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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/633,630	08/05/2003	Klaus Giese	39078-0005	6369
61263 7590 11/15/2007 PROSKAUER ROSE LLP 1001 PENNSYLVANIA AVE, N.W., SUITE 400 SOUTH WASHINGTON, DC 20004			EXAMINER CHONG, KIMBERLY	
			ART UNIT 1635	PAPER NUMBER
			MAIL DATE 11/15/2007	DELIVERY MODE PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No. 10/633,630*	Applicant(s) GIESE ET AL.	
	Examiner Kimberly Chong	Art Unit 1635	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 07 September 2007.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 12, 13, 15-17, 19, 29, 31, 32 and 34-62 is/are pending in the application.
- 4a) Of the above claim(s) _____ is/are withdrawn from consideration.
- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☒ Claim(s) 12, 13, 15-17, 19, 29, 31, 32 and 34-62 is/are rejected.
- 7) ☐ Claim(s) _____ is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☒ The drawing(s) filed on 04 February 2004 is/are: a) ☒ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☒ All b) ☐ Some * c) ☐ None of:
1. ☒ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. _____.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PC: Rule 17.2(a)).
- * See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- | | |
|---|---|
| 1) ☒ Notice of References Cited (PTO-892) | 4) ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date: _____ |
| 2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948) | 5) ☐ Notice of Informal Patent Application |
| 3) ☒ Information Disclosure Statement(s) (PTO/SB/08)
Paper No(s)/Mail Date <u>11/12/2007</u> . | 6) ☐ Other: _____ |

DETAILED ACTION

Request for Continued Examination

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 09/07/2007 has been entered.

Status of Application/Amendment/Claims

Applicant's response filed 09/07/2007 has been considered. Rejections and/or objections not reiterated from the previous office action mailed 06/13/2007 are hereby withdrawn. The following rejections and/or objections are either newly applied or are reiterated and are the only rejections and/or objections presently applied to the instant application. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

With entry of the amendment filed on 09/07/2007 claims 12-13, 15-17, 19, 29, 31-32 and 34-62 are pending and currently under examination. Applicant has canceled claims 1-11, 14, 18, 20-28, 30 and 33.

Information Disclosure Statement

The submission of the Information Disclosure Statement on 11/12/2007 is in compliance with 37 CFR 19.7. The information disclosure statements has been considered by the examiner and signed copies have been placed in the file.

Claim Objections

Claim 34 is objected to because of the following informalities: The claim recites "said molecules" when the claim is only drawn to a single double stranded ribonucleic acid molecule. Appropriate correction is required.

Claim 38 is objected to because of the following informalities: The claim recites "each stretch is 18-23 base pairs long". The use of the phrase "base pairs" is inconsistent with the context of the claim because a stretch in the claim is referring to an individual strand of nucleotides. Changing "base pairs" to "nucleotides", for example, would obviate this rejection. Appropriate correction is required.

Claim Rejections - 35 USC § 112

Claims 29 and 41 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a composition comprising the dsRNA molecule of claims 34 and 44 in a pharmaceutically acceptable carrier or diluent, does not reasonably provide enablement for mediating RNAi via introducing dsRNA molecules and a resultant treatment effect. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with these claims.

Specifically, the language "pharmaceutical composition" in claims 29 and 41 implies a therapeutic or treatment benefit that is not enabled. The *in vivo* inhibition and treatment effects described in the specification involve prophetic examples only and

have not been reduced to practice. Amendment of the claims to read "A composition comprising the dsRNA molecule of claims 34 or 44 and a pharmaceutically acceptable carrier or diluent", for example, would obviate this rejection.

Factors to be considered in a determination of lack of enablement include, but are not limited to:

- (A) The breadth of the claims;
- (B) The nature of the invention;
- (C) The state of the prior art;
- (D) The level of one of ordinary skill;
- (E) The level of predictability in the art;
- (F) The amount of direction provided by the inventor;
- (G) The existence of working examples; and
- (H) The quantity of experimentation needed to make or use the invention based on the content of the disclosure.

In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988)

The instant invention is drawn to a pharmaceutical composition comprising the siRNA molecule of claim 1 in an acceptable carrier or diluent.

There is no guidance in the specification as filed that teaches how to mediate RNA interference and a resultant treatment effect *in vivo*. Applicant is not enabled for mediating RNA interference *in vivo* with the instantly recited pharmaceutical composition, as delivery is known in the art to be unpredictable with regards to dsRNA duplexes.

The references cited herein illustrate the state of the art for therapeutic *in vivo* applications using dsRNA. Scherer et al. (Nat. Biotechnol., 2003, 21(12), pages 1457-

1465) teach that antisense oligonucleotides (ODNs), ribozymes, DNAzymes and RNA interference (RNAi) each face remarkably similar problems for effective application: efficient delivery, enhanced stability, minimization of off-target effects and identification of sensitive sites in the target RNAs. Scherer et al. teach that these challenges have been in existence from the first attempts to use antisense research tools, and need to be met before any antisense molecule can become widely accepted as a therapeutic agent.

Mahato et al. (Expert Opinion on Drug Delivery, January 2005, Vol. 2, No. 1, pages 3-28) teach that antisense oligodeoxynucleotides and double-stranded small interfering RNAs have great potential for the treatment of many severe and debilitating diseases. Mahato et al. teach that efforts have made significant progress in turning these nucleic acid drugs into therapeutics, and there is already one FDA-approved antisense drug in the clinic. Mahato et al. teach that despite the success of one product and several other ongoing clinical trials, challenges still exist in their stability, cellular uptake, disposition, site-specific delivery and therapeutic efficacy. Mahato et al. teach that in order for siRNAs to be used as therapeutic molecules several problems have to be overcome, including: the selection of the best sequence-specific siRNA for the gene to be targeted and the ability to minimize degradation in the body fluids and tissues.

Zhang et al. (Current Pharmaceutical Biotechnology 2004, vol. 5, p.1-7) reviews the state of the art with regard to RNAi and has this to say about use in mammalian cells. "Use of siRNA in mammalian cells could be just as far-reaching, with the applications extending to functional genomics and therapeutics. But various technical

issues must be addressed, especially for large-scale applications. For instance, dsRNA can be delivered to *C. elegans* by feeding or soaking, but effective delivery of siRNAs to mammalian cells will not be so simple."

As outlined above, it is well known that there is a high level of unpredictability in the RNAi art for therapeutic *in vivo* therapeutic applications. The scope of the claims in view of the specification as filed together do not reconcile the unpredictability in the art to enable one of skill in the art to make and/or use the claimed invention, namely a broad method of mediating RNA interference encompassing *in vivo* treatment effects with the instantly recited pharmaceutical composition.

Given the teachings of the specification as discussed above, one skilled in the art could not predict *a priori* whether introduction of RNA *in vivo* by the broadly disclosed methodologies of the instantly specification with the instantly claimed pharmaceutical composition would result in successful RNA interference and a therapeutic effect. To practice the claimed invention, one of skill in the art would have to *de novo* determine; the stability of the molecule *in vivo*, delivery of the molecule to the whole organism, specificity to the target tissue *in vivo*, dosage and toxicity *in vivo*, and entry of the molecule into the cell *in vivo* and the effective action therein. Without further guidance, one of skill in the art would have to practice a substantial amount of trial and error experimentation, an amount considered undue and not routine, to practice the instantly claimed invention.

The following is a quotation of the second paragraph of 35 U.S.C. 112:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claim 35 is rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 35 recites the limitation "said first stretch" and "said second stretch". There is insufficient antecedent basis for this limitation in the claim because claim 34 from which claim 35 depends only refers to a stretch of nucleotides in each strand and does not identify each stretch as being a first or second stretch of nucleotides. For purposes of prior art and examination, the claim will be interpreted to mean the first strand comprises a first stretch and the second strand comprises a second stretch, which is consistent with language in the currently amended claims.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 12-13, 15-17, 19, 29, 31-32 and 34-62 are rejected under 35 U.S.C. 103(a) as being unpatentable over McSwiggen et al. (of record, PTO 892 filed 01/12/2006) and Tuschl et al. (of record, PTO 892 filed 01/12/2006).

The instant claims are drawn to a double stranded ribonucleic acid molecule wherein the first strand is complementary to a target molecule, the second strand is complementary to a said first strand, wherein each strand comprises a stretch of alternating single 2'-modified and unmodified nucleotides. The claims are further drawn to a double stranded ribonucleic acid molecule comprising a first and second strand comprising a first and second stretch of nucleotides wherein each stretch consists of an alternating pattern of a plurality of groups of 2'-modified nucleotides linked by a single unmodified nucleotide, wherein the modified nucleotide on the first stretch is complementary to an unmodified nucleotide on the second stretch. Additionally, the claims are drawn to a ribonucleic acid molecule comprising a double stranded structure having a first and second strand comprising a first and second stretch of nucleotides each comprising a pattern of modifications wherein a 2'-modified nucleotide is linked to a non-modified nucleotide and wherein the modified nucleotide on one strand is complementary to a non-modified nucleotide on the other strand. The claims are further limited wherein the nucleotides are linked to phosphodiester bonds, each strand comprise 15 or more nucleotides, 17-23 or 18-23 nucleotides in length, wherein the RNA molecule is blunt ended on one or both strands, wherein the 2' modifications is from a group as listed in claims 16 and 39, wherein the first nucleotide of each strand or the last nucleotide of each strand is 2'-modified and a pharmaceutical composition comprising said double stranded ribonucleic acid.

McSwiggen et al. teach double stranded RNA comprising a sense and an antisense strand capable of silencing expression of a target gene (see paragraph 0016).

McSwiggen et al. teach specific embodiments of introduction of chemically modified nucleotides in numerous positions throughout the RNA strands and teach such modified dsRNA provide a powerful tool in overcoming potential limitations of *in vivo* stability and bioavailability inherent to native RNA molecules (see paragraph 0037). McSwiggen et al. teach various motifs, configuration and ways of designing dsRNA comprising various chemical modifications and teach specific embodiments wherein the dsRNA comprises about 19-25 nucleotides and said dsRNA can optionally comprise 1-3 nucleotides at the 3' terminus (see paragraph 0035). McSwiggen et al. teach the dsRNA can comprise chemical modifications on one or both strands and teach 1 or more nucleotides on each strand can comprise such modifications comprising phosphodiester internucleoside linkages or 2'-deoxy, 2'-O-methyl, and/or 2'-fluoro modifications (see paragraphs 0174 and 0051-0059). McSwiggen et al. do not specifically teach an embodiment of a dsRNA comprising patterns of a plurality of alternating 2' modified and unmodified nucleotides wherein each group is linked by a single unmodified nucleotide. However, McSwiggen et al. clearly recognize the importance of incorporating 2'- modifications, such as 2'-O-methyl into dsRNA to enhance the stability of said molecule.

Tuschl et al. teach a double stranded RNA, 19-25 nucleotides in length and teach the dsRNA is least 85% complementary and more preferably 100% complementary to the target and that the dsRNA are capable of mediating degradation of homologous RNAs (see paragraph 0017-0019). Tuschl et al. teach dsRNA having blunt ends are capable of mediating interference of gene expression and found dsRNA comprising overhangs on at least one strand are also capable of mediating interference (see Figure

11). Tuschl et al. additionally teach dsRNA molecules may contain nucleotides that are be modified at the 2' position of the ribose sugar. Preferred modifications are listed in paragraph 16 and include 2'-O-alkyl modifications and 2' fluoro modifications. Specific embodiments of modified siRNAs are taught at paragraph 166 and Fig. 14, which describe and show results obtained with 2' modified siRNAs and teach siRNA comprising minimal modifications retain RNAi activity and further teach modification of the entire double stranded RNA with 2'-O-methyl or 2'-deoxy is not well tolerated. Tuschl et al. teach compositions comprising said double stranded RNA molecule and a pharmaceutically acceptable carrier (see paragraphs 0031-0033) and teach a cell or organism comprising said double stranded RNA molecule (see paragraphs 0029 and 0040 and 0043).

Tuschl et al. do not explicitly teach the optimum number and placement of 2'-sugar modifications such that siRNA activity is retained and do not specifically teach a siRNA comprising alternating pattern of 2' modified and unmodified oligonucleotides. However, Tuschl et al. clearly recognize and teach that 2'-modifications enhance the nuclease stability of siRNA molecules and that more extensive 2'-deoxy or 2'-O-methyl modifications reduce the ability of siRNAs to mediate RNAi. Thus, Tuschl et al. appear to recognize that certain chemical modification of the 2'-OH results in enhanced nuclease resistance as well as modulation of RNAi activity.

It would have been obvious to one of ordinary skill in the art at the time the invention was made to make a double stranded RNA molecule comprising 2' modified nucleotides, as taught by McSwiggen et al. and Tuschl et al. and further it would have

been obvious and a matter of routine experimentation to use the general conditions of incorporating various chemical modification in various configurations, as taught by McSwiggen et al. to discover the optimal number and placement of 2'-sugar modifications in any dsRNA molecule, such that the resulting dsRNA molecule was endowed with maximum stability and functionality.

One would have been motivated to test all possibilities and configurations of 2' modified and unmodified nucleotides in one or both strands because it was well known in the art that incorporation of known modifications, such as 2'-O-methyl for example, impart increased stability and functionality in any RNA molecule, such as a dsRNA. MPEP 2144.05 states in part that "[W]here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation." *In re Aller*, 220 F.2d 454, 456, 105 USPQ 233, 235 (CCPA 1955).

Moreover, because Tuschl et al. teach a dsRNA comprising eight 2'-O-methyl modified nucleotides had RNAi activity and fully modifying all 42 nucleotides with 2'-O-methyl abolished RNAi activity, one would have been motivated to search for the optimum number and placement of the 2' modifications within this range by routine experimentation to see how well the modifications were tolerated with respect to stability and functionality of the dsRNA. Because there is a finite number of identified and predictable configurations of 2' modified and unmodified nucleotides known to impart increased stability and functionality in dsRNA as taught by Tuschl et al., one of ordinary skill in the art would have been motivated to incorporate modifications in various

configurations with a reasonable expectation of success at finding a functional motif with increased stability. Furthermore, it would have been routine to one of skill in the art to test these known modifications in various configurations to identify a dsRNA with increased stability and functionality, such as modifying only the first strand of the dsRNA while the strand is unmodified, or modifying every other nucleotide on each strand, for example. Further, since dsRNAs are taught by Tuschl et al. as being useful in cell culture and in whole organisms for elucidating gene function and for therapeutic use and since the chemical modifications of dsRNA are taught by McSwiggen et al. as being capable of improving cellular uptake and stability of dsRNA, one would therefore be motivated to chemically enhance the dsRNAs resistance to nucleases while preserving or maximizing its activity in order to most effectively target the desired gene.

One would have a reasonable expectation of success given that McSwiggen et al. teach numerous configurations and motifs comprising 2' modified and unmodified nucleotides in various patterns that were capable of eliciting RNAi. Further, one would have expected success at incorporating the chemical modifications into a dsRNA given Tuschl et al. teach how to make and use virtually any dsRNA to any gene provided the target sequence is known. One of skill in art would have had a reasonable expectation of success at identifying dsRNA comprising an alternating pattern of 2' modified and unmodified nucleotides on one or both strands of RNA from the finite number of possible modifications of a dsRNA taught by Tuschl et al. that retain RNAi activity.

