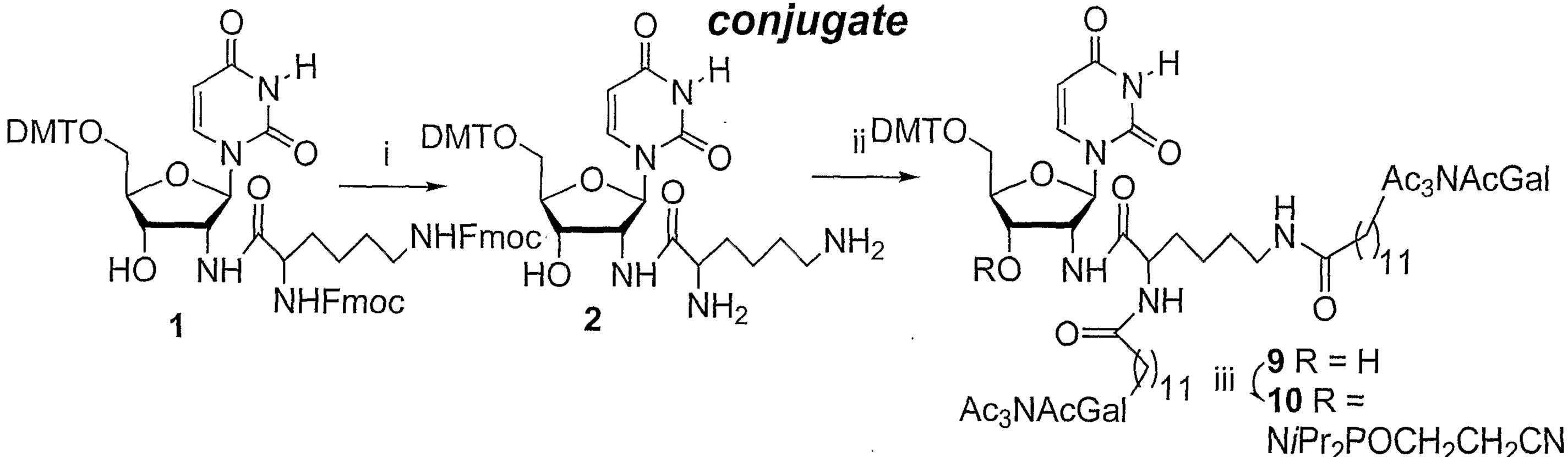
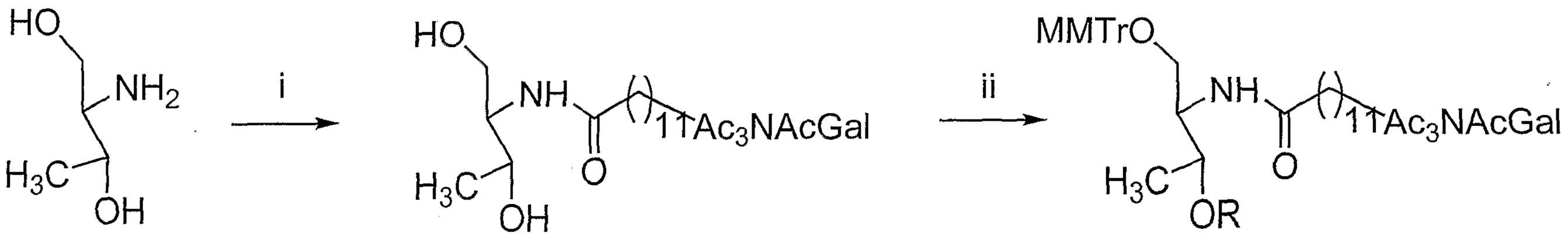


Figure 46: Synthesis of N-acetyl-D-galactosamine-2'-aminouridine conjugate



Reagents and Conditions: (i) diethylamine, DMF, (ii) **8**, diisopropylethylamine, DMF, (iii) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, 1-methylimidazole, DIPEA, CH_2Cl_2, (iv) Ac_2O, TEA, CH_3CN, (v) HCl, Ac_2O, (vi) $Hg(CN)_2$, MS 4A, CH_3NO_2-toluene 1:1, (vii) H_2, 5% Pd-C, ethanol, (viii) *N*-hydroxysuccinimide, DCC, THF.

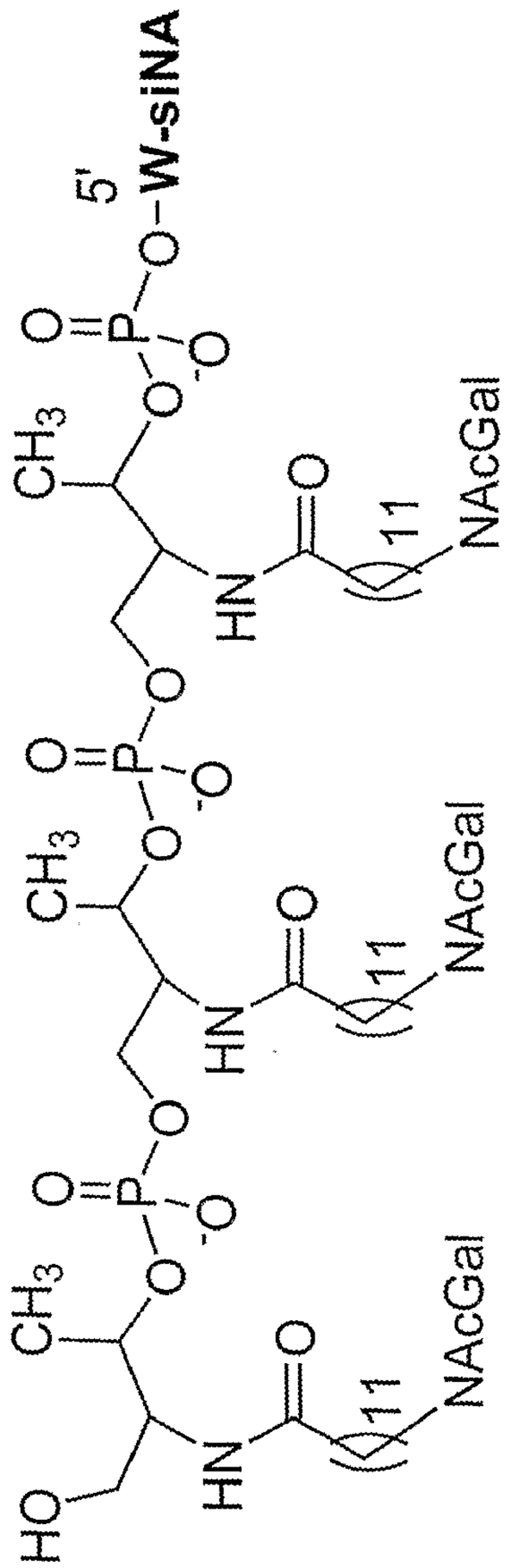
Figure 47: Synthesis of N-acetyl-D-galactosamine-D-threoninol conjugate



D-threoninol

Reagents and Conditions: (i) **7**, DCC, *N*-hydroxysuccinimide, (ii) MMTr-Cl, pyridine, (iii) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, 1-methylimidazole, DIPEA, CH_2Cl_2.

Figure 48: Conjugation of targeting ligands to the 5'-end of a siNA molecule



N-acetyl-D-galactosamine conjugate

Figure 49: Synthesis of dodecanoic acid linker

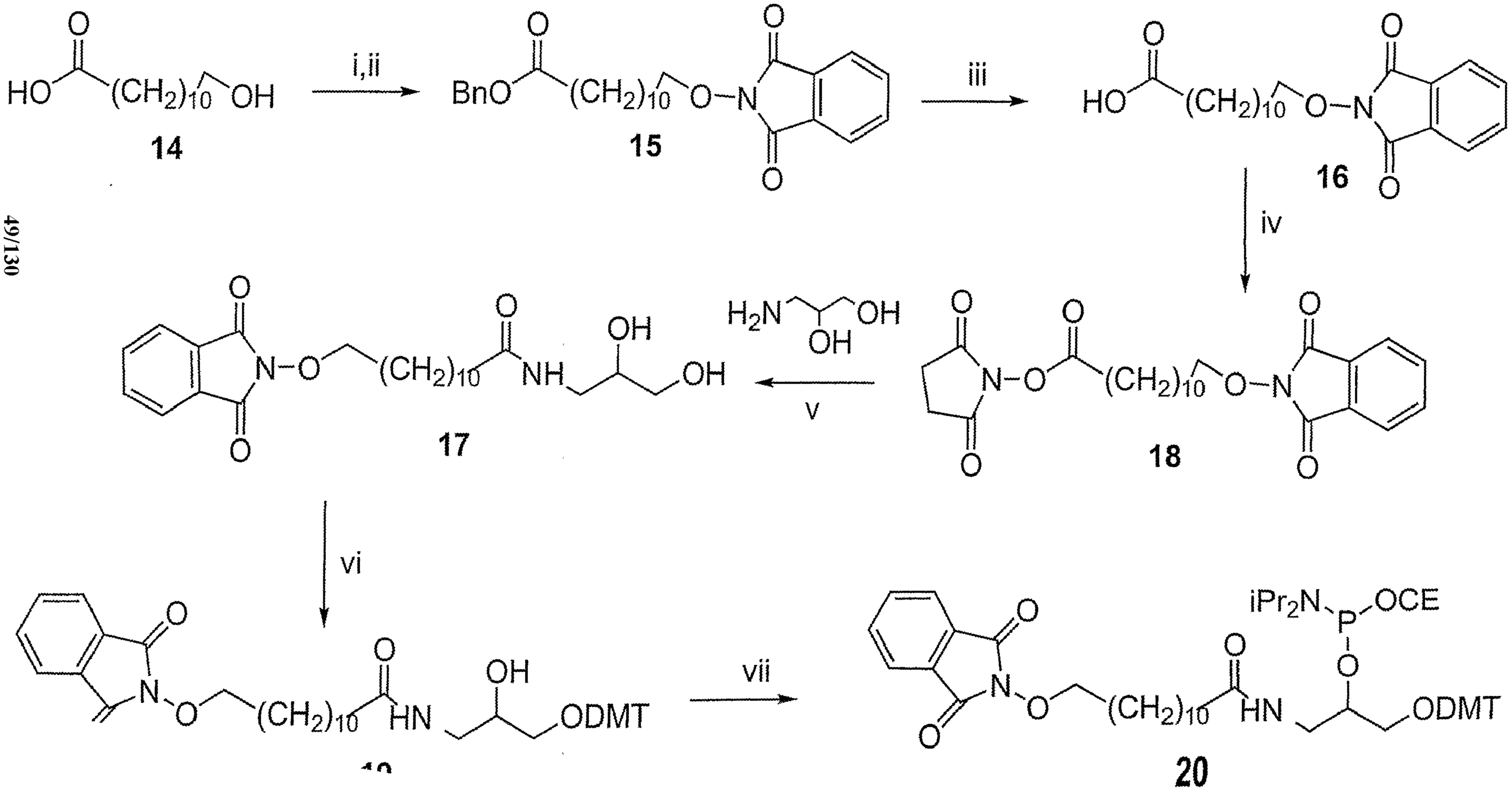


Figure 50: Oxime linked siNA/Peptide Conjugate

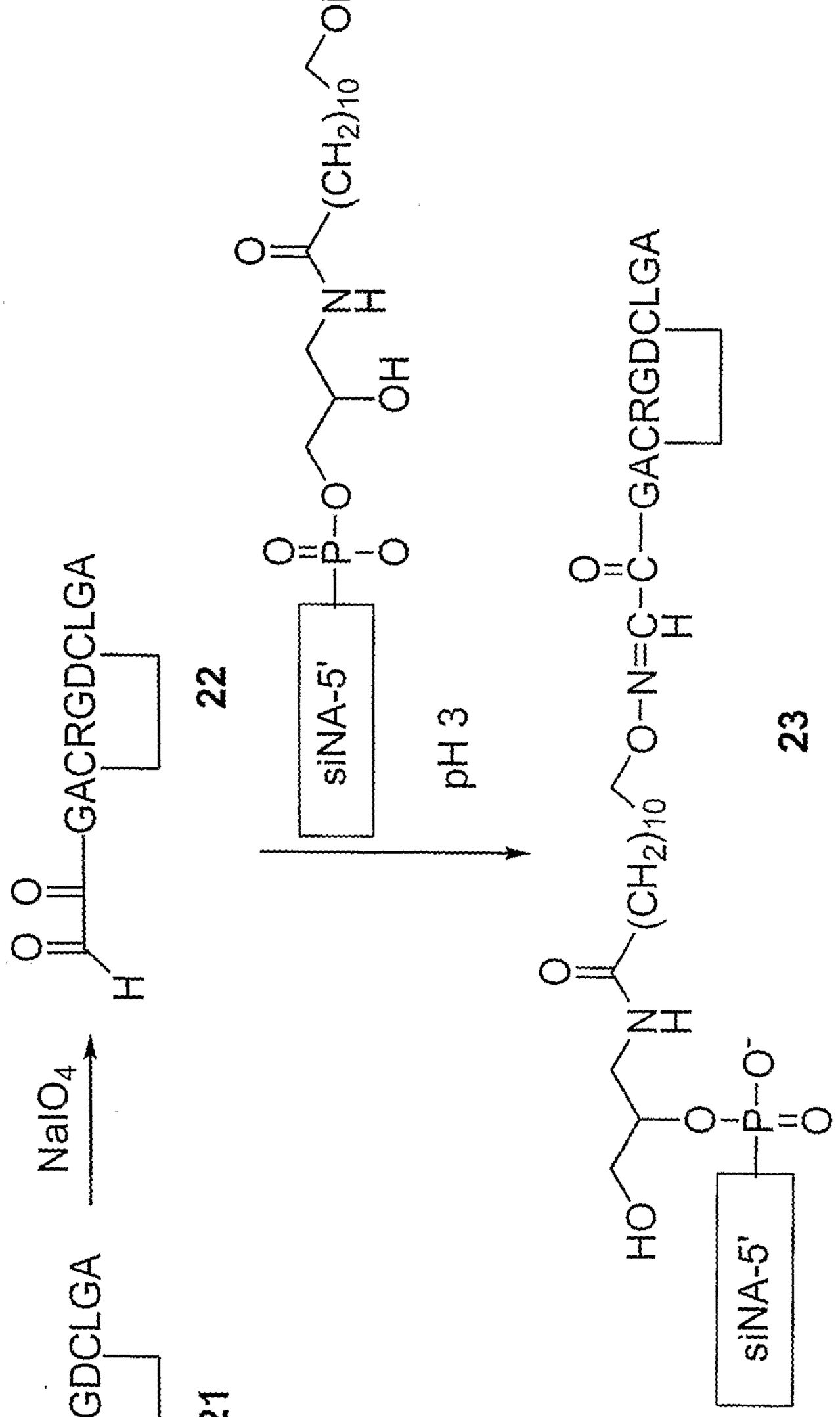
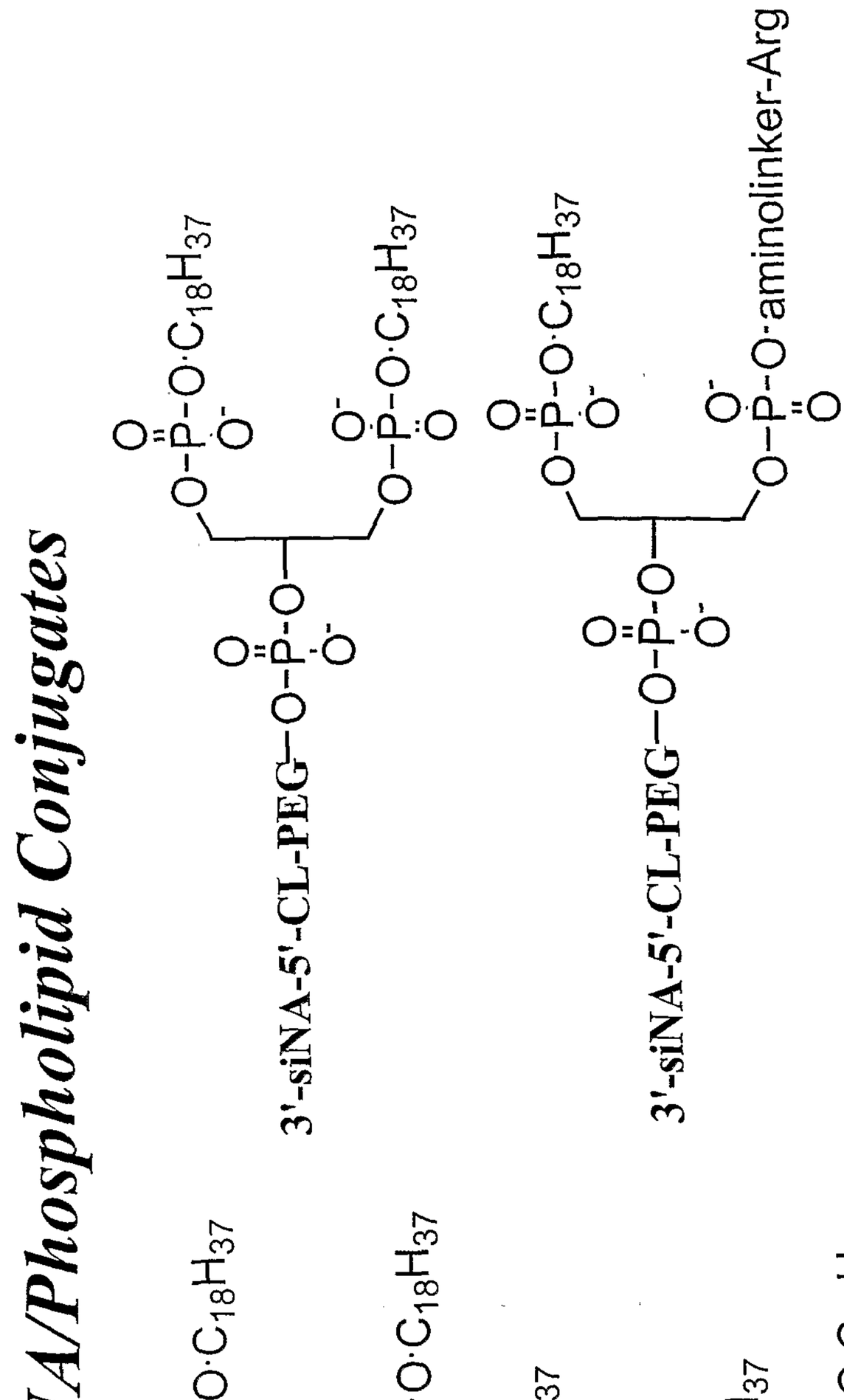
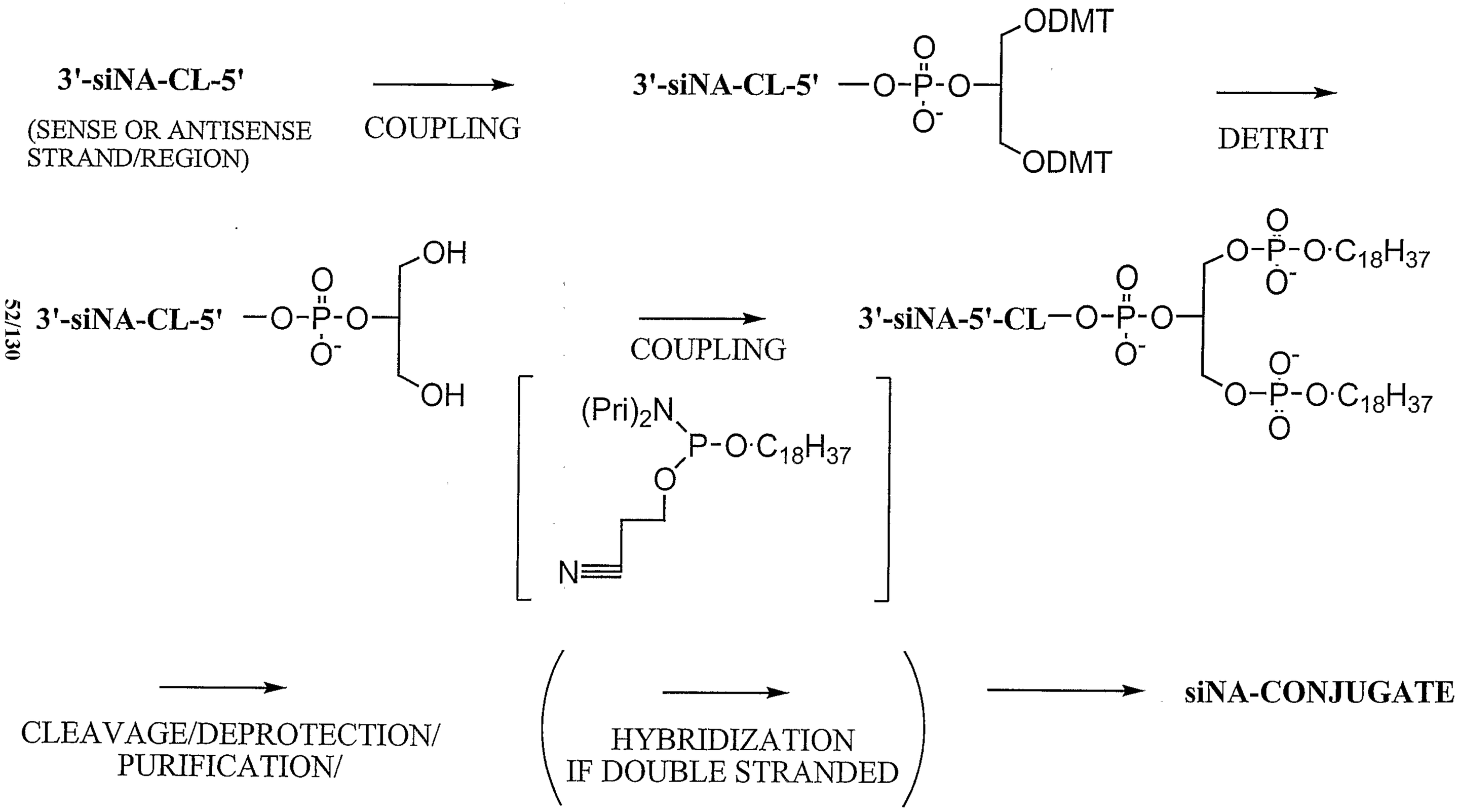


Figure 51: siNA/Phospholipid Conjugates



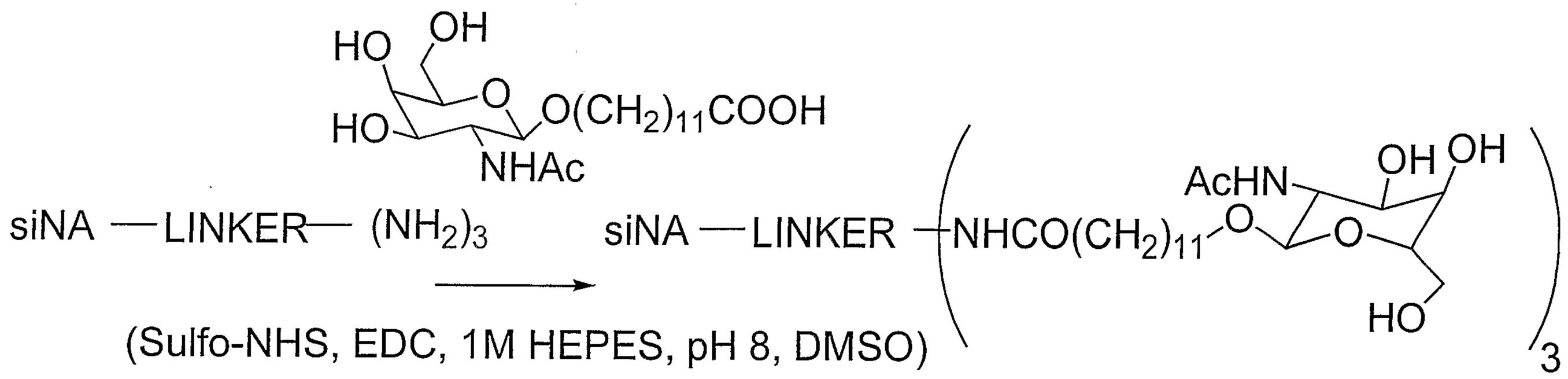
PEG=polyethylene glycol
CL=cleavable linker (e.g. A-dT, C-dT)
siNA= short interfering nucleic acid molecule or a portion thereof

Figure 52: siNA Phospholipid Conjugate

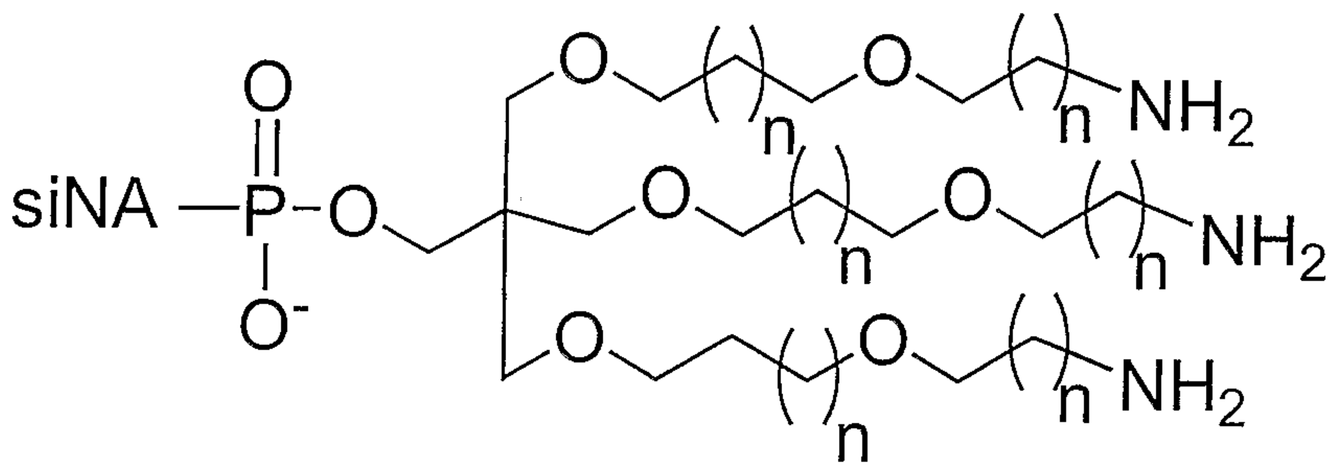


CL = CLEAVABLE LINKER, E.G. ADENOSINE-THYMIDINE DIMER THAT IS OPTIONALLY PRESENT

Figure 53: siNA-NAcGalactosamine post-synthetic coupling



FOR EXAMPLE: OLIGO-LINKER =



Where n is an integer from 1 to 20

Figure 54: siNA Cholesterol Conjugate

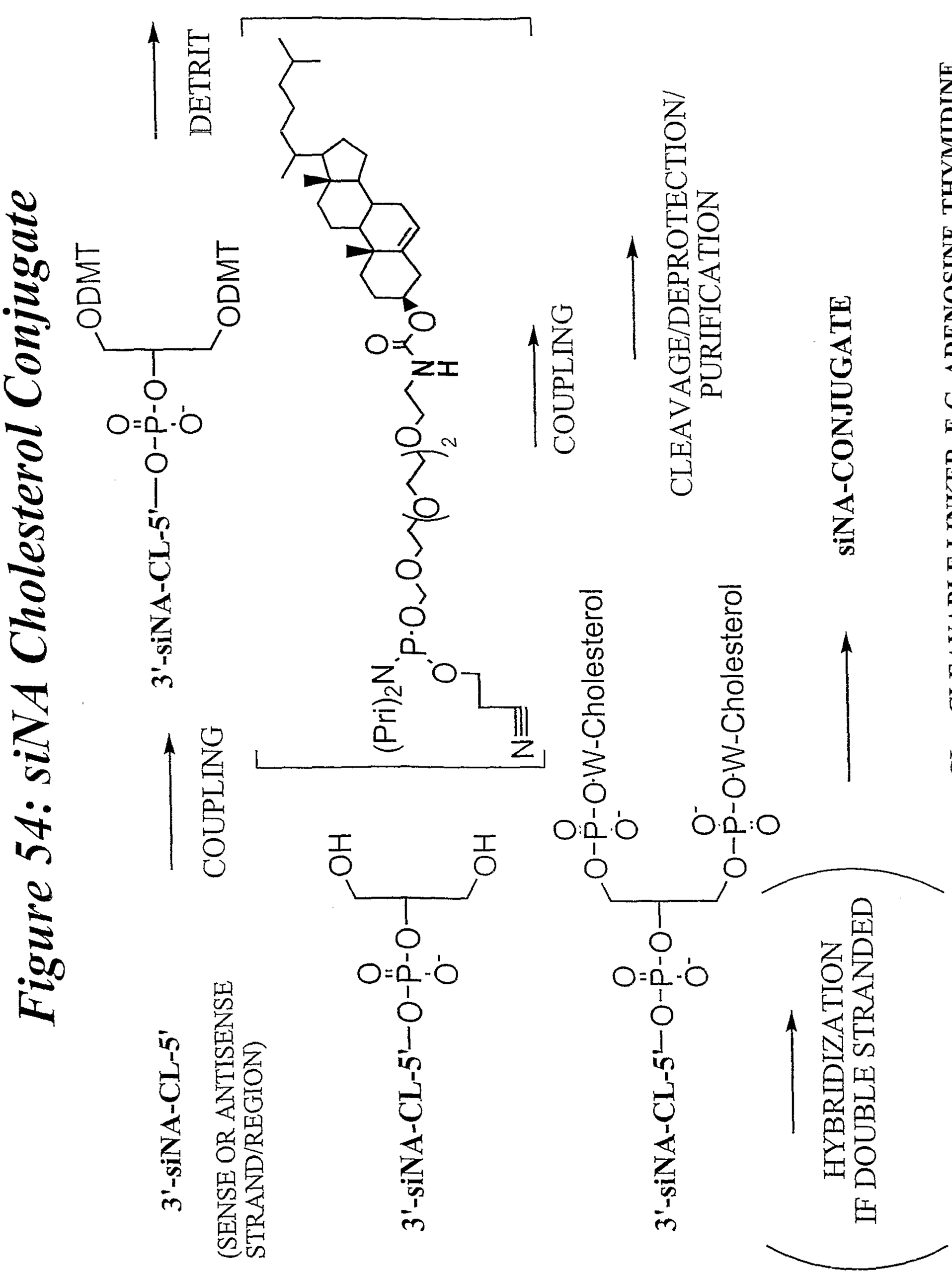
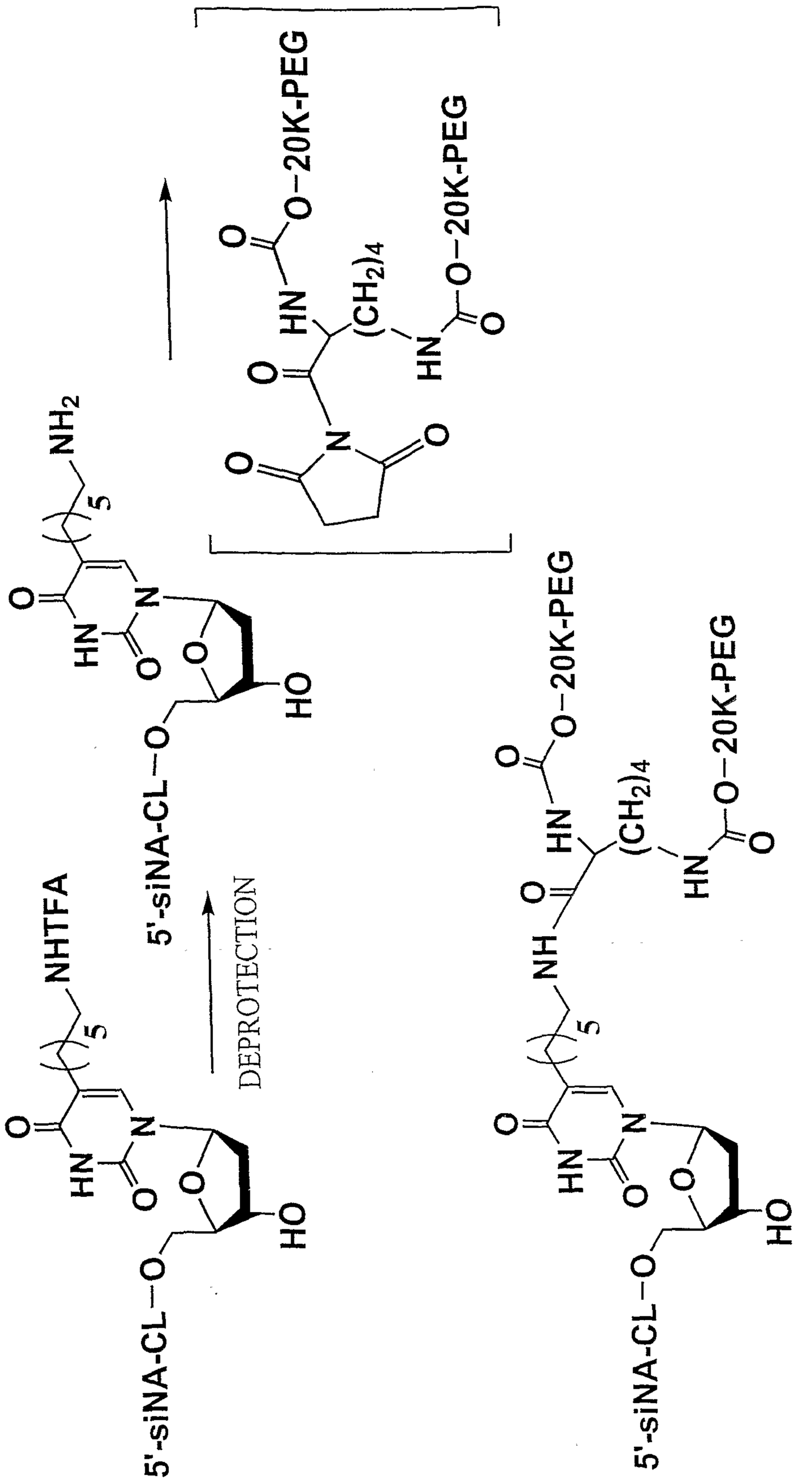