Thus in the absence of evidence to the contrary, the invention as a whole would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made.

Claims 12-13, 15-17, 19, 29, 31-32 and 34-62 are rejected under 35 U.S.C. 103(a) as being unpatentable over Cook et al. (U.S. Patent No. 5,955,589), Damha et al. (US 2005/014535) and Tuschl et al. (of record, PTO 892 filed 01/12/2006).

The instant claims are drawn to a double stranded ribonucleic acid molecule wherein the first strand is complementary to a target molecule, the second strand is complementary to a said first strand, wherein each strand comprises a stretch of alternating single 2'-modified and unmodified nucleotides. The claims are further drawn to a double stranded ribonucleic acid molecule comprising a first and second strand comprising a first and second stretch of nucleotides wherein each stretch consists of an alternating pattern of a plurality of groups of 2'-modified nucleotides linked by a single unmodified nucleotide, wherein the modified nucleotide on the first stretch is complementary to an unmodified nucleotide on the second stretch. Additionally, the claims are drawn to a ribonucleic acid molecule comprising a double stranded structure having a first and second strand comprising a first and second stretch of nucleotides each comprising a pattern of modifications wherein a 2'-modified nucleotide is linked to a non-modified nucleotide and wherein the modified nucleotide on one strand is complementary to a non-modified nucleotide on the other strand. The claims are further limited wherein the nucleotides are linked to phosphodiester bonds, each strand

comprise 15 or more nucleotides, 17-23 or 18-23 nucleotides in length, wherein the RNA molecule is blunt ended on one or both strands, wherein the 2' modifications is from a group as listed in claims 16 and 39, wherein the first nucleotide of each strand or the last nucleotide of each strand is 2'-modified and a pharmaceutical composition comprising said double stranded ribonucleic acid.

Cook et al. teach antisense oligonucleotides comprising alternating segments of 2' modified nucleotides and unmodified nucleotides called gapmers or gapped antisense oligonucleotides (see abstract). Cook et al. found that substituting the 2' position on nucleotides increased the binding affinity of the antisense to that target gene and enhanced inhibition of expression (see column 8). Cook et al. teach each nucleotide can be linked with phosphodiester bonds (see Tables 3 and 4 and column 17). Cook et al. do not specifically teach antisense oligonucleotides with alternating 2' modified and unmodified nucleotides and do not teach double stranded oligonucleotides comprising patterns of alternating 2' modified and unmodified nucleotides.

Damha et al. teach antisense oligonucleotides with alternating segments of modified and unmodified nucleotides (see paragraphs 0009-0042, especially paragraph 0014). Damha et al. specifically teach an antisense oligonucleotide consisting of alternating modification linked to an unmodified deoxyribonucleotide and teach the first nucleotide can be a 2' modified nucleotide or can be unmodified and teach the last nucleotide in the strand can be a 2' modified nucleotide or an unmodified nucleotide (see paragraphs 0033-0034). Damha et al. teach the nucleotides can be linked with various internucleoside linkages, such as phosphodiester (see paragraph 0018) and

teach 2' modifications such as methoxy and fluoro (see paragraph 0042). Damha et al. unexpectedly discovered antisense oligonucleotides consisting of this pattern of alternating segments of modified and unmodified nucleotides were able to elicit gene inhibition more efficiently than antisense oligonucleotides without this alternating pattern (see paragraph 0111).

Tuschl et al. teach a double stranded RNA, 19-25 nucleotides in length and teach the dsRNA is least 85% complementary and more preferably 100% complementary to the target and that the dsRNA are capable of mediating degradation of homologous RNAs (see paragraph 0017-0019). Tuschl et al. teach dsRNA having blunt ends are capable of mediating interference of gene expression and found dsRNA comprising overhangs on at least one strand are also capable of mediating interference (see Figure 11). Tuschl et al. additionally teach dsRNA molecules may contain nucleotides that are be modified at the 2' position of the ribose sugar. Preferred modifications are listed in paragraph 16 and include 2'-O-alkyl modifications and 2' fluoro modifications. Specific embodiments of modified siRNAs are taught at paragraph 166 and Fig. 14, which describe and show results obtained with 2' modified siRNAs and teach siRNA comprising minimal modifications retain RNAi activity and further teach modification of the entire double stranded RNA with 2'-O-methyl or 2'-deoxy is not well tolerated. Tuschl et al. teach compositions comprising said double stranded RNA molecule and a pharmaceutically acceptable carrier (see paragraphs 0031-0033) and teach a cell or organism comprising said double stranded RNA molecule (see paragraphs 0029 and 0040 and 0043).

Tuschl et al. do not explicitly teach the optimum number and placement of 2'-sugar modifications such that siRNA activity is retained and do not specifically teach a siRNA comprising alternating pattern of 2' modified and unmodified oligonucleotides. However, Tuschl et al. clearly recognize and teach that 2'-modifications enhance the nuclease stability of siRNA molecules and that more extensive 2'-deoxy or 2'-O-methyl modifications reduce the ability of siRNAs to mediate RNAi. Thus, Tuschl et al. appear to recognize that certain chemical modifications of the 2'-OH results in enhanced nuclease resistance as well as modulation of RNAi activity.

It would have been obvious to one of ordinary skill in the art at the time the invention was made to make a double stranded RNA molecule comprising 2' modified nucleotides, as taught by Tuschl et al. and further it would have been obvious and a matter of routine experimentation to use the general conditions of incorporating alternating segments of modified and unmodified nucleotides, as taught by Cook et al. and Damha et al. to discover the optimal number and placement of 2' modifications in any dsRNA molecule, such that the resulting dsRNA molecule was endowed with maximum stability and functionality.

One would have been motivated to test all possibilities and configurations of 2' modified and unmodified nucleotides in one or both strands because it was well known in the art that incorporation of known modifications, such as 2'-O-methyl for example, impart increased stability and functionality in any inhibitor nucleotides, such as a dsRNA. MPEP 2144.05 states in part that "[W]here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable

ranges by routine experimentation." *In re Aller*, 220 F.2d 454, 456, 105 USPQ 233, 235 (CCPA 1955).

Further, given that Cook et al. teach oligonucleotide gapmers comprising alternating segments of 2' modified that flank a segment of unmodified nucleotides and given that Damha et al. unexpectedly found that an antisense oligonucleotide comprising alternating modified and unmodified nucleotides increase target specificity, one would have clearly been motivated to create a dsRNA with one or both strands comprising this alternating pattern of 2' modified and unmodified nucleotides for the purpose of increasing the dsRNA molecules target specificity and increased nuclease stability. Moreover, because Tuschl et al. teach a dsRNA comprising eight 2' modified nucleotides had RNAi activity and a fully 2'-O-methyl modified dsRNA having all 42 nucleotides modified abolished RNAi activity one would have been motivated to search for the optimum number and placement of the 2' modifications in this range by routine experimentation to see how well the modifications were tolerated with respect to stability and functionality of the dsRNA.

Because both Cook et al. and Damha et al. identify alternating patterns of modified and unmodified nucleotides in antisense oligonucleotides and because Tuschl et al. teach dsRNA comprising at least eight 2' modified nucleotides were capable of eliciting RNAi while fully modified dsRNA comprising 42 nucleotides, there is a finite number of identified and predictable configurations of 2' modified and unmodified nucleotides such that one of ordinary skill in the art would have been motivated to incorporate such patterns of modifications with a reasonable expectation of success and

it would have been routine to one of skill in the art to test these known patterns of modifications to identify a dsRNA with increased stability and functionality. Further, since dsRNAs are taught by Tuschl et al. as being useful in cell culture and in whole organisms for elucidating gene function and therapeutic use one would therefore be motivated to chemically enhance the dsRNAs resistance to nucleases while preserving or maximizing its activity in order to most effectively target the desired gene.

One would have a reasonable expectation of success given that Tuschl et al. teach how to make and use virtually any siRNA to any gene provided the target sequence is known. Further one would have expected incorporation of alternating patterns of modified and unmodified nucleotides as taught by Cook et al. and Damha et al. as this ability to incorporate modifications and testing such modifications is routine to one of skill in the art. One of skill in art would have had a reasonable expectation of success at creating a dsRNA comprising an alternating pattern of 2' modified and unmodified nucleotides on each strand of said RNA from the finite number of possible modifications of a dsRNA taught by Tuschl et al. that retain RNAi activity.

Thus in the absence of evidence to the contrary, the invention as a whole would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made.

Response to Applicant' Arguments

Re: Claim Rejections - 35 USC § 102

The rejection of record of claims 11-16 and 21-23 under 35 U.S.C. 102(b) as being anticipated by Crooke et al. (cited on PTO Form 892 filed 01/12/2006) is

withdrawn in view of the new grounds of rejection above. Therefore response to Applicant's arguments filed 09/07/2007 is obviated.

The rejection of record of claims 11-19, 21-23, 25-27, 29 and 31-33 under 35 U.S.C. 102(e) as being anticipated by McSwiggen et al. (cited on PTO Form 892 filed 01/12/2006) was not addressed by Applicant but is withdrawn in view of the new grounds of rejection above.

Re: Claim Rejections - 35 USC § 103

The rejection of claims 11-23, 25-27, 29 and 31-33 under 35 U.S.C. 103(a) as being unpatentable over McSwiggen et al. (cited on PTO Form 892 filed 01/12/2006) in view of Holen et al. (Nucleic Acids Research 04/30/2002) is withdrawn in view of the new grounds of rejection above. Therefore response to Applicant's arguments filed 09/07/2007 is obviated.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Kimberly Chong whose telephone number is 571-272-3111. The examiner can normally be reached Monday thru Friday between 7-4 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, James Schultz can be reached at 571-272-0763. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight (EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent application status and history information. It also enables applicants to view the scanned images of their own application file folder(s) as well as general patent information available to the public. For more information about the PAIR system, see <http://pair-direct.uspto.gov>.

For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.

/Kimberly Chong/
Examiner
Art Unit 1635

Interview Summary	Application No. 10/633,630	Applicant(s) GIESE ET AL.	
	Examiner Kimberly Chong	Art Unit 1635	

All participants (applicant, applicant's representative, PTO personnel):

- (1) Kimberly Chong, Sean McGarry, James Schultz. (3) Klaus Giese, Jorg Kaufmann.
 (2) Paul Booth, John Lucas. (4) _____.

Date of Interview: 28 November 2007.

Type: a) ☐ Telephonic b) ☐ Video Conference
 c) ☒ Personal [copy given to: 1) ☐ applicant 2) ☐ applicant's representative]

Exhibit shown or demonstration conducted: d) ☐ Yes e) ☒ No.
 If Yes, brief description: _____.

Claim(s) discussed: of record and proposed.

Identification of prior art discussed: _____.

Agreement with respect to the claims f) ☐ was reached. g) ☐ was not reached. h) ☒ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: Discussed 103 rejection of record and whether there was a reasonable expectation of success at modifying siRNA in the middle of the strands with 2'-O-methyl. Discussed proposed claim amendments to obviate rejection of record.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.


 Examiner's signature, if required



Docket No. 14677-003

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Klaus GIESE, et al.	)	Confirmation No: 6369
	)	
Application Serial No.: 10/633,630	)	Group Art Unit: 1635
	)	
Filed: August 5, 2003	)	Examiner: Kimberly Chong
	)	
For: INTERFERING RNA MOLECULES	)	

United States Patent and Trademark Office
Randolph Building
401 Dulany Street
Alexandria, Virginia 22314

AMENDMENT AND RESPONSE UNDER 37 CFR § 1.111

Sir:

This is a response to the Official Action mailed November 15, 2007 in the captioned application. Please charge all fees due for filing this response to Deposit Account 50-3840.

Please amend the application as set forth below:

IN THE CLAIMS

1-62. (Canceled)

63. (New) A nucleic acid molecule comprising a first strand and a second strand,
wherein said first strand comprises a first stretch that is complementary to a target nucleic acid,

wherein said second strand comprises a second stretch that is the same length as said first stretch and that is complementary to said first stretch,

wherein said first strand and said second strand form a double-stranded structure consisting of said first stretch and said second stretch,

wherein said first stretch and said second stretch each consist of contiguous alternating single 2'-O-methyl modified and single unmodified ribonucleotides,

wherein said 2'-O-methyl modified and unmodified ribonucleotides in each stretch are linked by phosphodiester bonds,

wherein each modified ribonucleotide in said first stretch is base paired with a said unmodified ribonucleotide in said second stretch,

wherein each modified ribonucleotide in said second stretch is base paired with a said unmodified ribonucleotide in said first stretch, and

wherein each stretch consists of 15-23 ribonucleotides.

64. (New) The nucleic acid molecule according to claim 63, wherein said first strand and said second strand are each 15-23 nucleotides long.

65. (New) The nucleic acid molecule according to claim 63, wherein said first strand and said second strand are each 17-23 nucleotides long.

66. (New) The nucleic acid molecule according to claim 63, wherein said first strand and said second strand are each 19 nucleotides long.

67. (New) The nucleic acid molecule according to claim 63, wherein said first strand and said second strand are each 21 nucleotides long.

68. (New) The nucleic acid molecule according to claim 63, wherein said first strand and said second strand are each 23 nucleotides long.

69. (New) The nucleic acid molecule according to claim 63, wherein said nucleic acid is blunt ended at both ends.

REMARKS

Claims 1-62 have been canceled, and claims 63-69 have been added. The added claims are fully supported by the original claims and the specification.

In the November 15, 2007, Office Action, claims 34 and 38 were objected to for an informality. Claims 29 and 41 were rejected under 35 USC § 112, first paragraph, for lack of enablement. Claim 35 was rejected under § 112, second paragraph, as indefinite. Claims 12-13, 15-17, 19, 29, 31-32 and 34-62 were rejected under 35 USC 103(a) as obvious over McSwiggen and Tuschl. Claims 12-13, 15-17, 19, 29, 31-32 and 34-62 were rejected under 35 U.S.C. § 103(a) as obvious over Cook, Damha and Tuschl. The specific grounds for rejection, and applicants' response thereto, are set forth in detail below.

Support for amendments

The added claims are fully supported by the specification and original claims. Specifically, claim 63 is supported at page 6, line 29, page 23, line 10, page 24, lines 17-23, page 25, lines 4-13, Figure 2B, Figure 12B, and original claims 11-13 and 15. Claim 64 is supported at page 6, line 29. Claim 65 is supported at page 4, line 9 and page 6, lines 23-30. Claims 66 and 67 are supported at page 6, lines 25-30, and in Figure 12B. Claim 68 is supported at page 6, line 29. Claim 69 is supported at page 2, lines 27-28, page 3, lines 6-8, page 6, lines 3-5, Figure 12B, and original claims 1 and 4.

Rejection Under 35 U.S.C. §112

Claims 29 and 41 are rejected under 35 USC § 112, first paragraph, for lack of enablement. Claim 35 is rejected under § 112, second paragraph, as indefinite. Specifically, the Examiner asserts that the language "pharmaceutical composition" as recited in claims 29 and 41 "implies" a therapeutic or treatment benefit that allegedly is not enabled. Claims 34 and 38 are rejected due to alleged informalities. Applicants respectfully traverse.