Figure 55: siNA 3'-PEG Conjugate



CL = CLEAVABLE LINKER, E.G. ADENOSINE-THYMIDINE DIMER
THAT IS OPTIONALLY PRESENT

Figure 56: siNA 3'-Cholesterol Conjugate

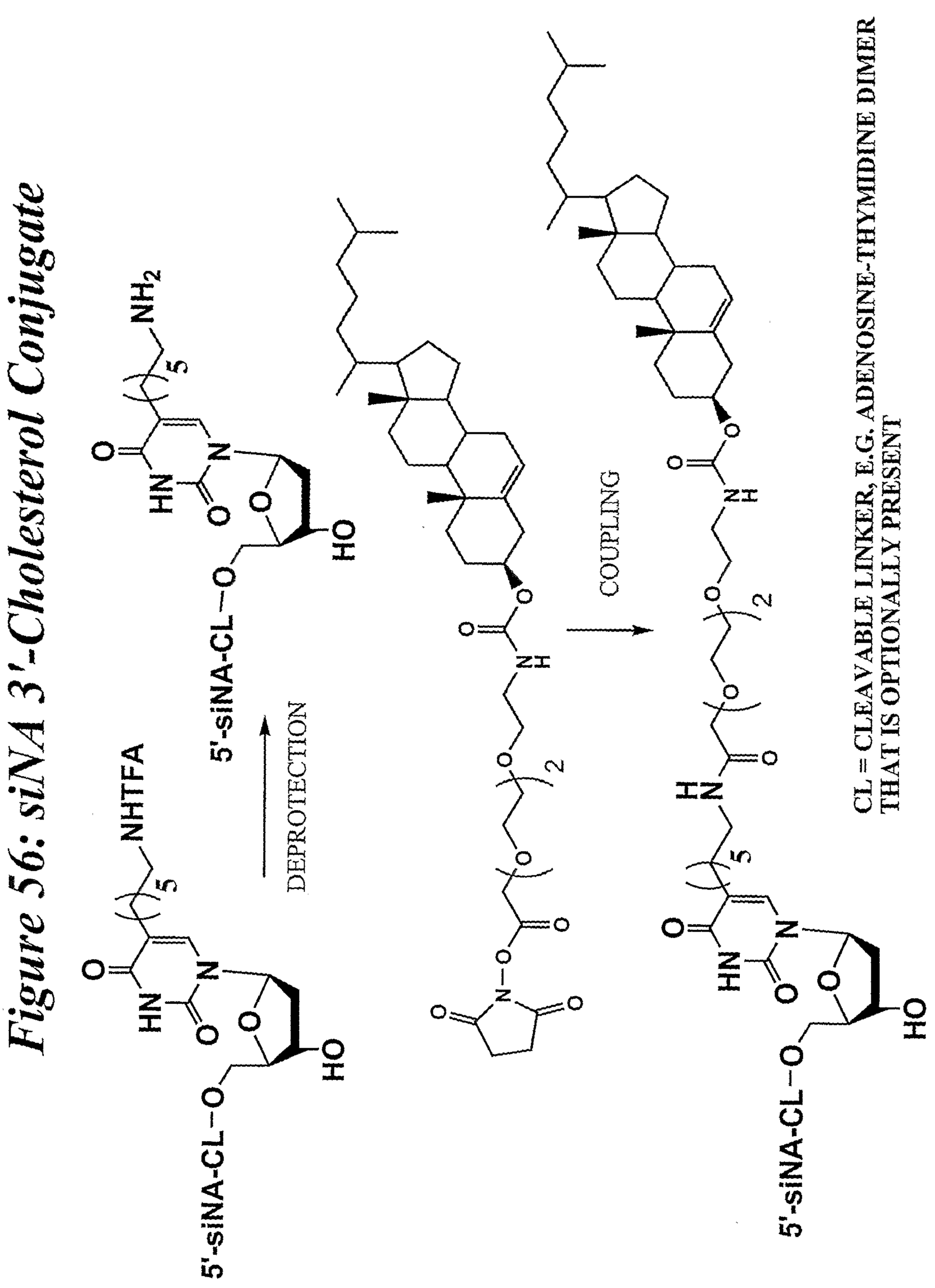
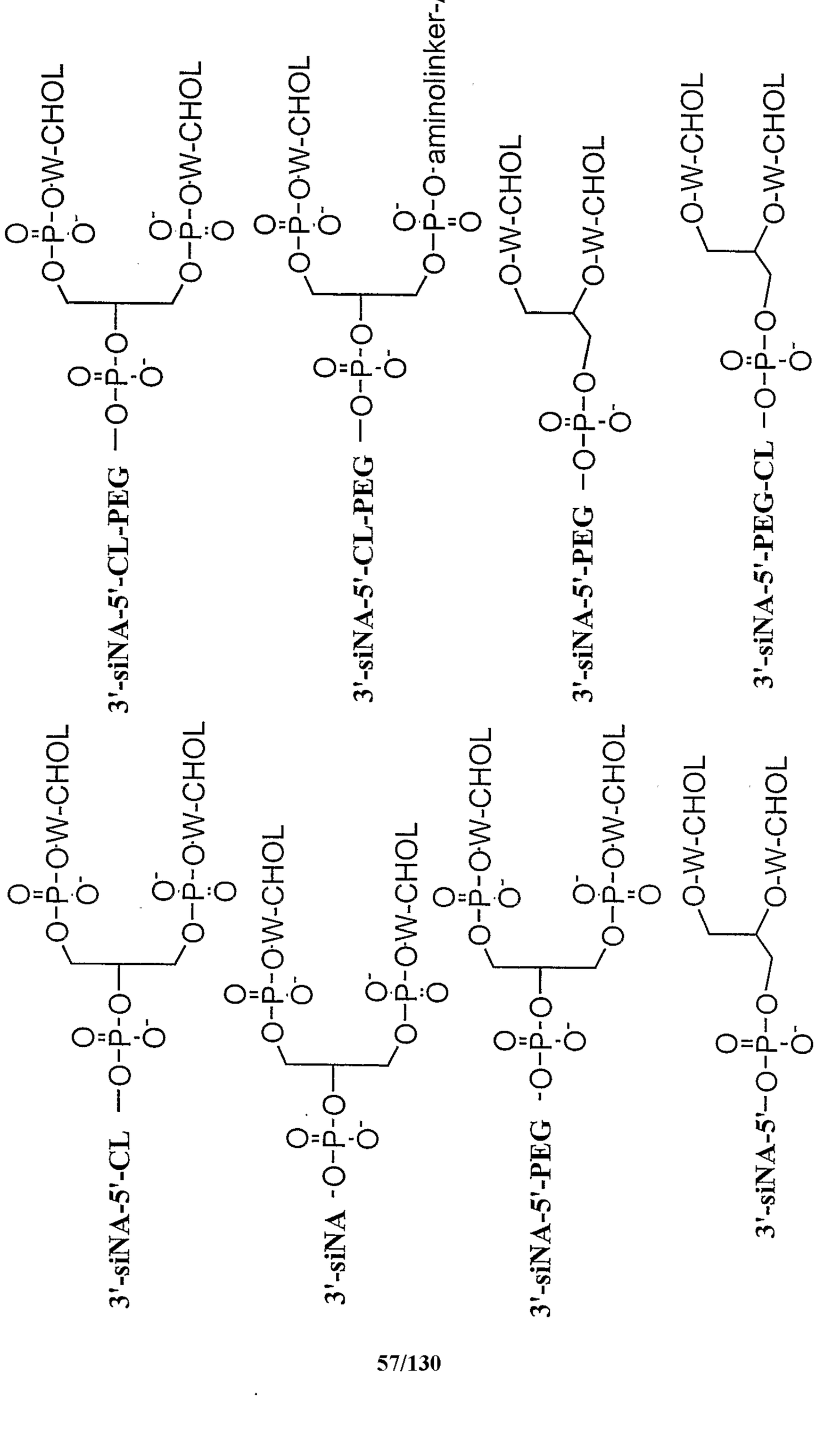
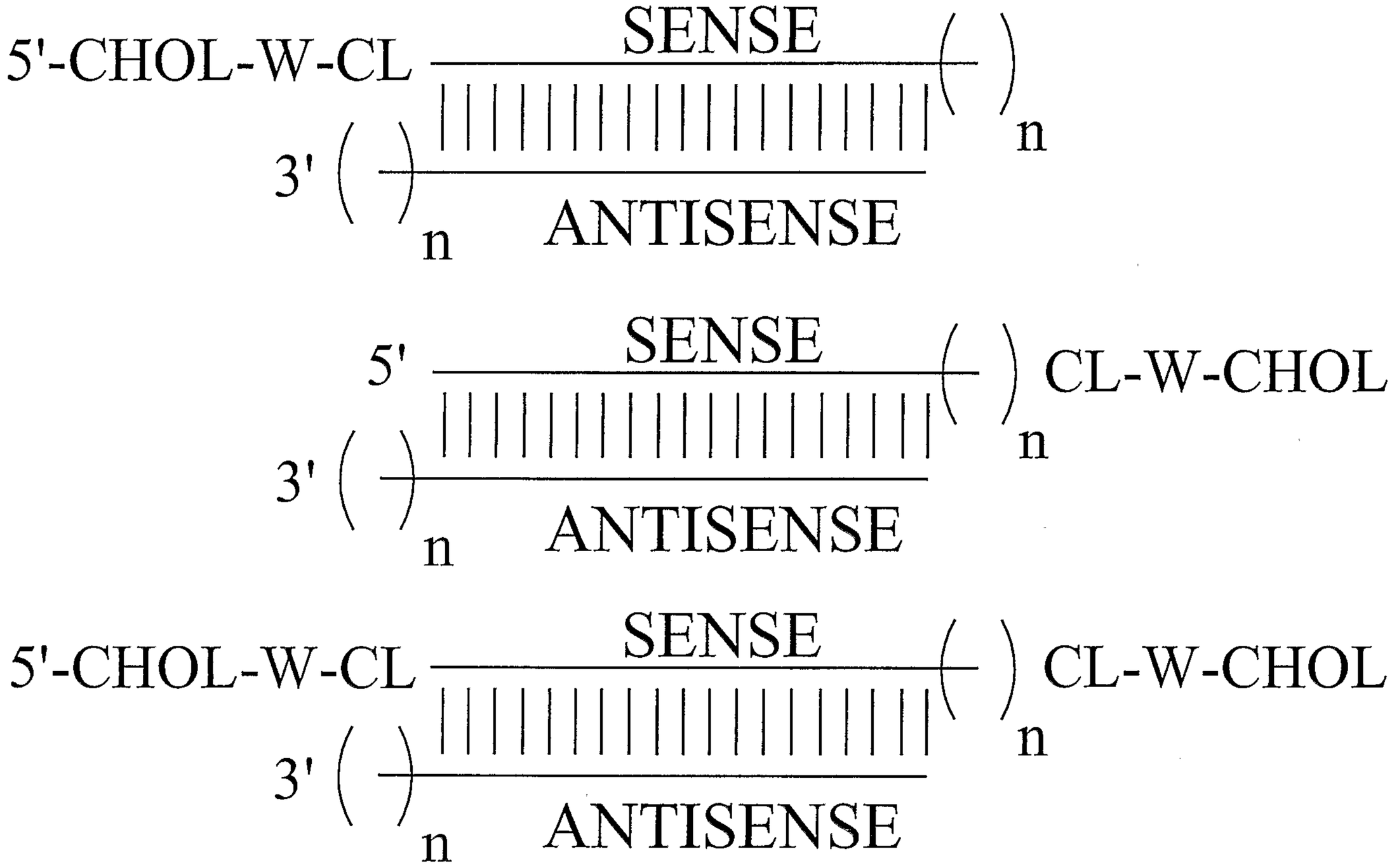


Figure 57: Nucleic Acid Cholesterol Conjugates



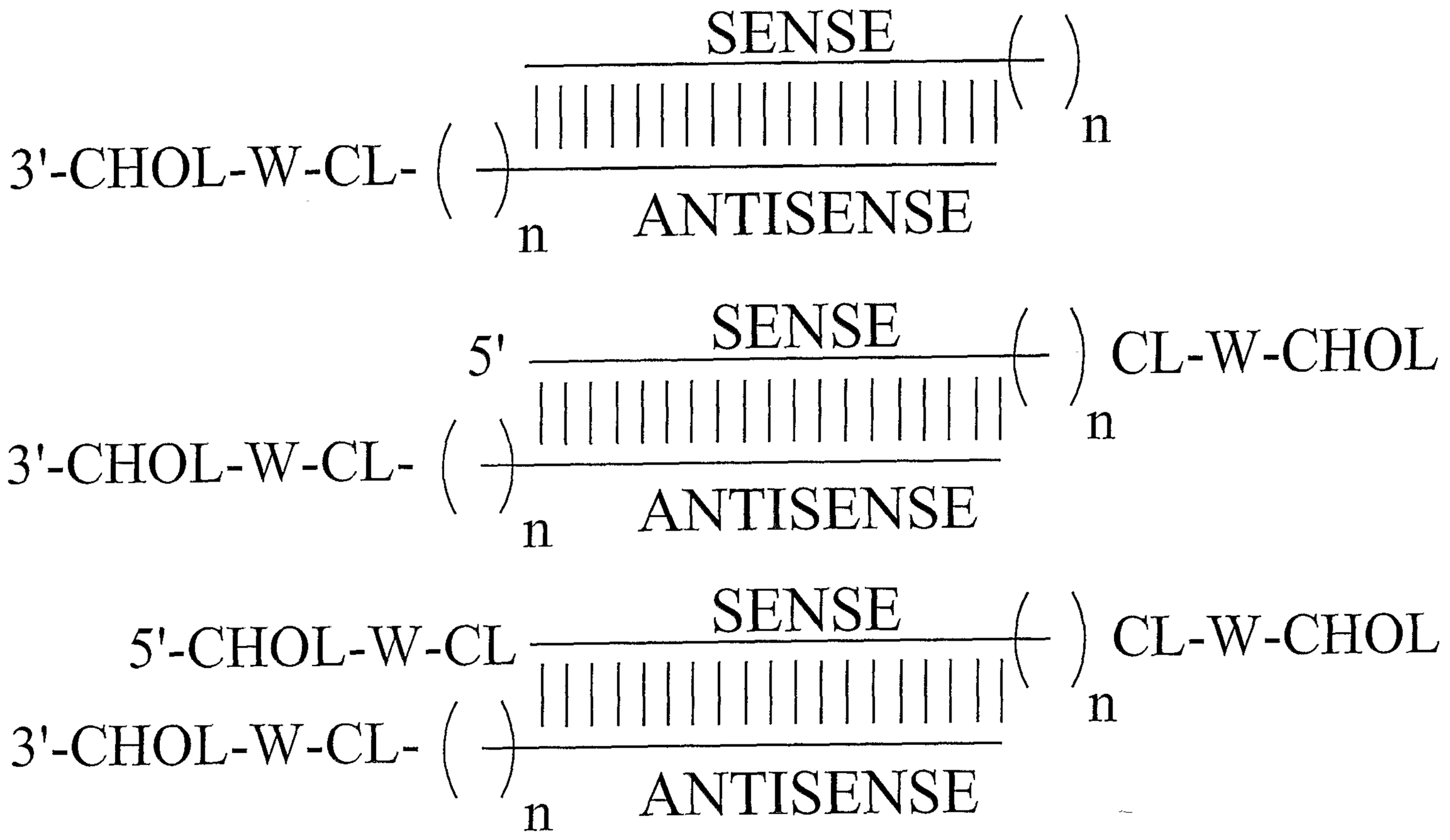
PEG=polyethylene glycol
CL=cleavable linker (e.g. A-dT, C-dT)
siNA= short interfering nucleic acid molecule or a portion thereof
CHOL=cholesterol or an analog or metabolite thereof
W= linker molecule (see for example Formulae 109 or 112)

Figure 58: siNA Cholesterol Conjugates



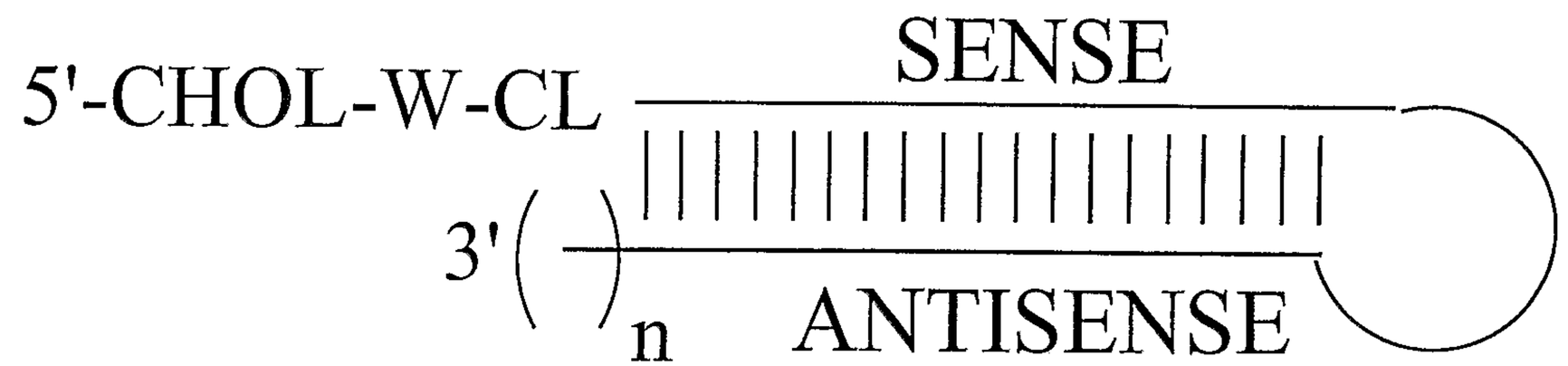
CL=cleavable linker (e.g. A-dT, C-dT) that is optionally present
CHOL=cholesterol or an analog or metabolite thereof
W= linker molecule (see for example Formulae 107, 108, 109 or 115)
n = integer, e.g. 1, 2, or 3

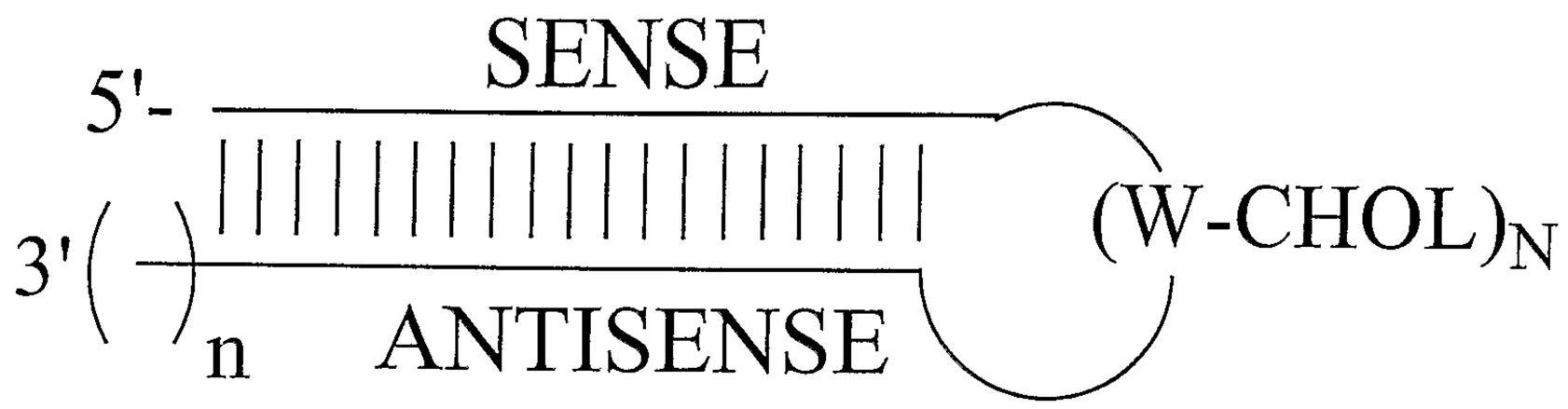
Figure 59: siNA Cholesterol Conjugates

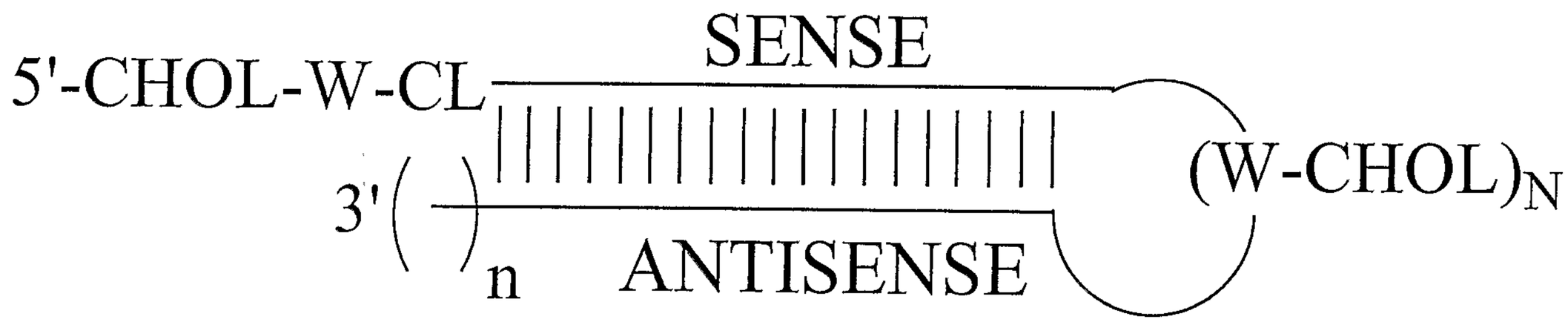


CL=cleavable linker (e.g. A-dT, C-dT) that is optionally present
CHOL=cholesterol or an analog or metabolite thereof
W= linker molecule (see for example Formulae 107, 108, 109 or 115)
n = integer, e.g. 1, 2, or 3

Figure 60: siNA Cholesterol Conjugates

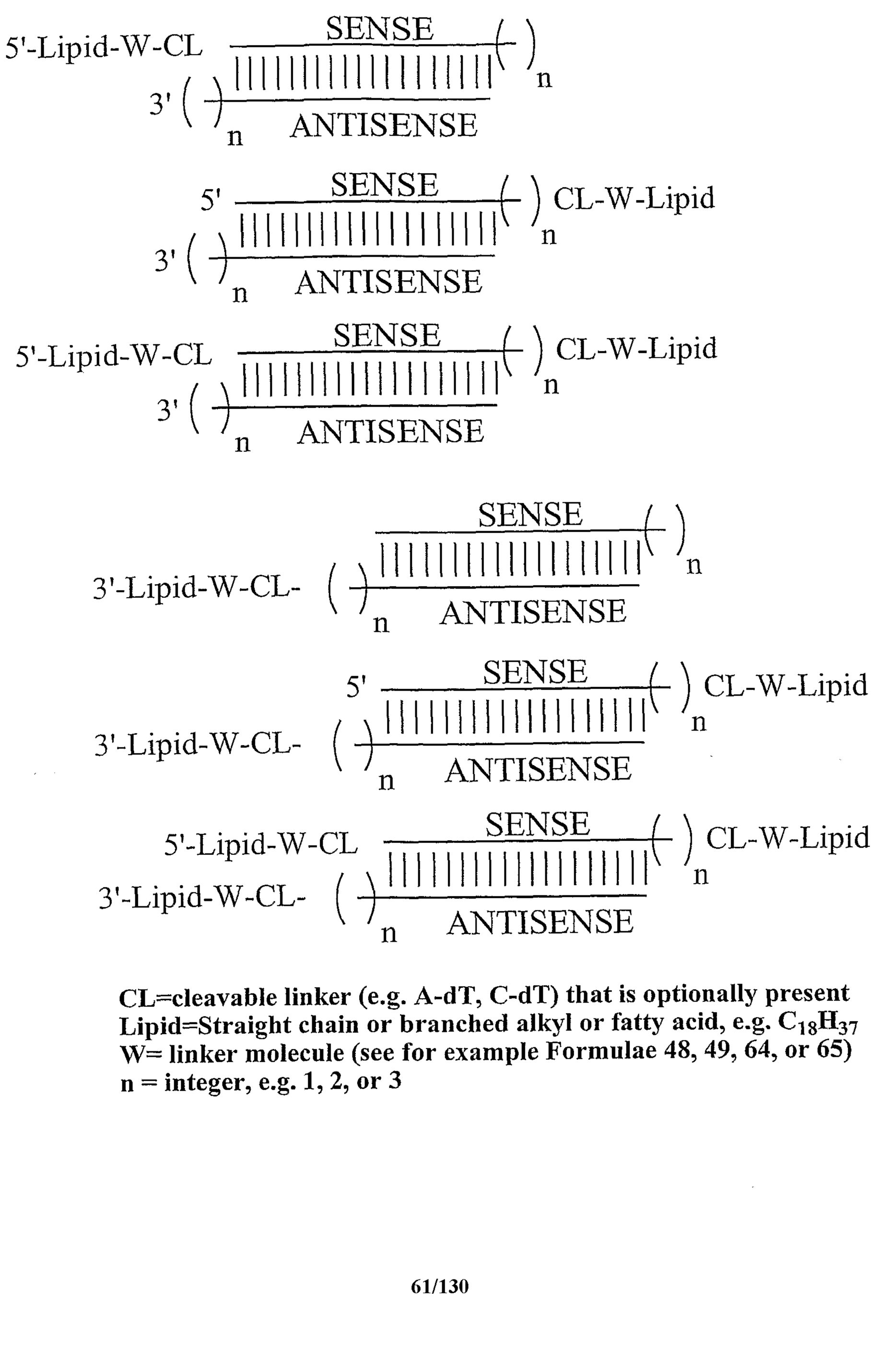






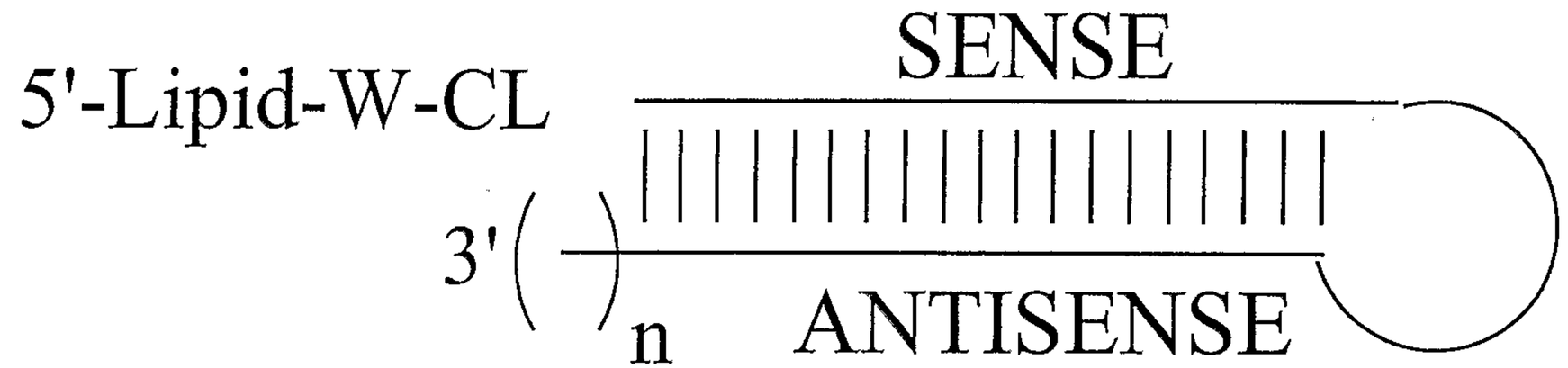
CL=cleavable linker (e.g. A-dT, C-dT) that is optionally present
CHOL=cholesterol or an analog or metabolite thereof
W= linker molecule (see for example Formulae 107, 108, 109 or 112)
n = integer, e.g. 1, 2, or 3
N=integer, e.g. 1, 2, 3, or 4

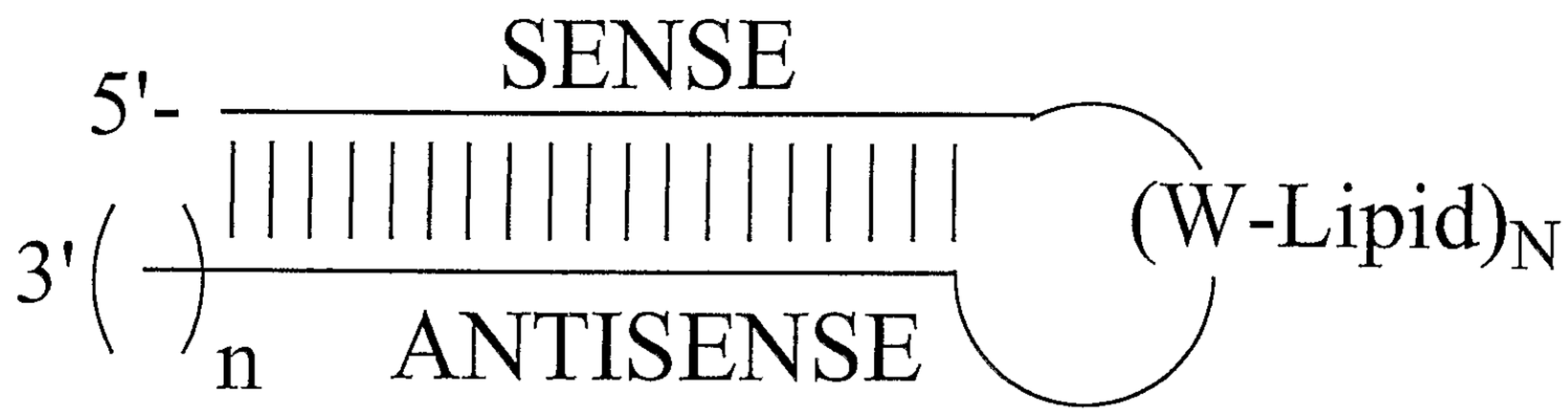
Figure 61: siNA Lipid Conjugates

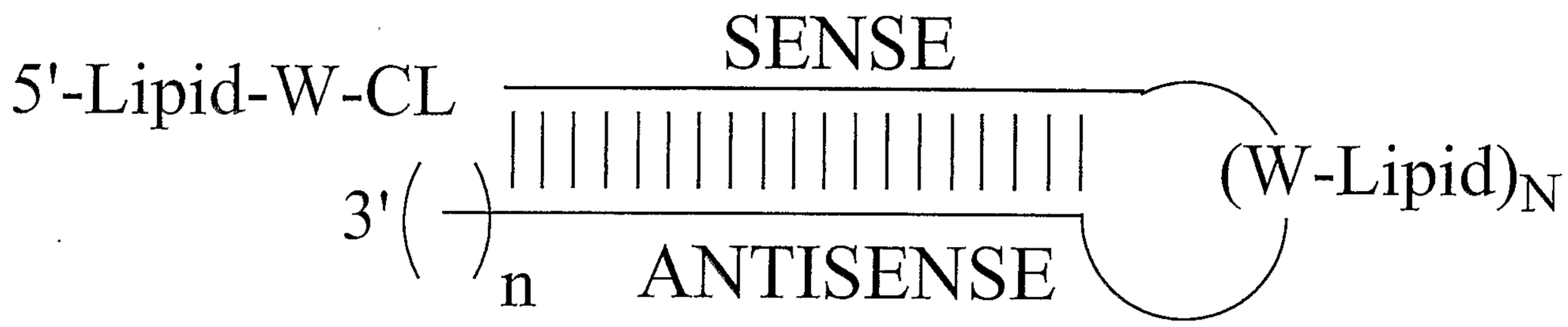


CL=cleavable linker (e.g. A-dT, C-dT) that is optionally present
Lipid=Straight chain or branched alkyl or fatty acid, e.g. $C_{18}H_{37}$
W= linker molecule (see for example Formulae 48, 49, 64, or 65)
n = integer, e.g. 1, 2, or 3

Figure 62: siNA Lipid Conjugates

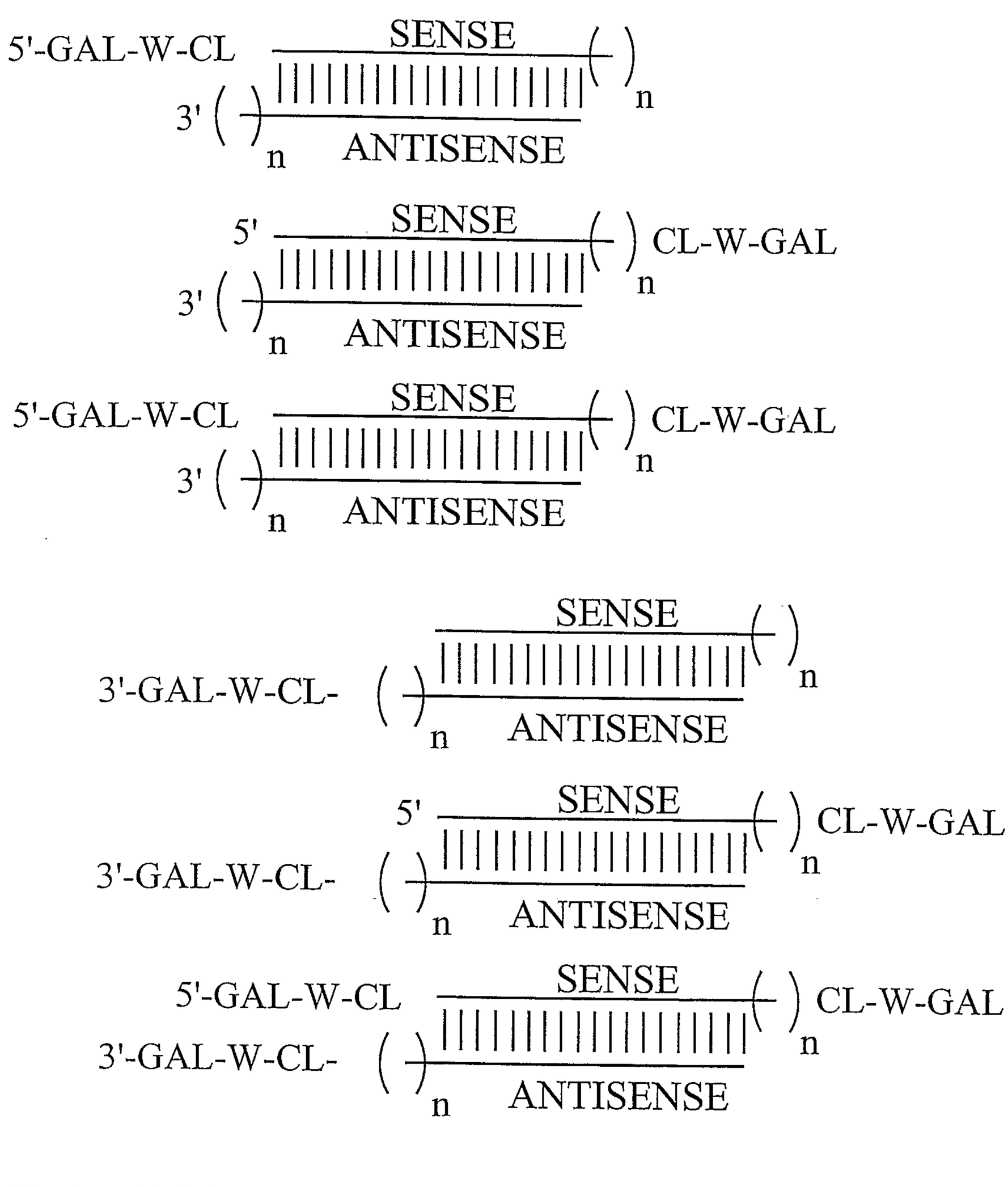






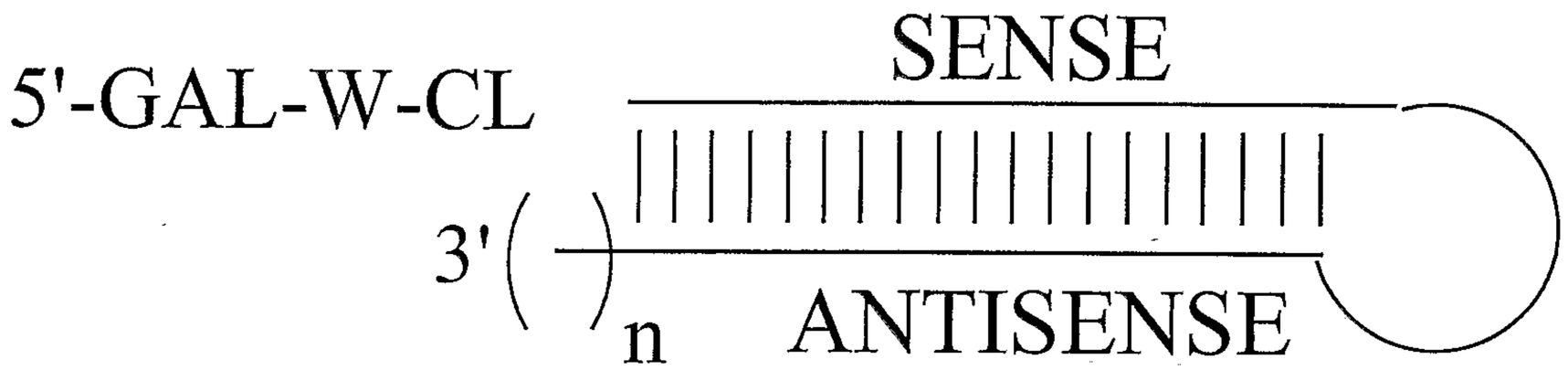
CL=cleavable linker (e.g. A-dT, C-dT) that is optionally present
Lipid=Straight chain or branched alkyl or fatty acid, e.g. $C_{18}H_{37}$
W= linker molecule (see for example Formulae 48, 49, 64, or 65)
n = integer, e.g. 1, 2, or 3
N=integer, e.g. 1, 2, 3, or 4

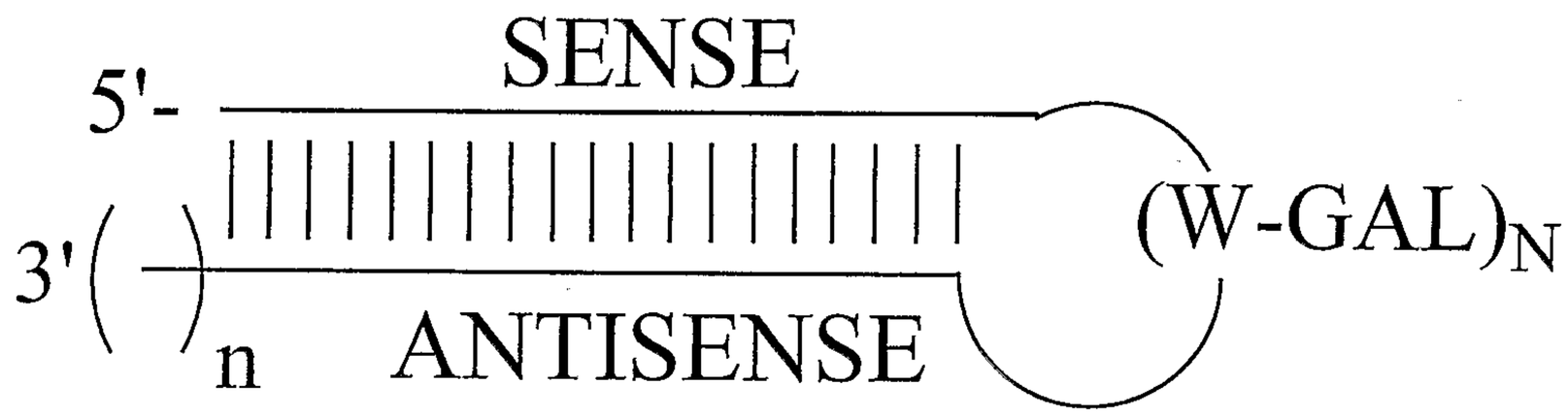
Figure 63: siNA Galactosamine Conjugates

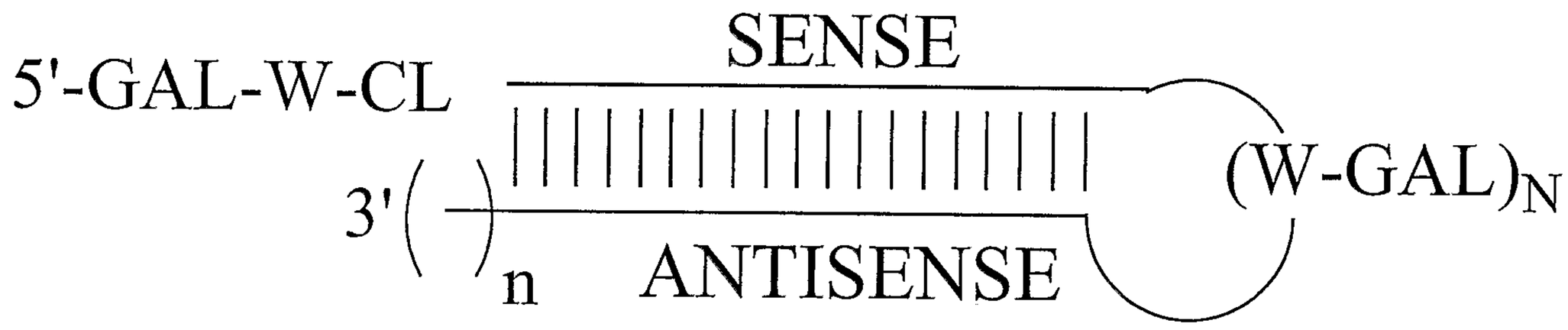


CL=cleavable linker (e.g. A-dT, C-dT) that is optionally present
GAL=GALACTOSAMINE; e.g. compounds having Formulae 51-56, 86, 92, 99, 100, 103, 105, 106
W= linker molecule (see for example Formulae 102 or 103)
n = integer, e.g. 1, 2, or 3

Figure 64: siNA Galactosamine Conjugates



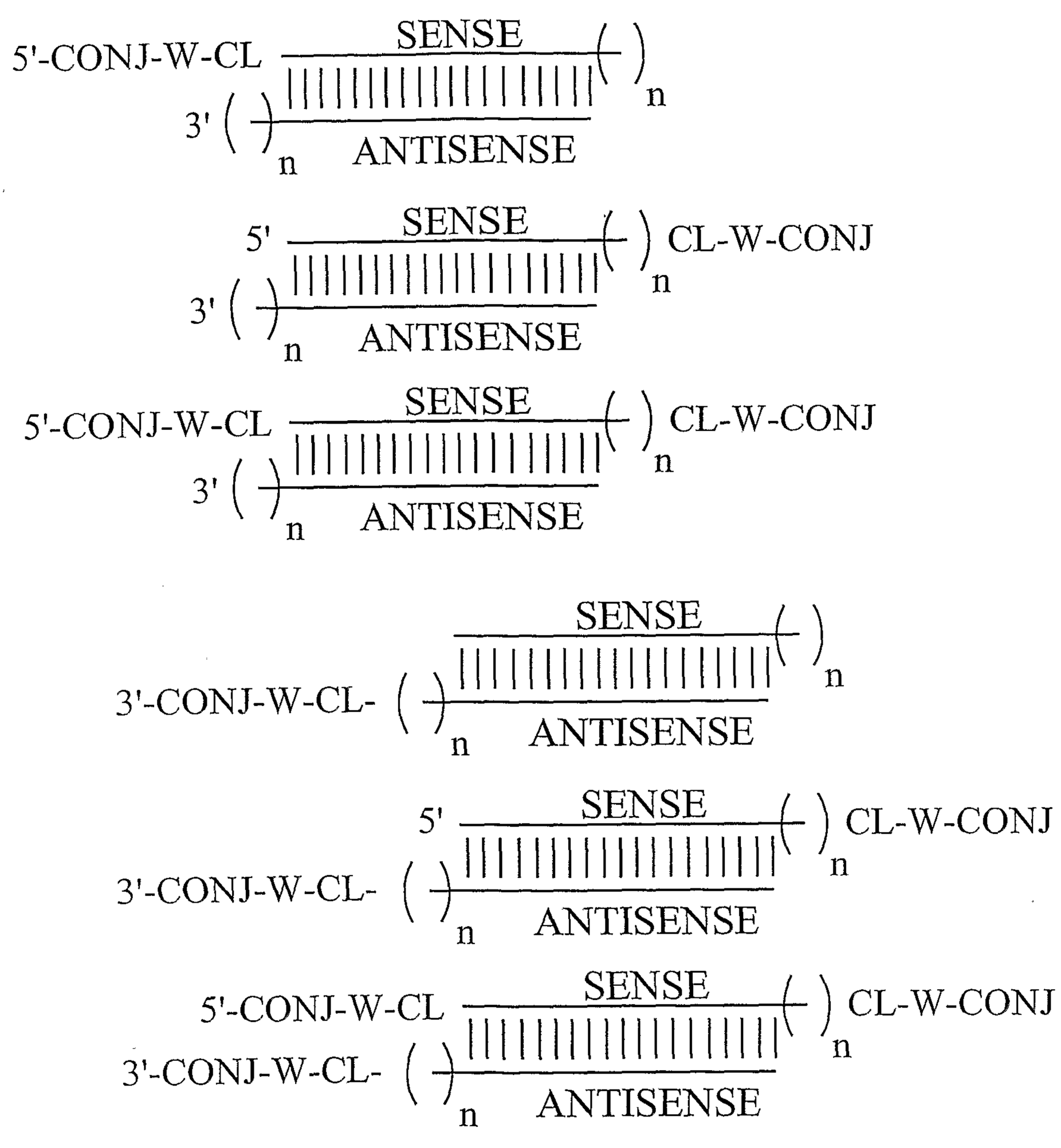




CL=cleavable linker (e.g. A-dT, C-dT) that is optionally present
GAL=GALACTOSAMINE; e.g. compounds having Formulae 51-56, 86, 92, 99, 100, 103, 105, 106
W= linker molecule (see for example Formulae 102 or 103)
n = integer, e.g. 1, 2, or 3
N=integer, e.g. 1, 2, 3, or 4

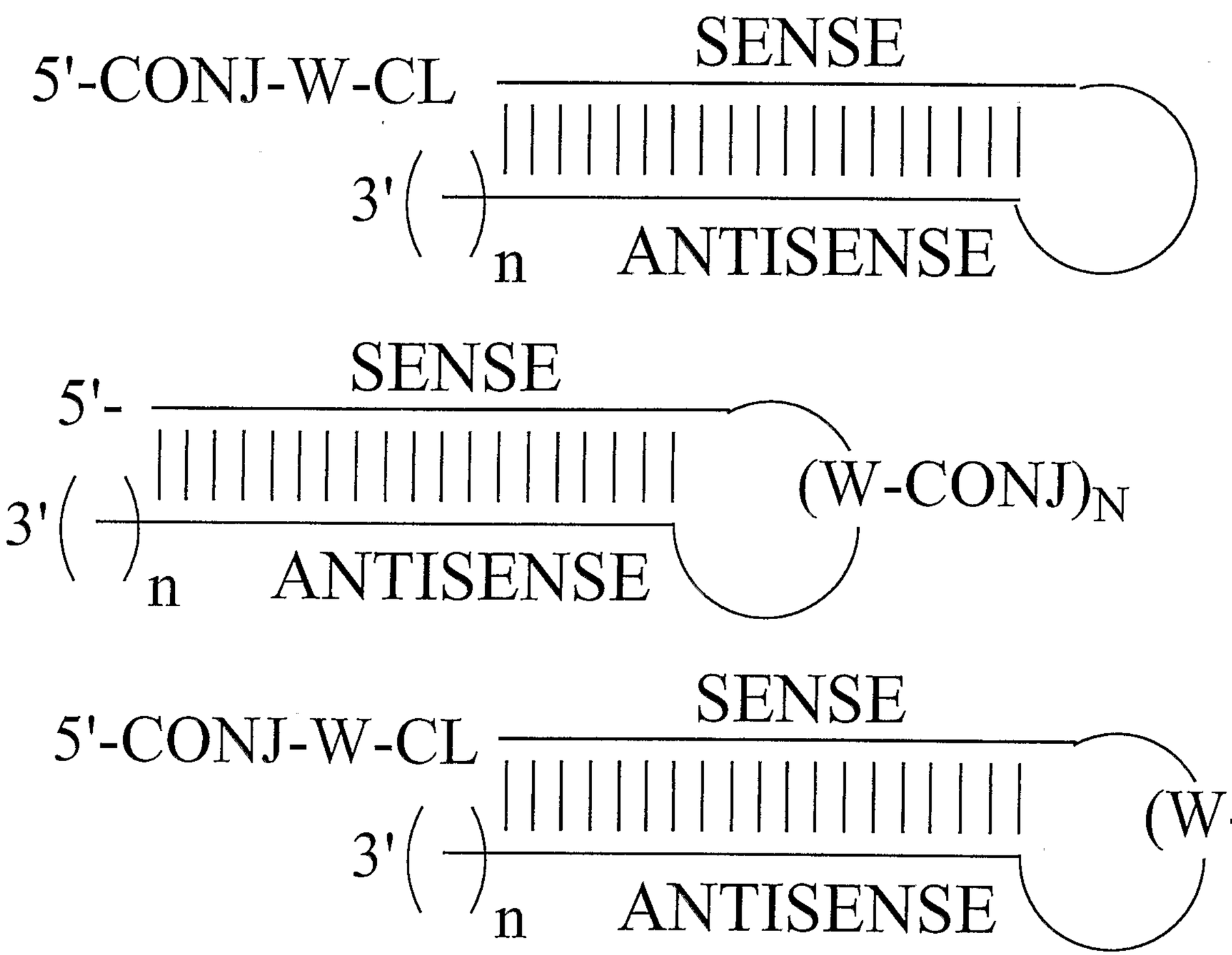
CA 02526831 2005-11-22

Figure 65: Generalized siNA Conjugate Design



CONJ=any biologically active molecule or conjugate as described herein
CL=cleavable linker (e.g. A-dT, C-dT) that is optionally present
W= linker molecule
n = integer, e.g. 1, 2, or 3

Figure 66: Generalized siNA Conjugate design



CONJ=any biologically active molecule or conjugate as described herein
CL=cleavable linker (e.g. A-dT, C-dT) that is optionally present
W= linker molecule
n = integer, e.g. 1, 2, or 3
N=integer, e.g. 1, 2, 3, or 4

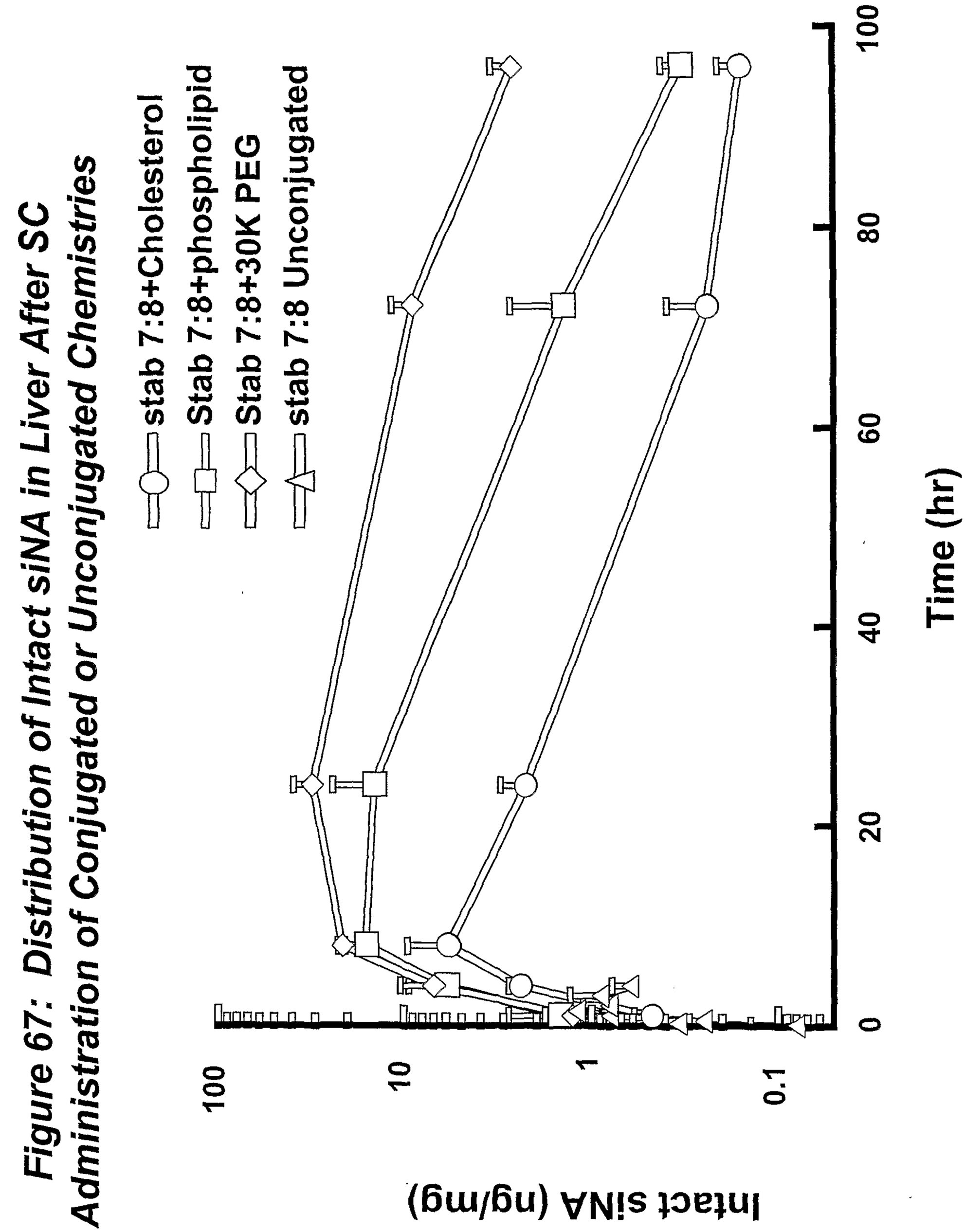


Figure 67: Distribution of Intact siNA in Liver After SC Administration of Conjugated or Unconjugated Chemistries

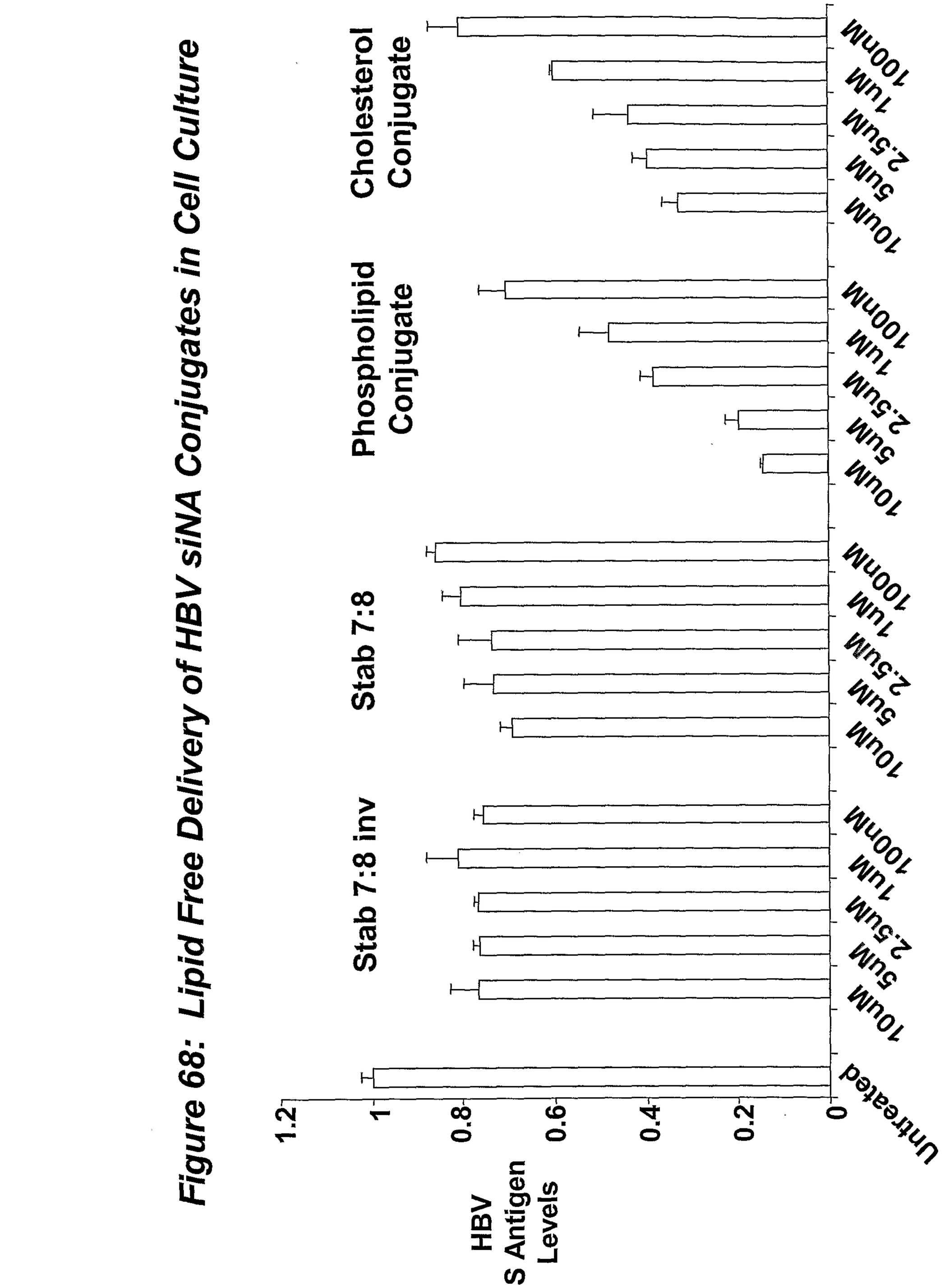


Figure 68: Lipid Free Delivery of HBV siNA Conjugates in Cell Culture
HBV S Antigen Levels
1.2
1
0.8
0.6
0.4
0.2
0
Untreated
10nM
5uM
2.5uM
1uM
100nM
Stab 7:8 inv
Cholesterol Conjugate
Phospholipid Conjugate
Stab 7:8

Figure 69: Scale-up of "mono" Galactosamine phosphoramidite

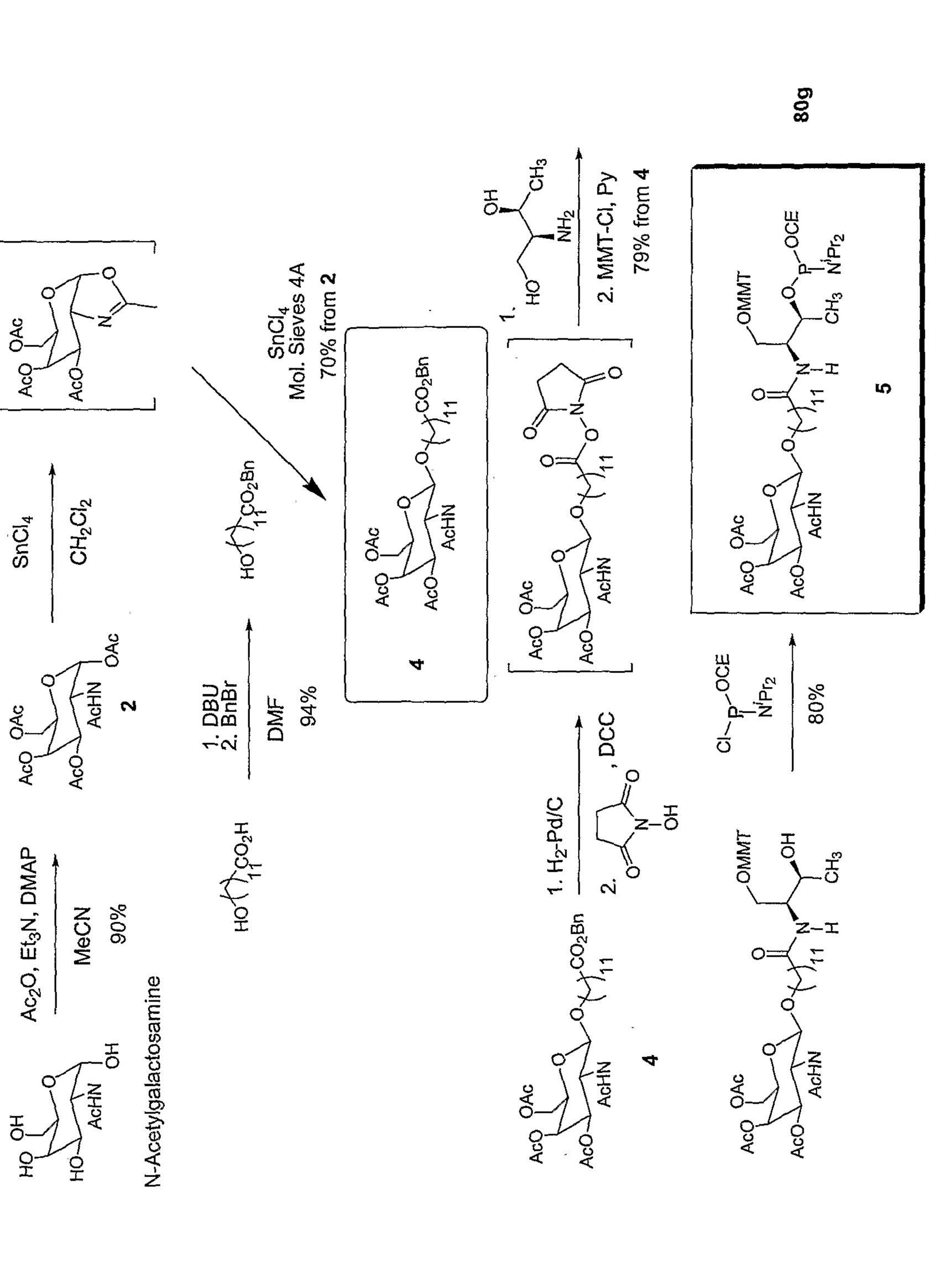


Figure 70: Synthesis of "tri" Galactosamine phosphoramidite

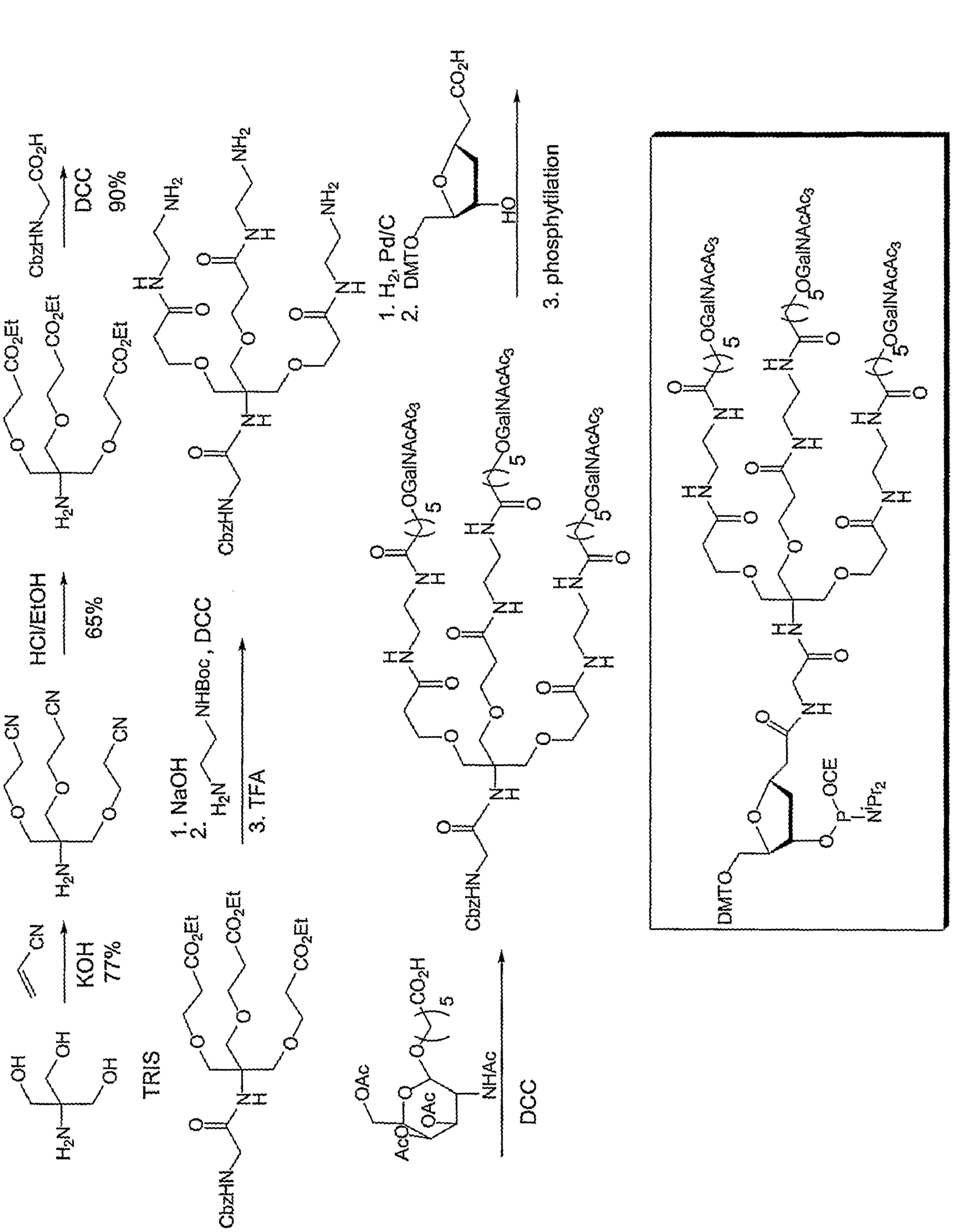


Figure 71: Synthesis of another Tri-Galactosamine Conjugate

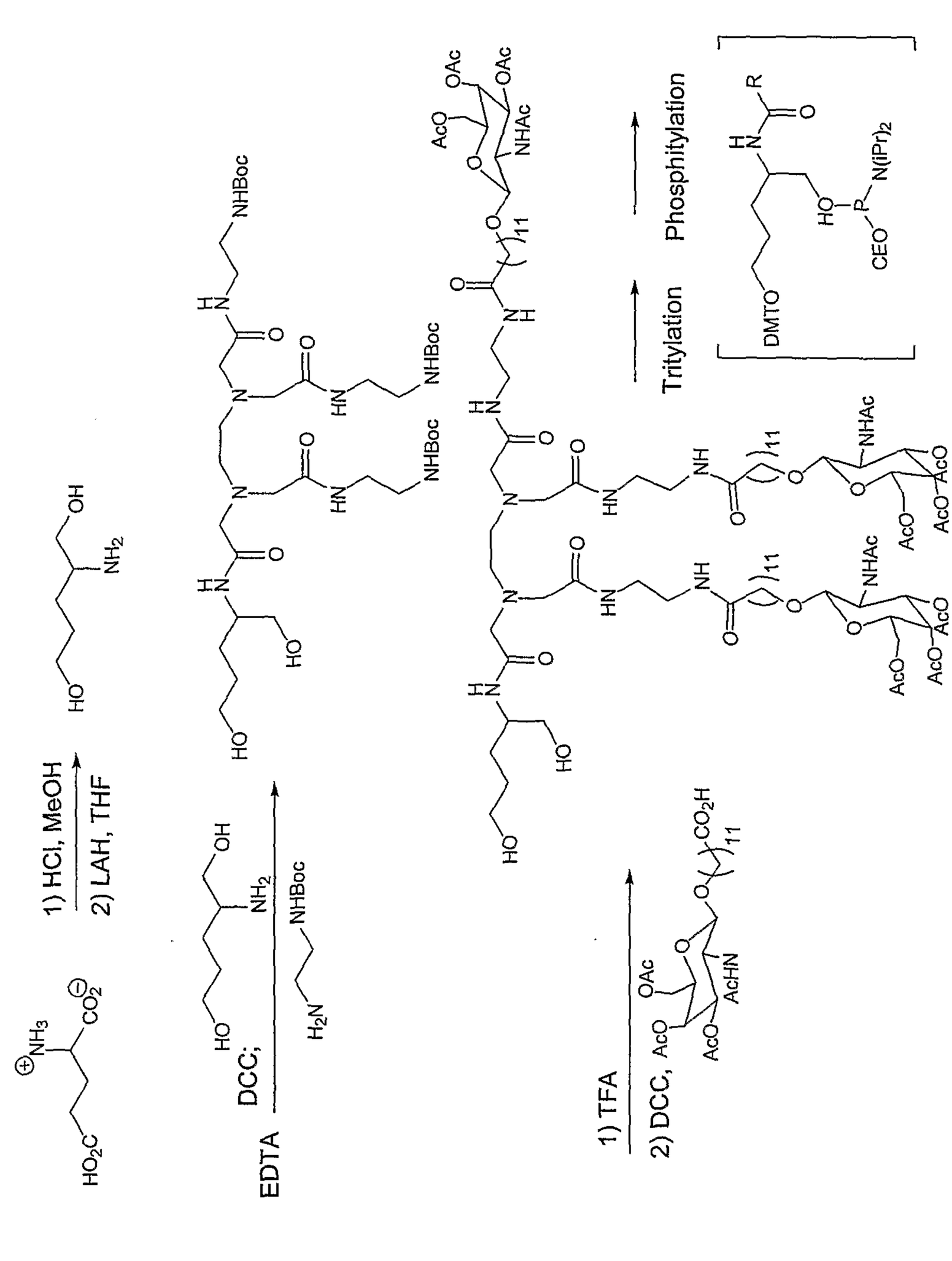


Figure 72: Alternate Synthesis of Tri-Galactosamine Conjugate

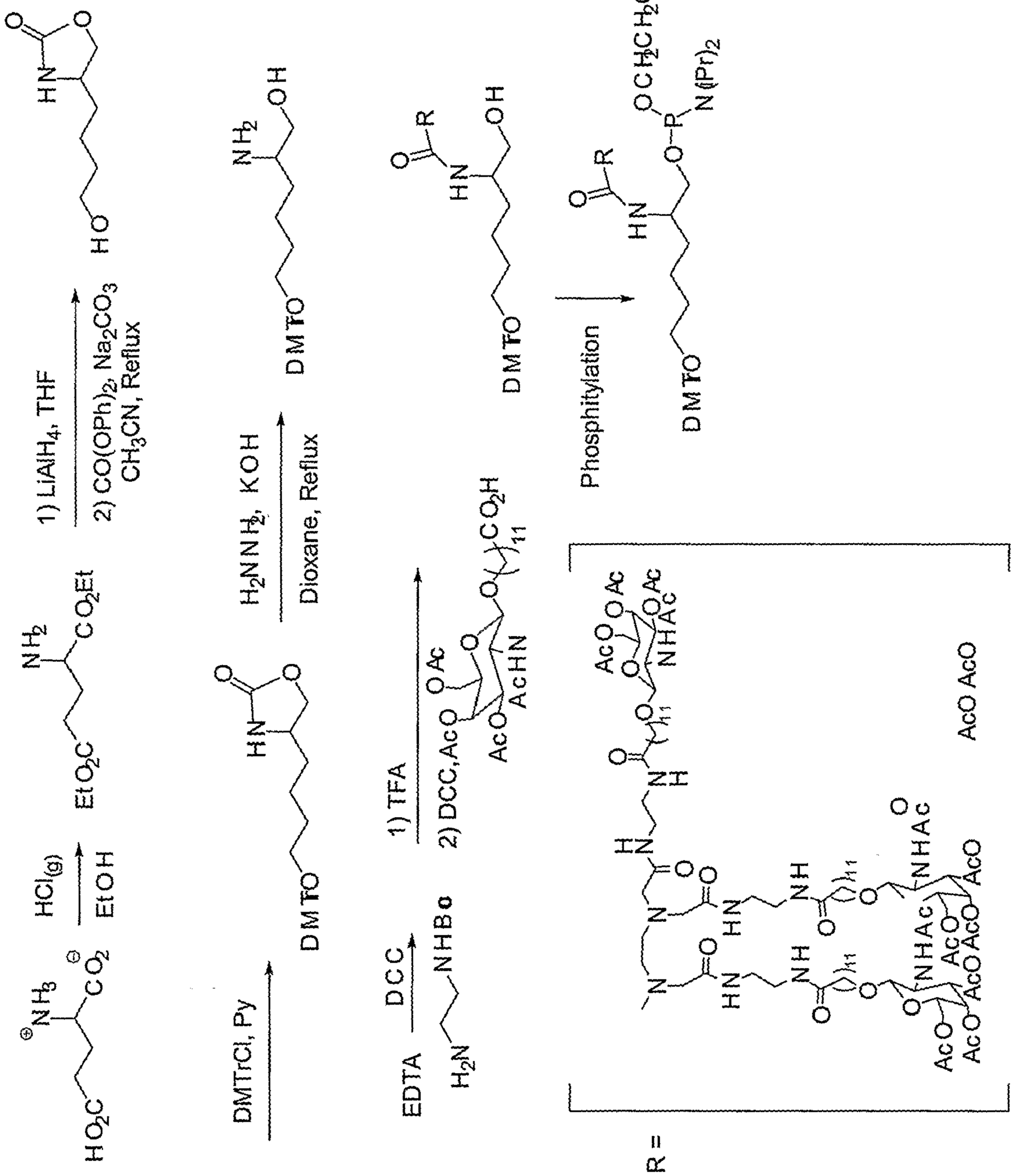


Figure 73: Synthesis of NHS Cholesterol Conjugate

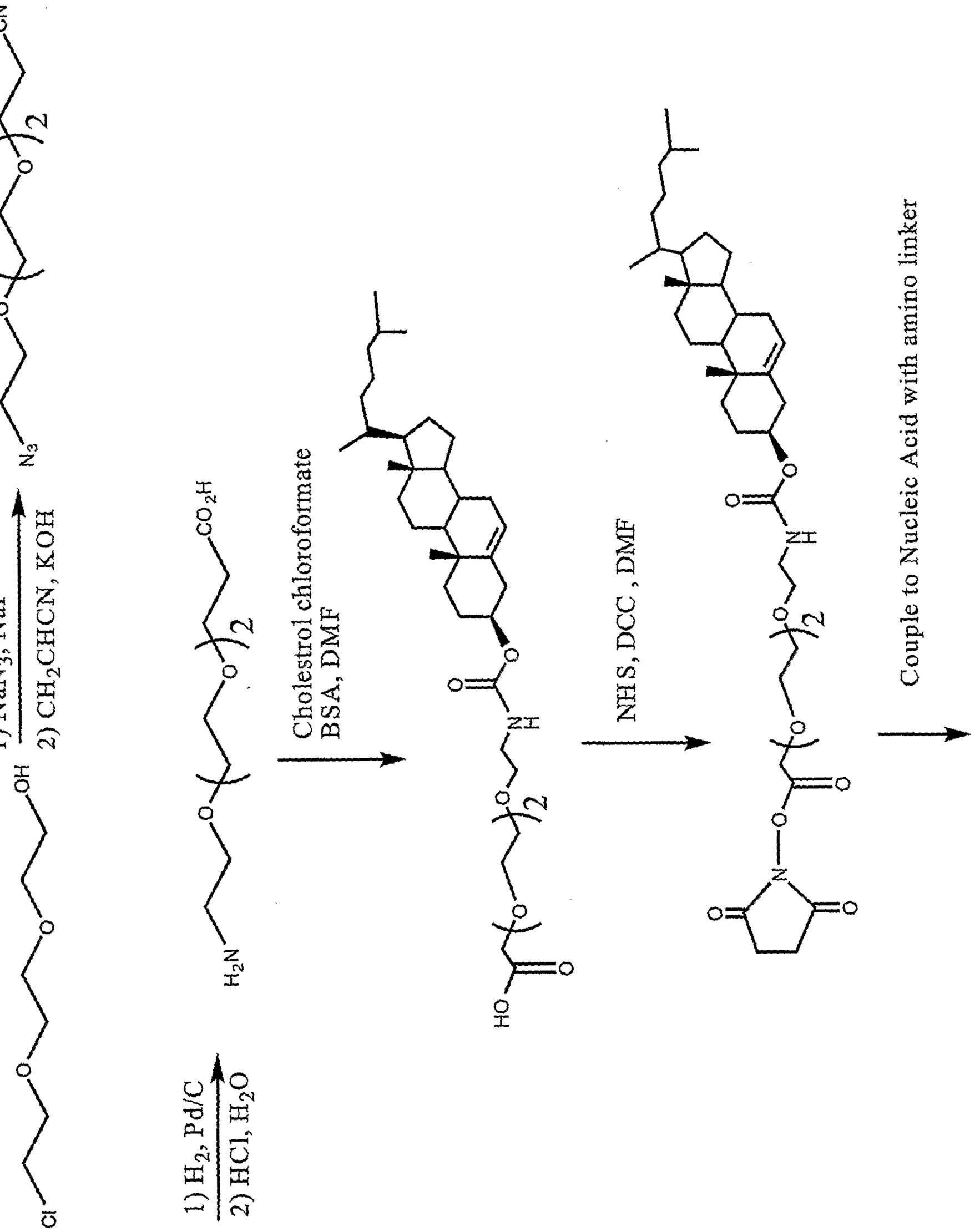


Figure 74: Phosphorylated siNA constructs

Figure 75: 5'-phosphate modifications

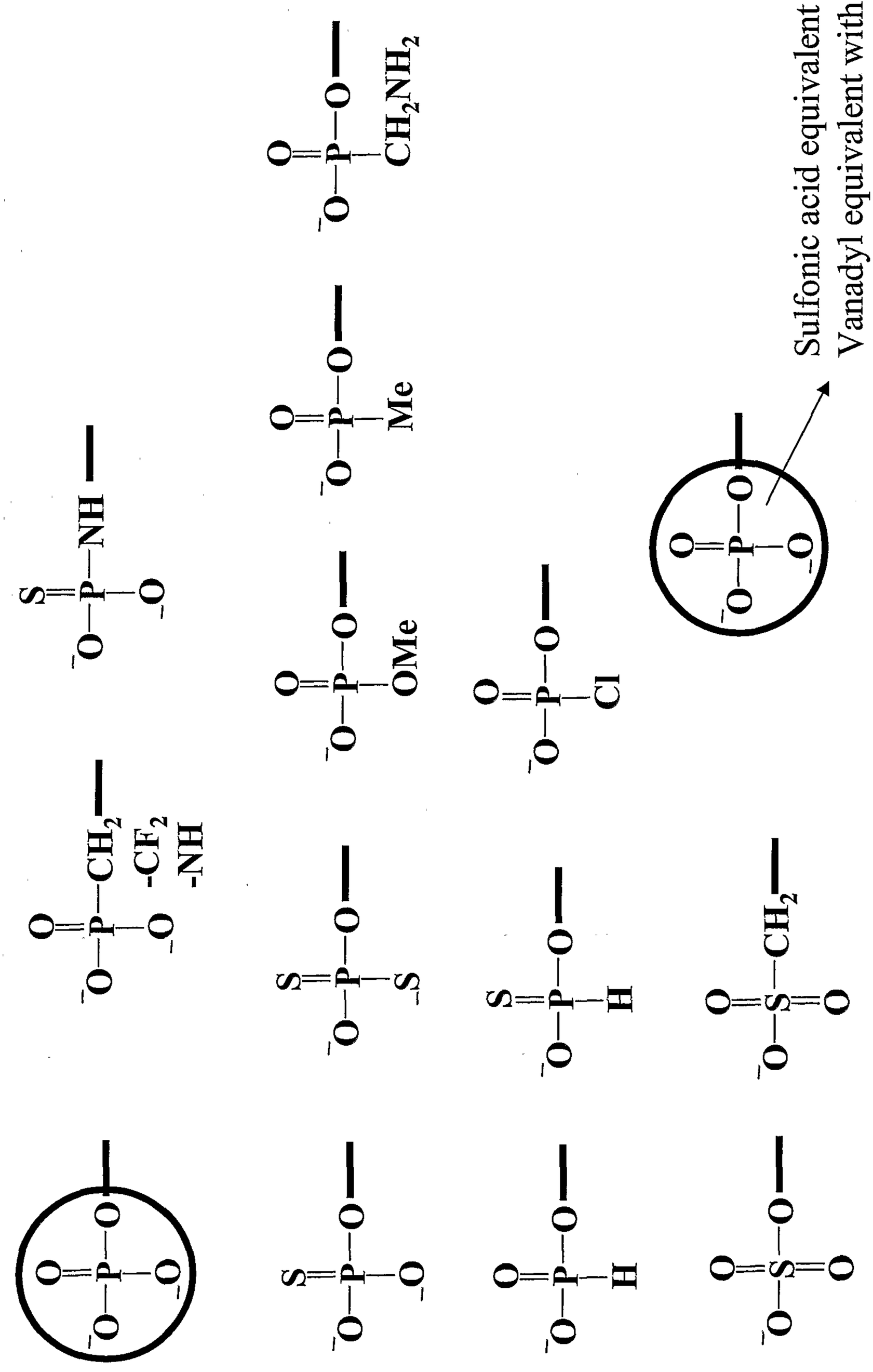
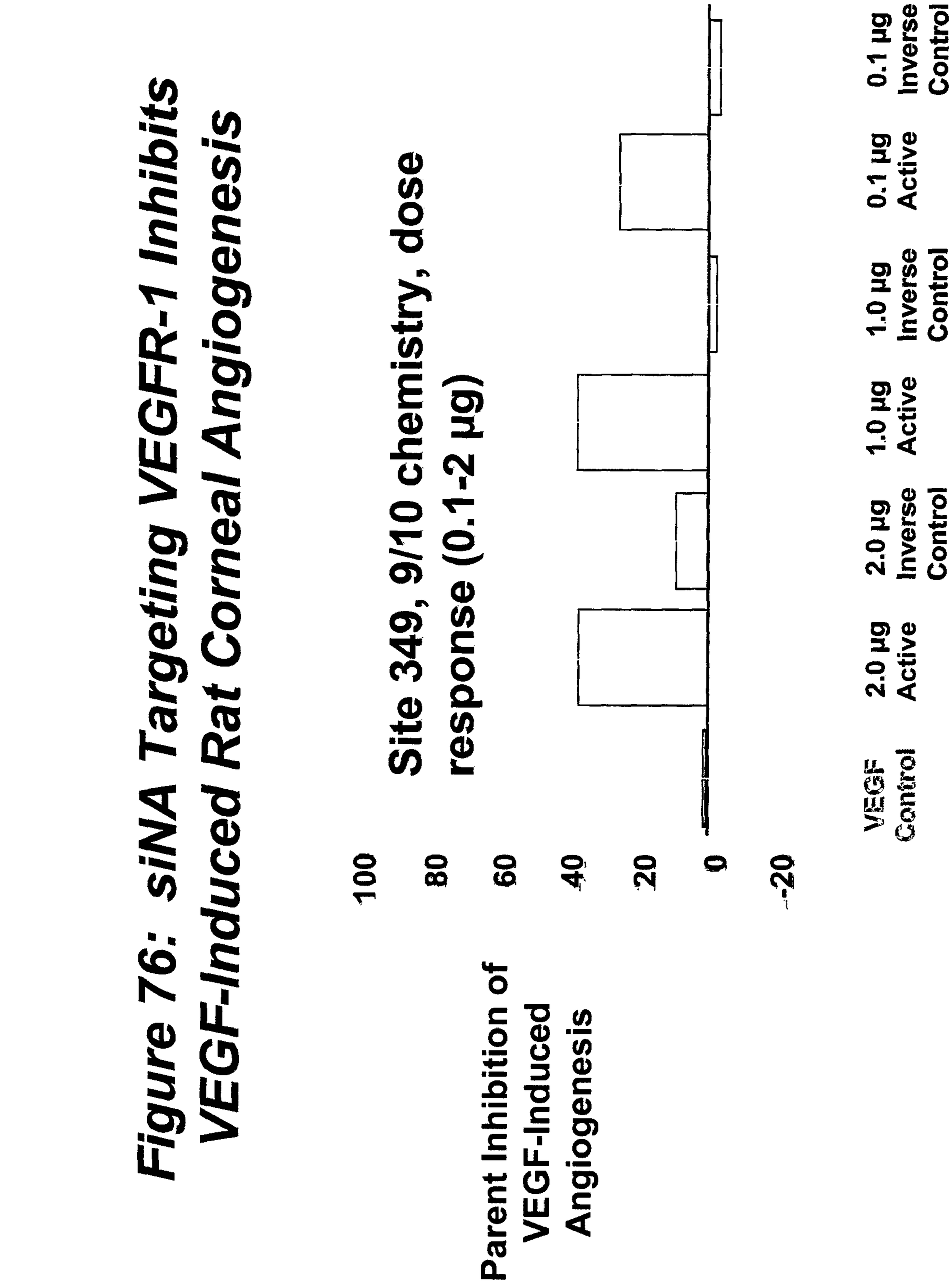