As the Examiner correctly notes, claims 29 and 41 are directed to compositions and not methods. It is axiomatic that the "enablement requirement is met if the description enables any mode of making and using the invention." Engel Indus., Inc. v. Lockformer Co., 946 F.2d 1528, 1533, 20 USPQ2d 1300, 1304 (Fed. Cir. 1991). The compositions of claims 29 and 49 can be

used *in vitro* regardless of their “implied” use in methods of treatment. This use of the claimed compositions is fully enabled by the working examples provided in the instant specification. Accordingly, the claims are fully enabled and the rejection should be withdrawn.

In any event, claims 29, 34, 38 and 41, have been canceled and, accordingly, the rejections are moot.

Rejections Under 35 U.S.C. §103(a)

Claims 12-13, 15-17, 19, 29, 31-32 and 34-62 were rejected under 35 USC 103(a) as obvious over McSwiggen and Tuschl. Claims 12-13, 15-17, 19, 29, 31-32 and 34-62 were rejected under 35 U.S.C. § 103(a) as obvious over Cook, Damha and Tuschl. The rejections are addressed below in the order set forth in the Office Action.

McSwiggen and Tuschl

The Examiner asserts that McSwiggen (US Patent Application US2003/0190635) teaches a chemically modified 19-25 nucleotide dsRNA capable of silencing expression of a target gene. The dsRNA may be modified at one or more nucleotides using various types of chemical modification, including modifications to the ribose sugar, the base and/or the phosphate backbone, either on one or both strands. Tuschl is cited as teaching blunt ended dsRNA that can induce RNA interference and also the use of modified nucleotides. The Examiner asserts that in light of these references it would have been obvious to make a dsRNA molecule comprising 2' modified nucleotides, and it would also have been obvious and “a matter of routine experimentation” to arrange 2'- modifications in any dsRNA molecule so as to provide maximum stability and functionality.

Moreover, the Examiner alleges that the references would have motivated one of ordinary skill in the to “test all possibilities and configurations” of 2' modified and unmodified nucleotides in one or both strands because it was well known that such modifications, “impart increased stability and functionality in any RNA molecule.” The Examiner further relies on *In re Aller*, (220 F.2d 454, 456, 105 USPQ 233, 235 (CCPA 1955)) for the proposition that “where the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation.” Finally, the Examiner asserts that

there is a finite number of ways to configure 2' modified and unmodified nucleotides “known” to increase stability and functionality in dsRNA, and that there would have been a reasonable expectation of success in identifying dsRNA having an alternating pattern of 2' modified and unmodified nucleotides on one or both strands of RNA “from the finite number of possible modifications of a dsRNA taught by Tuschl et al. that retain RNAi activity.”

Applicants respectfully traverse because there was no motivation in the art to prepare a nucleic acid having the 2'-O-methyl modifications recited in the instant claims. There was also no reasonable expectation that the 2'-O-methyl modifications recited in the claims would provide a nucleic acid that would either retain RNAi activity or increase stability, let alone both increase stability and maintain activity. Moreover, the instantly claimed nucleic acids have the unexpected property of having both a remarkable increase in stability and high degree of functional activity in promoting gene silencing. For at least these reasons, applicants respectfully submit that the rejection is improper and should be withdrawn.

A. McSwiggen is not available as prior art

McSwiggen has a filing date of 25 July 2002 and claims priority to provisional applications 60/358,580 (filed February 20, 2002), 60/363,124 (filed March 11, 2002), and 60/386,782 (filed June 6, 2002). McSwiggen was not published until 9 October 2003. The earliest filing date of the instant application is 5 August 2002. Accordingly, because McSwiggen was not published until after the earliest priority date of the instant application, it is available as a reference only under 35 USC § 102(e) and may properly be antedated by an appropriate evidentiary showing of prior invention.

The declaration by the inventors pursuant to 37 CFR § 1.131 (appended hereto as EXHIBIT A) clearly demonstrates that the captioned application was conceived and reduced to practice prior to McSwiggen's earliest priority date and therefore prior to the effective prior art date of McSwiggen. Accordingly, McSwiggen is not prior art against the instant application and all rejections based on McSwiggen should be withdrawn.

B. The Examiner has misread the disclosures of McSwiggen and Tuschl

The Examiner admits that McSwiggen fails to teach a dsRNA having alternating 2'-modified and unmodified nucleotides linked by a phosphodiester bond. The Examiner also admits that Tuschl fails to teach the number and placement of 2'-modified nucleotides such that siRNA activity is retained, and does not specifically teach a siRNA comprising an alternating pattern of 2' modified and unmodified oligonucleotides. However, the Examiner asserts that Tuschl teaches that "more extensive" 2'-deoxy or 2'-O-methyl modifications reduce the ability of siRNAs to mediate RNAi. Applicants respectfully submit that neither McSwiggen nor Tuschl disclose so much as the Examiner supposes.

(i) McSwiggen fails to disclose dsRNA molecules containing any pattern of modifications

Specifically, McSwiggen does not teach or suggest any pattern of 2'-O-methyl modified nucleotides that can both increase the stability of a dsRNA and retain RNAi activity. Indeed McSwiggen fails to teach or suggest any pattern of 2'-O-methyl modified nucleotides of a dsRNA that retains RNAi activity. Nor does McSwiggen teach or suggest any pattern of 2'-O-methyl modified nucleotides that will increase stability of a dsRNA. In fact, McSwiggen does not teach or suggest a dsRNA containing any pattern of 2'-O-methyl modified nucleotides whatsoever. In addition, McSwiggen fails to teach or suggest any specific 2'-O-methyl modifications spanning the antisense strand that will increase the stability of a dsRNA, let alone increase stability while simultaneously retaining functionality.

The Examiner states that "...the chemical modifications of dsRNA as taught by McSwiggen [are] capable of improving cellular uptake and stability of dsRNA." Applicants respectfully submit that this statement ignores the key question of whether or not the modifications eliminate the ability to elicit RNAi. A non-functional molecule might in theory be more stable and better taken up by a cell than a functional molecule, but it will still be non-functional because it will fail to elicit RNAi. Thus, any teaching or suggestion regarding modifications that may or may not stabilize a dsRNA molecule or increase its cellular uptake is insufficient to present a *prima facie* case of obviousness here without an additional teaching of the relationship between the chemical modifications effect on stability and activity. McSwiggen contains no such teaching.

(ii) *Tuschl fails to teach any 2'-O-methyl modified dsRNA molecules that retain RNAi activity*

Tuschl (WO 02/44321) is cited as teaching 19-25 nucleotide dsRNA molecules, modified at the 2'-position of the ribose sugar, that are capable of mediating degradation of homologous RNAs and the use of modified nucleotides. The Examiner admits that Tuschl does not teach the number and placement of 2'-modified nucleotides such that siRNA activity is retained and also does not specifically teach an siRNA comprising alternating pattern of 2'-modified and unmodified oligonucleotides.

Applicants respectfully submit that the Examiner has misread several aspects regarding the teachings of Tuschl. First, the Examiner asserts that Tuschl teaches that “modifications of the entire double stranded RNA with 2'-O-methyl or 2'-O-deoxy is not well tolerated.” The phrase “not well tolerated” suggests activity is reduced but not eliminated. However, Tuschl teaches that such modifications actually completely abolish RNAi activity (page 46, lines 13-14).

Second, the Examiner asserts that Tuschl teaches that dsRNA comprising eight 2'-O-methyl modified nucleotides had RNAi activity and that fully modifying all 42 nucleotides with 2'-O-methyl abolished RNAi activity. While it is correct that complete modification with 2'-O-methyl abolishes RNAi activity, Tuschl does not teach a dsRNA comprising eight 2'-O-methyl modified nucleotides that has RNAi activity.

Third, the statement by the Examiner that “Tuschl et al. clearly recognizes and teach that 2'-modifications enhance the nuclease stability of siRNA molecules and that more extensive 2'-deoxy or 2'-O-methyl modifications reduce the ability of siRNAs to mediate RNAi” could be misconstrued in two important ways. The statement first implies that Tuschl demonstrates a 2'-O-methyl modified dsRNA with increased stability. This is not correct. Tuschl describes no 2'-O-methyl modified dsRNA other than fully 2'-O-methyl modified dsRNA, which is completely inactive. By using the term “reduce” the statement secondly implies that extensively 2'-O-methyl- or 2'-deoxy-modified dsRNA retains some activity. The only extensively 2'-O-methyl or 2'-deoxy modified dsRNA taught by Tuschl is fully modified dsRNA and Tuschl clearly and dispositively states that “[c]omplete substitution of one or both siRNA strands by 2'-deoxy residues, however, abolished RNAi, as did substitution by 2'-O-methyl residues” (page 46, lines 13-14). A fairer reading of Tuschl, therefore, would be that more extensive 2'-O-methyl modification would be expected to eliminate, rather than merely reduce, RNAi activity.

Fourth, the Examiner states that “Tuschl et al. do not explicitly teach the optimum number and placement of 2’-sugar modifications such that siRNA activity is retained....” The term “optimum” as used here implies that Tuschl somehow teaches multiple 2-O-methyl modified dsRNAs with varying degrees of activity. However, with respect to 2’-O-methyl modified dsRNA, the only actual example provided by Tuschl is completely inactive. Tuschl prophetically suggests modifying the ends of a dsRNA to stabilize the molecule but does not teach what level of activity is retained by these molecules. More particularly, however, Tuschl does not teach placement of 2’-O-methyl groups anywhere in the middle region of a dsRNA in a manner that retains RNAi activity.

C. *McSwiggen recognizes the deficiencies in the art but fails to remedy those deficiencies*

The Examiner alleges that one of ordinary skill in the art would have been motivated to “test all possibilities and configurations of 2'-modified and unmodified nucleotides in one or both strands because it was well known that modifications, such as 2'-O-methyl modifications, “impart increased stability and functionality in any RNA molecule.” Applicants address the practical impossibility of this assertion in detail below, but first point out that the Examiner’s statement serves to make that point that neither McSwiggen nor Tuschl, alone or in combination, point one of skill in the art to the claimed invention. If either of the two references, either alone or in combination, provided any guidance or direction to one skilled in the art to the claimed invention, then one would *not* have been motivated to make each and every possible combination of modifications.

Specifically, McSwiggen fails to teach or suggest how to use 2’-O-methyl modifications that both impart both increased stability and functionality in terms of mediating the cleavage of target mRNA. As stated above, a non-functional dsRNA, i.e., a dsRNA that is unable to mediate RNAi, but that has increased stability and increased cellular uptake, is still non-functional.

Moreover, McSwiggen explicitly acknowledges in paragraph [0006] the well known facts that the prior art, in referring, in part, to 2'-O-methyl modifications on a dsRNA “fails to show to what extent these modifications are tolerated in siRNA molecules nor do they provide any examples of such modified siRNA.” McSwiggen likewise fails to show to what extent these modifications are or are not tolerated in siRNA molecules nor does it provide any data describing

results obtained with such modified siRNA. In other words, McSwiggen recognizes the limitations of the prior art yet nothing in McSwiggen would have made one of ordinary skill in the art any the wiser with respect to those limitations. Without a teaching or suggestion of how to place 2'-O-methyl modifications on a dsRNA, particularly across the antisense strand, in a manner that would both increase stability and retain activity, there would have been no motivation to make the claimed invention and no reasonable expectation of success.

D. Nothing in either McSwiggen or Tuschl would have motivated one of ordinary skill in the art to make 2'-O-methyl modified dsRNA having the pattern recited in the claims

There is simply no connection between the modifications of McSwiggen and those of the present invention. Tuschl provides no teaching that would remedy this lack of connection. The modifications of the present invention are based simply on the order of the nucleotides in a dsRNA molecule and are arranged such that an alternating pattern of modifications is formed. By contrast, McSwiggen relates to modifications based on the chemical identity of each nucleotide in a dsRNA molecule, not the order in which the nucleotides appear in that molecule. More specifically, McSwiggen teaches modification of pyrimidines. By way of background, RNA is made up of 4 different types of nucleotides, A, G, C and U. Two of these, A and G, belong to the chemical class of nucleotides called purines. The other two, C and U, belong to the chemical class of nucleotides called pyrimidines. Each dsRNA molecule will have a different sequence, or arrangement, of each of the A, G, C and U nucleotides, based on the sequence of the target mRNA. Thus, the placement of McSwiggen's modifications will change with essentially every sequence chosen without any reference to the order of each nucleotide in the sequence and without any regard to the formation of any specific pattern of modifications. In light of this one of ordinary skill in the art would not have been motivated to make modifications based on any pattern but, at most, would have tried modifications based on the specific chemical identity of each base.

E. There was no motivation in the art to make the claimed dsRNA molecules: The state of the art at the time of the invention

The rejection also relies upon a misrepresentation of the state of the art at the time the invention was made. Thus, the Examiner asserts that McSwiggen stands for the proposition that

modified ribonucleotides can be used to stabilize dsRNA molecules and that Tuschl teaches that “more extensive” 2'-deoxy or 2'-O-methyl modifications reduce the ability of siRNAs to mediate RNAi. Based upon these assertions, the Examiner alleges that the instantly claimed molecules were merely “optimized” and obvious variants of those molecules allegedly taught by McSwiggen and Tuschl. However, the Examiner reads far more into the references than is there, as is shown by the Declaration of Dr. Mike Gait, appended hereto as EXHIBIT B. A proper consideration of the state of the art at the time the present invention was made clearly leads to the conclusion that the instantly claimed molecules are nonobvious and therefore withdrawal of the rejection respectfully is requested.

Notably, as Dr. Gait explains, in 2002 the identity and nature of the nucleases that degrade dsRNA was not well understood. There was a further prejudice in the art that exonucleases, rather than endonucleases, were likely to be the primary causes of dsRNA degradation. This prejudice is shown in the nature of the modifications described by Tuschl which focused on modifications at the ends or termini of the dsRNA strands, a classic strategy for inhibiting exonuclease digestion. By contrast, the present inventors surprisingly discovered that endonucleases are primarily responsible for degradation of dsRNA of the type used in RNA interference.

In the only discussion of modifications in the interior of the strand, Tuschl states that a molecule containing a fully 2'-O-methyl modified antisense strand was inactive in causing RNA interference. The Examiner uses this statement to assert that one skilled in the art therefore would somehow have been motivated to use a less extensively modified dsRNA to avoid this loss of activity. However, the Examiner's assertions reflect a lack of understanding of the art at the time the present invention was made, lack factual foundation, and rely completely on prohibited hindsight.

A proper consideration of Tuschl's results would not have led one of ordinary skill in the art to make a less extensively modified dsRNA for RNAi purposes. Rather, the reasonable and scientifically appropriate conclusion would have been to avoid making modifications to the interior of the antisense strand of the dsRNA so as to avoid inhibiting binding of the antisense strand in the RISC complex. Thus, Tuschl describes preparing end-modified molecules that were successful in inducing RNAi, but compares the results of end-modification to the complete loss of activity caused by completely modifying the antisense strand:

The siRNA User Guide

Efficiently silencing siRNA duplexes are preferably composed of 21-nt antisense siRNAs, and should be selected to form a 19 bp double helix with 2-nt 3' overhanging ends. 2'-deoxy substitutions of the 2-nt 3' overhanging ribonucleotides do not affect RNAi, but help to reduce the costs of RNA synthesis and may enhance RNase resistance of siRNA duplexes. More extensive 2'-deoxy or 2'-O-methyl modifications, however, reduce the ability of siRNAs to mediate RNAi, probably by interfering with protein association for siRNAP assembly.

See Tuschl at page 49. Contrary to the Examiner's assertions, this does not suggest that there somehow might be some less extensive modification that would be successful at stabilizing dsRNA while mediating RNAi; rather, it is a clear suggestion that modifications *other than* modifications at the termini of the molecules would be expected to inhibit formation of the RISC complex and therefore prevent RNAi activity. Rather than a suggestion to make less extensively modified molecules, it is a clear warning that molecules that are more extensively modified than end-modified dsRNA are unlikely to be active in mediating RNAi. The conclusion that the Examiner draws from Tuschl is not only incorrect, but it is the exact *opposite* of the clear statements made by Tuschl. For at least these reasons, applicants respectfully submit that the rejection is improper and should be withdrawn.

F. McSwiggen fails to overcome the prejudice in the art against 2'-O-methyl modifications in the antisense strand.

As described above, McSwiggen is not available as prior art against the instantly claimed invention and therefore McSwiggen cannot cure the many deficiencies of Tuschl. However, even if it were available, nothing in McSwiggen, either alone or in combination with Tuschl, would have led one of ordinary skill in the art to the present invention. More specifically, McSwiggen fails to describe any data showing that molecules having 2'-O-methyl modifications in the interior of the antisense strand are active in mediating RNAi.

Thus, McSwiggen speculates that a certain number of nucleotides on each strand of a dsRNA might be 2'-modified by any one of a number of possible modifications, including fluoro and 2'-O-methyl groups. The only teaching provided by McSwiggen regarding the placement of these modifications is to suggest modification at pyrimidines. Nothing in McSwiggen teaches or suggests the pattern of 2'-O-methyl modified and unmodified nucleotides recited in the instant

claims. More specifically, McSwiggen provides no data that would have overcome the prejudice of one skilled in the art regarding the nature of modifications that should be avoided in preparing dsRNA for RNAi applications.

As explained by Dr. Gait, the prejudice in the art in 2002 was that modification of the antisense strand of dsRNA molecules by larger groups, such as 2'-O-methyl, would cause steric hindrance in the RISC complex that would inhibit or prevent RNAi activity. McSwiggen provides a large table of single stranded RNA molecules that contain various modifications, but provides precious little data regarding activity of these molecules. Moreover, although McSwiggen's table shows a very small number of 2'-O-methyl modified antisense strands, no data are shown for either the stability or RNAi activity of these strands. The only data shown by McSwiggen in which the antisense strand is modified at positions across its entire length are when the modification is 2'-fluoro. It is well known that a fluorine atom is much *smaller* than a hydroxyl group, whereas a methoxy group is clearly *much larger*. It is telling that, when McSwiggen provides data using a modified antisense strand, it is for strands containing the fluoro substituent. The clear conclusion is that McSwiggen is conforming to the prejudice in the art in 2002 – namely, that large substituents should be avoided in the center of the antisense strand.

Finally, any reasonable review of McSwiggen would convince one of ordinary skill in the art that the Examiner's position that mere "routine experimentation" is all that is required to obtain the instantly claimed compounds, even if legally relevant, is factually unfounded. McSwiggen shows literally hundreds of RNA sequences that purport to be useful for mediating RNAi (albeit with little actual data). Why, then, does McSwiggen fail to describe a single molecule that remotely resembles the instantly claimed molecules? If the instantly claimed molecules can be obtained by mere optimization of known molecules, why did McSwiggen fail to obtain such molecules? The answer of course is that the present invention is directed to molecules that are not taught or suggested by the prior art and that could not have been obtained by "routine experimentation." Accordingly, withdrawal of the rejection respectfully is requested.

G. The Examiner's assertions regarding a finite number of possible modifications of dsRNA molecules are improper

The Examiner makes the remarkable statement that one would have been motivated to test “all possibilities and configurations of 2’ modified and unmodified nucleotides in one or both strands” because it was well known that such modifications “increased stability and function in any inhibitor nucleotides, such as a dsRNA.” This statement misapplies the relevant law and evinces a failure to properly consider the factual background of the instant invention.

As shown in the accompanying declaration by Dr. Jörg Kaufmann, appended hereto as EXHIBIT C, there are approximately 10^{17} or 100,000,000,000,000,000 possible ways of arranging 2’ modified and unmodified nucleotides on antisense and sense strands of a dsRNA 23 nucleotides in length. This number assumes the dsRNA contain no modifications other than 2’-O-methyl modified nucleotides. The number therefore does not even take into account the possibility of including other possible 2’-modifications taught by McSwiggen which includes groups where the 2’-OH is substituted with H, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF₃, OCF₃, 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, ONO₂, NO₂, N₃, NH₂, aminoalkyl, aminoacid, aminoacyl, ONH₂, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, and substituted silyl modifications. When these possibilities are included the number of possible arrangements becomes unimaginably high. In light of these numbers it is clear that the “routine” testing proposed by the Examiner is impossible and the rejection relies on a grossly mistaken factual premise.

Moreover, it appears that the legal basis for the rejection relies on the recent *KSR* case, since the Examiner’s reference to a “finite” number of possibilities closely follows the language in that case. See 82 USPQ2d 1385 (2007). The Examiner misapplies the teachings of that case however.

In *KSR* the Supreme Court states that:

When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely the product not of innovation but of ordinary skill and common sense.

(Emphasis supplied). Thus, in the very special case where there are a knowable and reasonable number of *identified* and *predictable* solutions then it might be that the solutions are obvious. For example, if there are 10 ways of arranging a brake pedal and the results of those arrangements are predictable, then the *KSR* cases concludes that each one of the 10 arrangements is *prima facie* obvious.

The instant case could not be more different from the situation contemplated in *KSR*. Here, the results of modifying a dsRNA molecule are not predictable, and the nature and placement of the modifications has not been identified in the art. In the absence of any identified and predictable solutions, the *KSR* analysis described above simply is inapposite and the rejection is improper and should be withdrawn.

H. There was no reasonable expectation of success in making the claimed dsRNA molecules

As described above, all that was known in the art at the time that the present invention was made was that modifying the terminus of a dsRNA did not abolish RNAi activity, while complete modification of the antisense strand did abolish that activity. Nothing in the art would have led of ordinary skill in the art to conclude that a dsRNA having the structure recited in the instant claims would be highly active in mediating RNAi while showing significant stability against degradation. In the absence of any reasonable expectation of success, no *prima facie* case of obviousness exists, and the rejection should be withdrawn.

I. The claimed compounds have unexpected properties

For the reasons set forth above, the Examiner has failed to set forth a *prima facie* case of obviousness against the instantly claimed invention. However, even if it is assumed, purely for the sake of argument, that a *prima facie* exists, it is rebutted by the unexpectedly superior results shown by the claimed invention. In particular, nothing in the art taught or suggested that it was possible to prepare modified dsRNA molecules that retain high RNAi activity while providing high stability against nucleases *in vivo*.

The declaration by Dr. Klaus Giese, appended hereto as EXHIBIT D, describes results that show that dsRNA molecules having the structure recited in the instant claims are both highly

active and stable in serum. Moreover, this result is a general one that has been demonstrated in dsRNA molecules directed against over 100 gene targets.

Accordingly, applicants respectfully submit that the surprising and superior results described by Dr. Giese further demonstrate the patentability of the instantly claimed molecules. Accordingly, withdrawal of the rejection respectfully is requested.

J. The rejection is based on a misapplication of the law

The Examiner admits that neither of the cited references describe the claimed dsRNA molecules and, more specifically, admits that neither references teaches or suggests dsRNA molecules containing an alternating pattern of 2'-O-Me modified and unmodified RNA nucleotides on both strands, where the strands are arranged as set forth in the claims. Nevertheless, the Examiner bases the rejection on the notion that McSwiggen and Tuschl teach that modification of some number of unidentified nucleotides in unidentified positions (as in McSwiggen) or at the terminus of the strands (as in Tuschl) renders obvious the instantly claimed molecules because the claimed molecules could be obtained by "routine experimentation," citing *In re Aller* in support of this proposition. Applicants respectfully traverse because the grounds for the rejection are contrary to the express language of 35 USC §103, and ignore the express instructions laid down in the MPEP regarding this type of rejection.

The express language of 35 USC 103 provides that "patentability shall not be negated by the manner in which the invention was made." This language in the statute was inserted to make it clear that inventions are fully patentable even though they arise from routine research and development programs involving typical trial and error methods. The CCPA made it plain that rejections that rely on the notion that an invention is obvious merely because it could have been obtained by routine experimentation are contrary to law ("the emphasis upon routine experimentation is contrary to the last sentence of section 103," *In re Yates*, 211 USPQ 1149 (CCPA)). Accordingly, withdrawal of the rejection respectfully is requested.

The MPEP sets forth the very limited and specific types of situation in which an invention may be rejected as obvious based upon the notion of routine experimentation. See MPEP 2144.05. Notably, the instantly claimed invention does not remotely resemble the type of situation contemplated in the MPEP or in *In re Aller*, relied on by the Examiner. Thus the MPEP

states that the type of rejection sustained in *Aller* applies to ranges of common reaction variables such as concentration or temperature. Se MPEP 2144.05.II.A. Nowhere, either in the MPEP or in the case law, is there a single case standing for the proposition that *changes in chemical structure* are obvious because they allegedly could be arrived at by “routine experimentation.” Indeed, if it were otherwise, no patents on new pharmaceuticals would issue, since the unpredictable relationship between chemical structure and biological activity requires progress through trial and error and variation and substitution of functional groups in a manner that the Examiner deems “routine experimentation.” For at least these reasons, the basis for the rejection that the claimed structures were merely optimized positions arrived at by routine experimentation is improper and should be withdrawn.

Cook, Damha and Tuschl

Cook is cited as teaching “gapmer” antisense oligonucleotides containing 2'-modified nucleotides where each nucleotide can be linked with phosphodiester bonds. The Examiner cites Cook as teaching that substitution at the 2' position increased binding affinity and enhanced activity. Damha is cited as teaching antisense oligonucleotides with alternating modified and unmodified nucleotides where unmodified deoxyribonucleotide alternates with a 2'-modified nucleotide and that the nucleotides can be linked with phosphodiester bonds.

The Examiner admits that Tuschl does not teach an siRNA having alternating 2' modified and unmodified oligonucleotides, but asserts that Tuschl recognizes that certain chemical modifications of the 2'-OH result in enhanced nuclease resistance as well as modulation of RNAi activity. Based upon these alleged facts, the Examiner asserts that it would have been obvious to make a dsRNA molecule comprising 2'-modified nucleotides, as taught by Tuschl and it would have been “a matter of routine experimentation to use the general conditions of incorporating alternating segments of modified and unmodified nucleotides, as taught by Cook et al. and Damha et al. to discover the optimal number and placement of 2'-modifications in any dsRNA molecule, such that the resulting dsRNA molecule was endowed with maximum stability and functionality.” Applicants respectfully traverse.

Damha is not available as prior art

Damha was not published until after the filing date of the instant application but has a claimed priority date of February 1, 2002. Accordingly, Damha is available as a reference only under 35 USC § 102(e) and may properly be antedated by an appropriate evidentiary showing of prior invention. The accompanying declaration by the inventors clearly demonstrates that the captioned application was reduced to practice prior to February 1, 2002, the earliest effective filing date of Damha. Accordingly, Damha is not available as a reference, and the rejection must stand or fall on the combination of Cook and Tuschl. In any event, for the reasons set forth herein, even if Damha were available, the cited references fail to teach or suggest the instantly claimed invention and the rejection should be withdrawn.

Cook teaches away from the claimed invention

Cook is limited to single stranded antisense molecules but, in any event, fails to teach modification to the inner region of the RNA molecule that is responsible for activity. In fact, Cook specifically states that internal modifications to the RNA using 2'-O-methyl groups inactivate the antisense molecule (column 16, Table 1). The molecules of the present invention necessarily have modifications that span the center of the molecule, on both strands. However, one of ordinary skill in the art, reading Cook, would learn to avoid making modifications in the center of an oligonucleotide, lest the molecule be inactive. Accordingly, Cook directly teaches away from the instantly claimed invention, and the rejection is improper and should be withdrawn.

Damha also teaches away from the claimed invention

Damha is cited as teaching antisense oligonucleotides with alternating modified and unmodified nucleotides where unmodified deoxyribonucleotide alternates with a 2'-modified nucleotide and that the nucleotides can be linked with phosphodiester bonds. The Examiner asserts "Damha et al. unexpectedly discovered antisense oligonucleotides consisting of this pattern of alternating segments of modified and unmodified nucleotides were able to elicit gene inhibition more efficiently than antisense oligonucleotides without this alternating pattern." The Examiner however, leaves out two important facts.

First, Damha does not teach the unexpected results using 2'-O-methyl modifications. The unexpected results of Damha were the result of a different chemical modification (an isomeric form of the ribose sugar) to a different nucleic acid (DNA rather than RNA). Specifically, Damha's molecules are single stranded molecules containing alternating *deoxyribonucleotides* and *arabonucleotides*.

Second, Damha does describe alternating 2'-O-methyl modification of antisense molecules, but these molecules "showed only poor ability to elicit RNase H degradation of target RNA" (paragraph [0149] and Figure 3). Thus, rather than showing unexpectedly good results, the 2'-O-methyl modified nucleic acid of Damha showed poor results. Accordingly, Damha directly teaches away from the instantly claimed invention, and the rejection is improper and should be withdrawn.

The combination of Cook and Tuschl fails to teach or suggest the claimed invention

For the reasons described above, Damha is not available as a reference and therefore the rejection must stand or fall on the combination of Cook, which teaches away from the claimed invention, and Tuschl, whose deficiencies are discussed in detail above. Here, Tuschl is cited as teaching dsRNA capable of mediating interference of gene expression and also as teaching that siRNA comprising minimal 2'-O-modifications retain RNAi activity and that modification of the entire double stranded RNA with 2'-O-methyl or 2'-deoxy is not well tolerated. However, as discussed in detail above, the only 2'-O-methyl modified dsRNA made by Tuschl was not only not well tolerated, but was inactive. In this fashion, Tuschl can also be considered as teaching away from the claimed invention in that the only 2'-O-methyl modified molecules described by Tuschl, in which the modifications spanned the center section of the molecule, were inactive. One of ordinary skill in the art would consider this as mere further proof of the teachings of Cook and Damha that 2'-O-methyl modifications to the center of the molecule should be avoided if activity is to be retained.

The Examiner again asserts that one would have been motivated to test "all possibilities and configurations" of 2'-O-modified and unmodified nucleotides because it was "well known" that this would impart increased stability and functionality in "any inhibitor nucleotides, such as a dsRNA." As addressed above, this assertion lacks any basis in fact and is contradicted by the

teachings of Tuschl, Cook, and Damha which all show that 2'-O-methyl modification across the inner region of a nucleic acid results in loss of activity.