Figure 76: siNA Targeting VEGFR-1 Inhibits VEGF-Induced Rat Corneal Angiogenesis



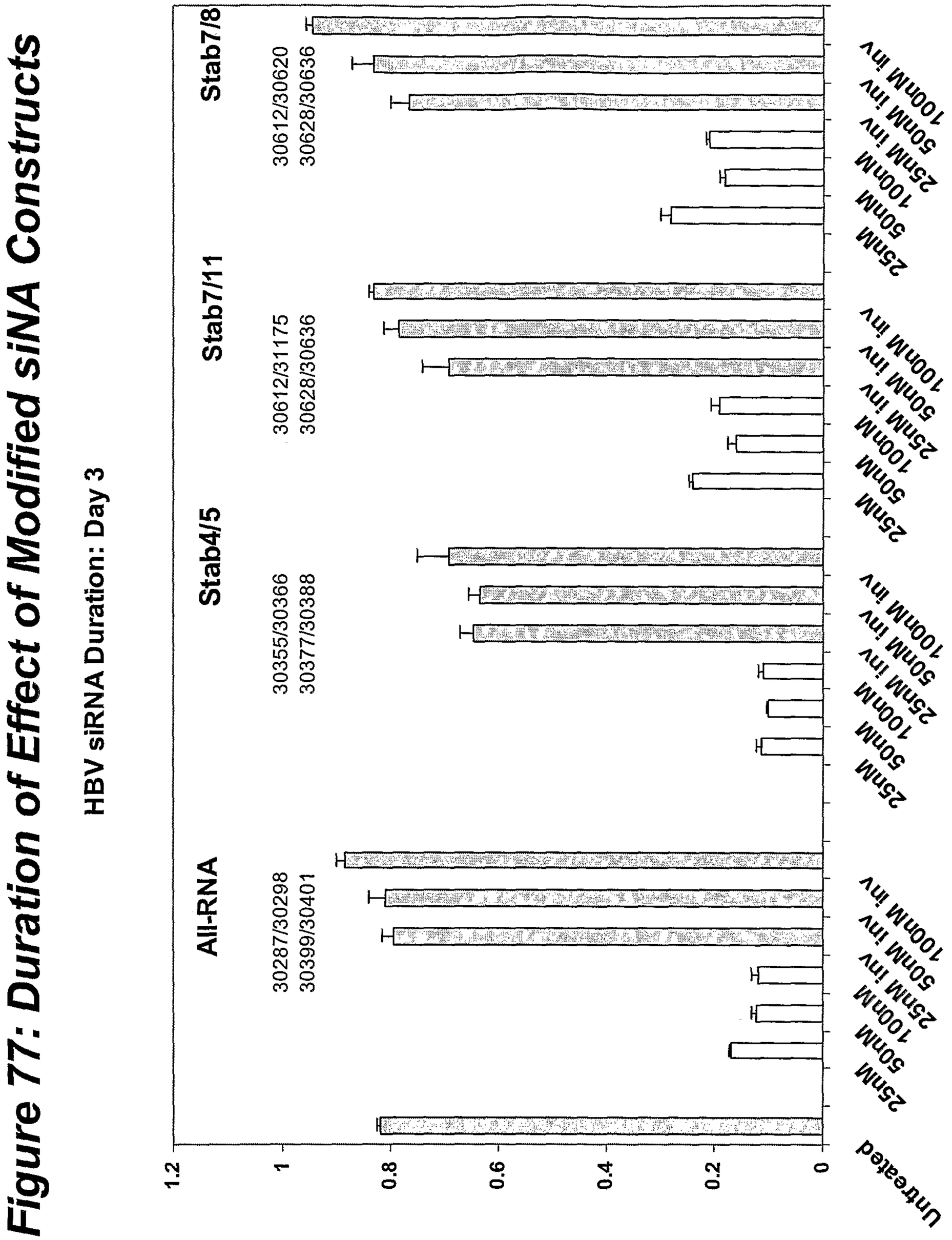


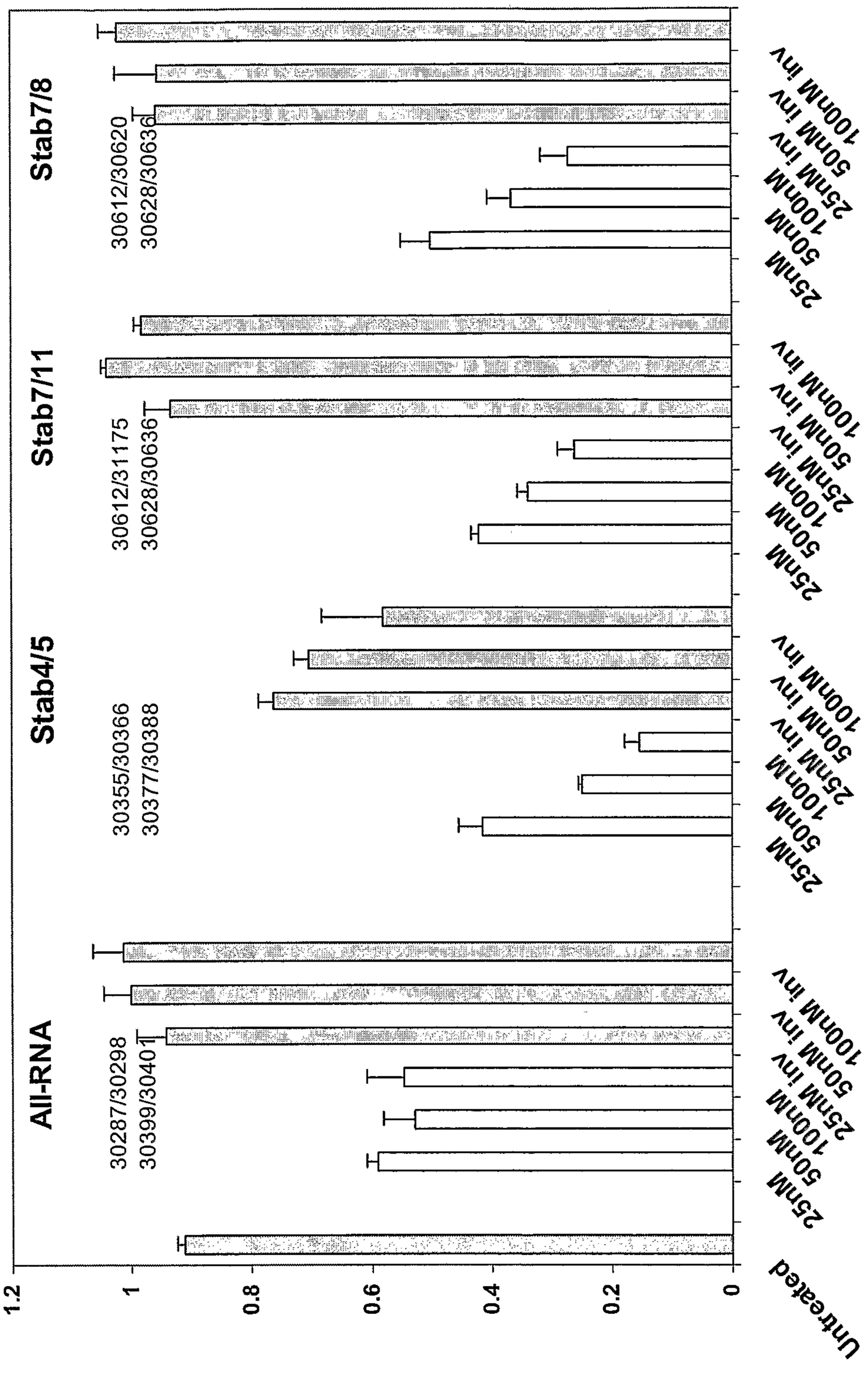
Figure 77: Duration of Effect of Modified siNA Constructs

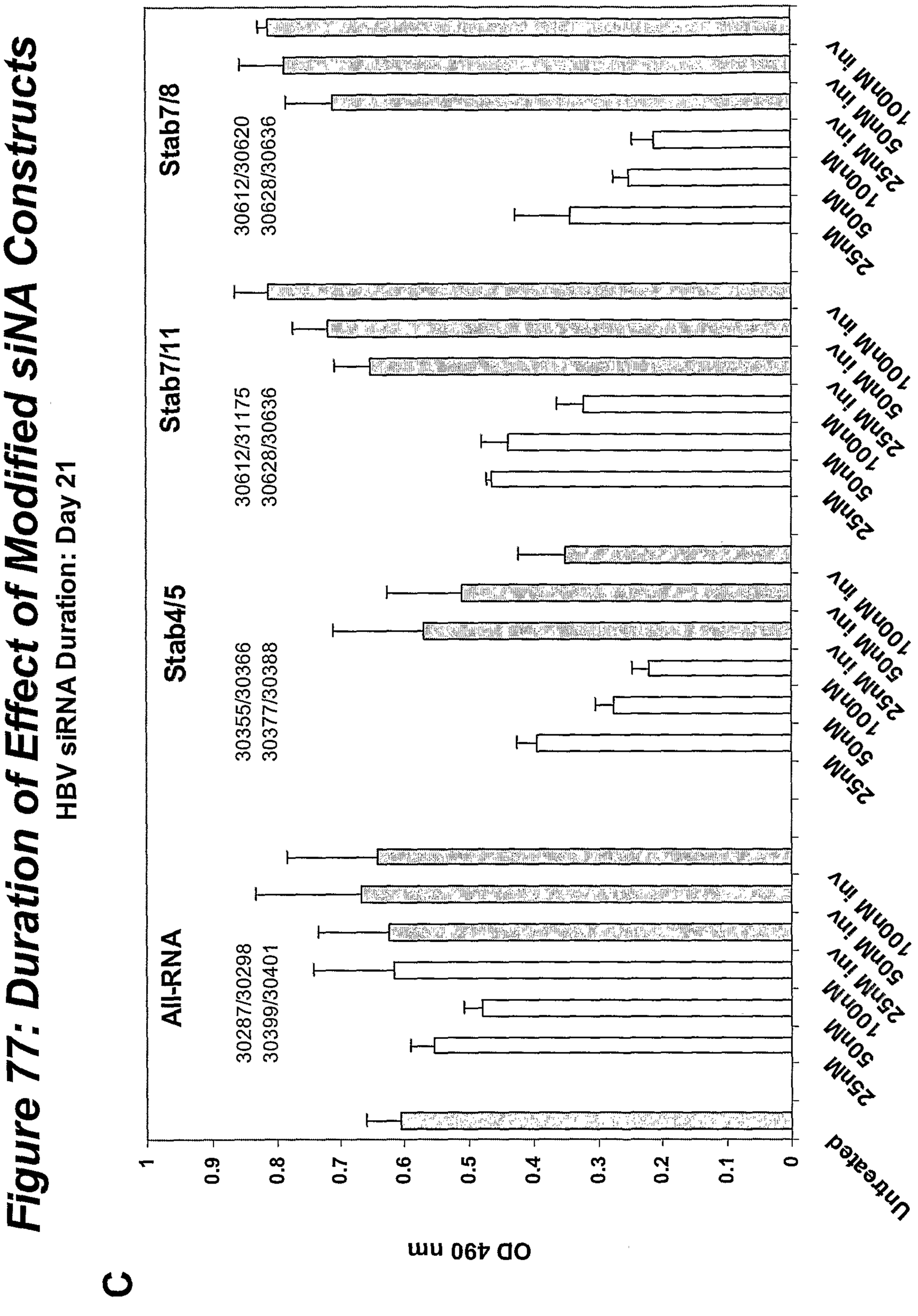
HBV siRNA Duration: Day 3

Figure 77: Duration of Effect of Modified siNA Constructs

HBV siRNA Duration: Day 9

B





C

Figure 77: Duration of Effect of Modified siNA Constructs

HBV siRNA Duration: Day 21

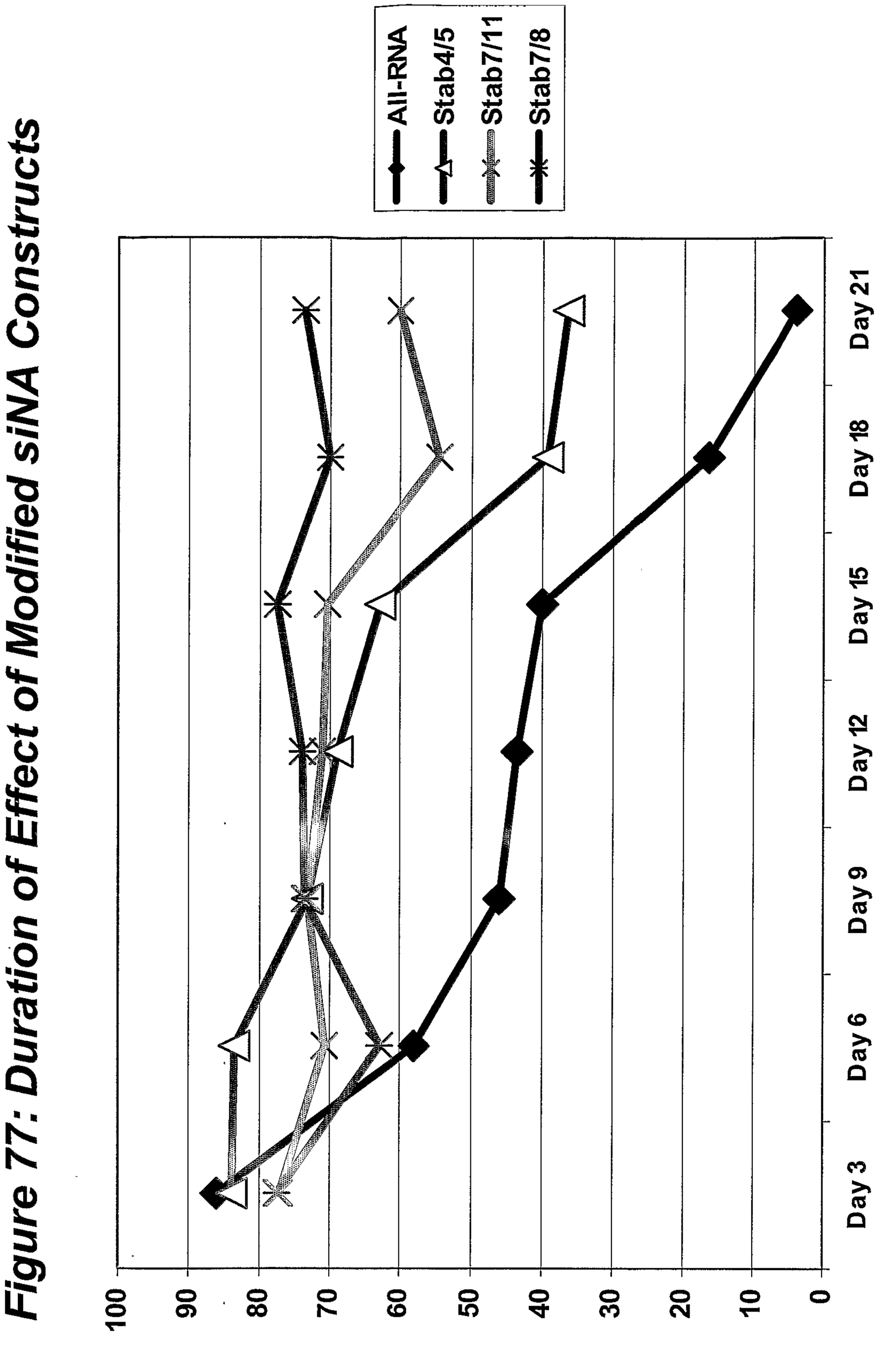


Figure 77: Duration of Effect of Modified siNA Constructs

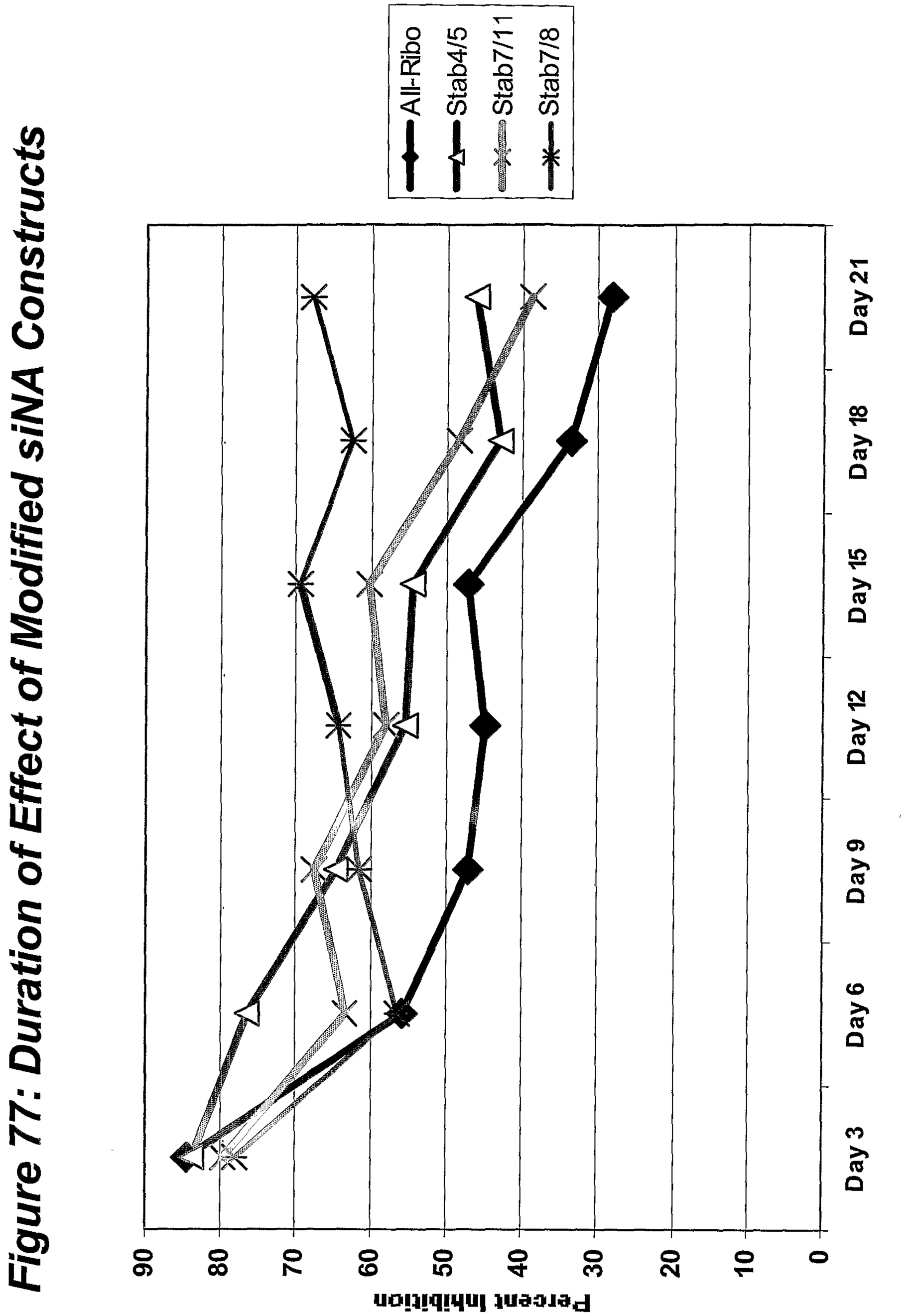


Figure 77: Duration of Effect of Modified siNA Constructs

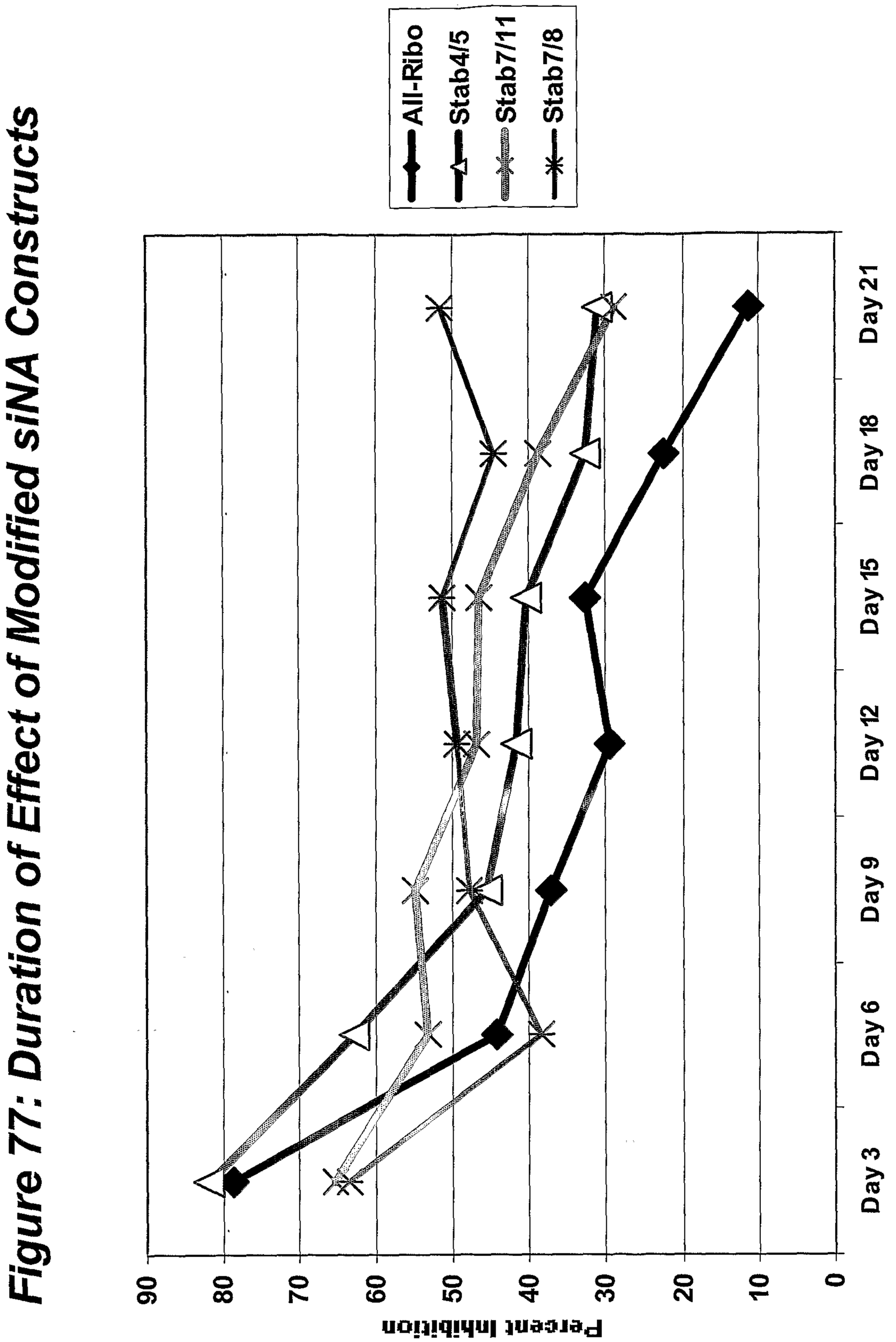
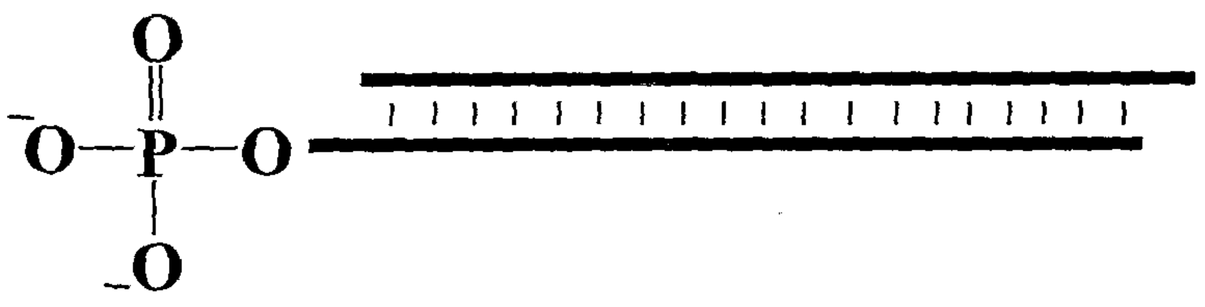
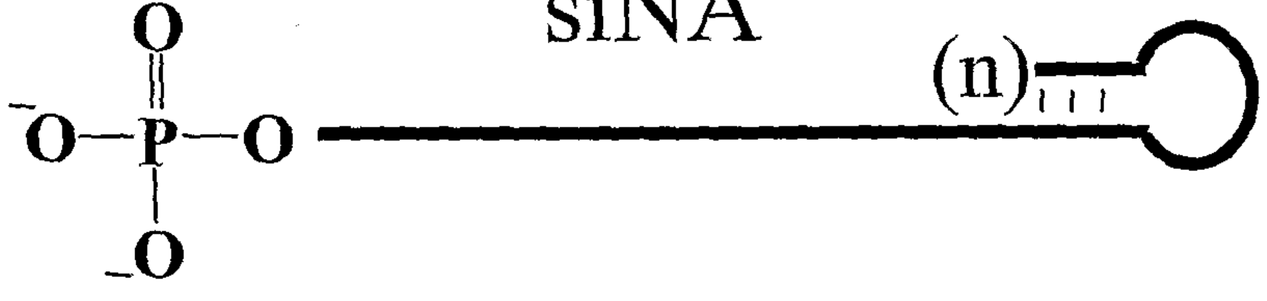


Figure 77: Duration of Effect of Modified siNA Constructs

Figure 78: Phosphorylated siNA constructs

Phosphates can be modified as described herein





Asymmetric hairpin siNA

Asymmetric duplex siNA

(n) = number of base pairs (e.g. 3-18 bp)

Figure 79: 5'-phosphate modifications

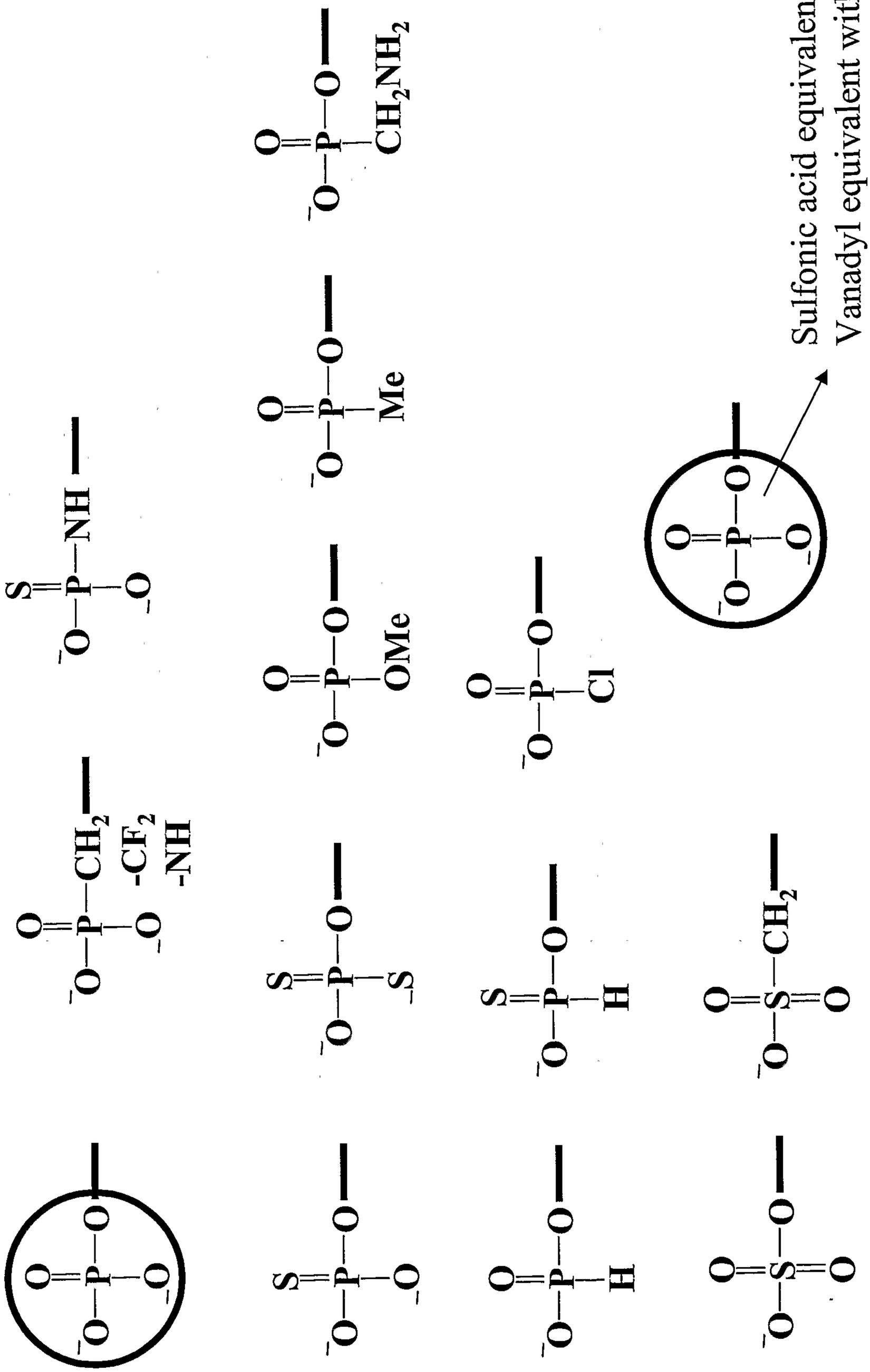


Figure 80: Serum HBV DNA in Mice Treated with siNA Via HDI

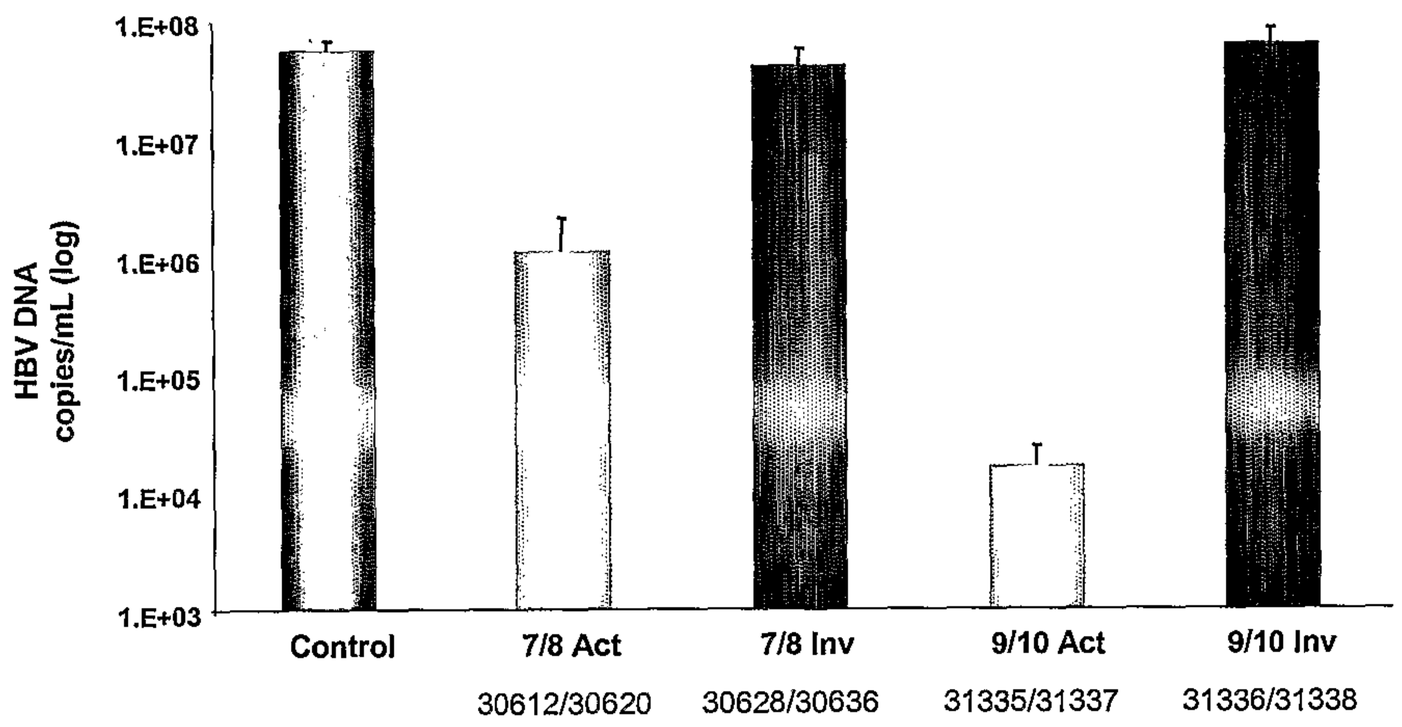


Figure 81: Serum HBsAg in Mice Treated with siNA Via HDI

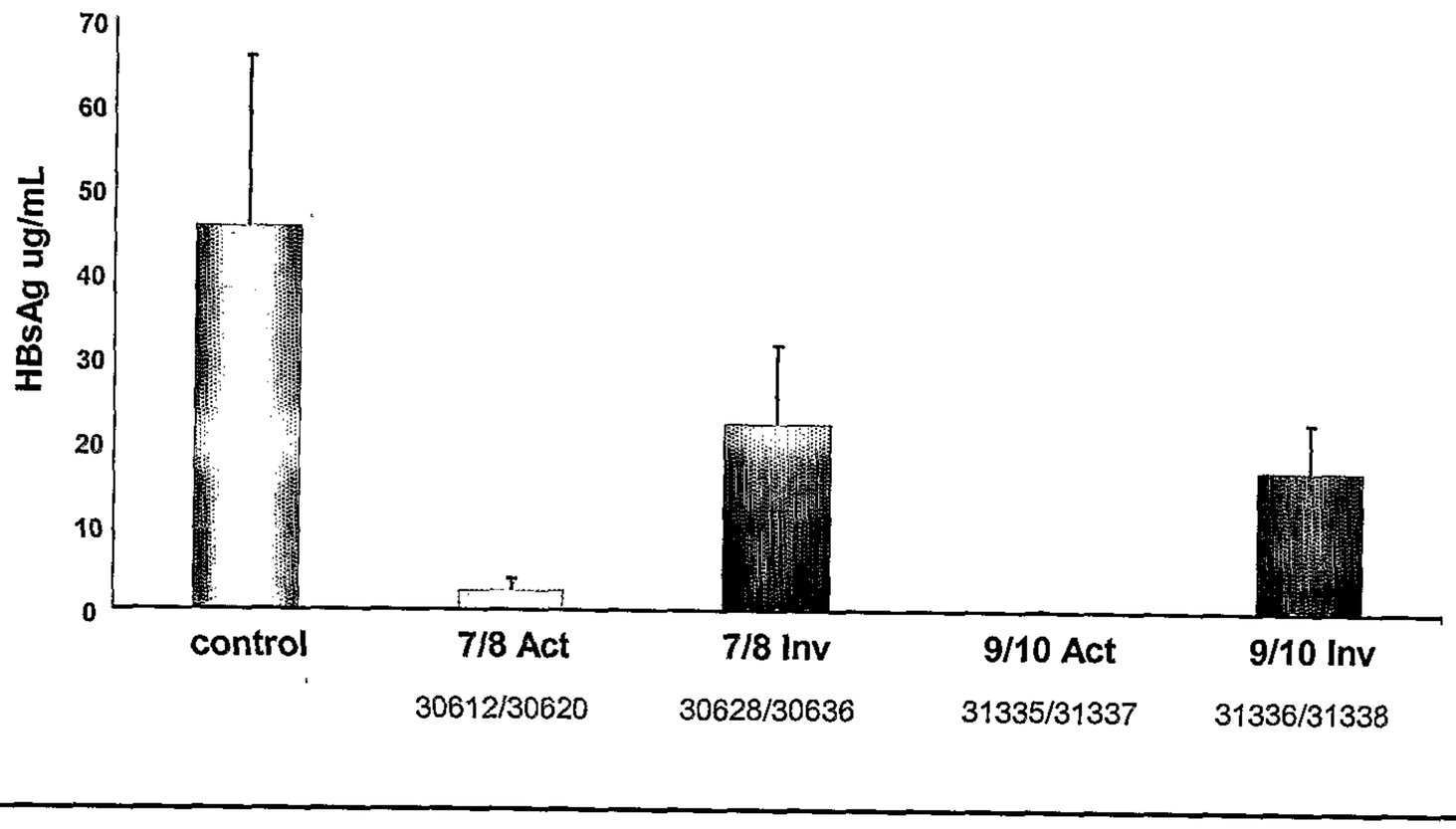
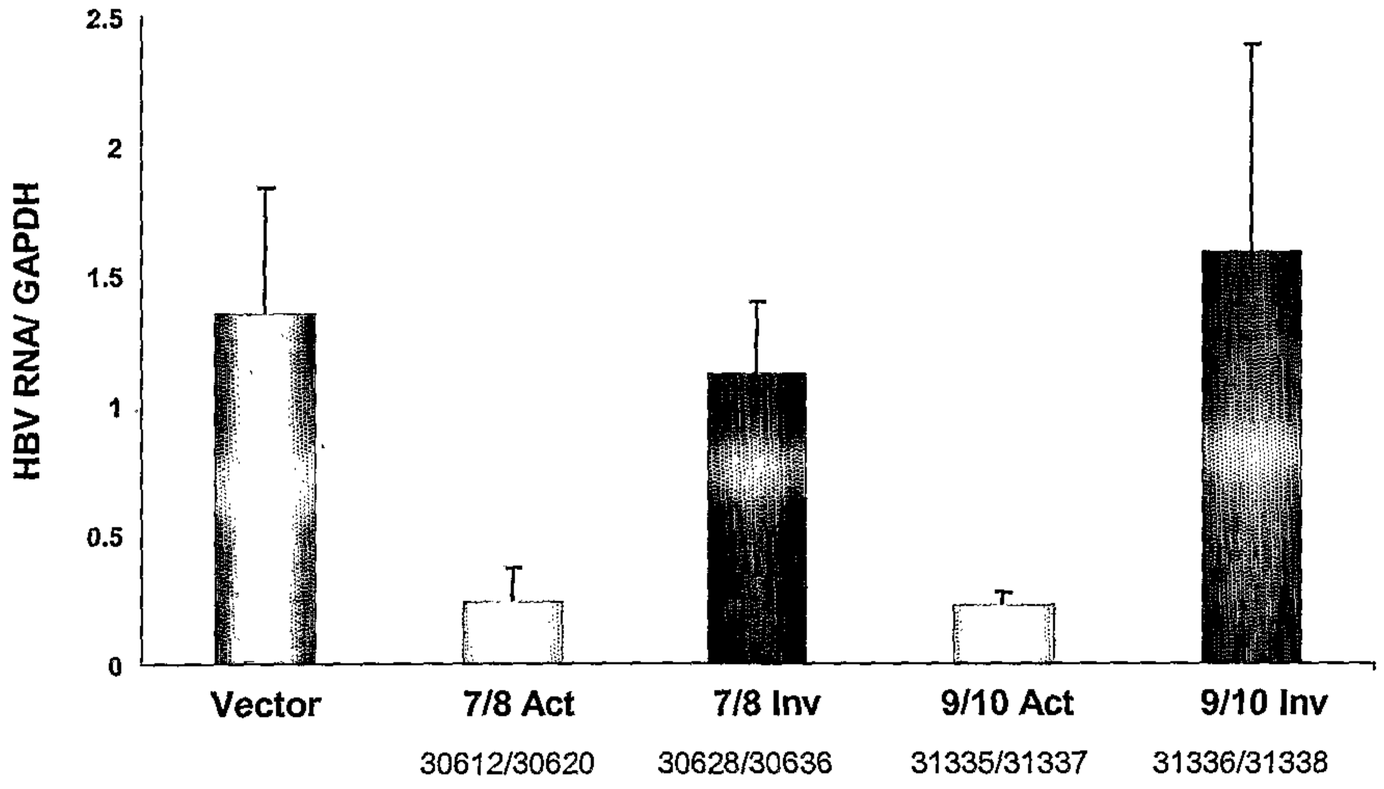


Figure 82: Liver HBV RNA in Mice Treated with siNA Via HDI



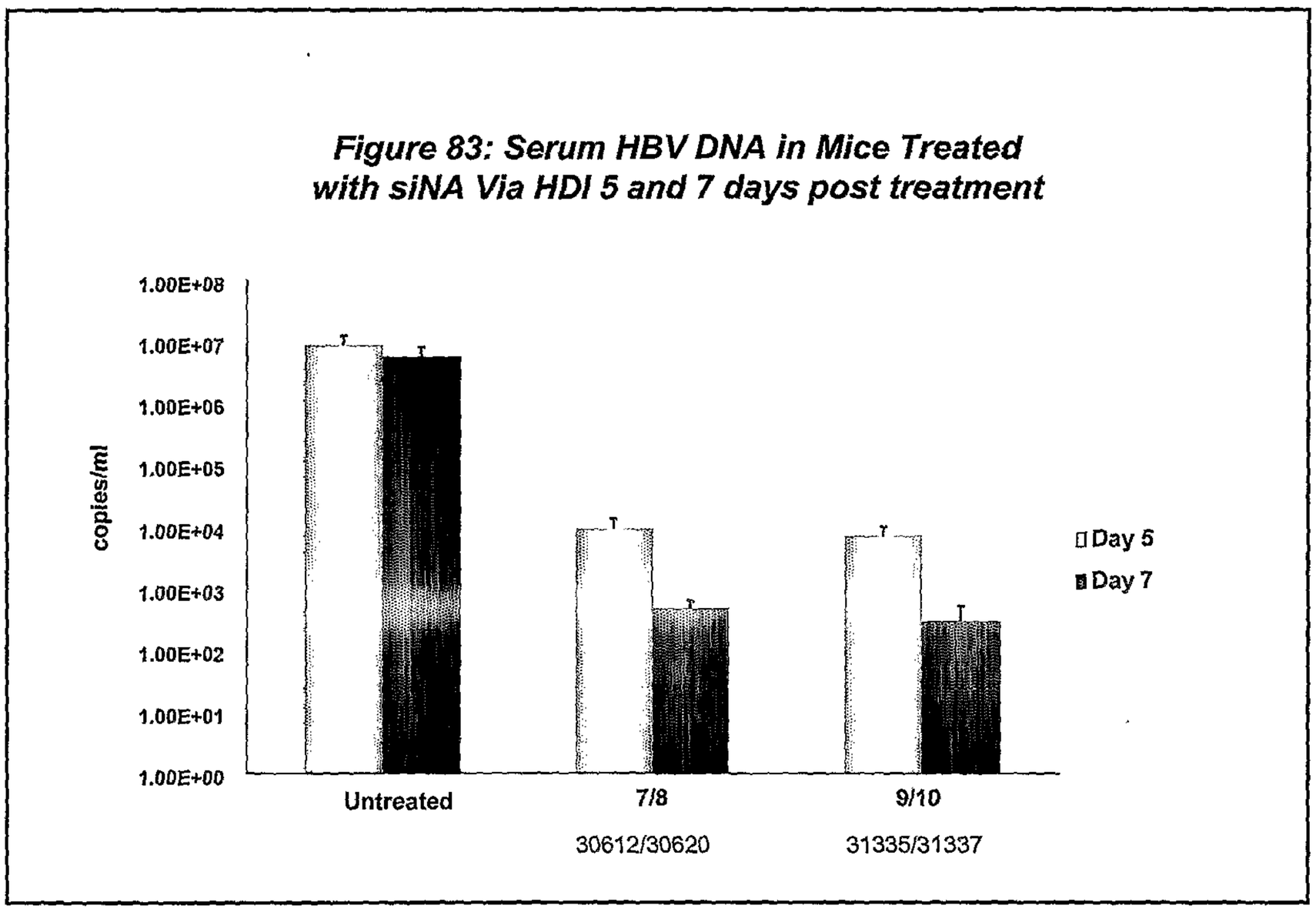


Figure 83: Serum HBV DNA in Mice Treated with siNA Via HDI 5 and 7 days post treatment
copies/ml
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Day 5
Day 7
Untreated
7/8
30612/30620
9/10
31335/31337

Figure 84: Luciferase Dose Response of select active siNA constructs

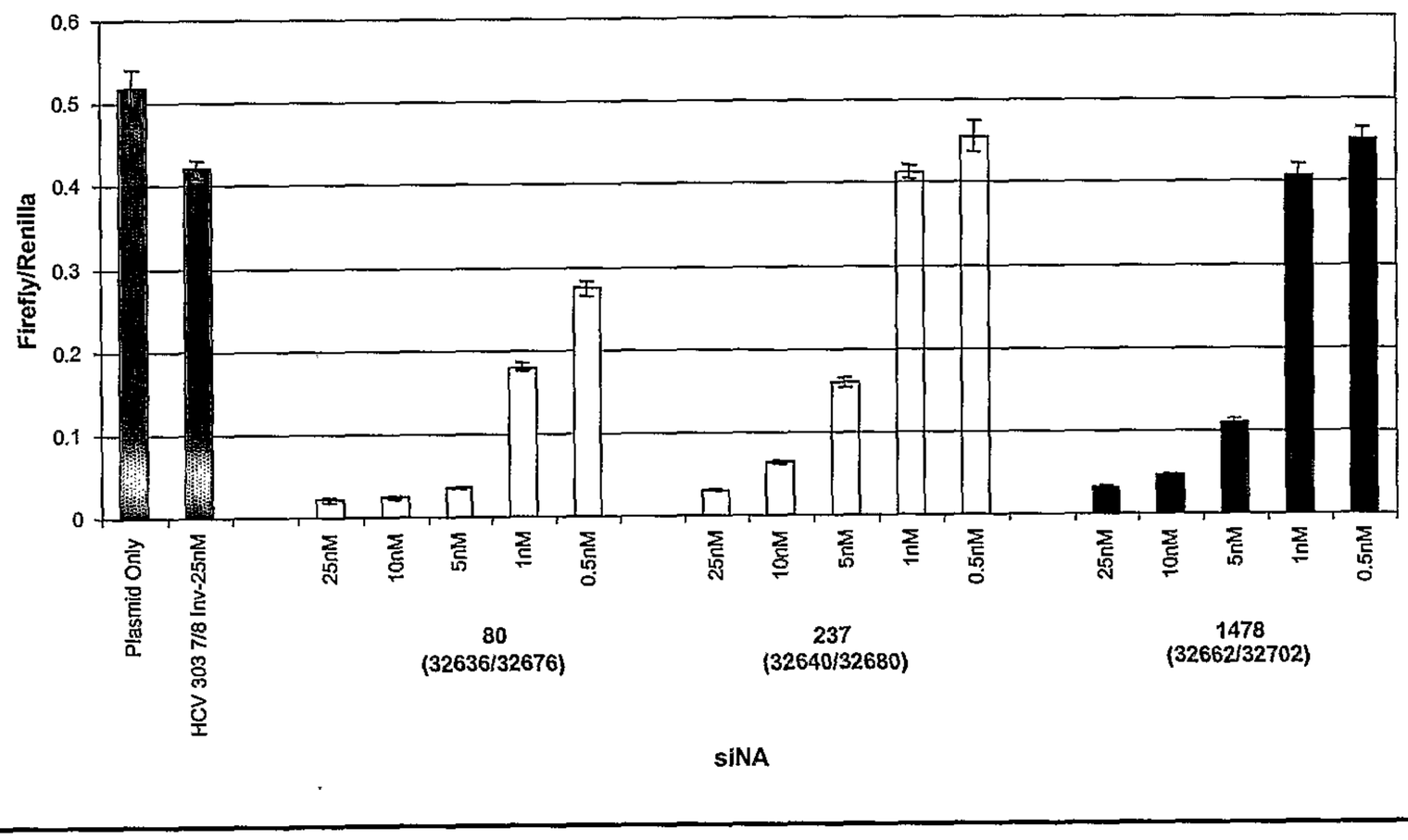
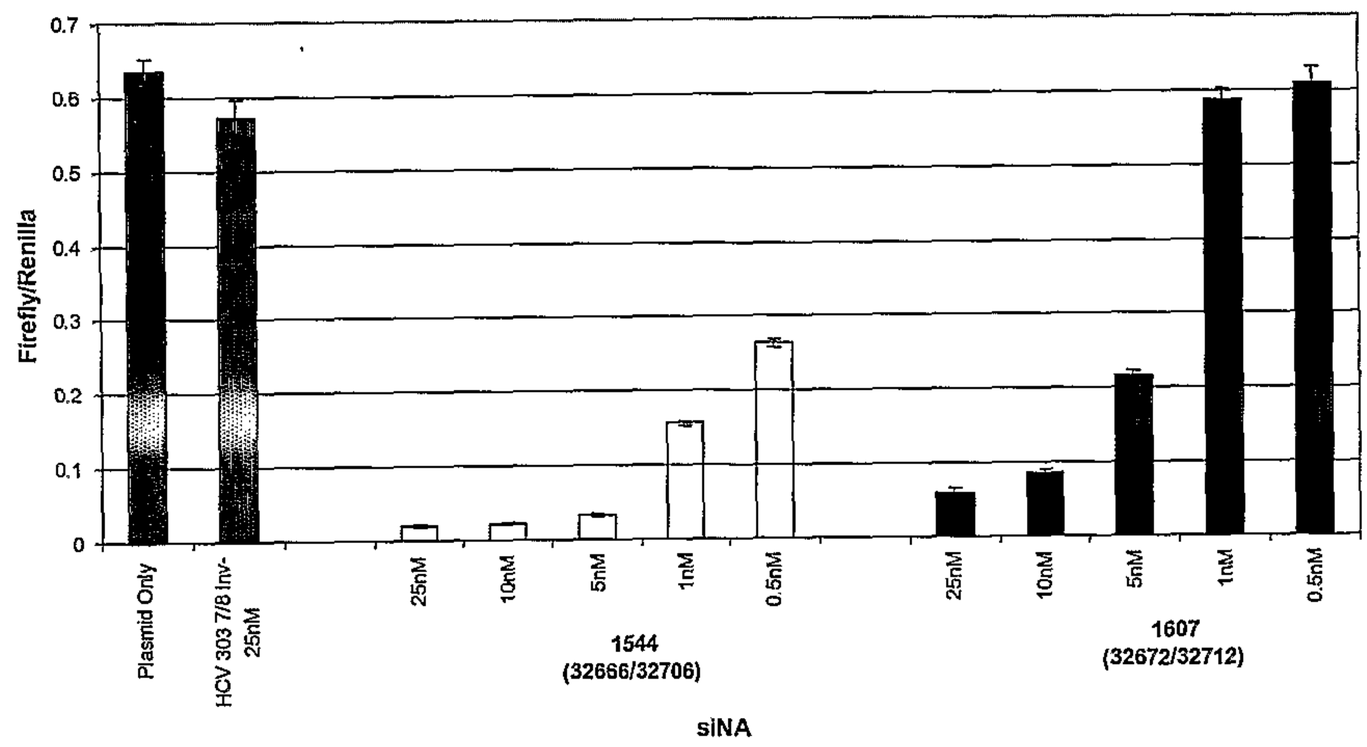


Figure 85: Luciferase Dose Response of select active siNA constructs



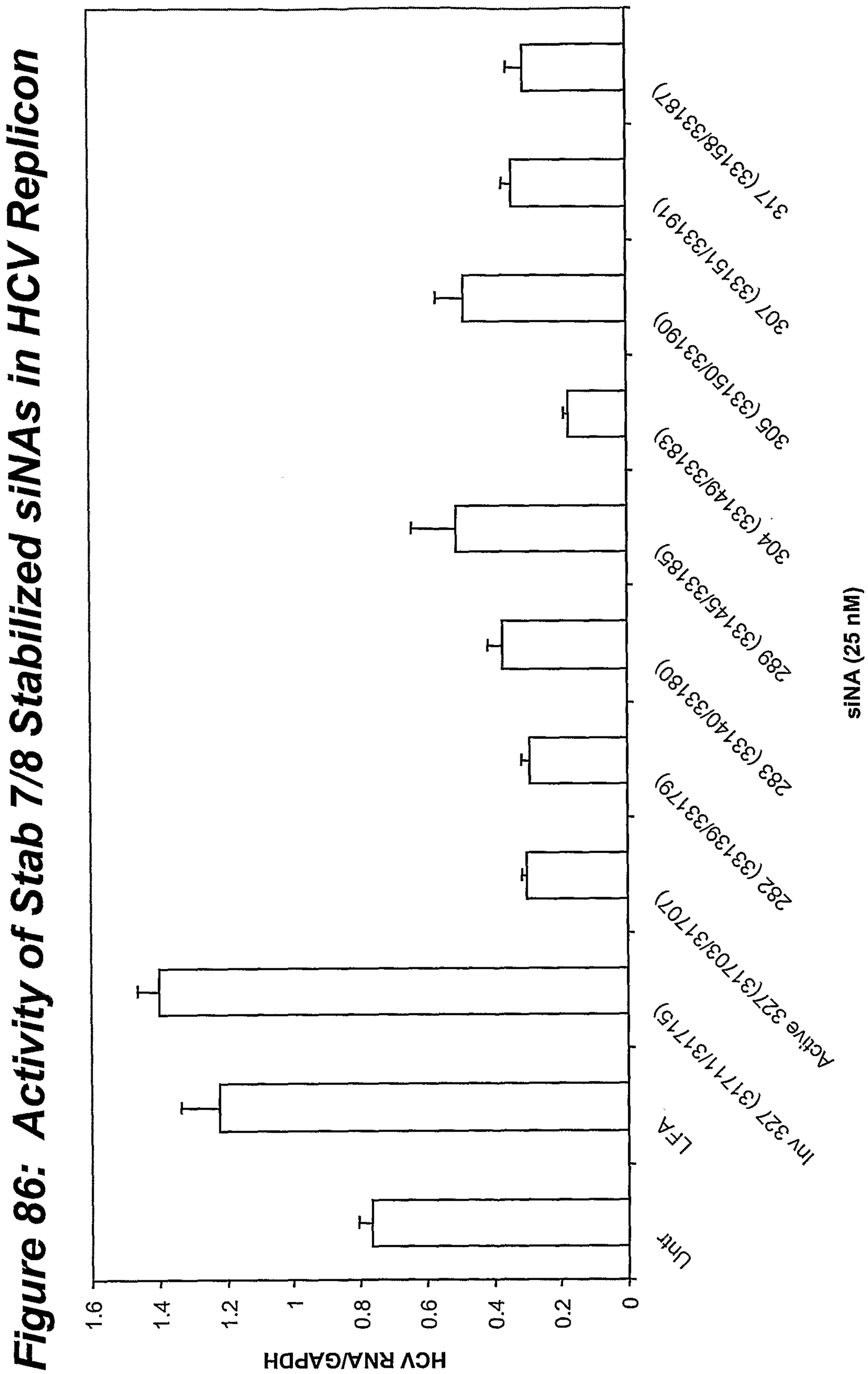


Figure 86: Activity of Stab 7/8 Stabilized siNAs in HCV Replicon

Figure 87: Activity of Stabilized 7/8 siNAs Against HBV in HepG2 Cells

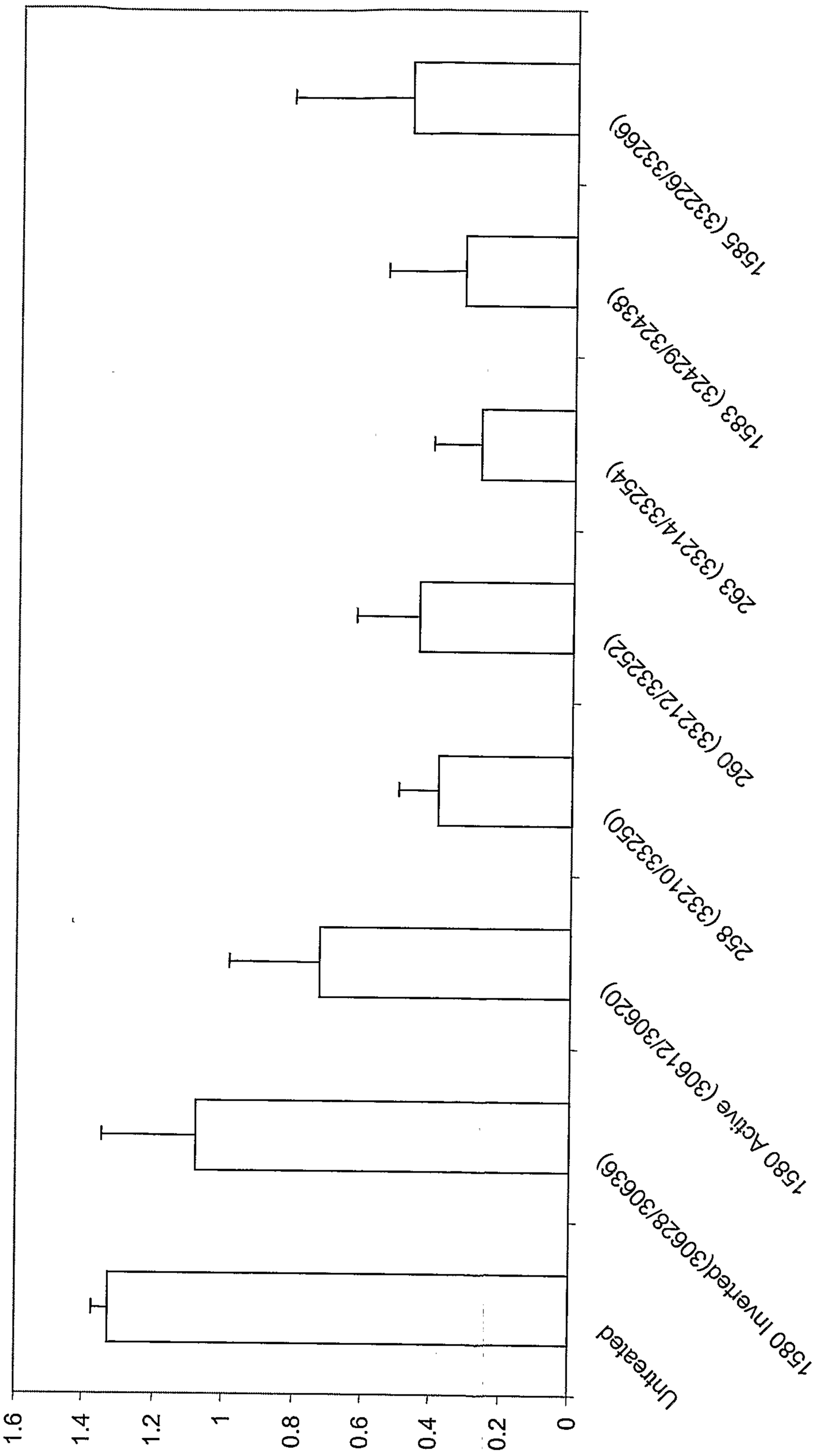


Figure 88: HBV/siNA to site 1580 Combination Constructs

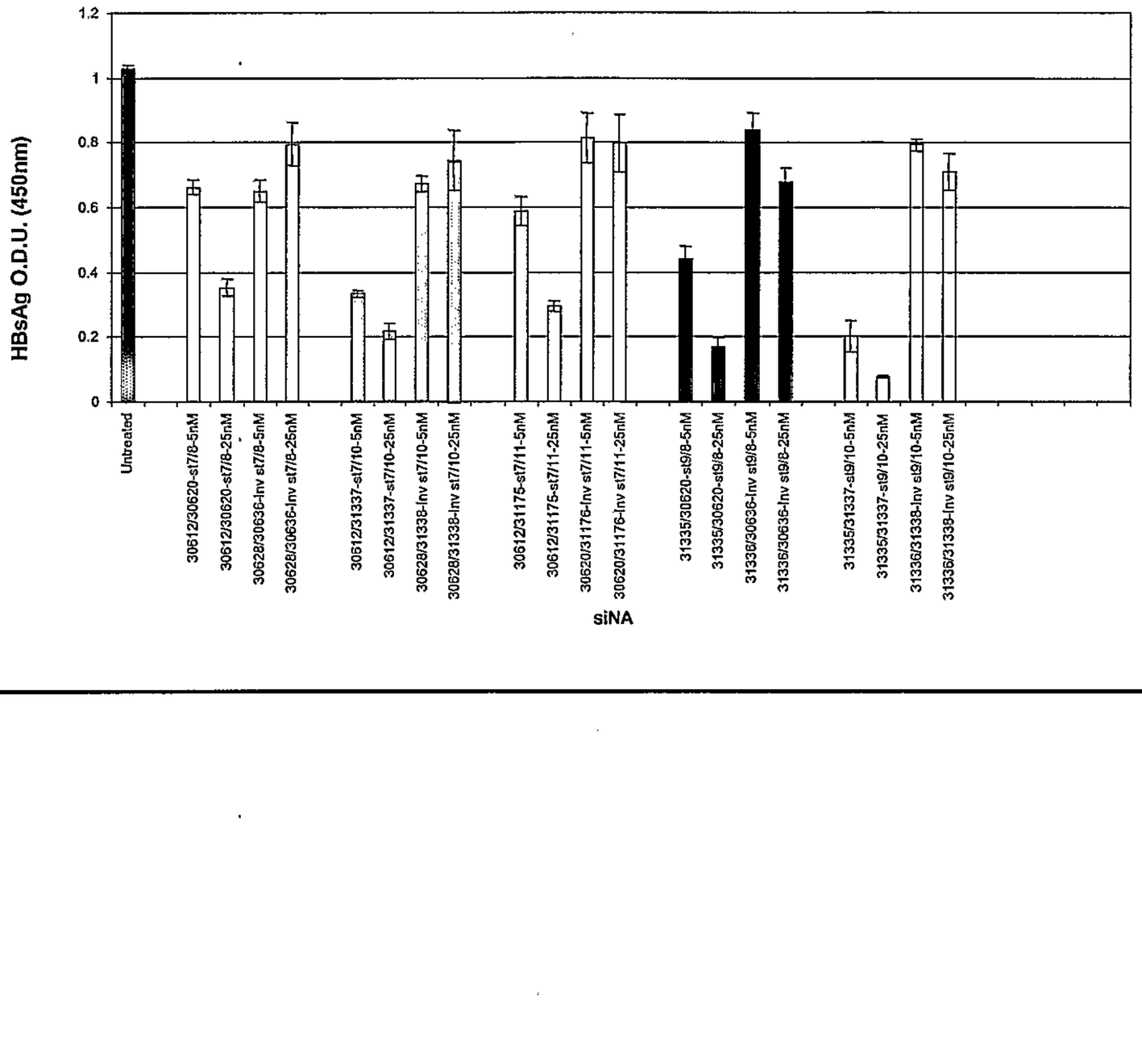


Figure 89: HBV/siNA to site 1580 Combination Constructs

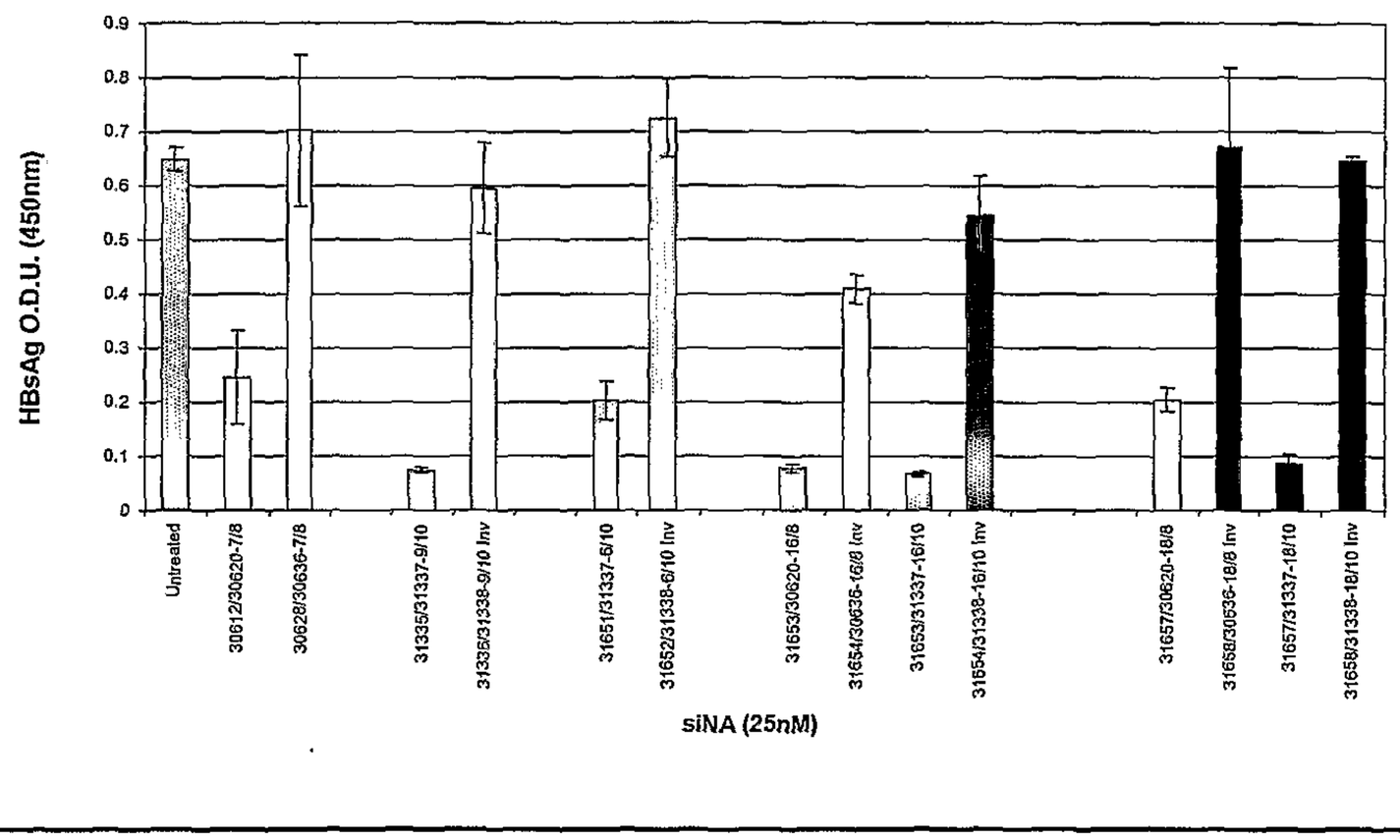
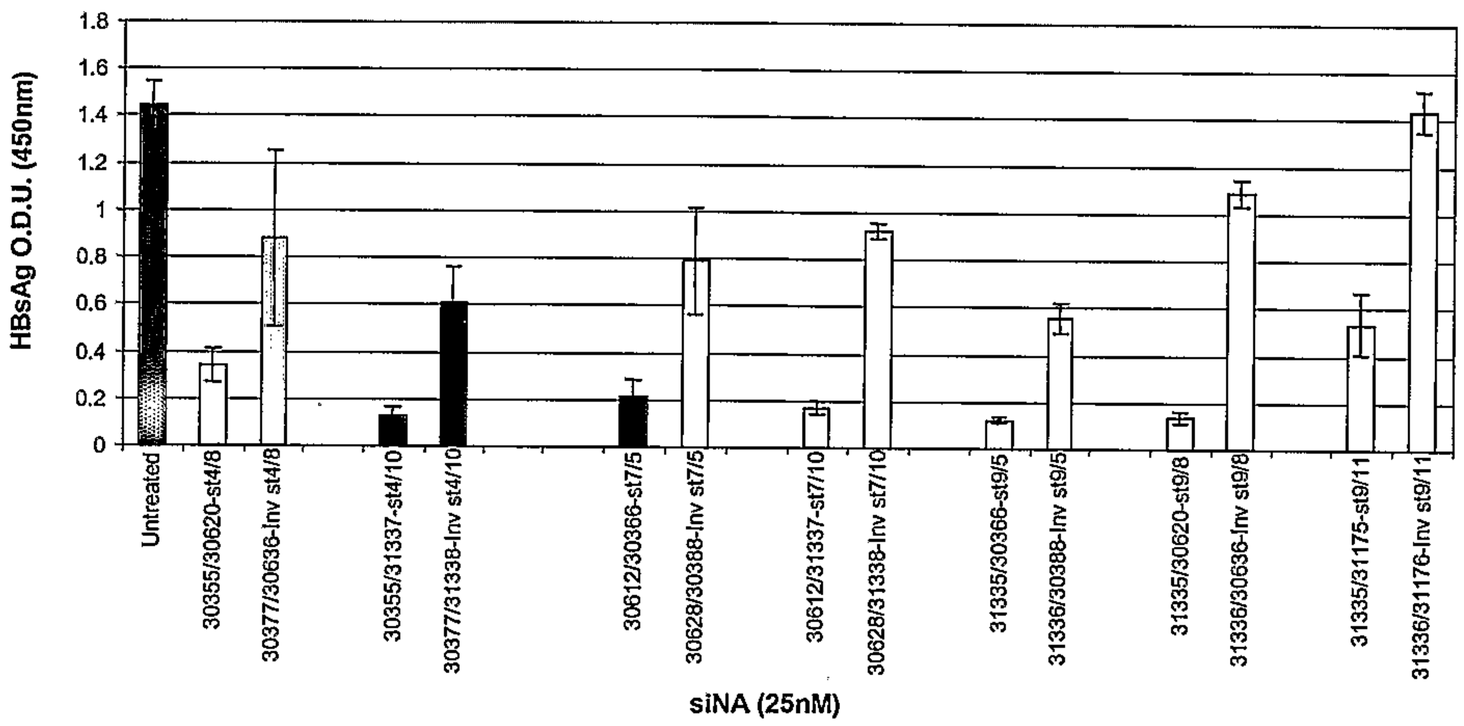


Figure 90: HBV/siNA to site 1580 Combination Constructs



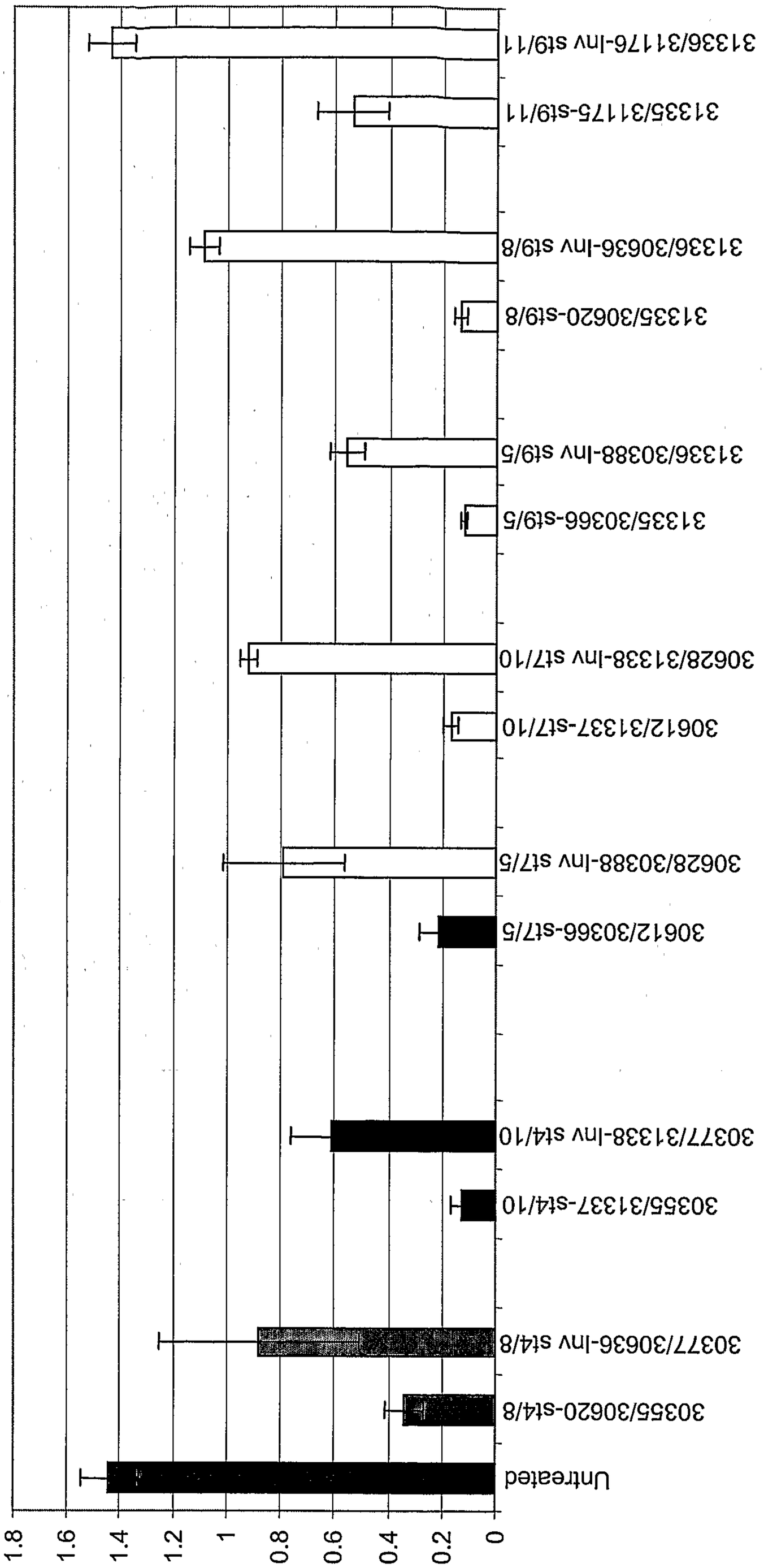


Figure 90: HBV/siNA to site 1580 Combination Constructs
HBsAg O.D.U. (450nm)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Untreated
30355/30620-st4/8
30377/30636-Inv st4/8
30355/31337-st4/10
30377/31338-Inv st4/10
30612/30366-st7/5
30628/30388-Inv st7/5
30355/30620-st9/8
30336/30636-Inv st9/8
30612/31337-st7/10
30628/31338-Inv st7/10
30355/30366-st9/5
30336/30388-Inv st9/5
siNA (25nM)
31335/31175-st9/11
31336/31176-Inv st9/11