The Examiner concludes that because Tuschl shows that a dsRNA having eight 2' modified nucleotides had RNAi activity while a fully 2'-O-methyl modified dsRNA abolished RNAi activity one skilled in the art would have been motivated to "search for the optimum number and placement of the 2' modifications in this range by routine experimentation to see how well the modifications were tolerated with respect to stability and functionality of the dsRNA." Applicants submit that while Tuschl may demonstrate dsRNA with eight 2'-deoxy modified nucleotides had RNAi activity, the only 2'-O-methyl modified dsRNA tested by Tuschl was inactive. Moreover, far from providing motivation, Tuschl's "siRNA User Guide" warns against making 2'-O-methyl modifications to the inner region of the dsRNA.

The Examiner repeats the argument made in the rejection over McSwiggen and Tuschl that there were "a finite number of identified and predictable configurations of 2' modified and unmodified nucleotides such that one of ordinary skill in the art would have been motivated to incorporate such patterns of modifications with a reasonable expectation of success and it would have been routine to one of skill in the art to test these known patterns of modifications to identify a dsRNA with increased stability and functionality." Applicants again assert that there is no teaching in the cited references of any pattern of 2'-O-methyl modified dsRNA and no teaching of any nucleic acid with 2'-O-methyl modifications across a functional strand with increased stability and retained functionality. In light of the lack of guidance as to the relationship between 2'-O-methyl chemical modification and functionality, including the lack of guidance as to the placements of 2'-O-methyl chemical modifications based on the position of a nucleoside in a dsRNA there could be no motivation and no reasonable expectation of successfully making the claimed nucleic acid.

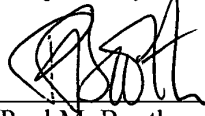
Moreover, for the reasons set forth in the Giese declaration, the instantly claimed molecules are both unexpectedly active in promoting gene silencing and also unexpectedly stable. For at least these reasons, applicants respectfully submit that the rejection is improper and should be withdrawn.

CONCLUSION

In view of the foregoing amendments and remarks, applicants respectfully submit that the application is in condition for allowance. Should the Examiner feel that there are any issues outstanding after consideration of this response, the Examiner is invited to contact the undersigned to expedite prosecution of the application.

The Commissioner is hereby authorized by this paper to charge any fees during the entire pendency of this application including fees due under 37 C.F.R. §§ 1.16 and 1.17 which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-3840. **This paragraph is intended to be a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).**

Respectfully submitted,



Paul M. Booth
Attorney for Applicant
Reg. No.: 40,244

Date: February 12, 2008

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EXHIBIT A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No: 14677-003

Applicant(s):	Klaus Giese <i>et al.</i>	Confirmation No.: 6369
Serial No.:	10/633,630	Examiner: Kimberly Chong
Filing Date:	August 5, 2003	Group Art Unit: 1635
Title:	INTERFERING RNA MOLECULES	

DECLARATION UNDER 37 C.F.R § 1.131

We, Klaus Giese, Anke Klippel-Giese and Jörg Kaufmann, do hereby declare as follows:

1. We understand that the claims in the captioned application have been rejected over U.S. patent application 2003/0190635, which lists February 20, 2002 as the earliest priority date, and U.S. patent application 2005/0142535, which lists February 1, 2002 as the earliest priority date.

2. We submit this declaration, based on our personal knowledge to demonstrate that the invention claimed in the captioned application was completed prior to February 2002 and therefore prior to the earliest prior art date of either U.S. patent application 2003/0190635, or 2005/0142535. All dates on the attached Exhibits have been masked out.

3. The delivery receipt attached as EXHIBIT 1 shows that two complementary oligoribonucleotide molecules, 94A2 and 94B1 were synthesized prior to February 1, 2002. 94A2 is the antisense strand and 94B1 is the sense strand. These molecules are described in the specification of the captioned application in Figure 15 where they are shown as PTENA V15 and PTENB V15.

4. In the delivery receipt, the lower case letters a, u, c, and g indicate the conventional unmodified ribonucleotides adenosine, uracil, cytosine, and guanosine, respectively. The number 5 indicates 2'O-methyl-ribo U, number 6 indicates 2'O-methyl-ribo A, number 7 indicates 2'O-

16

methy-ribo C, and number 8 indicates 2'-O-methyl-ribo G. Accordingly, the sequences of the two oligoribonucleotides are:

94A2: 5'-CuCcUuUuGuUuCuGcUaAcG-3' and

94B1: 5'-cGuUaGcAgAaAcAaAaGgAg-3'.

In these sequences a lower case letter indicates the unmodified nucleotide and the upper case indicates the 2'-O-methyl nucleotide. The nucleosides are linked by phosphodiester bonds.

5. Comparison of the sequences of 94A2 and 94B1 shows that they are both 21 nucleotides long, are complementary and have contiguous alternating 2'-O-methyl modified and single unmodified ribonucleotides, where a modified ribonucleotide on one strand is base paired with an unmodified ribonucleotide on the second strand and vice versa. 94A2 is complementary to a part of the PTEN gene. When 94A2 and 94B1 are combined, the resulting double stranded molecule is blunt ended.

6. Prior to February 1, 94A2 and 94B1 were combined to form a double stranded molecule designated 94A2/94B1 and demonstrated to inhibit expression of PTEN as shown in EXHIBIT 2. The results shown in EXHIBIT 2 were recorded prior to February 1, 2002.

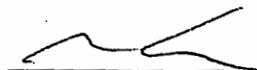
7. EXHIBIT 2 shows the results of an experiment on the ability of RNAi molecules to inhibit PTEN expression in HeLaB cells. PTEN mRNA was measured using quantitative real time PCR analysis and was normalized to p110a mRNA as control. The bar graph in EXHIBIT 2 shows the results obtained with various double stranded RNA molecules. The 80AB molecule was an unmodified blunt siRNA molecule, and represented a positive control. The 79AB molecule was a completely 2'-O-methyl modified siRNA molecule and served as a negative control. All molecules were tested at four different concentrations: 40 nM, 10 nM, 5 nM, 2.5 nM.

8. As can be seen from the data, molecule 94A2/94B1 shows activity that is very similar to the positive control 80AB, demonstrating that the molecule was effective at reducing PTEN mRNA expression in cells.

9. We hereby declare that all statements made herein of our own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements, and the like so made, are punishable by fine or imprisonment, or both, under Section 1001, Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Feb 10, 2008

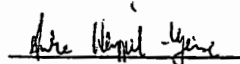
Date



Klaus Giese

Feb 10, 2008

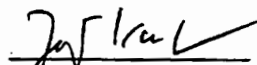
Date



Anke Klippel-Giese

Feb. 08/2008

Date



Jörg Kaufmann

EXHIBIT 1 of
EXHIBIT A



Lieferschein Delivery note	@oligo@klein@service Tel: 069 4109 2563 Fax: 069 4109 2564 mail: info@biospring.de
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Jörg Kaufmann					
atugen AG / Haus 80					
Robert-Rössle-Str.10					
D-13125 Berlin					
Ihre Bestnr. Lieferscheinnr. 3925			Kundennummer 10003 Bestellung vom [REDACTED]		
1 94A1					
5'-c5c 7u5 u5g 5u5 c5g 7u6 a7g-3'					
IDO	16893	OD	12,9 bei 260 nm	Länge	21-mer
Scale	0,2 µmol	Menge	35361 pmol	Molekular Gew.	5348,20
Reinigung	Modifikation		35 nmol	GC Gehalt	29 %
DNA-Typ	DNA		189 µg	Tm (GC)	39 °C
				ε (mM)	364,810
Zusammensetzung A=1,0 C=3,0 G=3,0 T=4,0 Modi=10,0					
Mod. 5'		Mod. 5 2'OMe-rU		Mod. 7 2'OMe-rC	
Mod. 3'		Mod. 6 2'OMe-rA		Mod. 8	
2 94A2					
5'-7u7 c5u 5u8 u5u 7u8 c5a 6c8-3'					
IDO	16894	OD	33,4 bei 260 nm	Länge	21-mer
Scale	0,2 µmol	Menge	88514 pmol	Molekular Gew.	4799,86
Reinigung	Modifikation		89 nmol	GC Gehalt	14 %
DNA-Typ	DNA		425 µg	Tm (GC)	34 °C
				ε (mM)	377,340
Zusammensetzung A=1,0 C=3,0 G=0,0 T=6,0 Modi=11,0					
Mod. 5'		Mod. 5 2'OMe-rU		Mod. 7 2'OMe-rC	
Mod. 3'		Mod. 6 2'OMe-rA		Mod. 8 2'OMe-rG	
3 94B1					
5'-c8u 5a8 c6g 6a6 c6a 6a8 g6g-3'					
IDO	16910	OD	18,9 bei 260 nm	Länge	21-mer
Scale	0,2 µmol	Menge	56445 pmol	Molekular Gew.	6523,12
Reinigung	Modifikation		56 nmol	GC Gehalt	29 %
DNA-Typ	DNA		368 µg	Tm (GC)	39 °C
				ε (mM)	334,840
Zusammensetzung A=4,0 C=3,0 G=3,0 T=1,0 Modi=10,0					
Mod. 5'		Mod. 5 2'OMe-rU		Mod. 7	
Mod. 3'		Mod. 6 2'OMe-rA		Mod. 8 2'OMe-rG	

BioSpring
Gesellschaft für Biotechnologie mbH

Geschäftsführer:
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Konto: 134295 BLZ: 50190000
Amtsgericht: Frankfurt am Main
Handelsregisternummer: HRB 43255
Ust-IdNr. DE187592327

4 94B2						
5'-7g5 u6g 7a8 a6a 7a6 a6g 8a8-3'						
IDO	16911	OD	32,7 bei	260 nm	Länge	21-mer
Scale	0,2 µmol	Menge		96665 pmol	Molekular Gew.	6555,12
Reinigung	Modifikation			97 nmol	GC Gehalt	14 %
DNA-Typ	DNA			634 µg	Tm (GC)	34 °C
					ε (mM)	338,280
Zusammensetzung		A=6,0	C=0,0	G=3,0	T=1,0	Modi=11,0
Mod. 5'		Mod. 5 2'OMe-rU			Mod. 7 2'OMe-rC	
Mod. 3'		Mod. 6 2'OMe-rA			Mod. 8 2'OMe-rG	

EXHIBIT 2 of
EXHIBIT A

47

120

► 96 well PTEC RNAi Transfection

ASST: PEN 2441 (Date:

Day 1: Set 2 pieces of Hydraz cords in 64 well TC plates: 2500 cells/well

Day 1

LEAD STOCKS: 2.0 ml of 10X Gird stock solution is Complementary Media

LIPID:	1X	2X	3X liquid	4X crystals media
	1.0	10	60	1980

COMPLEX: 2N4140ch 25W

- Prepare ICX GB samples in 96-well polystyrene U-bottom plate 40µl each

CD ₁	1X	10X	ul stock	ul storage media
-----------------	----	-----	----------	------------------

- ◆ Add 400 μ l of 10X lipid to the wells
- ◆ Shake plate
- ◆ Centrifuge in JY CO_2 incubator for 15-30 minutes
- ◆ Aspirate culture media from cells; add 200 μ l of H_2O media (no antibiotics)
- ◆ Add 100 μ l per well of the complexes in each replicate on T6 and T8 plates
- ◆ Incubate in CO_2 incubator at 37 for 24 hours

Day 1

- Arteria media completely gone walls no wash. (the same wall in (COAL) SLT (the better than China ice.

A	30 AB
B	19 AB
C	81 AB
D	64 AB
E	64 AB
F	
G	
H	
A	97A/97B
B	97A/97B
C	97A/97B
D	97A/97B
E	
F	
G	
H	

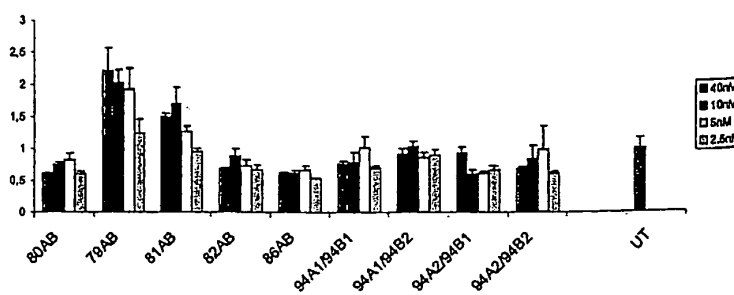
40 nH / 60 nH / 2.5 nH

* Tagihan 2x PEN / pmo

[illegible][illegible]

! Wiederholung d. Transfection erforderlich, da beim RNA Präp was schief gelaufen ist

Pten RNAi's in HelaB
(2500c/w NC 388 1µg/ml)



	2.5nm		
F3	0,09806335	0,60995611	0,03763394
F3	0,32695876	1,24788647	0,21298051
F7	0,08411727	0,85747478	0,04783074
I6	0,05928035	0,67125114	0,07974708
I7	0,05932739	0,53462942	0,01397018
I7	0,17560172	0,69505888	0,04158917
I3	0,0818074	0,89987118	0,09701874
I1	0,03013899	0,86374888	0,06892218
I3	0,36815269	0,81253536	0,03463899
I9	0,11221274		
I3	0,23545446		

Continued on Page

Read and Understood By

Ch. Technus

Signed

Date _____

Melanie Baker

Signed

Date _____

EXHIBIT B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Klaus GIESE, et al.	)	Confirmation No: 6369
	)	
Application Serial No.: 10/633,630	)	Group Art Unit: 1635
	)	
Filed: August 5, 2003	)	Examiner: Kimberly Chong
	)	
For: INTERFERING RNA MOLECULES	)	

United States Patent and Trademark Office
Randolph Building
401 Dulany Street
Alexandria, Virginia 22314

Declaration under 37 C.F.R. § 1.132

I, Dr. Michael Gait, declare and say:

1. I hold a PhD degree in Chemistry from the University of Birmingham, UK and a BSc in Chemistry, also from the University of Birmingham, UK. A copy of my Curriculum Vitae (CV) and reference list is attached to this declaration as EXHIBIT 1.

2. I am not an employee of or affiliated with Silence Therapeutics. I am being compensated by Proskauer Rose, LLP for the time spent in preparing this Declaration. I have extensive experience in the area of RNA biochemistry, including antisense and RNAi technology. I have published over 200 peer-reviewed publications in the field of oligonucleotide and RNA chemistry, biochemistry and applications in molecular biology. I was working directly in the field of RNAi at the time the captioned application was filed and have further studied degradation of siRNA in serum (see CV and reference list).

3. I have reviewed the specification of the captioned application. In addition to the relevant art, I have, in particular, reviewed the Official Action mailed November 15, 2007, together with the references cited in the Official Action. Specifically, I have reviewed the McSwiggen (US Patent Application US2003/0190635), Tuschl et al. (WO 02/44321), Cook et al. (US Patent 5,955,589) and Damha et al. (US Patent Application US2005/0142535) references.

4. I understand that the currently claimed invention is directed, in part, to a double stranded nucleic acid molecule where each strand contains a stretch of 15-23 ribonucleotides made up of contiguous alternating single unmodified ribonucleotides and single 2'-O-methyl modified ribonucleotides, with the ribonucleotides being linked by natural phosphodiester bonds. The modified ribonucleotides of each strand are "staggered" such that a 2'-O-methyl modified ribonucleotide on one strand base pairs with an unmodified ribonucleotide on the other strand. The double stranded molecule is highly resistant to degradation by nucleases whilst maintaining potent activity in gene silencing. The invention is further directed to the above double stranded nucleic acid molecule having blunt ends.

5. I also understand that the proper standard for considering the state of the RNAi art is from the viewpoint of a person of ordinary skill in the art at the time the invention was made.

6. At the time of the invention, it was known that nucleic acids could be modified by use of various chemical modifications to increase their resistance to cleavage by nucleases. It was also known that such chemical modifications to a nucleic acid could reduce or abolish its functionality (functional activity). It was therefore desirable to chemically modify nucleic acids to increase their resistance to nucleases, but only if the functionality could be maintained.

7. In the case of siRNA molecules, the person of ordinary skill in the art seeking to increase resistance to nucleases whilst retaining siRNA activity is faced with a paradox. This is because the modified double stranded siRNA molecules must be resistant to nucleases present in cells and biological fluids, e.g. serum, without inhibiting the activity of the specific endonuclease in the RISC complex that cleaves the target mRNA as a result of siRNA function. Thus, the effect of chemical modification of an siRNA molecule is highly unpredictable because it

involves decreasing the ability of the nucleic acid to act as a substrate for one or more undesired nucleases present in cells and biological fluids whilst retaining its ability to act as a substrate for the desired endonuclease in the RISC complex. Thus, the property sought to be reduced (nuclease cleavage of a double stranded RNA) is the same property sought to be maintained (nuclease cleavage of a double stranded RNA).

8. Further, the level of unpredictability involved in achieving this desired outcome was even greater, because little or nothing was known about the nuclease or nucleases involved in the unwanted degradation of the double stranded siRNA. Both endonucleases and exonucleases were known to be present in cells and biological fluids at the time of the invention. In 2002, when the captioned application was filed, no nucleases involved in the undesired degradation of double stranded siRNA molecules had been identified, let alone characterized in any manner. Moreover, little was known about RISC, the complex of proteins involved in cleavage of the target mRNA, except that the nuclease in RISC had been partially characterized as Argonaute 2, an endonuclease. A further unknown was how tolerant RISC would be to 2'-O-methyl and other modifications, especially modifications to nucleotides in the region of each strand of siRNA at or near the active cleavage site recognized by the RISC endonuclease. For at least these reasons, the level of unpredictability in identifying modifications to an siRNA molecule that would increase its stability to undesired endonucleases and exonucleases whilst maintaining its functionality to RISC was high. Neither McSwiggen nor Tuschl et al. teach which type or types of undesired nucleases (endonuclease or exonuclease) are responsible for siRNA cleavage in biological fluids (e.g., serum) or in cells.

9. Tuschl et al. (WO 02/44321) states that RNAi activity was abolished if all nucleotides on the sense, antisense or both the sense and antisense strands were substituted with 2'-O-methyl groups (p. 46, lines 13-14 and Figure 14, bars 7-9). Contrary to the examiner's assertions, Tuschl et al. do not describe any 2'-O-methyl modified siRNA that retain activity (p. 46, lines 5-14). Rather, the only modified molecules described by Tuschl et al. that retained RNAi activity contained 2'-deoxy substitutions at the 3' ends (p. 46, lines 8-12 and Figure 14, bars 2-6). Note that the placement of these modifications at the 3' ends is in line with the expectation that undesired nuclease activity on double stranded siRNA might be due to

exonucleases, as was well known for DNA oligonucleotides. Accordingly, the examiner's assertion that there are a "finite number of identified and predictable configurations of 2'-modified and unmodified nucleotides known to impart increased stability and functionality in dsRNA as taught by Tuschl" can apply, at most, to these 2'-deoxy substitutions. Tuschl et al. do not teach any actual or predictable 2'-O-methyl modified configurations that would maintain functionality. In view of Tuschl et al., one of ordinary skill in the art would have concluded that modification of siRNA molecules with 2'-O-methyl groups would reduce or abolish RNAi activity and, accordingly would not have been motivated to make 2'-O-methyl modified siRNA molecules, especially in the region of the active cleavage site recognized by the RISC endonuclease.

10. A group led by Tuschl published an academic paper (Elbashir et al. EMBO J. 20:6877 (2001)) (EXHIBIT 2) corresponding to work disclosed in Tuschl et al. (WO 02/44321). In Elbashir et al., the Tuschl group provides the reader with "The siRNA user guide" (page 6885, column 1). A similar "guide" appears in Tuschl et al. (WO 02/44321) at page 49, line 25, et seq. In both cases, the "guide" begins by pointing out that 2'-deoxy substitutions at the ends of the siRNA duplexes are useful but that "2'-O-methyl modifications reduce the ability of siRNAs to mediate RNAi, probably by interfering with protein association for siRNP assembly" (Elbashir et al. at page 6885, last sentence, first full paragraph; Tuschl et al. page 49, line 31 to page 50, line 2). Thus, Tuschl et al. warn against making 2'-O-methyl modifications, predicting that such modifications would reduce functionality. Elbashir et al. further states that the reason for loss of functionality caused by 2'-O-methyl modifications was "probably because methylation of the 2'-hydroxyls blocked hydrogen bond formation or introduced steric hindrance" (page 6886, column 2, last sentence of paragraph bridging columns 1 and 2). In view of the prior art, including Elbashir et al., one of ordinary skill in the art would have expected that making 2'-O-methyl modifications to an siRNA molecule, especially in the region of the active cleavage site recognized by the RISC endonuclease, would either abolish or greatly reduce functionality.

11. That Elbashir et al. remained the state of the art at the time of the captioned application was filed is further supported by Amarzguoui et al. NAR 31(2):589 (2003) (Exhibit 3) who noted that "siRNA with a general 2'-O-methylation in the either strand have no activity"

(page 591, column 1, lines 19-21). Amarzguioui et al. were then motivated to place 2'-O-methyl groups less extensively in the siRNA, but only at the 3' and 5' ends of each strand. Amarzguioui et al. were not motivated to try modifications in the central region and none are described. Similarly, Chiu and Rana, RNA 9:1034 (2003) (Exhibit 4) showed that incorporation of 2'-O-methyl nucleotides fully in either strand "greatly diminished EGFP gene silencing" (page 1037, column 2, line 4) and noted that "the bulkiness of the methyl group would likely be the cause of decreased RNAi activity" (page 1037, column 2, line 11-12). These authors noted that 2'-fluoro pyrimidine residues in both strands generally allowed reasonable functionality of the siRNA by contrast.

12. McSwiggen (US Patent Application US2003/0190635) provides no guidance to one of ordinary skill in the art as to the specific placement of 2'-O-methyl modifications that would both increase stability and maintain functionality. McSwiggen particularly lacks any specific statements or teachings regarding molecules containing 2'-O-methyl modifications within the antisense strand, which is the strand that ultimately binds to the target mRNA and directs its cleavage by the RISC endonuclease. This lack of a teaching or suggestion of making 2'-O-methyl modifications within the antisense strand is best understood when placed in the context of the generally accepted belief at the time of the invention that the structural requirements for the recognition of the antisense strand by RISC were more stringent than for the sense strand. This belief is reflected, for instance, in the comments in Elbashir et al. and Tuschl et al., described above, regarding the effects of 2'-O-methyl modification on protein association for siRNP assembly and in the comment by Elbashir et al. that "the chiral environment is distinct for sense and antisense siRNA, hence their function" (page 6885, column 2, first full paragraph). McSwiggen specifically points out the unpredictability in the state of the art at the time of his filing of siRNA chemical modification using 2'-O-methyl groups with the statement that the art fails to "show to what extent these modifications are tolerated in siRNA molecules nor provide any examples of such modified siRNA" (paragraph [0006]). McSwiggen also fails to provide any data regarding the activity of siRNA molecules that contain 2'-O-methyl modifications within the antisense strand. Even in the sense strand McSwiggen only teaches that pyrimidine nucleotides can be substituted by 2'-O-methyl, not purine.

13. Tuschl et al. at page 3, lines 22-27, states that “if both strands are *exactly* 21 or 22 nt, it is possible to observe *some* RNA interference when both ends are blunt (0 nt overhang)” (emphasis added). One of ordinary skill in the art would have interpreted this statement, when read in the context of the entire document, to mean that blunt end siRNA molecules 21 or 22 nt in length have significantly reduced activity and blunt ended siRNA molecules that are less than 21 nt or greater than 22 nt are not active. This is supported by Elbashir et al., for example at Figure 1, panels F-J, which show that all blunt end molecules have substantially reduced or no activity. Further support is provided on page 6884 which contains the opening statement under the heading “Sequence effects and 2'-deoxy substitutions in the 3' overhang” that reads “The 2 nt 3' overhang is critical for siRNA function” (emphasis added). The Tuschl group reinforces this assertion in the first sentence of the “The siRNA user guide” on page 6885 of Elbashir et al. stating that efficiently silencing siRNA “must be selected to form a 19 bp double helix with 2 nt 3' overhanging ends” (emphasis added).

14. At the time the captioned application was filed it was generally believed that double stranded siRNA was most vulnerable to cleavage by exonucleases (nucleases that degrade from the ends of the nucleic acid), rather than endonucleases (nucleases that cleave within a nucleic acid). Modifications at the ends of a nucleic acid were believed to be sufficient to protect against degradation by exonucleases. Thus, one of ordinary skill would not have been motivated to make modifications, particularly 2'-O-methyl modifications, to an siRNA molecule, other than possibly to the ends, because it was believed that modifications at positions other than at the ends of the molecules would severely reduce or abolish functionality. That exonucleases were thought primarily responsible for siRNA degradation at the time of the invention is shown by Soutschek et al. Nature 432(11):173-178 (2004) (EXHIBIT 5) which states “exonucleolytic degradation is the predominant mechanism for siRNA degradation in serum.”

15. It also should be noted that the effects of other types of potential 2'-modification, such as 2'-fluoro modification, are not predictive of the results of 2'-O-methyl modifications because the various modifying groups differ in their chemical and physical properties. For example, a 2'-fluoro group is much smaller than a 2'-O-methyl group and would thus not be expected to have the same issues with steric hindrance, as supported by Chiu and Rana.

16. Cook et al. (US Patent 5,955,589) and Damha et al. (US Patent Application US2005/0142535) provide no evidence against the position that at the time the captioned application was filed, one of ordinary skill in the art would have expected that an siRNA molecule containing 2'-O-methyl modifications within the central portion of the antisense strand involved in RISC binding and target cleavage, would have either greatly reduced or abolished functionality of the siRNA molecule.

17. Cook et al. do not report a double stranded RNA molecule, but only on a single stranded DNA molecule and how it may be substituted by 2'-O-methyl residues. It was known at the time the captioned application was filed that functionality of an antisense DNA oligonucleotide was reduced or abolished if the middle portion of the antisense DNA oligonucleotide, i.e., the portion recognized and cleaved by RNase H, was modified with 2'-O-methyl groups. Functionality was not affected if the same modifications were made at the ends of the antisense oligonucleotide. This gave rise to the creation of gapmers, such as those taught by Cook et al. With gapmers, the end portions, but not the middle portion, can be modified with 2'-O-methyl groups. Cook et al. even demonstrates how 2'-O-methyl modifications across the middle portion of an antisense oligonucleotide greatly reduce antisense functionality in Figure 2 (oligos 3975 and 3979). Cook et al. therefore teach that the central region of the DNA that is recognized by RNase H, an enzyme that recognizes a DNA/RNA hybrid (RNA target), cannot be substituted by 2'-O-methyl.

18. Damha et al. also do not report on a double stranded RNA molecule but instead show a single stranded DNA molecule and how it may be substituted by 2'-O-methyl. It was further known that antisense DNA oligonucleotides with alternating 2'-O-methyl modifications that spanned the middle portion of the antisense oligonucleotide also significantly reduced or abolished functionality. For example, Damha et al. states that an antisense oligo with alternating 2'-O-methyl modifications spanning the entire oligonucleotide "showed only poor ability to elicit RNase H degradation of target RNA (Fig. 3)." (paragraph [0149]) This severe reduction in functionality is demonstrated in Figure 3, sequence 8 with the sequence shown in Table 1 (p. 10, bottom of column 1).

19. Thus, together, Cook et al. and Damha et al. show that alternating 2'-O-methyl modifications of a nucleic acid have the same or similar effect on abolishing or severely reducing functionality as does 2'-O-methyl modifications on all nucleotides. Both fully modified and alternating modified antisense oligonucleotides similarly fail to elicit cleavage of a target mRNA by an endonuclease. Elbashir et al. shows that 2'-O-methyl modifications on all nucleotides of either siRNA strand fail to elicit cleavage by the RISC endonuclease of a target mRNA. One of ordinary skill in the art would have expected alternating 2'-O-methyl modifications of an siRNA molecule to have the same or similar effect as a fully 2'-O-methyl modified strand of an siRNA molecule, with the result of either severely reducing or abolishing cleavage of a target mRNA.

20. In summary, for the reasons set forth above, the state of the art of chemically modifying siRNA in a manner that would both increase resistance to cleavage by nucleases and maintain functionality at the time of the invention was unpredictable.

21. One of ordinary skill in the art would further not have been motivated to make the internal 2'-O-methyl modifications of the claimed nucleic acid because at the time of the invention it was believed that chemical protection against exonucleases rather than endonucleases was required to obtain a double stranded siRNA with suitable stability for use in biological fluids or cells.

22. In view of state of the art at the time of the invention, one of ordinary skill in the art would not have been motivated to make the nucleic acids claimed in the captioned application because alternating 2'-O-methyl modifications would have been expected to greatly reduce or abolish functionality (in terms of the ability of the siRNA to elicit cleavage of a target mRNA by RISC) in a manner similar to what was shown for full modification across the RISC recognition and cleavage site.

23. One of ordinary skill in the art would still further not have been motivated to make 2'-O-methyl modifications to the antisense strand because at the time of the invention it

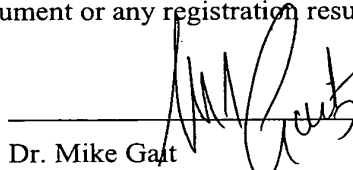
was believed that the antisense strand would be far less tolerant to modifications than the sense strand.

24. For these reasons, the ability of the claimed nucleic acid having alternating 2'-O-methyl modifications to be both highly resistant to nuclease activity as well as retaining a high level of RNAi activity, as shown, for example, in Figure 15 is unexpected.

25. In addition, the statements by Elbashir et al. described above that overhangs are "critical" for RNAi activity and that efficiently silencing siRNA "must be selected to form a 19 bp double helix with 2 nt 3' overhanging ends" mean that the ability of the claimed nucleic acid having blunt ends to retain full RNAi activity, as shown, for example, in Figure 16, oligonucleotides 155/156 and 161/162, is unexpected.

26. All statements made herein of my knowledge are true and all statements made on information and belief are believed to be true; and further, these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any document or any registration resulting therefrom.