Figure 91: PEI-Peg-Gal (3:1): Demonstration of Activity & Specificity HBV DNA

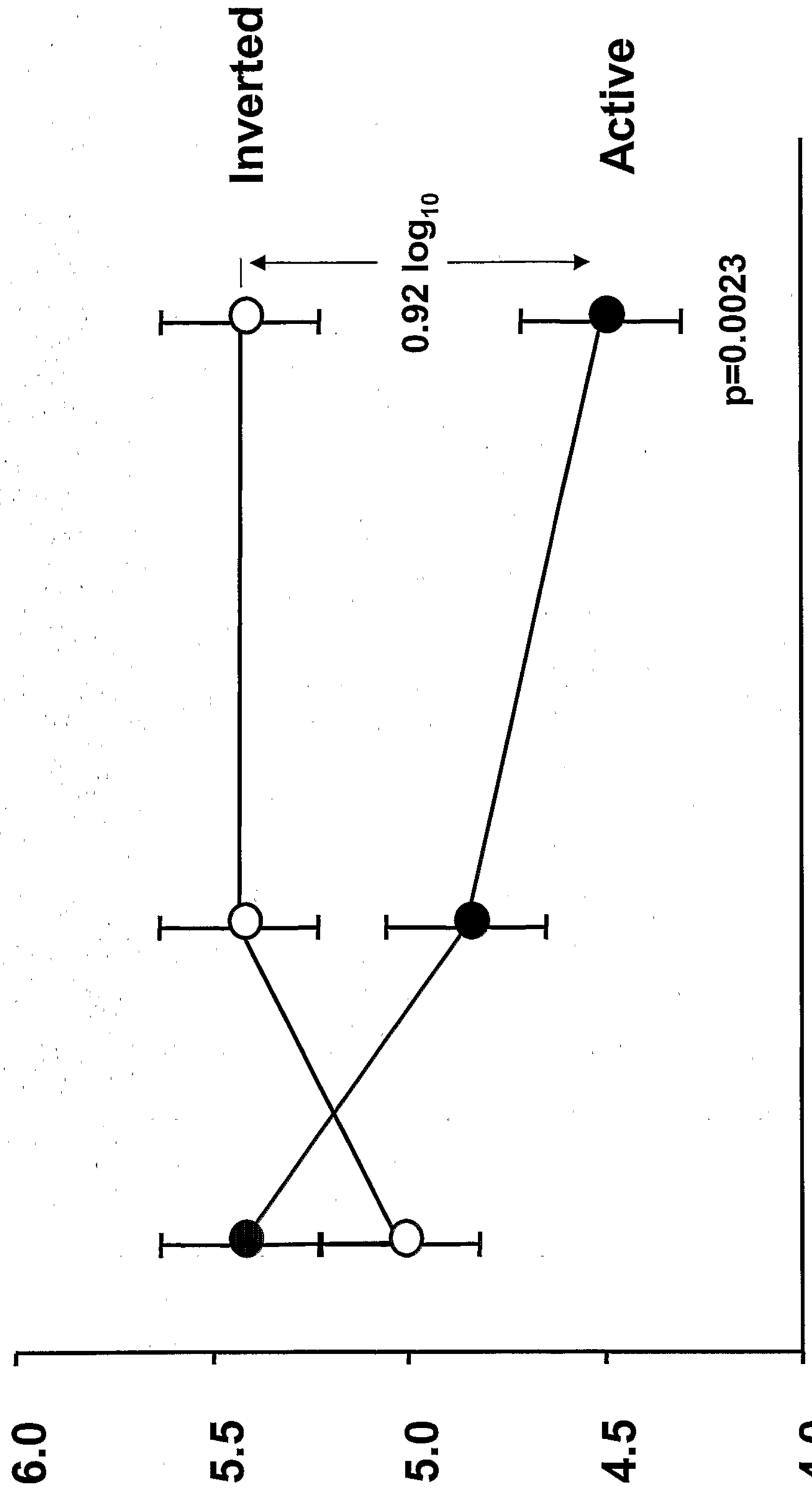


Figure 92: PEI-Peg-Gal (3:1): Demonstration of Activity & Specificity HBsAg

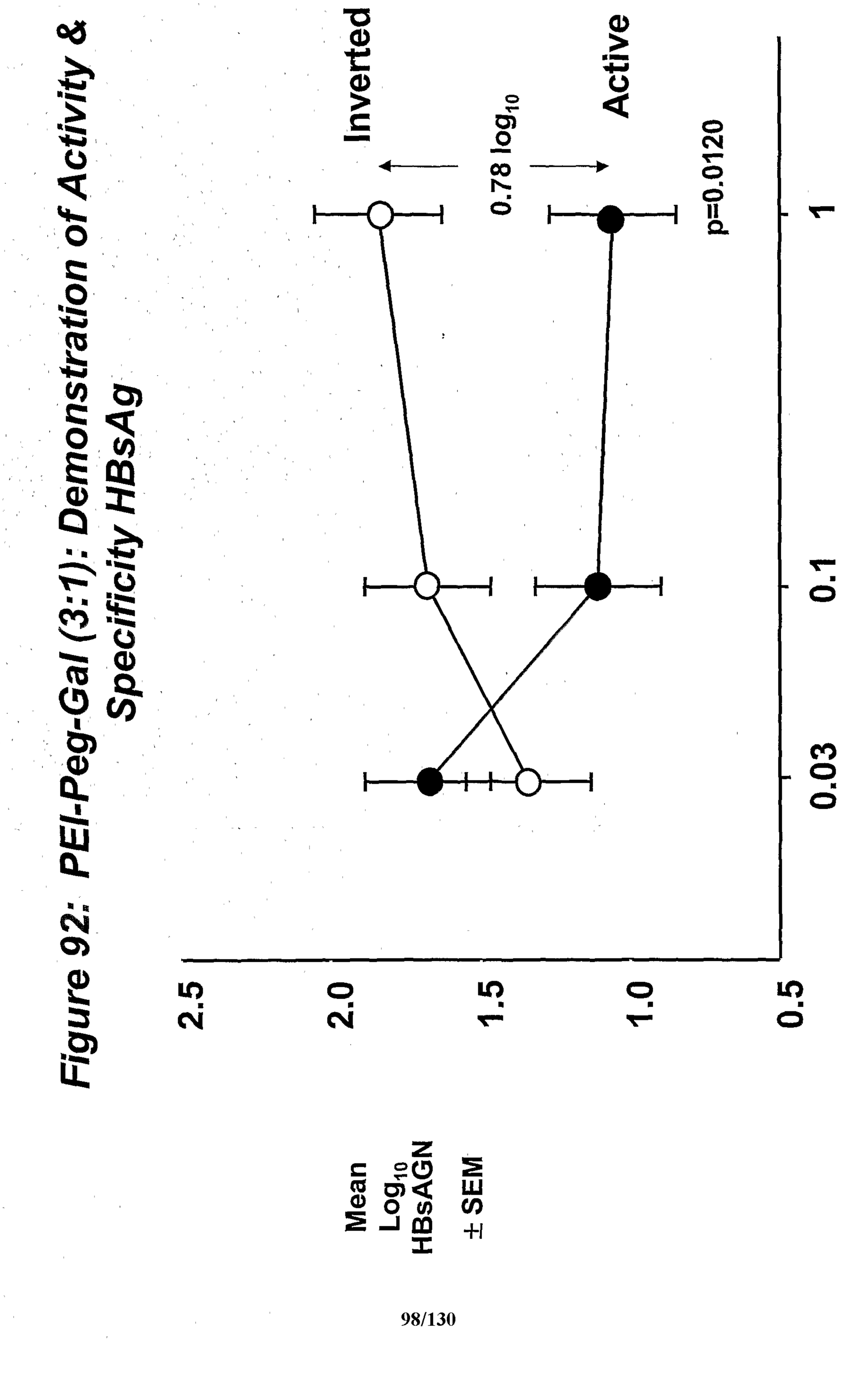


Figure 93: General Structure of PEI-PEG-TriGal with a lipid linker

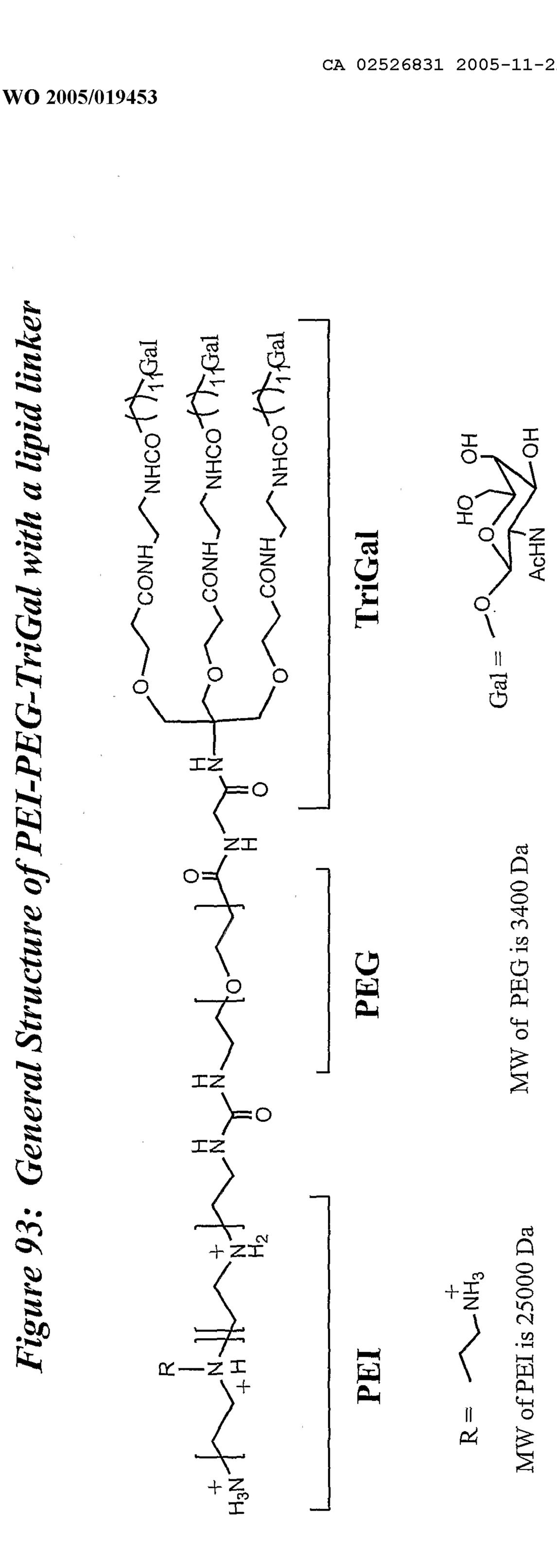
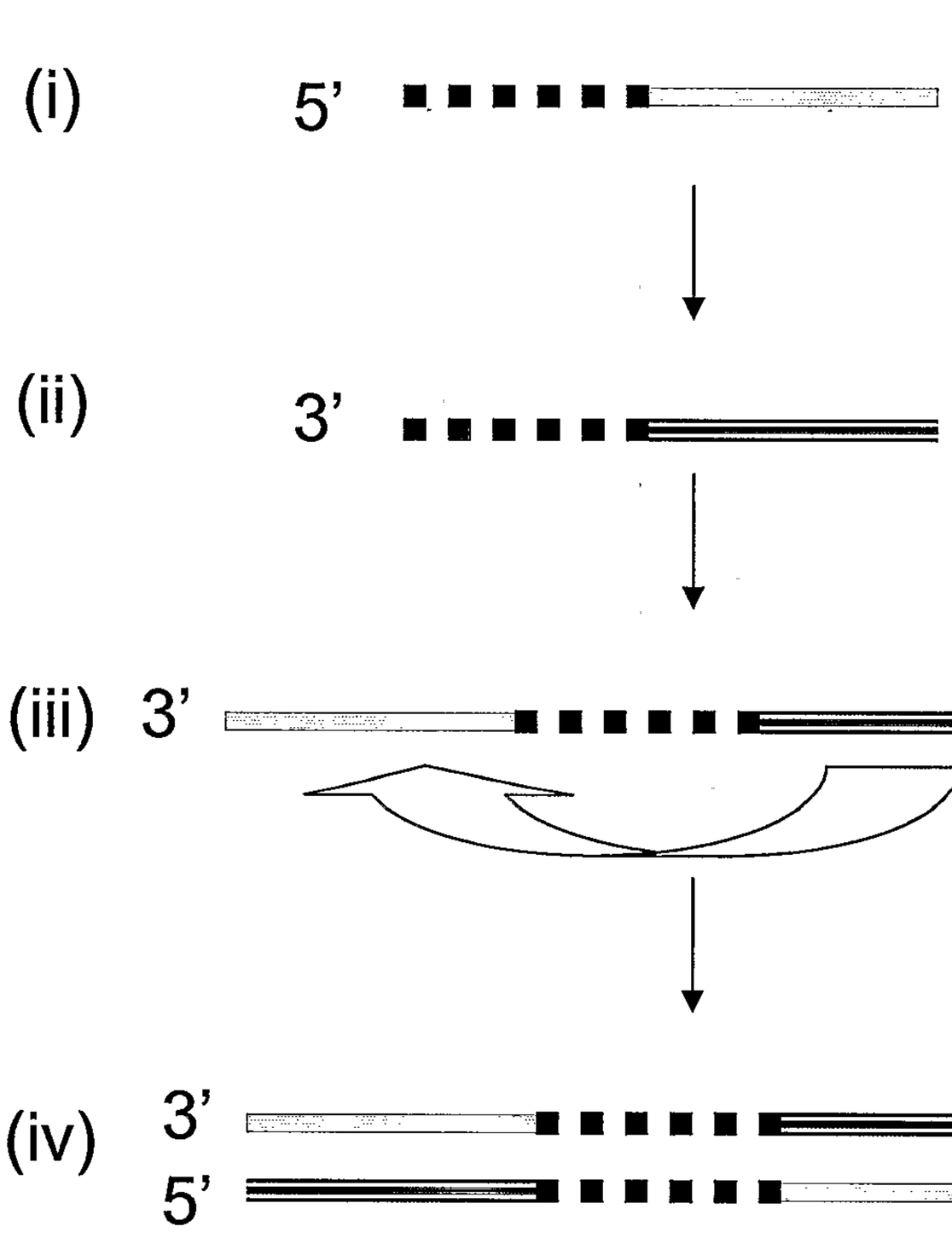


Figure 94A: Duplex forming oligonucleotide constructs that utilize palindrome or repeat sequences



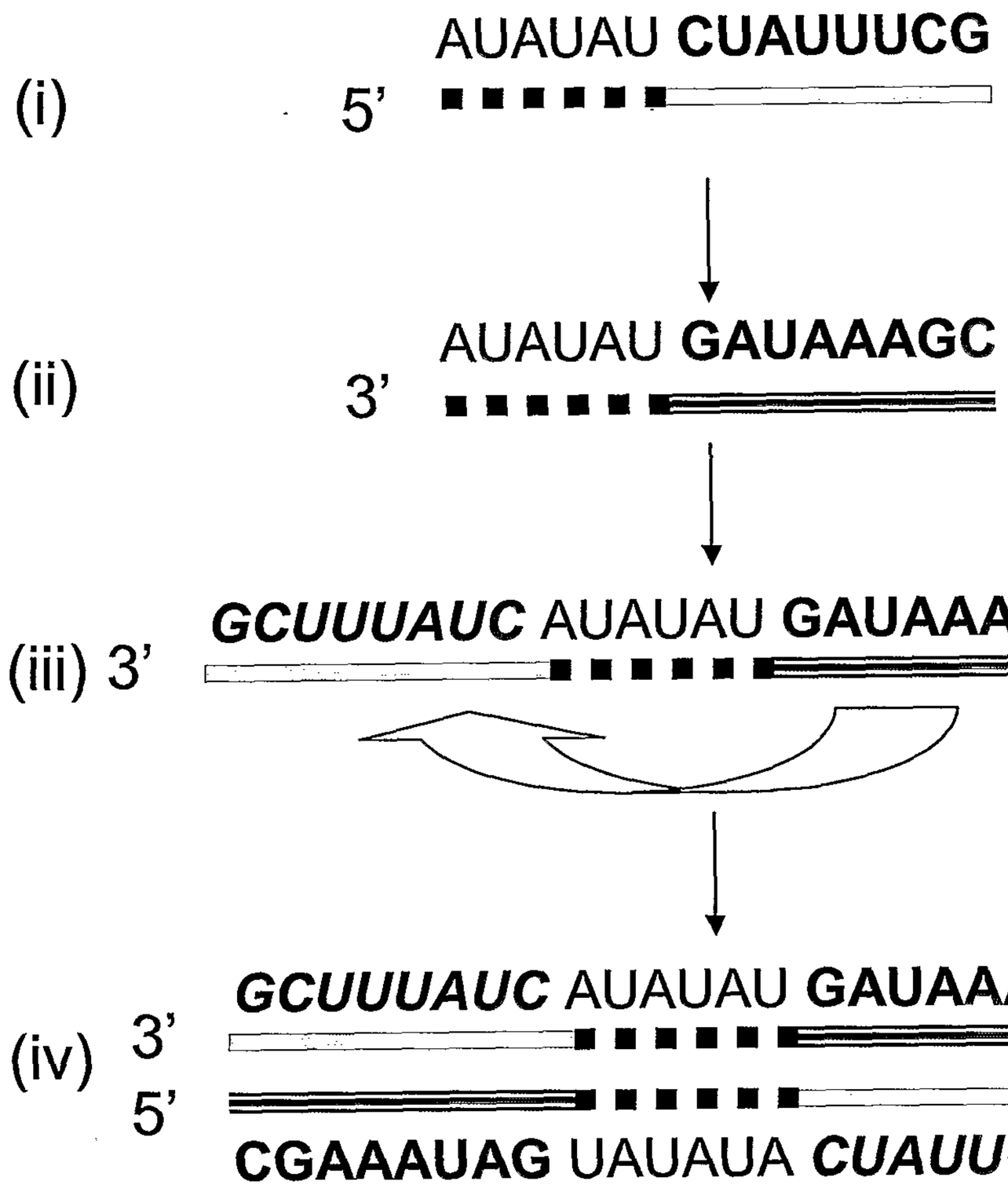
Identify Target Nucleic Acid sequence (e.g., 14 to 24 nucleotides in length) containing palindrome/repeat sequence at 5'-end (dashed portion)

Design Complementary Sequence to the Target Nucleic Acid sequence of (i) above

Append inverse sequence of the Non-palindromic Complementary Sequence of (ii) to 3'-end of complementary sequence

Self assembly of self complementary strands to form duplex construct

Figure 94B: Example of a duplex forming oligonucleotide sequence that utilizes a palindrome or repeat sequence



Identify Target Nucleic Acid sequence (e.g., 14 to 24 nucleotides in length) containing palindrome/repeat sequence at 5'-end (dashed portion)

Design Complementary Sequence to the Target Nucleic Acid sequence of (i) above

Append inverse sequence of the Non-palindromic Complementary Sequence of (ii) to 3'-end of complementary sequence

Self assembly of self complementary strands to form duplex construct (blunt ends)

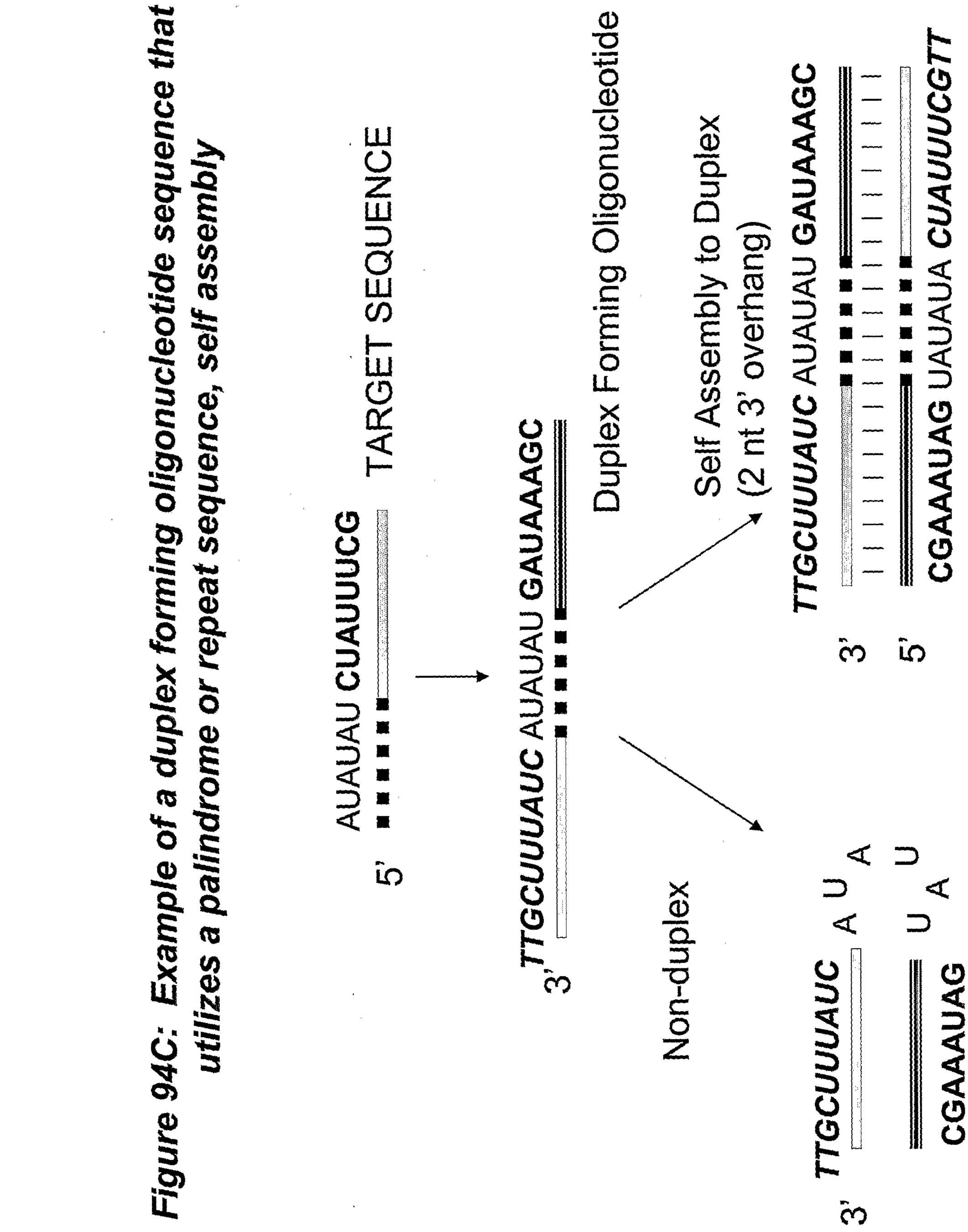


Figure 94C: Example of a duplex forming oligonucleotide sequence that utilizes a palindrome or repeat sequence, self assembly

Figure 94D: Example of a duplex forming oligonucleotide sequence that utilizes a palindrome or repeat sequence, self assembly and inhibition of Target Sequence Expression

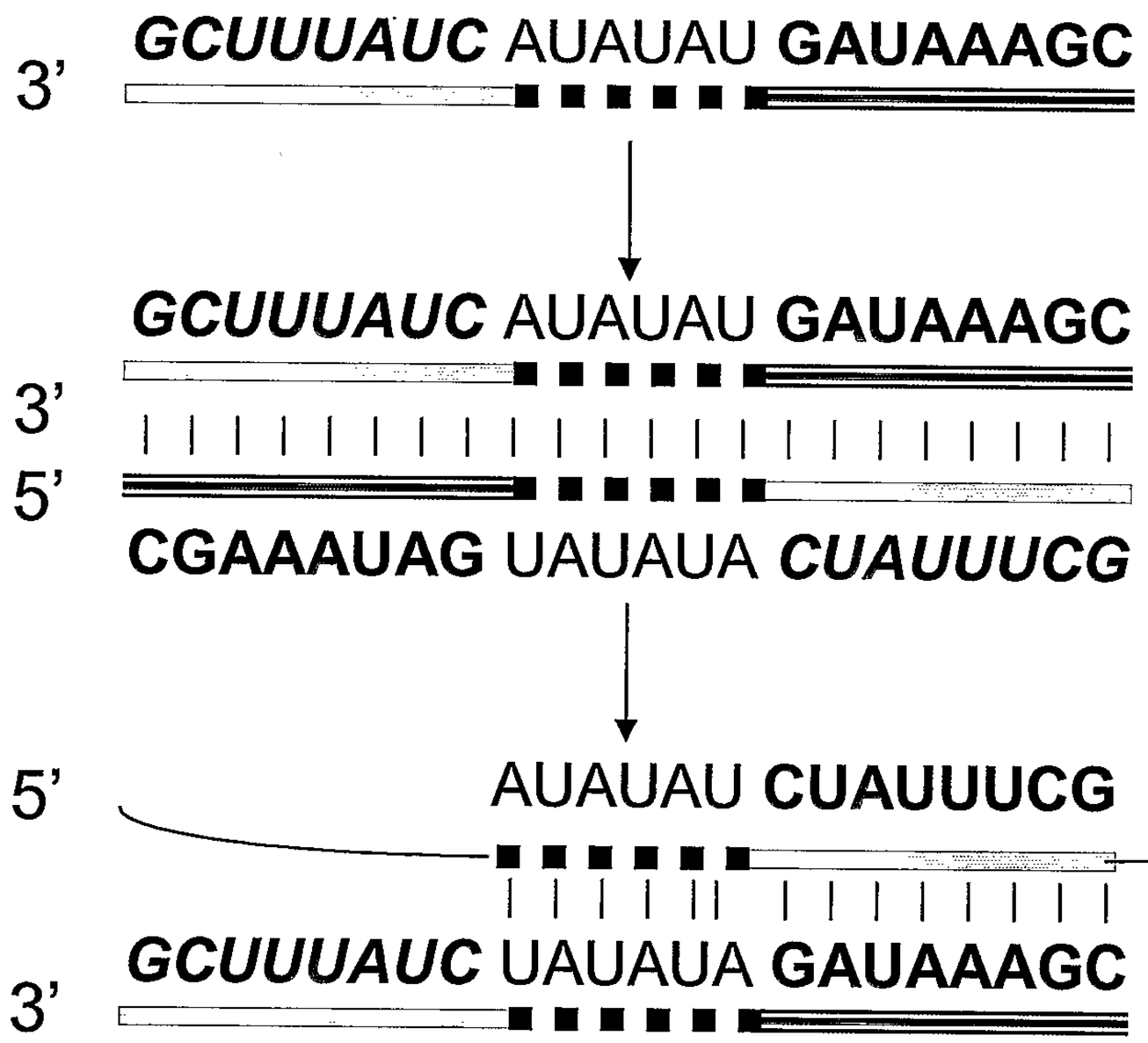
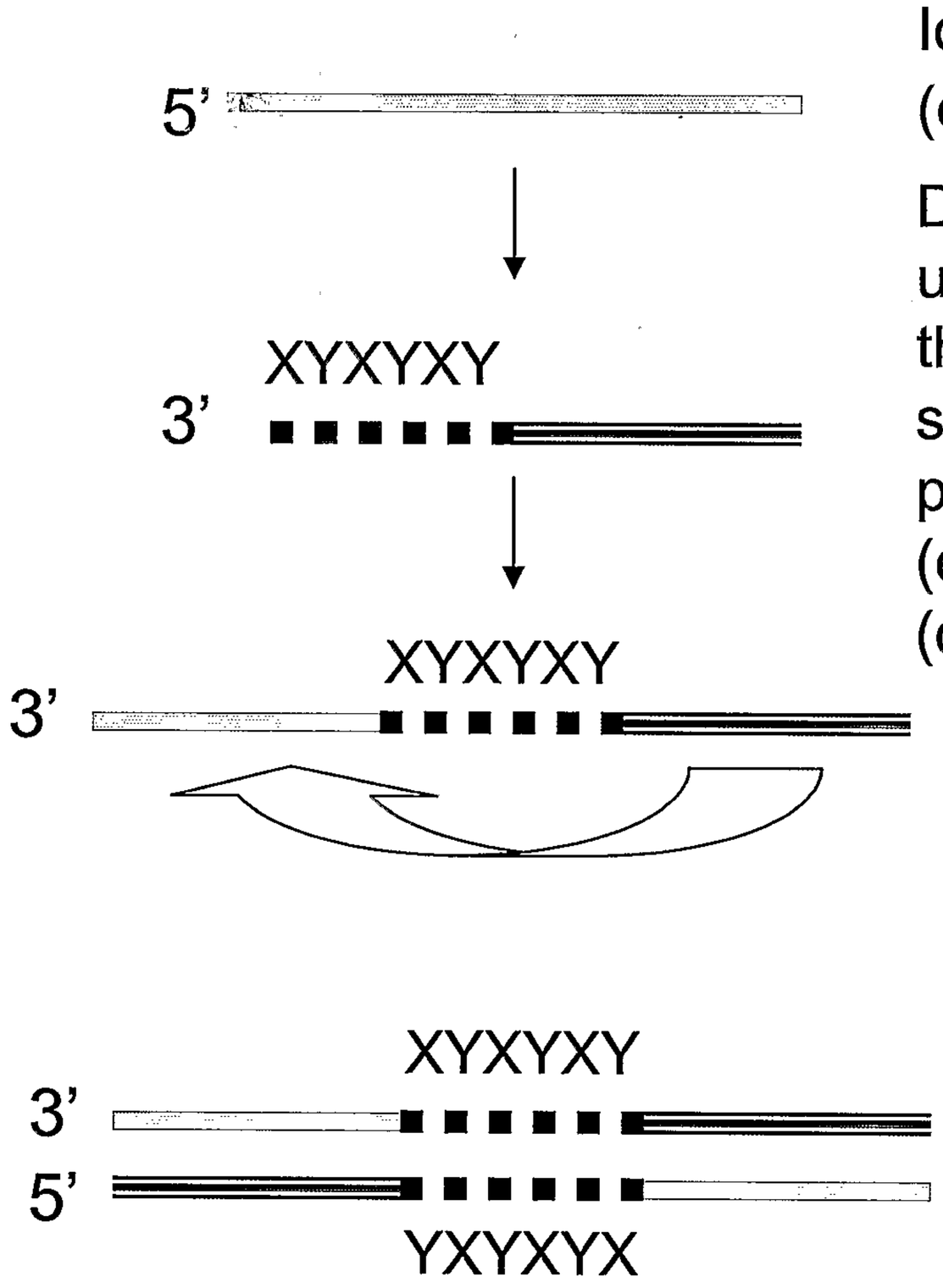


Figure 95: Duplex forming oligonucleotide constructs that utilize artificial palindrome or repeat sequences



Identify Target Nucleic Acid sequence (e.g., 14 to 24 nucleotides in length)

Design Complementary Sequence and utilize modified nucleotides (shown as X, Y) that interact with a portion of the target sequence and result in the formation of a palindrome/repeat sequence (e.g., 2 to 12 nucleotides) at 3'-end (dashed portion)

Append inverse sequence of Complementary region to 3'-end of palindrome/repeat sequence

Hybridize self complementary strands to form duplex siNA construct

Figure 96: Examples of double stranded multifunctional siNA constructs with distinct complementary regions

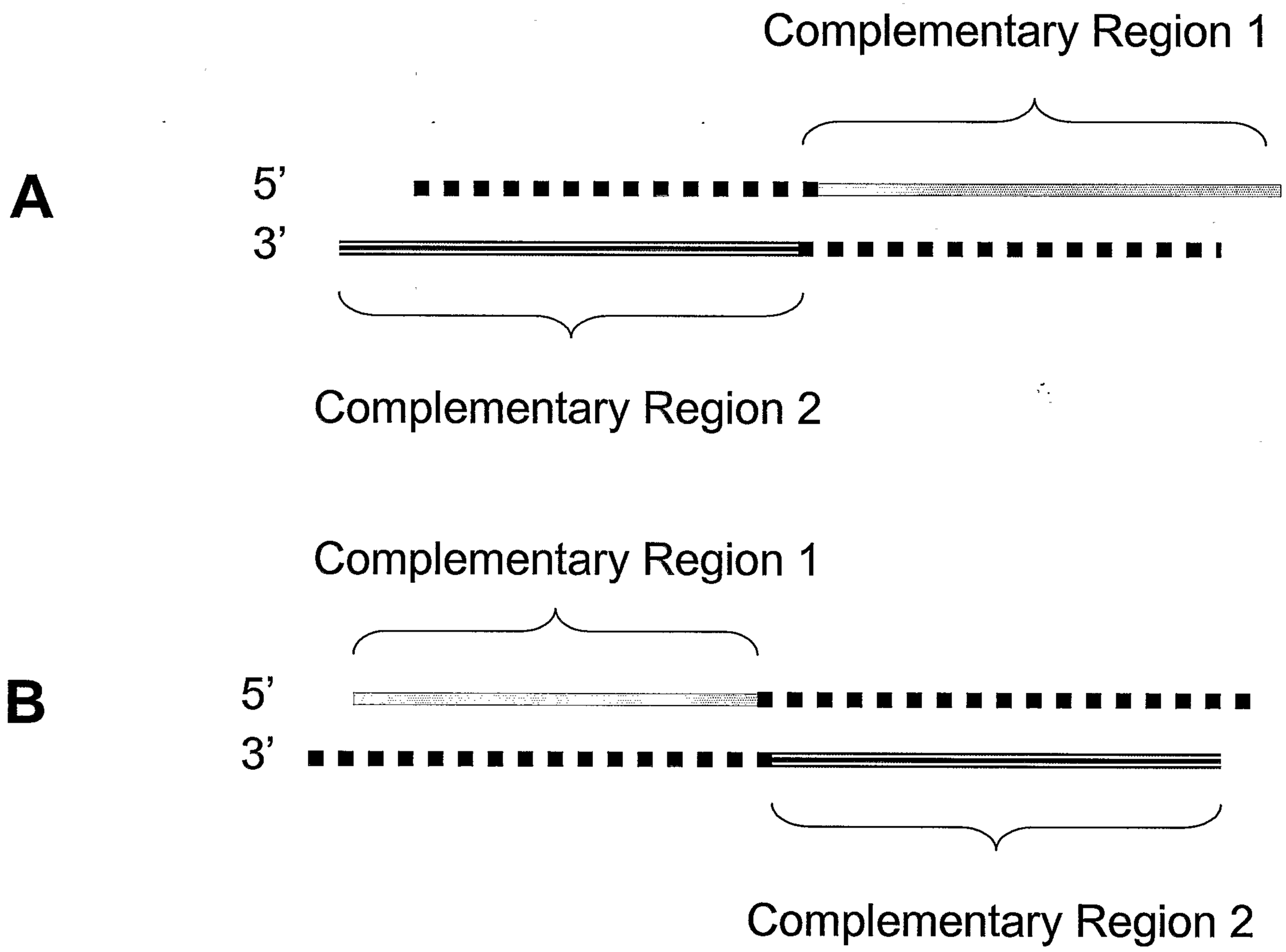


Figure 97: Examples of hairpin multifunctional siNA constructs with distinct complementary regions

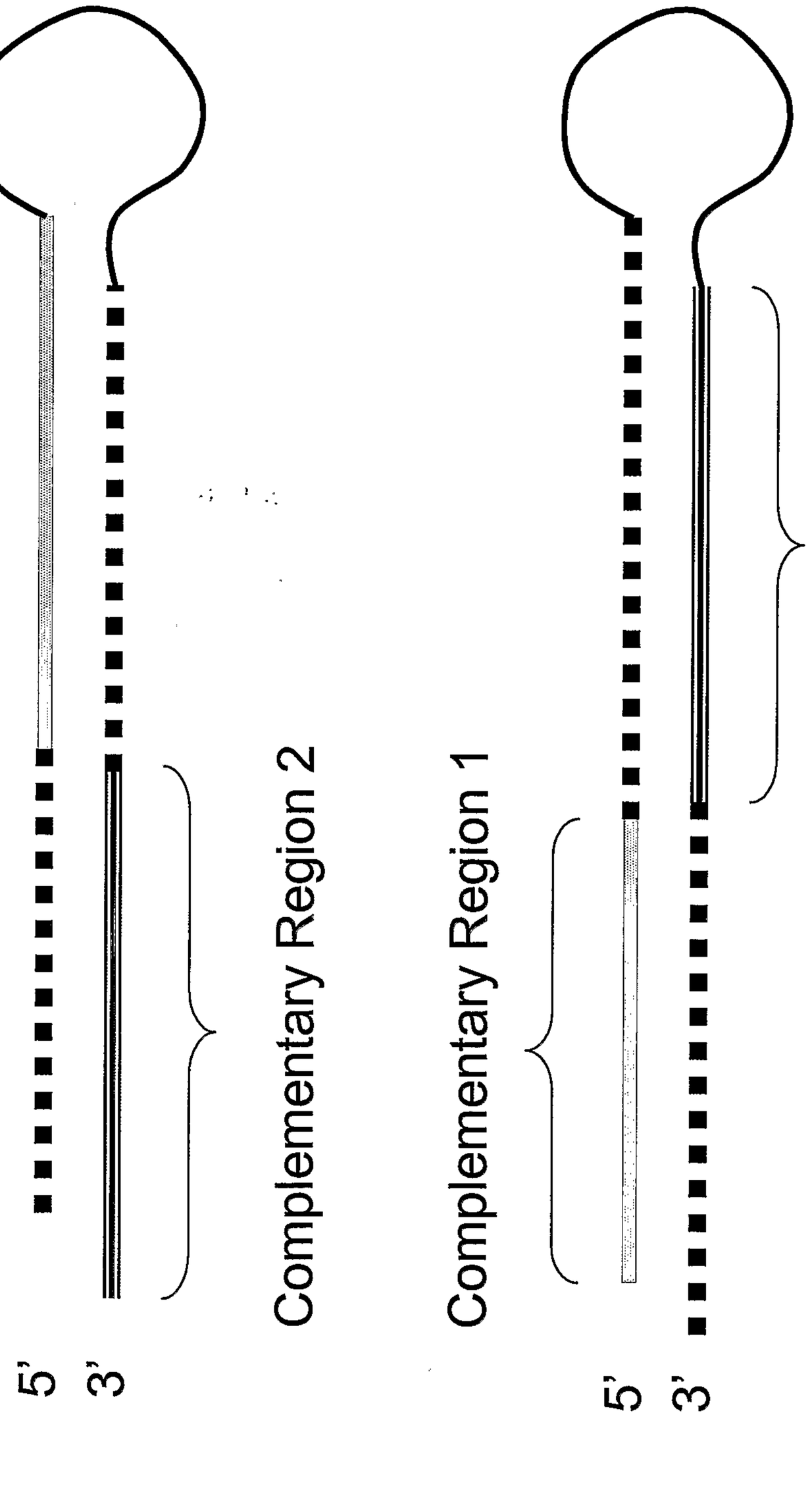


Figure 98: Examples of double stranded multifunctional siNA constructs with distinct complementary regions and a self complementary/palindrome region

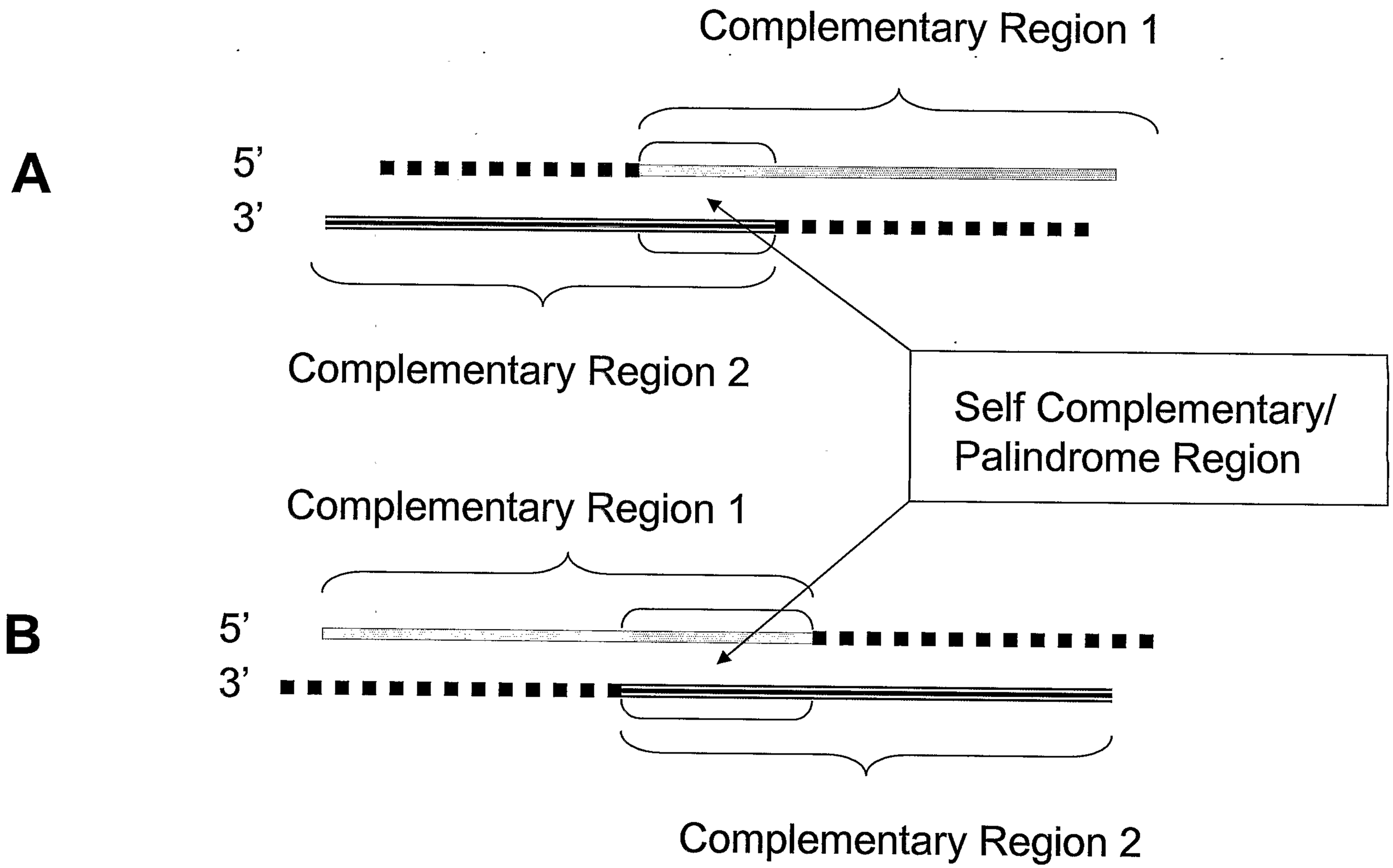
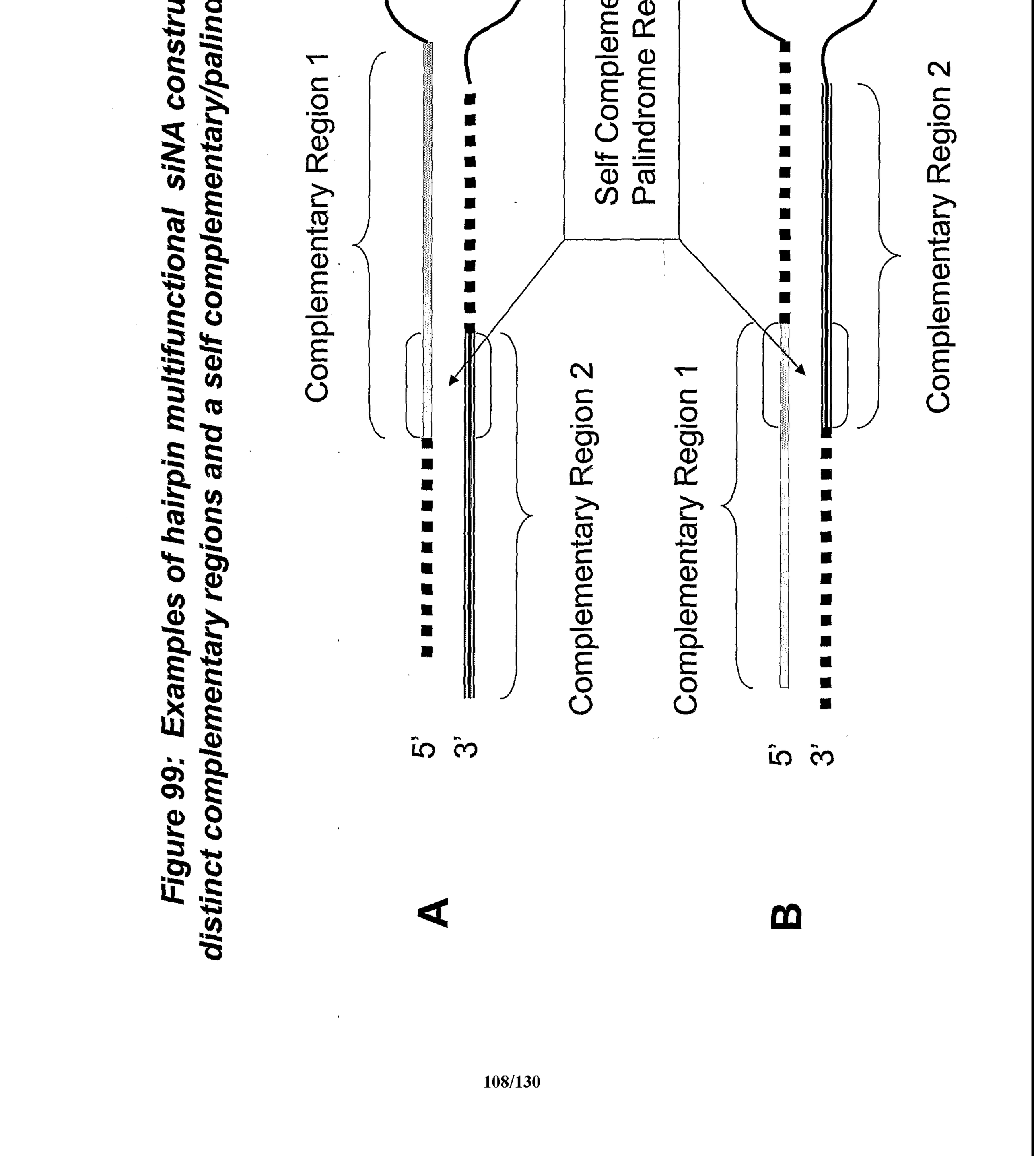