Date: February 4th 2008



Dr. Mike Galt

EXHIBIT 1 of
EXHIBIT B

CURRICULUM VITAE

Michael John Gait

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Citizenship: British

PRESENT EMPLOYMENT

4/94 - MRC Programme Leader

4/87 - 3/94 Senior Staff Scientist

9/75 - 4/87 Staff Scientist (tenured 1980)

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EDUCATION

1973 PhD, Chemistry, University of Birmingham, England

Supervisors: Prof A S Jones, Prof. R T Walker. Subject: Nucleic Acids

1970 BSc, Chemistry (Class I) University of Birmingham, England

OTHER EMPLOYMENT EXPERIENCE

9/82 - 9/83 Research Associate, Collaborative Research Inc., (leave of absence)
Waltham, Massachusetts, USA

9/73 - 8/75 Research Associate, Massachusetts Institute of Technology,
Cambridge, Massachusetts, USA (Department of Chemistry)
Project Director: Prof H. G. Khorana

CONSULTANCIES

04/07 - GlaxoSmithKline

09/06 - Panagene Inc. (Scientific Advisory Board)

7/97 - 6/03 RiboTargets Ltd, Cambridge, UK (Scientific Advisory Board)

7/89 - 6/03 Gilead Sciences, Foster City, CA, USA (Scientific Advisory Board)

5/94 - 4/95 Glaxo Group Research, Greenford, UK

9/86 - 9/92 British Bio-Technology Ltd, UK

9/86 - 10/88 Integrated Genetics/Gene-Trak Systems, Framingham, USA

8/81 - 4/82 Transgene, Strasbourg, France

3/81 - 3/82 Celltech Ltd, Slough, UK

10/79 - 4/85 Collaborative Research Inc., Waltham, MA, USA

AWARDS

Winner of the 2003 RSC Award in Nucleic Acids Chemistry

Elected an EMBO member 2006

SCIENTIFIC SOCIETIES

Royal Society of Chemistry:

Associate Member 1973-1987

Fellow of the RSC 2002-

Chemical Biology Forum Exec Committee Member 2003-2007

Awards Committee 2007-

Nucleic Acids Group Committee 1982-1994 and 2000-

(Secretary/Treasurer 1988-1992 Chairman 2000-2003)

M J Gait

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EXHIBIT 2 of
EXHIBIT B

Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate

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Duplexes of 21–23 nucleotide (nt) RNAs are the sequence-specific mediators of RNA interference (RNAi) and post-transcriptional gene silencing (PTGS). Synthetic, short interfering RNAs (siRNAs) were examined in *Drosophila melanogaster* embryo lysate for their requirements regarding length, structure, chemical composition and sequence in order to mediate efficient RNAi. Duplexes of 21 nt siRNAs with 2 nt 3' overhangs were the most efficient triggers of sequence-specific mRNA degradation. Substitution of one or both siRNA strands by 2'-deoxy or 2'-O-methyl oligonucleotides abolished RNAi, although multiple 2'-deoxynucleotide substitutions at the 3' end of siRNAs were tolerated. The target recognition process is highly sequence specific, but not all positions of a siRNA contribute equally to target recognition; mismatches in the centre of the siRNA duplex prevent target RNA cleavage. The position of the cleavage site in the target RNA is defined by the 5' end of the guide siRNA rather than its 3' end. These results provide a rational basis for the design of siRNAs in future gene targeting experiments.

Keywords: PTGS/RNA interference/small interfering RNA

Introduction

Post-transcriptional gene silencing (PTGS) mediated by double-stranded (ds) RNA represents an evolutionarily conserved cellular defence mechanism for controlling the expression of alien genes in protists, filamentous fungi, plants and animals (Fire, 1999; Bass, 2000; Cogoni and Macino, 2000; Carthew, 2001; Hammond *et al.*, 2001b; Sharp, 2001; Tuschl, 2001; Voinnet, 2001; Waterhouse *et al.*, 2001). It is believed that random integration of alien genes (such as transposons) or viral infection causes production of dsRNA, which activates sequence-specific degradation of homologous single-stranded mRNA or viral genomic RNA, thereby preventing expression or replication of the foreign genetic material. The dsRNA is used as the guide RNA in this sequence-specific RNA degradation process. In some cases, dsRNA may also be

involved in amplification of the silencing signal important for systemic spread (Palauqui *et al.*, 1997; Voinnet *et al.*, 1998) or long-term maintenance of silencing (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000; Smardon *et al.*, 2000). In animals, the dsRNA-triggered silencing effect is referred to as RNA interference (RNAi; Fire *et al.*, 1998).

One important feature of the mechanism of RNAi is the processing of long dsRNAs into duplexes of 21–25 nucleotide (nt) RNAs. These short RNA products were first detected in plant tissues that exhibited transgene- or virus-induced PTGS (Hamilton and Baulcombe, 1999), but were also found later in fly embryos and worms injected with long dsRNAs (Parrish *et al.*, 2000; Yang *et al.*, 2000) or in extracts from *Drosophila melanogaster* Schneider-2 (S2) cells that were transfected with dsRNA (Hammond *et al.*, 2000). The processing reaction of long dsRNAs to 21–23 nt RNAs was first recapitulated *in vitro*, in extracts prepared from *D.melanogaster* embryos (Zamore *et al.*, 2000) and later in extracts from S2 cells (Bernstein *et al.*, 2001). In the embryo lysate, it was observed that the target mRNA was cleaved in ~21 nt intervals (Zamore *et al.*, 2000) and that synthetic 21 and 22 nt RNA duplexes added to the lysate were able to guide efficient sequence-specific mRNA degradation, while duplexes of 30 bp dsRNA were inactive (Elbashir *et al.*, 2001b). The 21 nt RNA products were therefore named small interfering RNAs or silencing RNAs (siRNAs).

A ribonuclease III enzyme, dicer, is required for processing of long dsRNA into siRNA duplexes (Bernstein *et al.*, 2001). It was recently shown that dicer has an additional cellular function and is also required for excision of 21 and 22 nt small temporal RNAs (stRNAs) from ~70 nt stable stem-loop precursors (Grishok *et al.*, 2001; Hutvagner *et al.*, 2001). These tiny expressed RNA molecules are important regulators of developmental timing and control the translation of downstream regulatory genes (Ambros, 2000; Moss, 2000; Pasquinelli *et al.*, 2000). stRNAs are different from siRNAs in that the target mRNA is not degraded during silencing (Wightman *et al.*, 1993; Olsen and Ambros, 1999) and they are single stranded (Reinhart *et al.*, 2000), while siRNAs are believed to be double stranded (Elbashir *et al.*, 2001b; Hutvagner *et al.*, 2001).

In RNAi, a siRNA-containing endonuclease complex cleaves a single-stranded target RNA in the middle of the region complementary to the 21 nt guide siRNA of the siRNA duplex (Elbashir *et al.*, 2001b). This cleavage site is one helical turn displaced from the cleavage site that produced the siRNA from long dsRNA, suggesting dramatic conformational and/or compositional changes after processing of long dsRNA to 21 nt siRNA duplexes. The target RNA cleavage products are rapidly degraded because they either lack the stabilizing cap or poly(A) tail. A protein component of the ~500 kDa endonuclease or

RNA-induced silencing complex (RISC) was recently identified and is a member of the argonaute family of proteins (Hammond *et al.*, 2001a); however, it is currently unclear whether dicer is required for RISC activity.

It is also unknown whether RISC contains single- or double-stranded siRNAs. By analogy to stRNA excision, it may be envisaged that only one of the strands of a siRNA duplex is incorporated into a catalytic siRNP, but because of the symmetry of the siRNA duplex, two approximately equal populations of sense and antisense strand-containing catalytic siRNPs are produced. Synthetic siRNA duplexes cleaved sense as well as antisense target RNAs in the middle of the region covered by the siRNA duplex in *D.melanogaster* lysate (Elbashir *et al.*, 2001b). However, longer dsRNAs did not produce symmetric sense and antisense target RNA cleavage sites in embryo lysate (Elbashir *et al.*, 2001b), suggesting that the direction of processing of long dsRNA defined which of the strands of the resulting siRNA duplex could be used for guiding target degradation. Some protein, involved in the production of the 21 nt siRNA duplexes, may be deposited on the siRNA duplex to mark the strand that is going to be used for guiding target RNA cleavage.

Despite the lack of profound mechanistic understanding, RNAi has rapidly developed into an important tool for reverse genetics and has been widely applied in *Caenorhabditis elegans* (Fraser *et al.*, 2000; Gönczy *et al.*, 2000; Piano *et al.*, 2000; Maeda *et al.*, 2001), as well as in insects (see references in Lam and Thummel, 2000) and insect cell lines (Clemens *et al.*, 2000; Hammond *et al.*, 2000; Ui-Tei *et al.*, 2000). RNAi has also been shown to occur in a variety of vertebrates by targeting of mRNAs important for embryonic development. In differentiated mammalian cells, dsRNAs with >30 bp generally activate the interferon response, which leads to a global shut-off in protein synthesis as well as non-specific mRNA degradation (Stark *et al.*, 1998). This unspecific response to long dsRNAs can be bypassed using 21 nt siRNA duplexes, resulting in specific knock-down of the expression of the targeted gene (Elbashir *et al.*, 2001a; Hutvagner *et al.*, 2001), providing a new method for analysis of mammalian gene function in cultured cells.

Here we describe the results of a systematic analysis of the length, secondary structure, sugar backbone and sequence specificity of siRNA duplexes for RNAi, using the established *D.melanogaster* embryo *in vitro* system. The most potent siRNA duplexes are 21 nt long, comprising a 19 nt base-paired sequence with 2 nt 3'-overhanging ends. The 5' end of the target-complementary siRNA strand (guide siRNA) sets the ruler to define the position of target RNA cleavage. Furthermore, we find that target recognition is extremely specific, as even single

nucleotide mismatches between the siRNA duplex and the target mRNA abolish interference. These results provide a rational basis for the design of siRNAs for future gene targeting experiments.

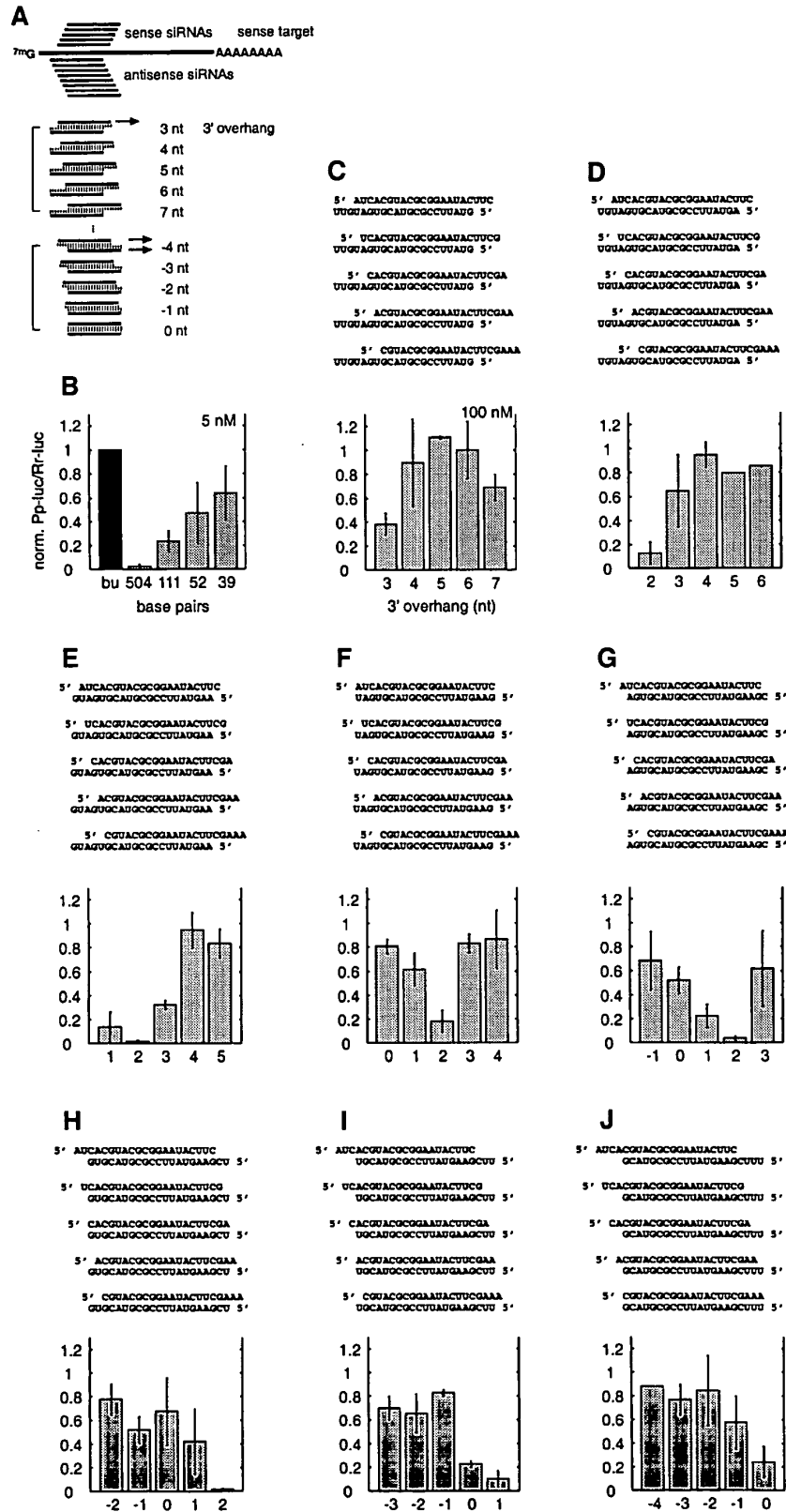
Results

Variation of the 3' overhang in duplexes of 21 nt siRNAs

We reported previously that two or three unpaired nucleotides at the 3' end of siRNA duplexes were more efficient in target RNA degradation than blunt-ended duplexes (Elbashir *et al.*, 2001b). To perform a more comprehensive analysis of the function of the terminal nucleotides, we synthesized five 21 nt sense siRNAs, each displaced by one nucleotide relative to the target RNA, and eight 21 nt antisense siRNAs, each displaced by one nucleotide relative to the target (Figure 1A). By combining these sense and antisense siRNAs, a series of eight siRNA duplexes with symmetric overhanging ends were generated spanning a range from 7 nt 3' overhang to 4 nt 5' overhang. The interference was measured using the dual luciferase assay system (Tuschl *et al.*, 1999; Zamore *et al.*, 2000). siRNA duplexes were directed against firefly luciferase mRNA and sea pansy luciferase mRNA was used as internal control. The luminescence ratio of target to control luciferase activity was determined in the presence of siRNA duplex and was normalized to that observed in its absence. For comparison, the interference ratios of long dsRNAs (39–504 bp) are shown in Figure 1B (Elbashir *et al.*, 2001b). The interference ratios were determined at concentrations of 5 nM for long dsRNAs (Figure 1A) and at 100 nM for siRNA duplexes (Figure 1C–J). The 100 nM concentration of siRNAs was chosen because complete processing of 5 nM 504 bp dsRNA would result in 120 nM total siRNA duplexes.