Figure 99: Examples of hairpin multifunctional siNA constructs with distinct complementary regions and a self complementary/palindrome region



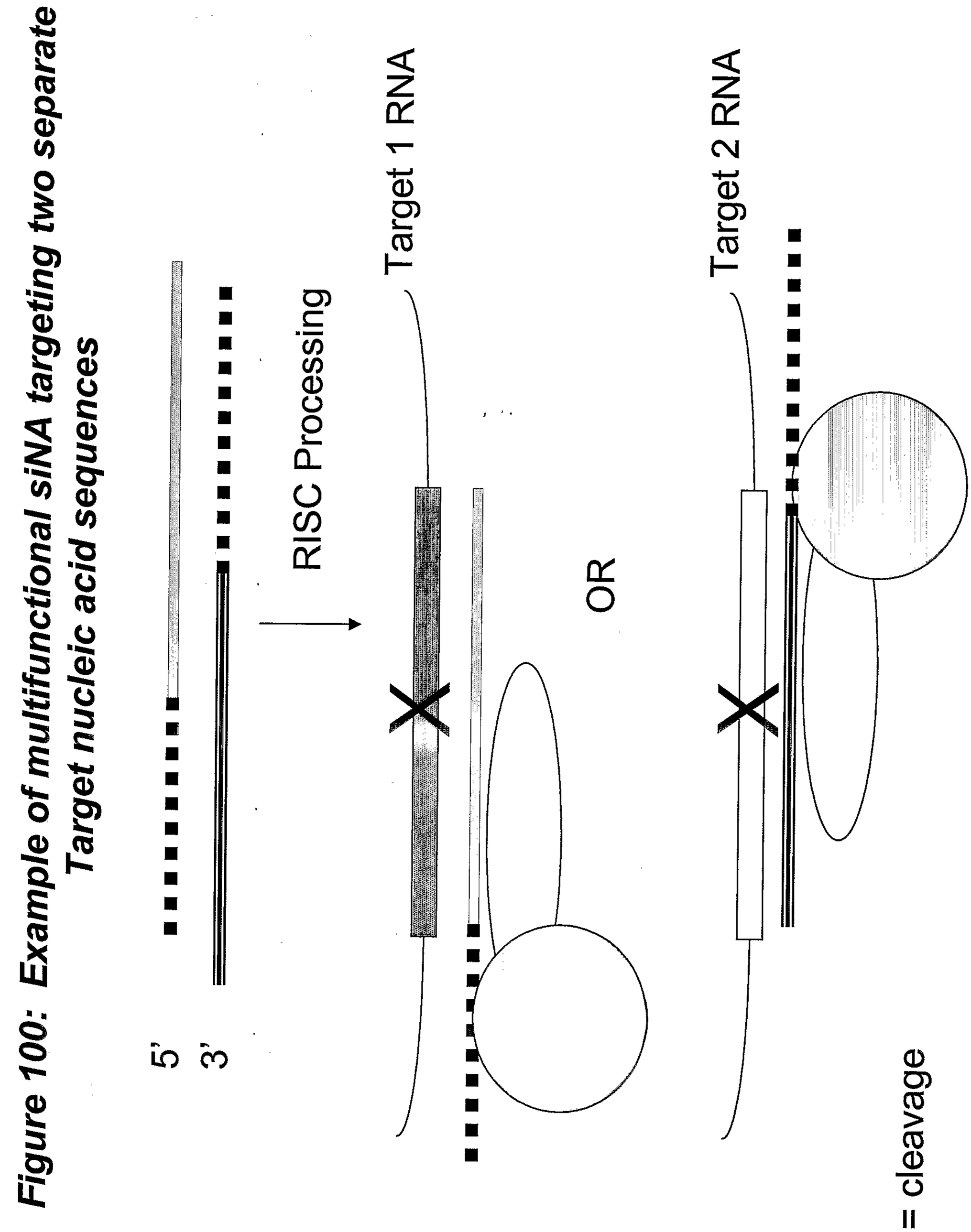


Figure 100: Example of multifunctional siNA targeting two separate Target nucleic acid sequences

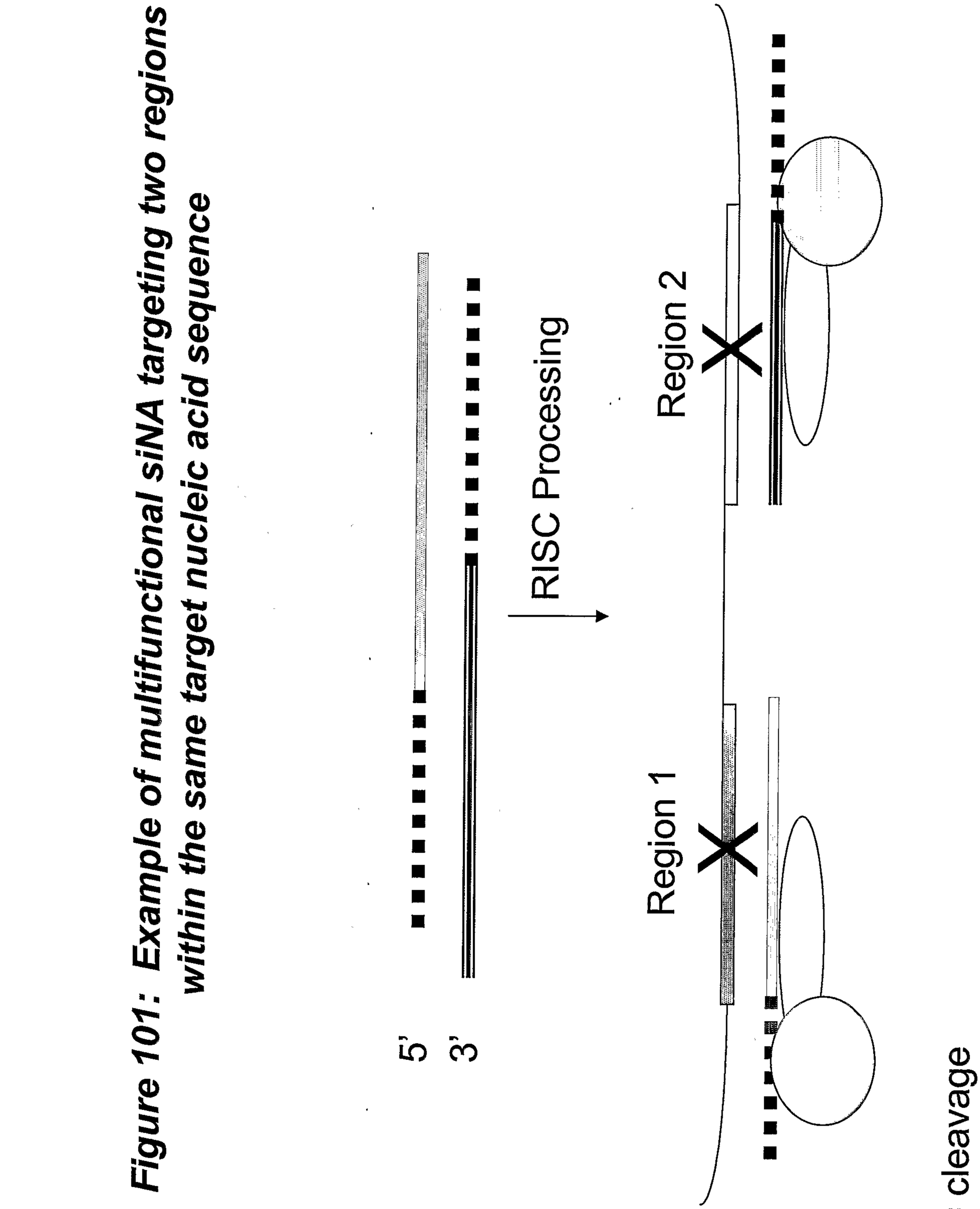


Figure 101: Example of multifunctional siNA targeting two regions within the same target nucleic acid sequence

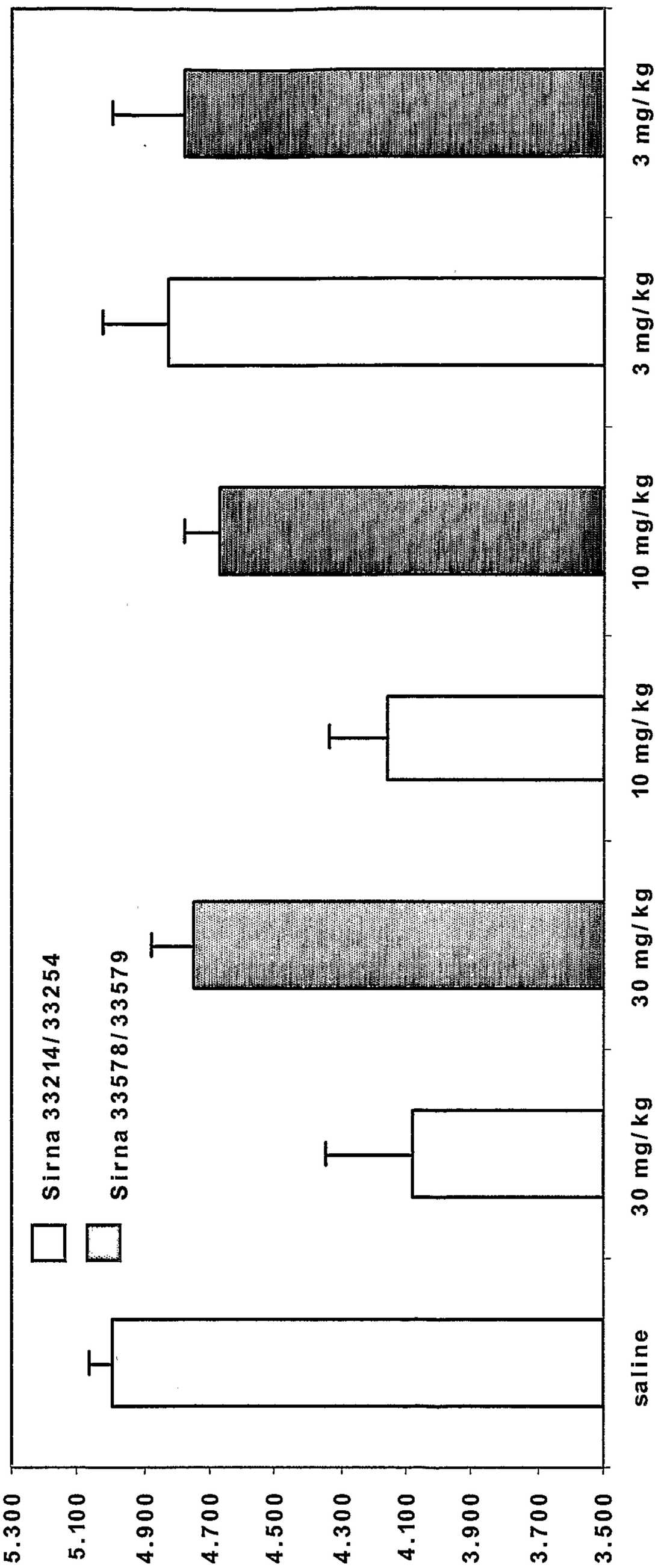


Figure 102: Activity of Systemically Administered Stabilized siNA in HBV Mouse Model: Serum HBV DNA Levels

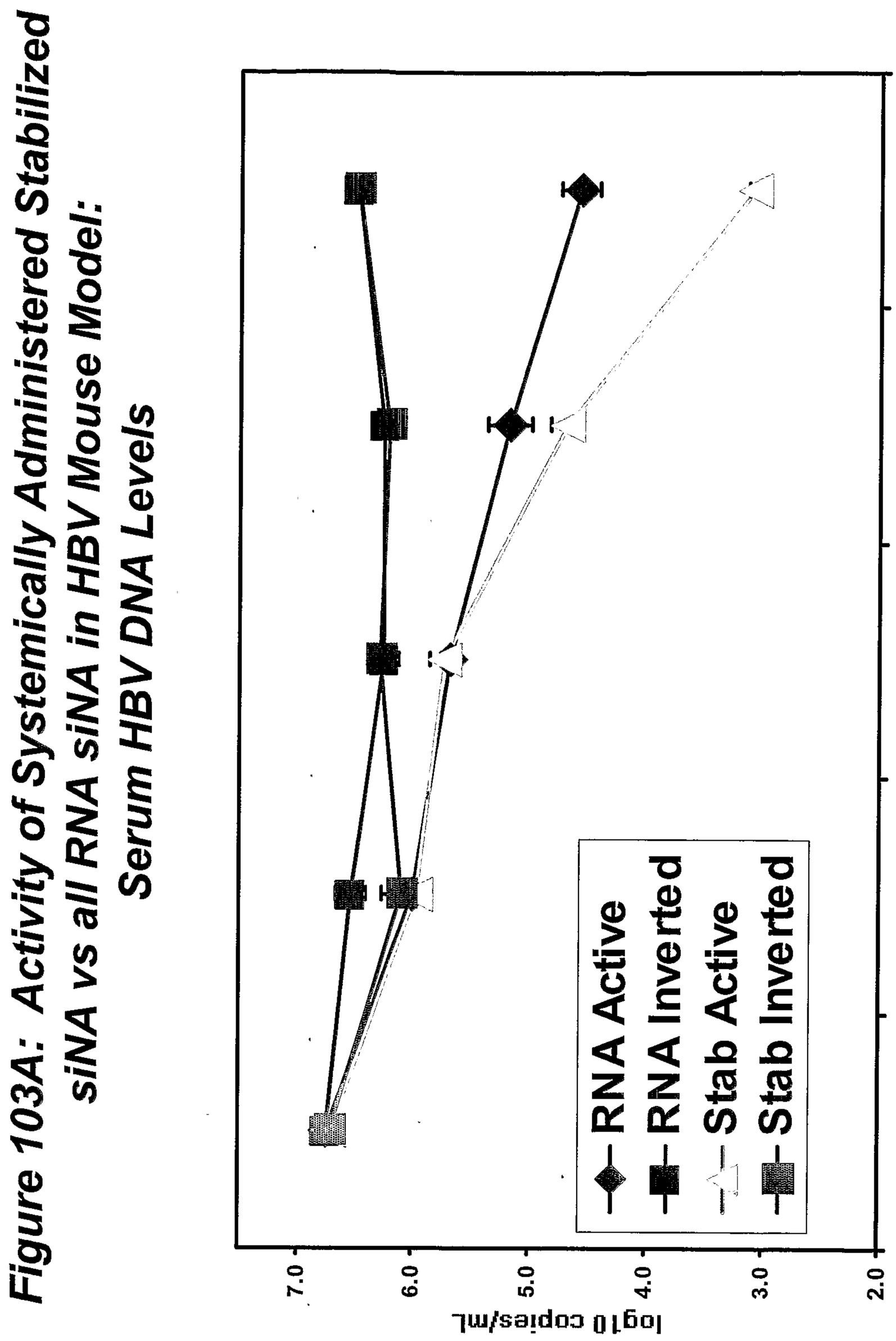


Figure 103A: Activity of Systemically Administered Stabilized siNA in HBV Mouse Model: siNA vs all RNA siNA on Serum HBV DNA Levels

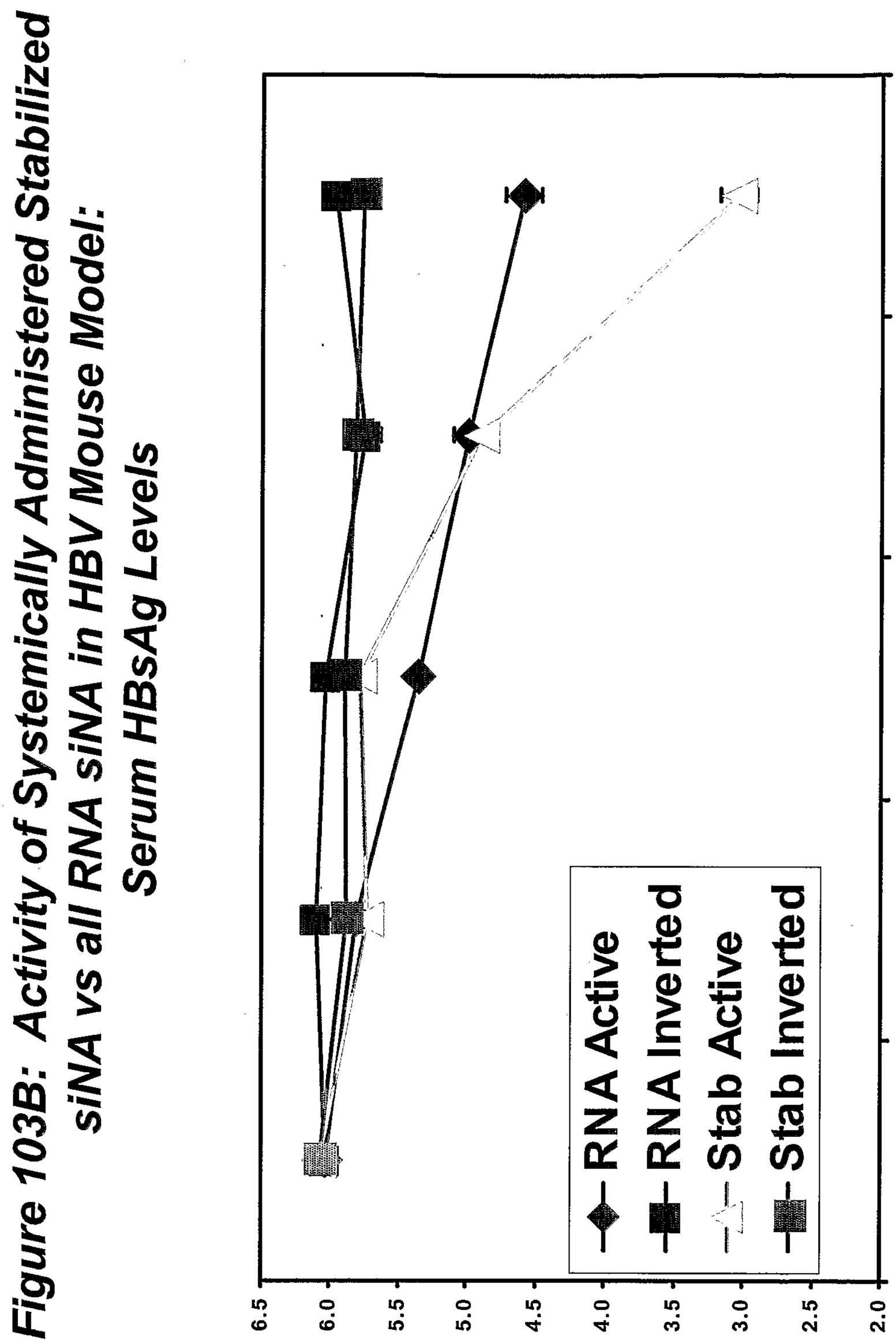
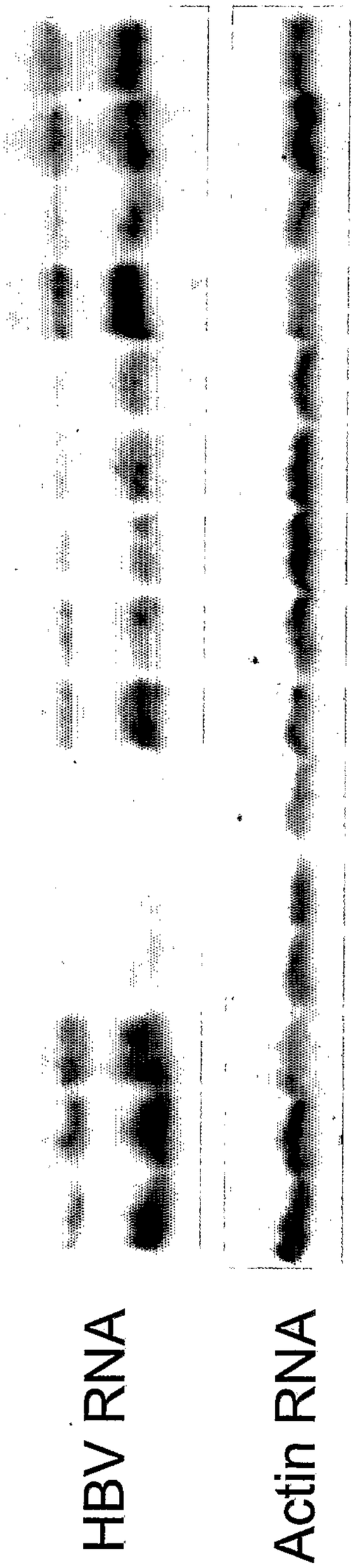


Figure 103B: Activity of Systemically Administered Stabilized siNA vs all RNA siNA in HBV Mouse Model: Serum HBsAg Levels

Figure 103C: Activity of Systemically Administered Stabilized siNA vs all RNA siNA in HBV Mouse Model: Liver HBV RNA Levels



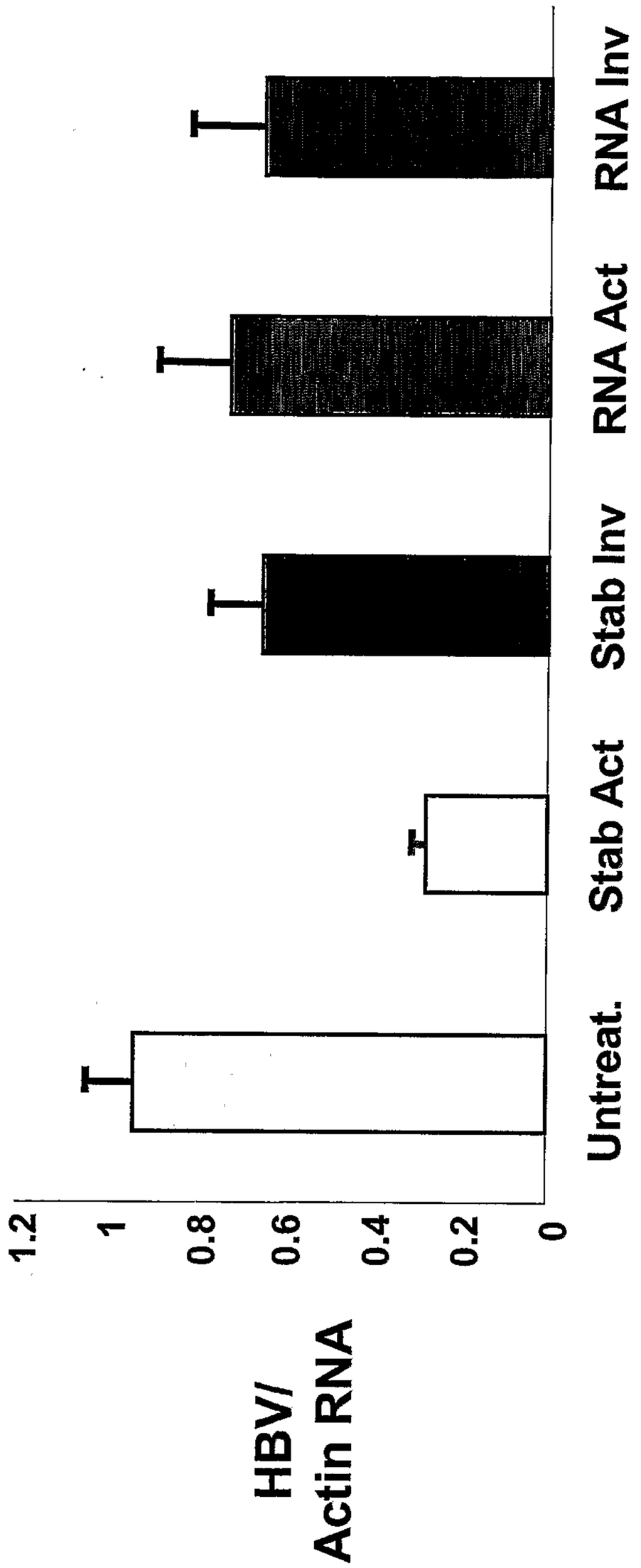
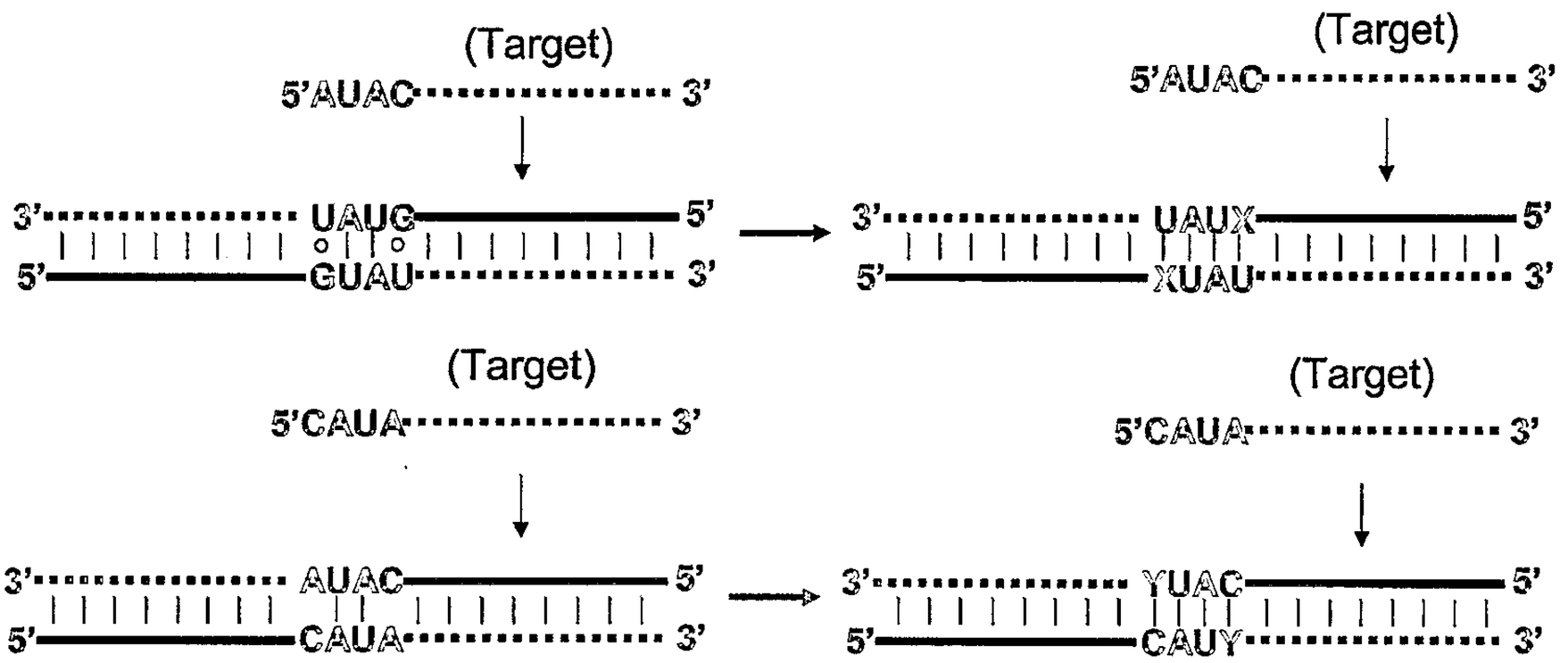


Figure 104: Examples of introducing artificial palindrome sequences in DFO constructs with a target nucleic acid not having a palindrome/repeat



X = 2-aminopurine
Y = 2-amino-1,6-dihydropurine

Figure 105: Self-complimentary DFO targeting VEGFR1 RNA

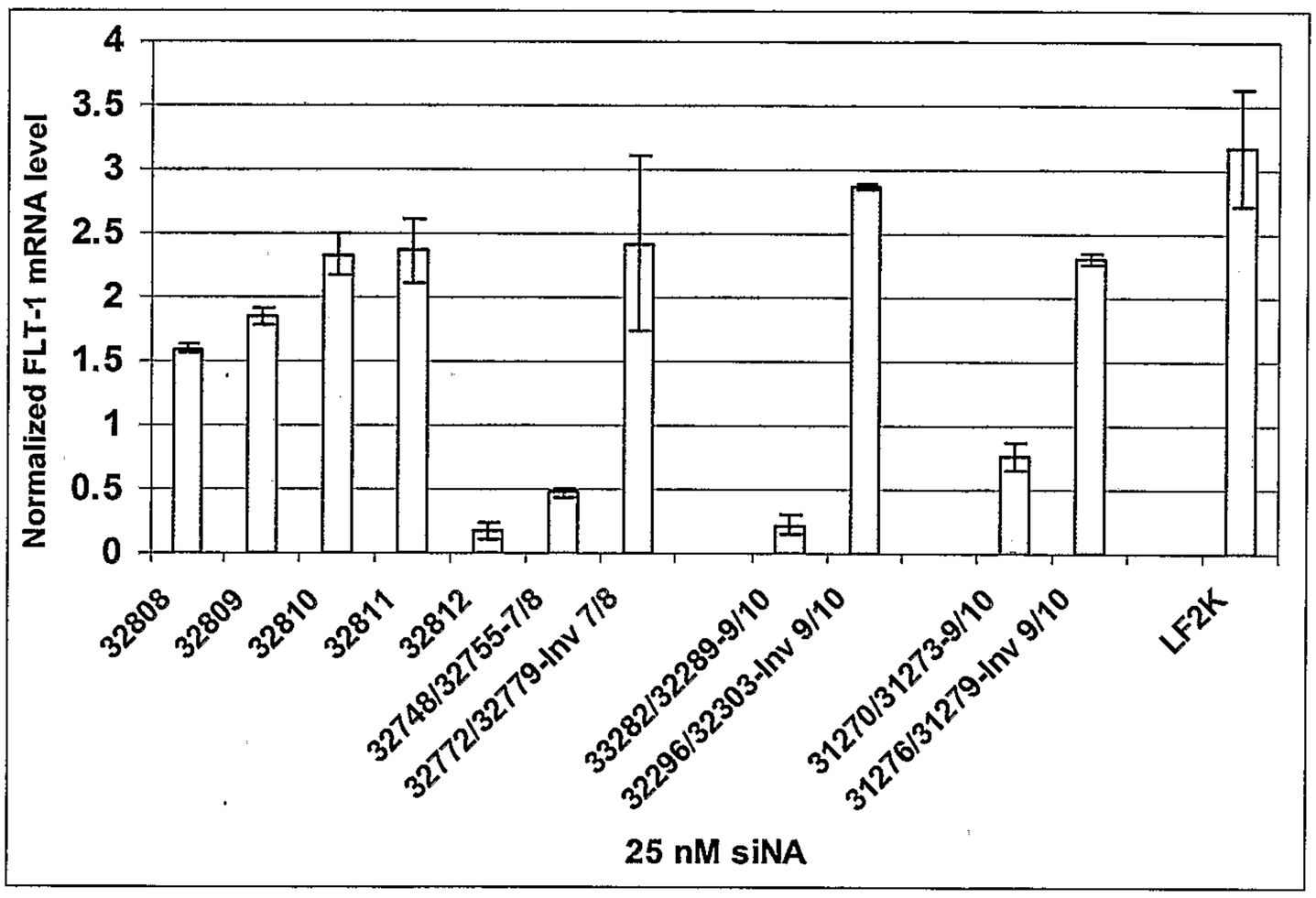
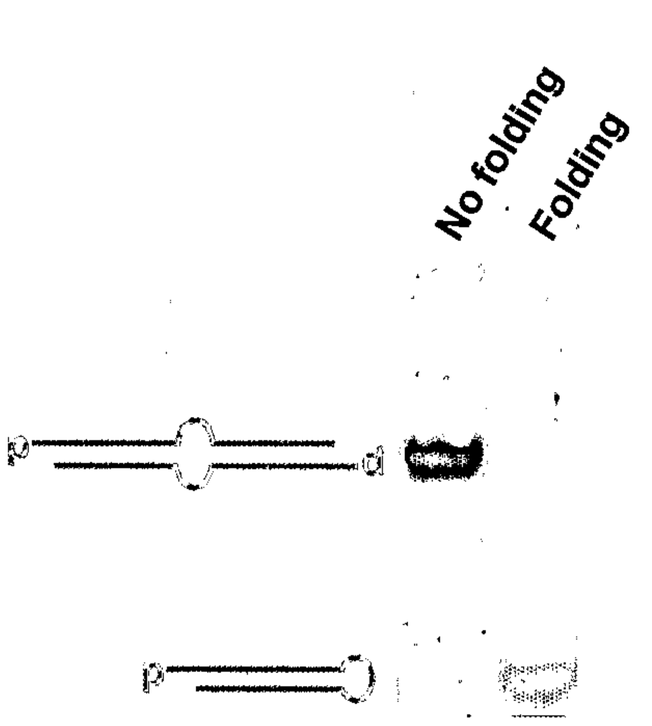


Figure 106: Self-complimentary DFO targeting HBV RNA



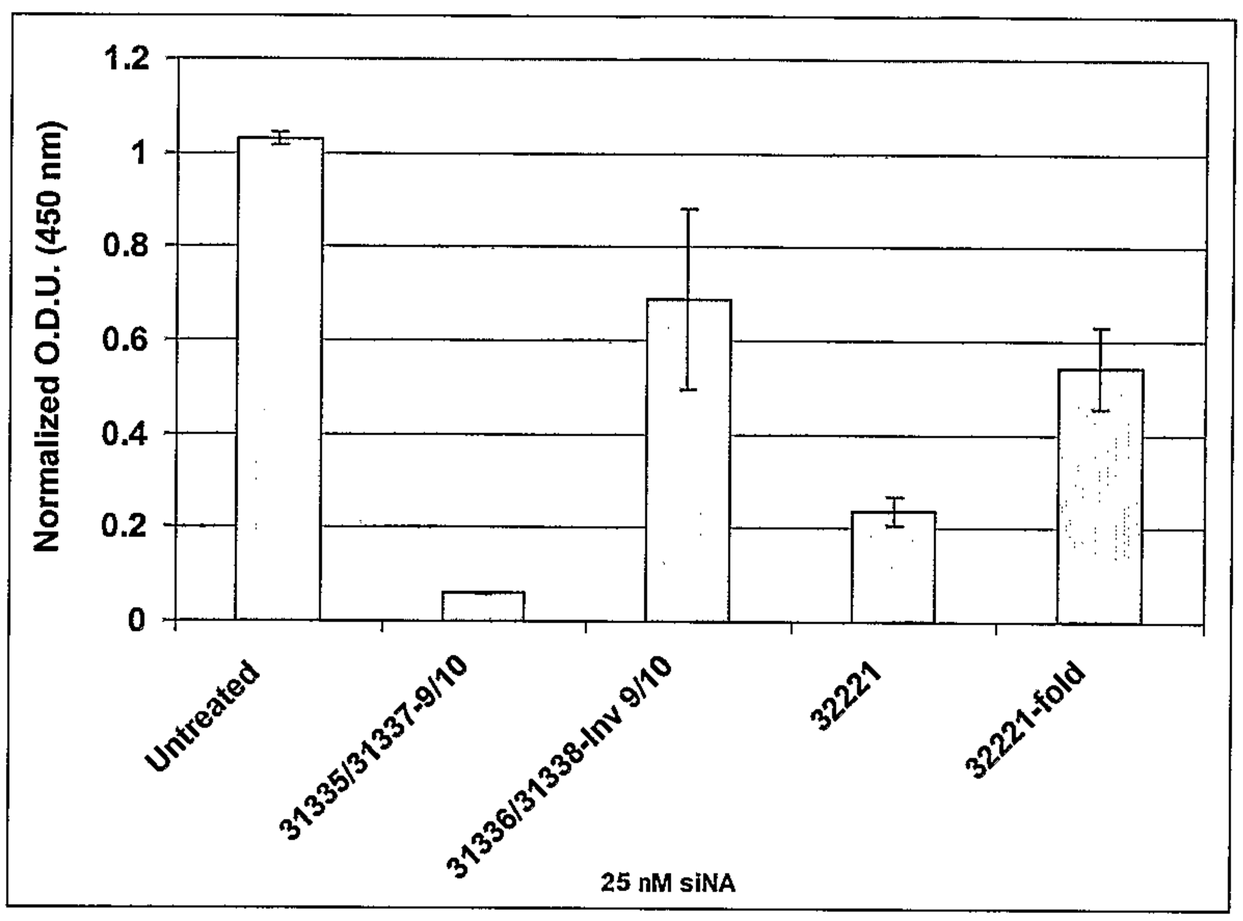


Figure 107: DFO mediated inhibition of TGF-beta receptor RNA expression

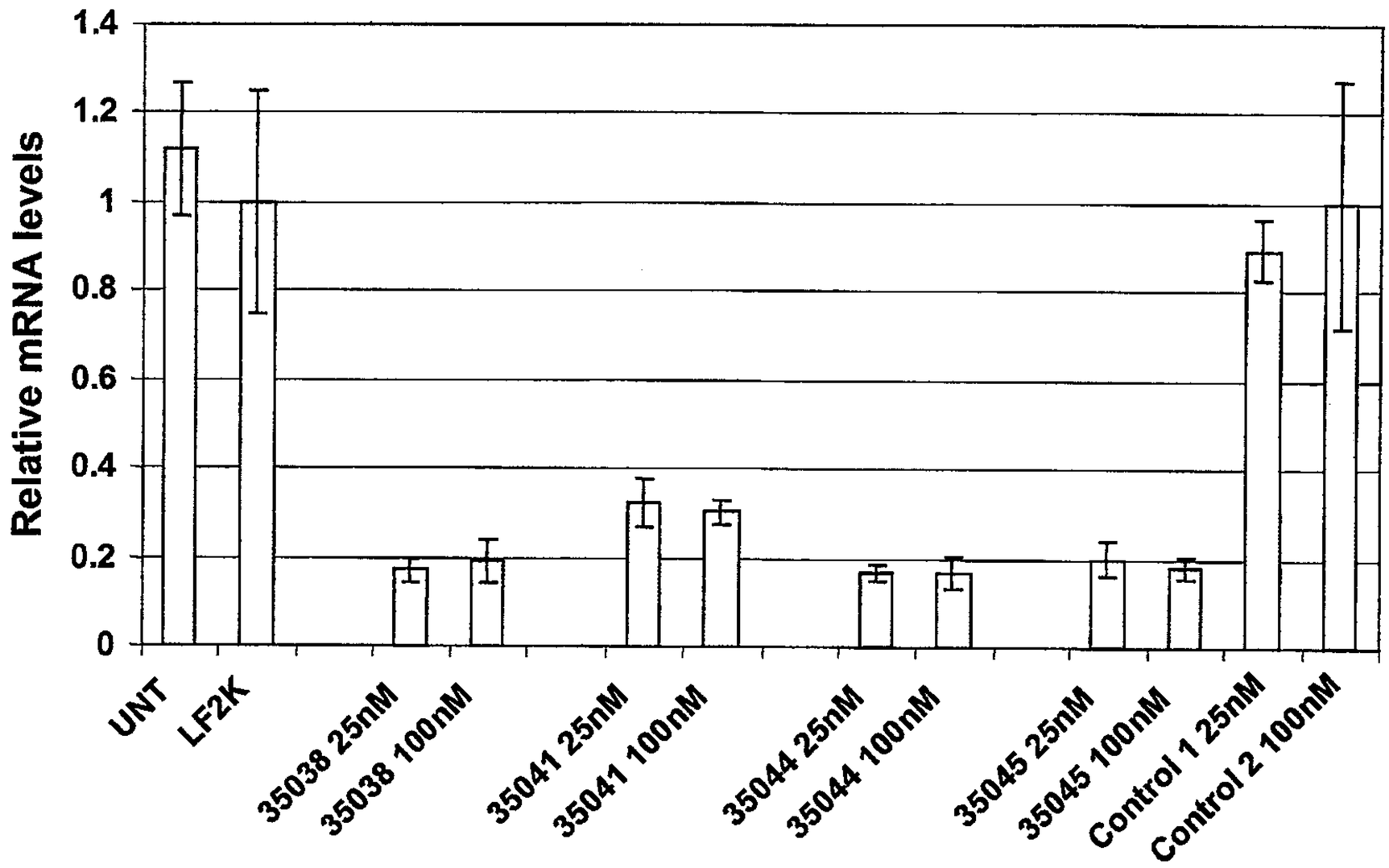


Figure 108: Multifunctional siNA mediated inhibition of HBV RNA expression

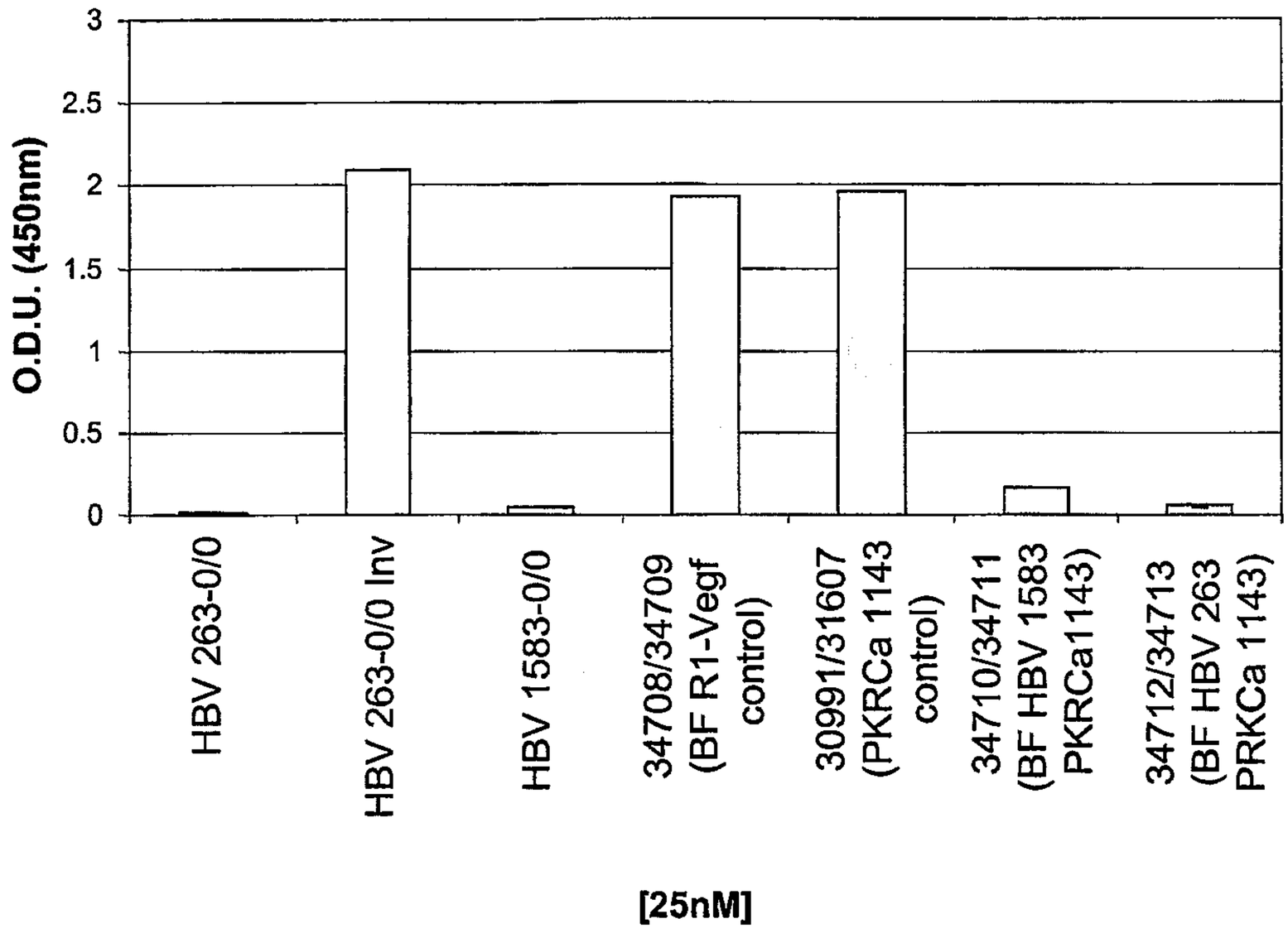


Figure 109: Multifunctional siNA mediated inhibition of PKC-alpha RNA expression

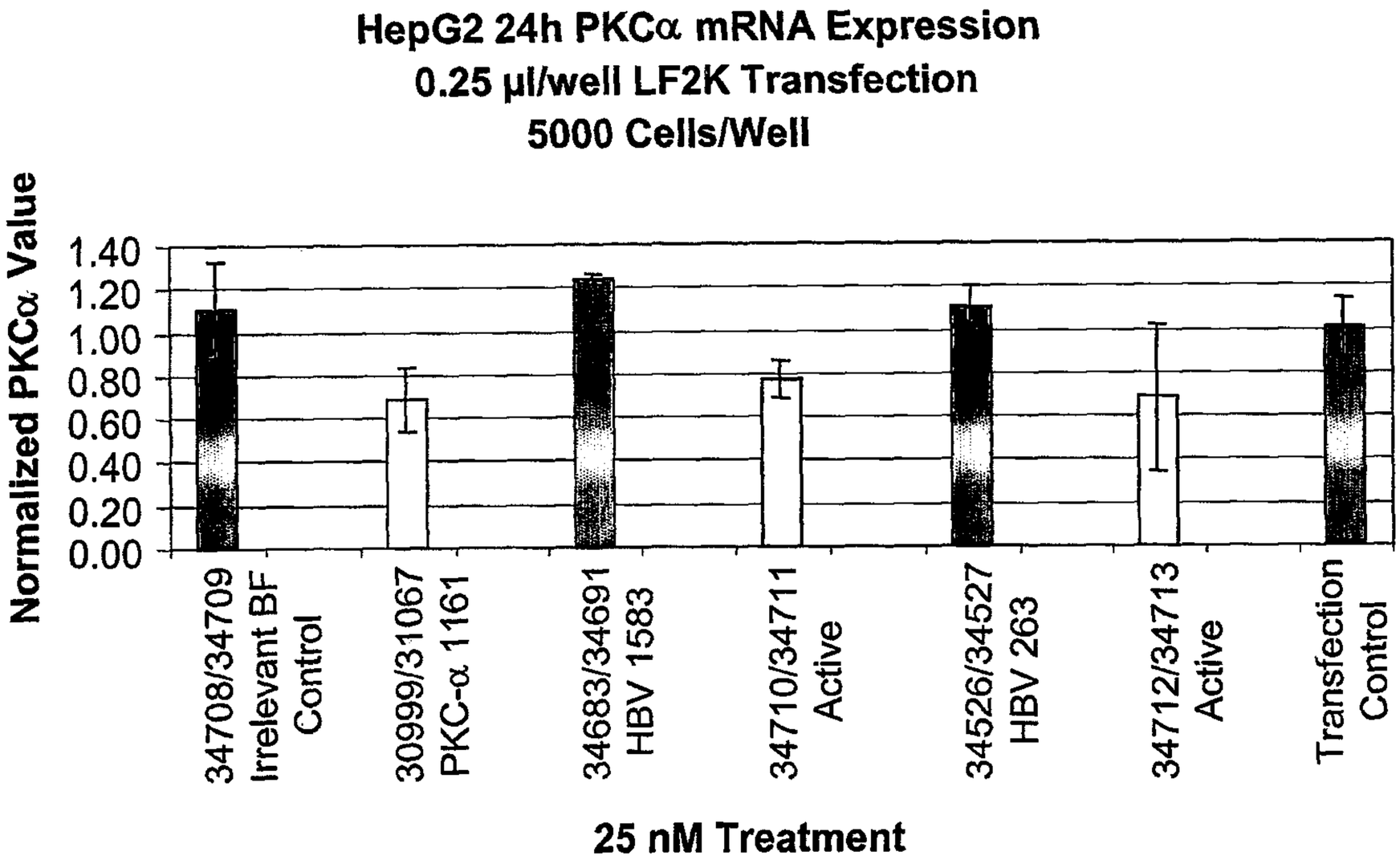
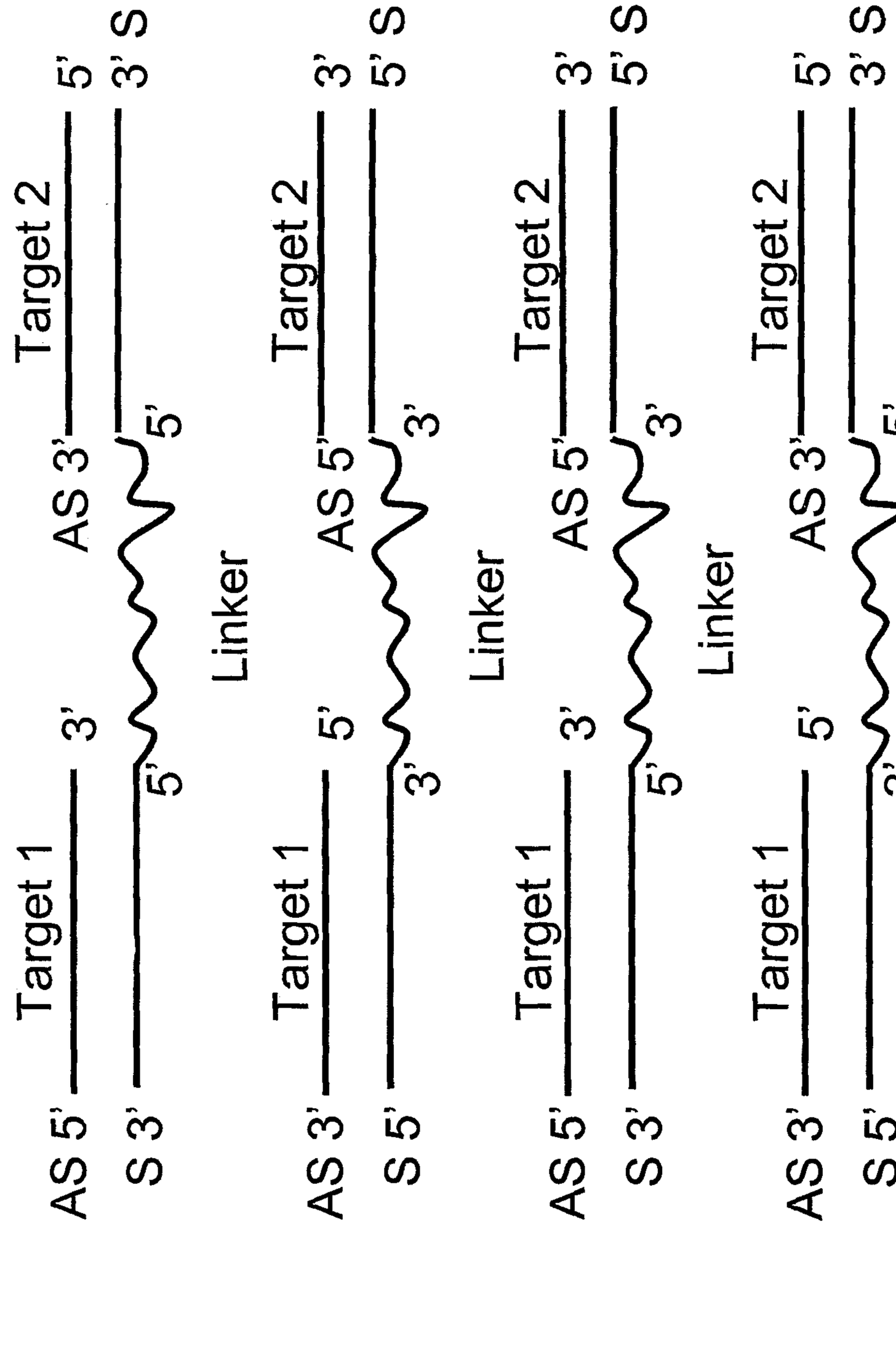


Figure 110: Tethered Multifunctional siNA designs



S = sense, AS = antisense
Linker region can be nucleotide or non-nucleotide linker, and can optionally be decorated, for example with conjugates polymers or aptamers, such as for delivery purposes.

Figure 110: Tethered Multifunctional siNA design

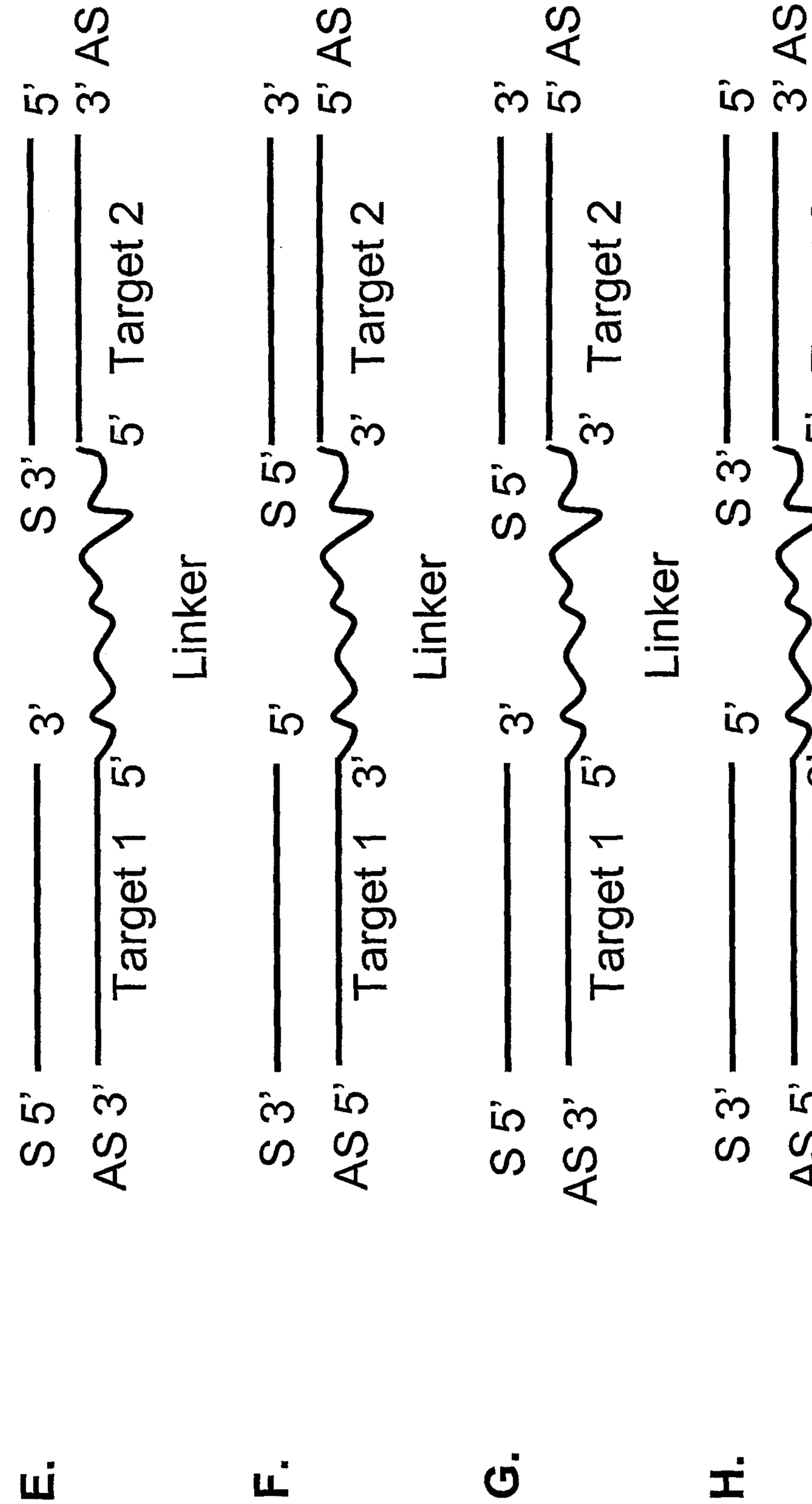


Figure 111: Dendrimer Multifunctional siNA designs

Figure 112: Supramolecular Multifunctional siNA designs

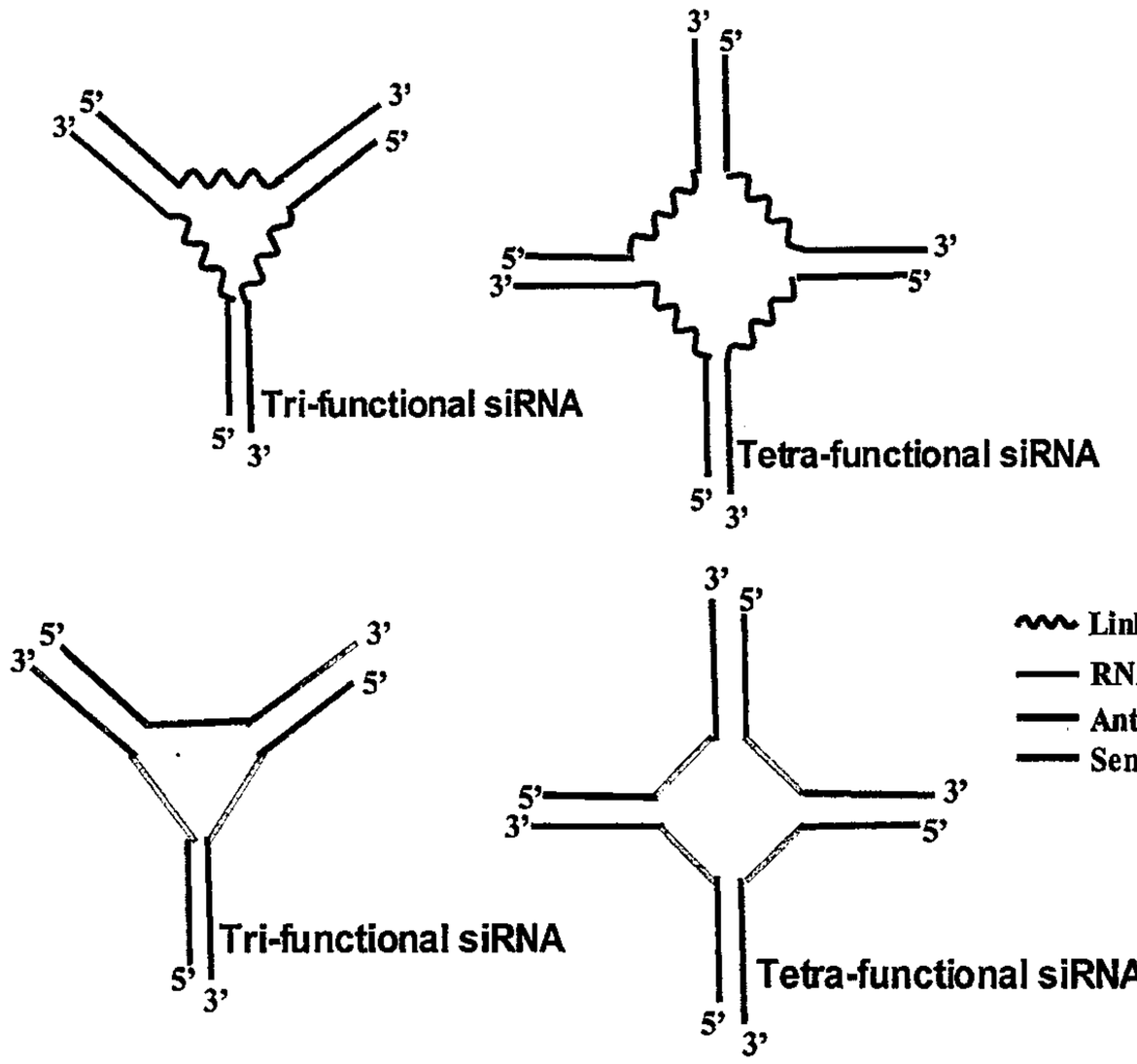


Figure 113: Dicer enabled multifunctional siNA design

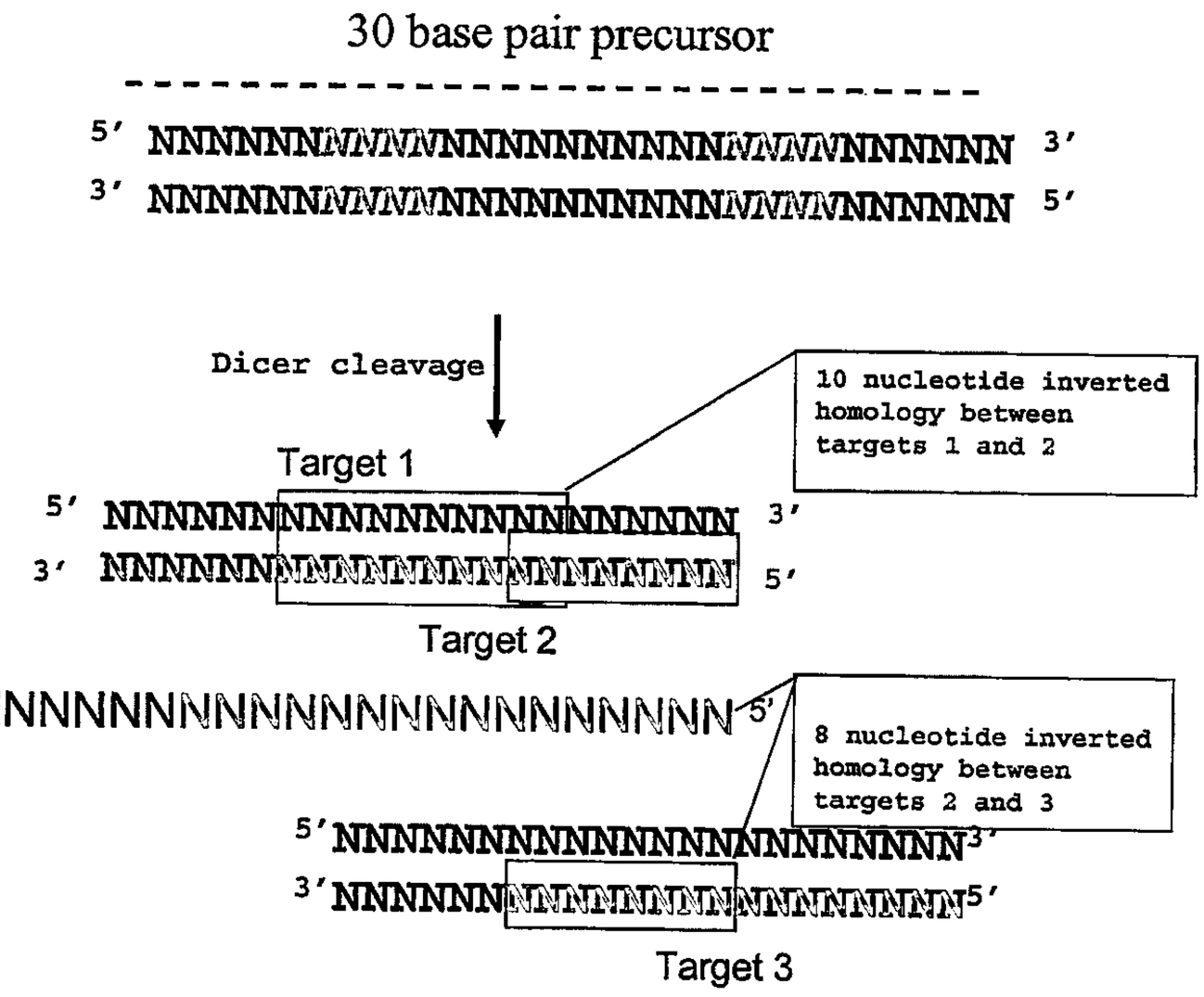


Figure 114: Dicer enabled multifunctional siNA design

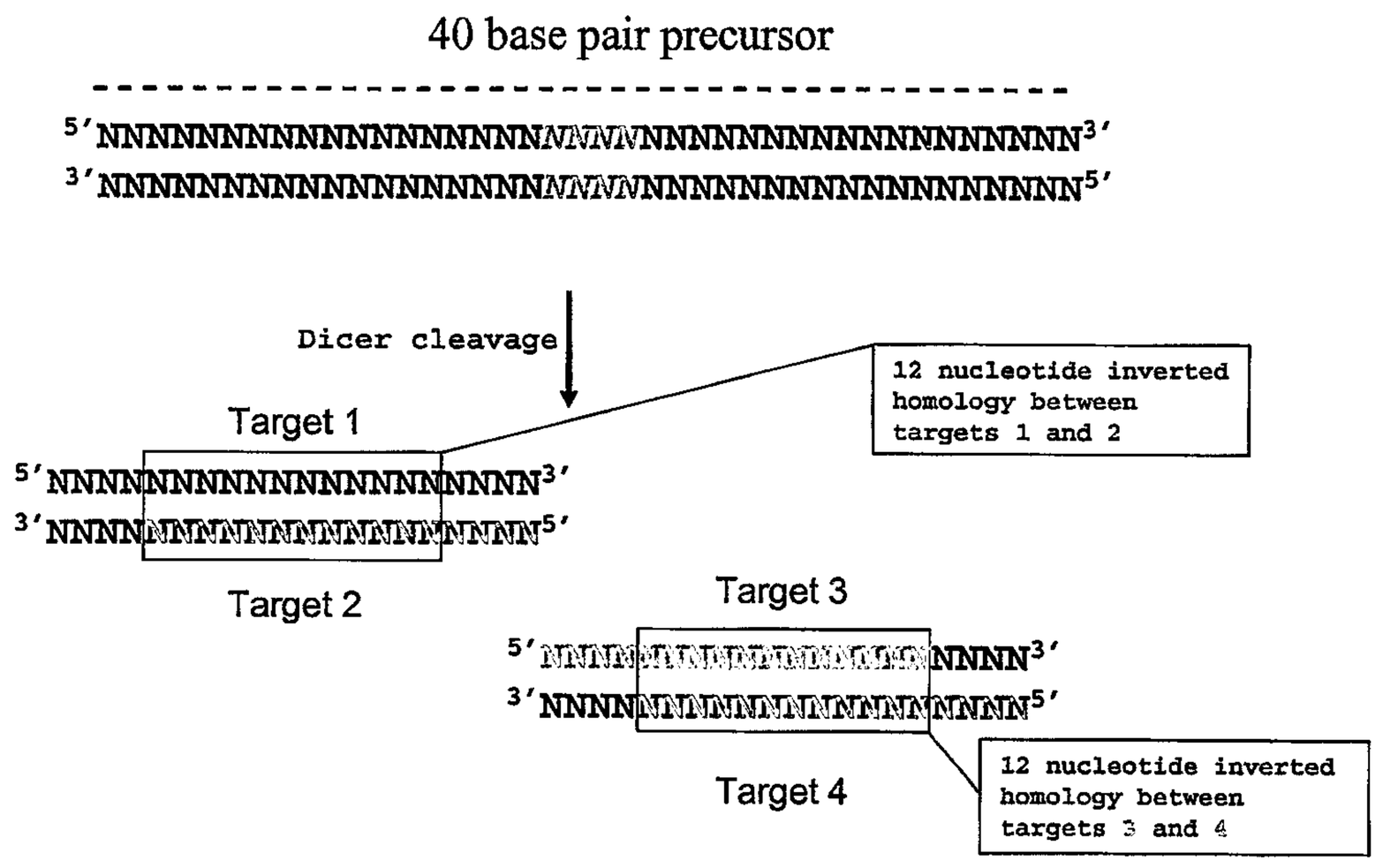


Figure 115: siNA base pair walk

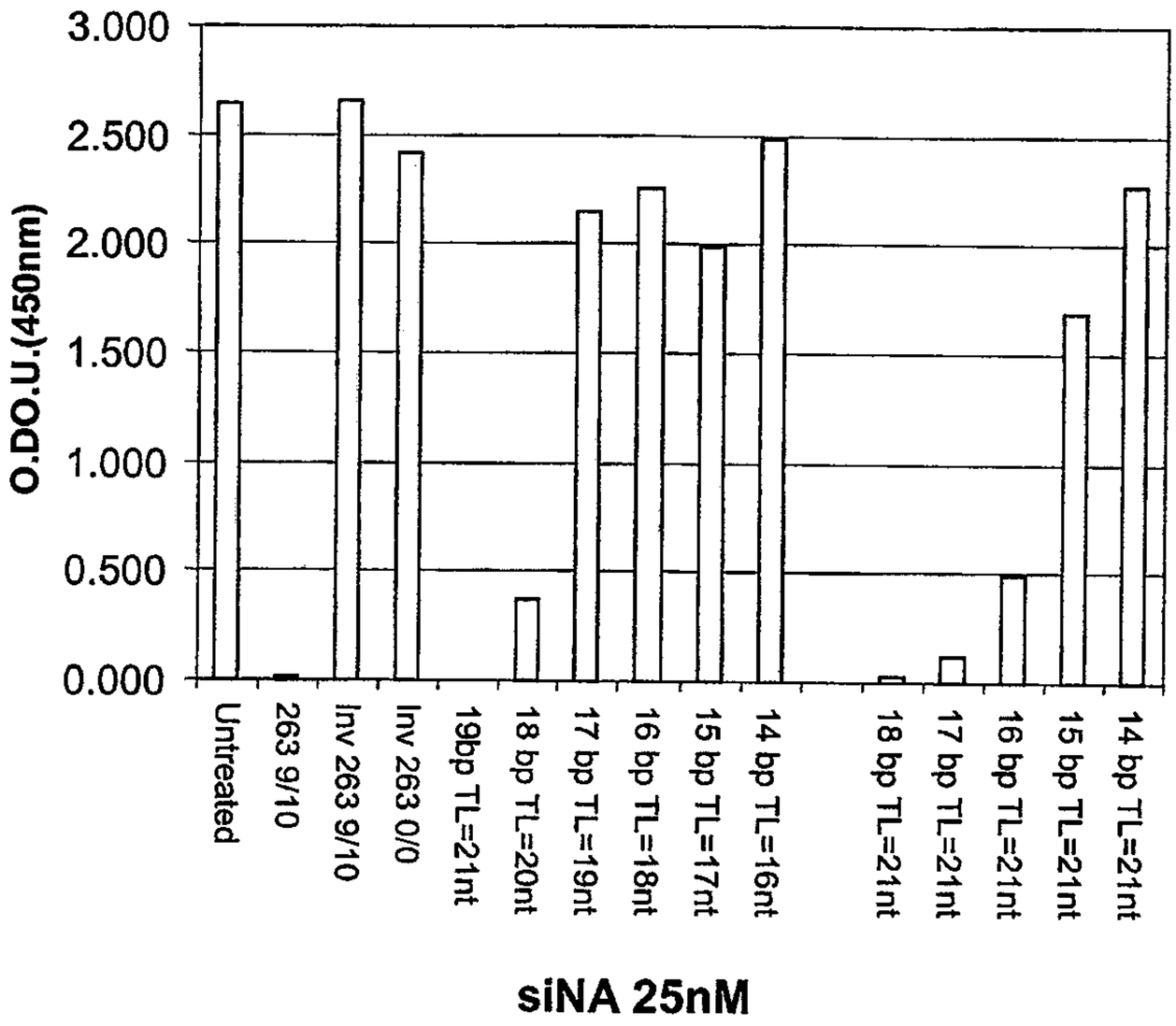


Figure 116: Additional Multifunctional siNA designs

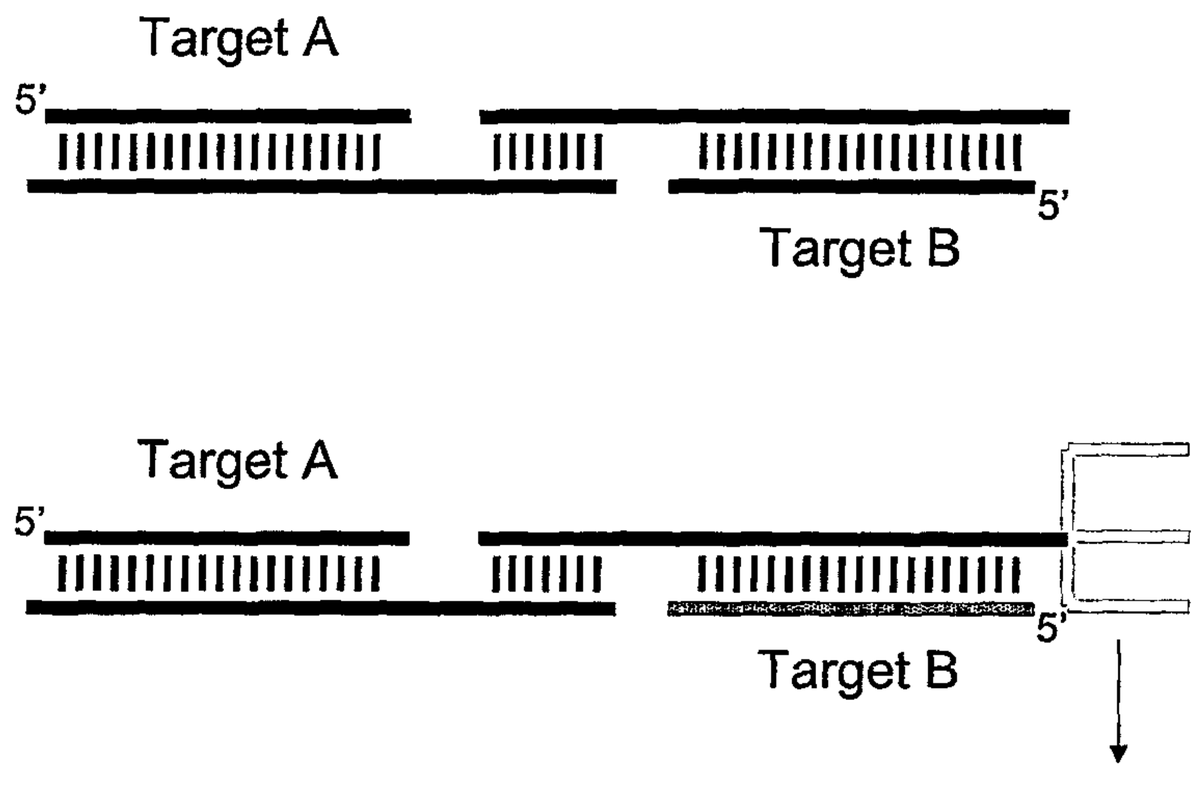


Figure 117: Additional Multifunctional siNA designs

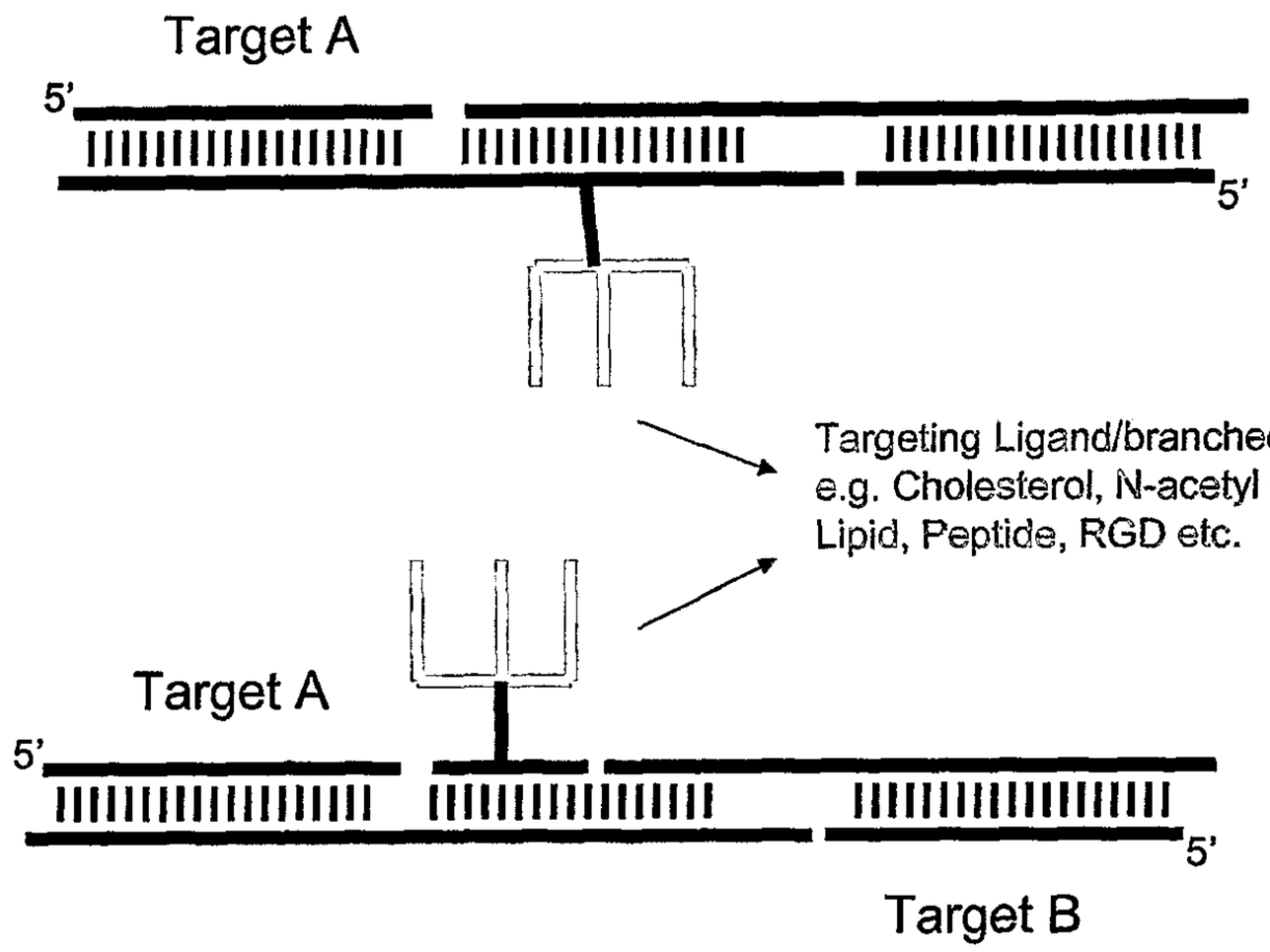


Figure 118: siNA length walk