The ability of 21 nt siRNA duplexes to mediate RNAi is dependent on the number of overhanging nucleotides or base pairs formed. Duplexes with 4–6 3'-overhanging nucleotides were unable to mediate RNAi (Figure 1C–F), as were duplexes with two or more 5'-overhanging nucleotides (Figure 1G–J). The duplexes with 2 nt 3' overhangs were most efficient in mediating RNA interference, although the efficiency of silencing was also sequence dependent and up to 12-fold differences were observed for different siRNA duplexes with 2 nt 3' overhangs (compare Figure 1D–H). Duplexes with blunted ends, 1 nt 5' overhang or 1–3 nt 3' overhangs were sometimes functional and sometimes completely inactive. The small silencing effect observed for the siRNA duplex with 7 nt 3' overhang (Figure 1C) may be due to an antisense effect of the long 3' overhang rather than to

Fig. 1. Variation of the 3' overhang of duplexes of 21 nt siRNAs. (A) Outline of the experimental strategy. The capped and polyadenylated sense target mRNA is depicted and the relative positions of sense and antisense siRNAs are shown. Eight series of duplexes according to the eight different antisense strands were prepared. The siRNA sequences and the number of overhanging nucleotides were changed in 1 nt steps. (B) Normalized relative luminescence of target luciferase (*Photinus pyralis*, Pp-luc) to control luciferase (*Renilla reniformis*, Rr-luc) in *D.melanogaster* embryo lysate in the presence of 5 nM blunt-ended dsRNAs (Elbashir *et al.*, 2001b). The luminescence ratios determined in the presence of dsRNA were normalized to the ratio obtained for a buffer control (bu; black bar). Normalized ratios less than 1 indicate specific interference. (C–J) Normalized interference ratios for eight series of 21 nt siRNA duplexes. The sequences of siRNA duplexes are depicted above the bar graphs. Each part shows the interference ratio for a set of duplexes formed with a given antisense guide siRNA and five different sense siRNAs. The number of overhanging nucleotides (3' overhang, positive numbers; 5' overhang, negative numbers) is indicated on the x-axis. Data points were averaged from at least three independent experiments. Error bars represent standard deviations.



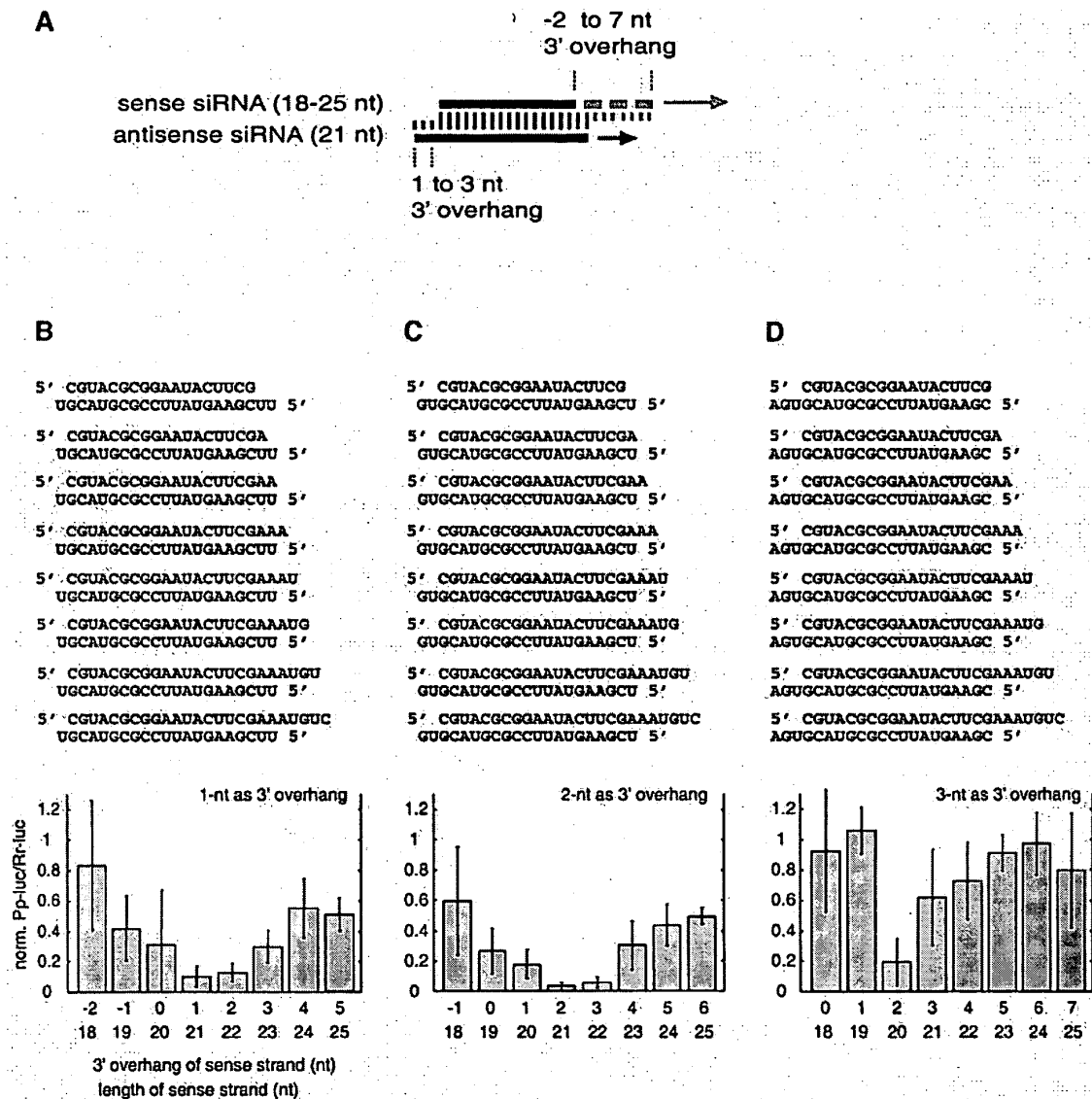


Fig. 2. Variation of the length of the sense strand of siRNA duplexes. (A) Representation of the experiment. Three 21 nt antisense strands were paired with eight sense siRNAs. The siRNAs were changed in length at their 3' end. The 3' overhang of the antisense siRNA was 1 nt (B), 2 nt (C) or 3 nt (D), while the sense siRNA overhang was varied for each series. The sequences of the siRNA duplexes and the corresponding interference ratios are indicated.

RNAi. Comparison of the efficiency of RNAi between long dsRNAs (Figure 1B) and the most effective 21 nt siRNA duplexes (Figure 1E, G and H) indicates that a single siRNA duplex at 100 nM concentration can be as effective as 5 nM 504 bp dsRNA.

Length variation of the sense siRNA paired to an invariant 21 nt antisense siRNA

In order to investigate the effect of the length of siRNAs on RNAi, we prepared three series of siRNA duplexes, combining three 21 nt antisense strands with eight 18–25 nt sense strands. The 3' overhang of the antisense siRNA was fixed to 1, 2 or 3 nt in each siRNA duplex series, while the

sense siRNA was varied at its 3' end (Figure 2A). Independently of the length of the sense siRNA, we found that duplexes with 2 nt 3' overhang of antisense siRNA (Figure 2C) were more active than those with 1 or 3 nt 3' overhang (Figure 2B and D). In the first series, with 1 nt 3' overhang of antisense siRNA, duplexes with 21 and 22 nt sense siRNAs, carrying a 1 and 2 nt 3' overhang of sense siRNA, respectively, were most active. Duplexes with 19–25 nt sense siRNAs were also able to mediate RNAi, but to a lesser extent. Similarly, in the second series, with 2 nt overhang of antisense siRNA, the 21 nt siRNA duplex with 2 nt 3' overhang was most active and any other combination with the 18–25 nt sense siRNAs was active to

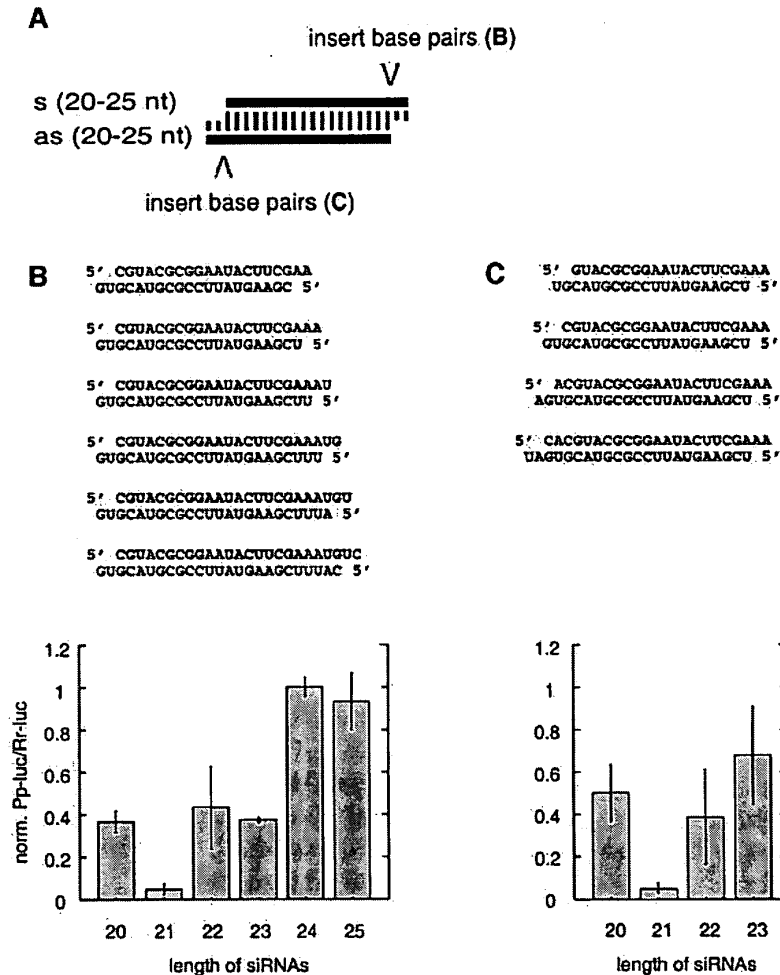


Fig. 3. Variation of the length of siRNA duplexes with preserved 2 nt 3' overhangs. (A) Graphic representation of the experiment. The 21 nt siRNA duplex is identical in sequence to the one shown in Figures 1H and 2C. The siRNA duplexes were extended to the 3' side of the sense siRNA (B) or the 5' side of the sense siRNA (C). The siRNA duplex sequences and the respective interference ratios are indicated.

a significant degree. In the last series, with 3 nt antisense siRNA 3' overhang, only the duplex with a 20 nt sense siRNA and 2 nt sense 3' overhang was able to reduce target RNA expression. Together, these results indicate that the length of the siRNA as well as the length of the 3' overhang are important, and that duplexes of 21 nt siRNAs with 2 nt 3' overhang are optimal for RNAi.

Length variation of siRNA duplexes with a constant 2 nt 3' overhang

We then examined the effect of simultaneously changing the length of both siRNA strands by maintaining symmetrical 2 nt 3' overhangs (Figure 3A). Two series of siRNA duplexes were prepared, including the 21 nt siRNA duplex of Figure 1H as reference. The length of the duplexes was varied between 20 and 25 bp by extending the base-paired segment at the 3' end of the sense siRNA (Figure 3B) or at the 3' end of the antisense siRNA (Figure 3C).

Duplexes of 20–23 bp caused specific repression of target luciferase activity, but the 21 nt siRNA duplex was at least 8-fold more efficient than any of the other duplexes. siRNA duplexes of 24 and 25 nt did not result in any detectable interference. Sequence-specific effects were minor as variations on both ends of the duplex produced similar effects.

2'-deoxy- and 2'-O-methyl-modified siRNA duplexes

To assess the importance of the siRNA ribose residues for RNAi, duplexes with 21 nt siRNAs and 2 nt 3' overhangs with 2'-deoxy- or 2'-O-methyl-modified strands were examined (Figure 4). Substitution of the 2 nt 3' overhangs by 2'-deoxynucleotides had no effect and even the replacement of two additional ribonucleotides by 2'-deoxyribonucleotides adjacent to the overhangs in the paired region produced significantly active siRNAs. Thus, 8 out of 42 nt of a siRNA duplex were replaced by DNA

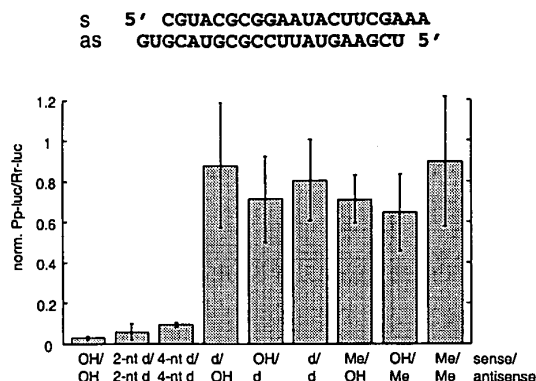


Fig. 4. Substitution of the 2'-hydroxyl groups of the siRNA ribose residues. The 2'-hydroxyl groups (OH) in the strands of siRNA duplexes were replaced by 2'-deoxy (d) or 2'-O-methyl (Me). 2 and 4 nt 2'-deoxy substitutions at the 3' ends are indicated as 2- and 4-nt d, respectively. Uridine residues were replaced by 2'-deoxythymidine.

residues without loss of activity. Complete substitution of one or both siRNA strands by 2'-deoxy residues, however, abolished RNAi, as did complete substitution by 2'-O-methyl residues.

Definition of target RNA cleavage sites

Target RNA cleavage positions were previously determined for 22 nt siRNA duplexes and for a 21 and 22 nt duplex (Elbashir *et al.*, 2001b). The position of target RNA cleavage was located in the centre of the region covered by the siRNA duplex, 11 or 12 nt downstream of the first nucleotide that was complementary to the 21 or 22 nt siRNA guide sequence. Five distinct 21 nt siRNA duplexes with 2 nt 3' overhang (Figure 5A) were incubated with 5' cap-labelled sense or antisense target RNA in *D.melanogaster* lysate (Tuschl *et al.*, 1999; Zamore *et al.*, 2000). The 5' cleavage products were resolved on sequencing gels (Figure 5B). The amount of sense target RNA cleaved correlated with the efficiency of siRNA duplexes determined in the translation-based assay, and siRNA duplexes 1, 2 and 4 (Figures 5B, 1E, G and H) cleaved target RNA faster than duplexes 3 and 5 (Figures 5B, 1D and F). Notably, the sum of radioactivity of the 5' cleavage product and the input target RNA were not constant over time and the 5' cleavage products did not accumulate. Presumably, the cleavage products, once released from the siRNA–endonuclease complex, were rapidly degraded due to the lack of either the poly(A) tail or the 5' cap.

The cleavage sites for both sense and antisense target RNAs were located in the middle of the region spanned by the siRNA duplexes. The cleavage sites for each target produced by the five different duplexes varied by 1 nt according to the 1 nt displacement of the duplexes along the target sequences. The targets were cleaved precisely 11 nt downstream of the target position complementary to the 3'-most nucleotide of the sequence-complementary guide siRNA (Figure 5).

In order to determine whether the 5' or the 3' end of the guide siRNA sets the ruler for target RNA cleavage, we devised the experimental strategy outlined in Figure 6A

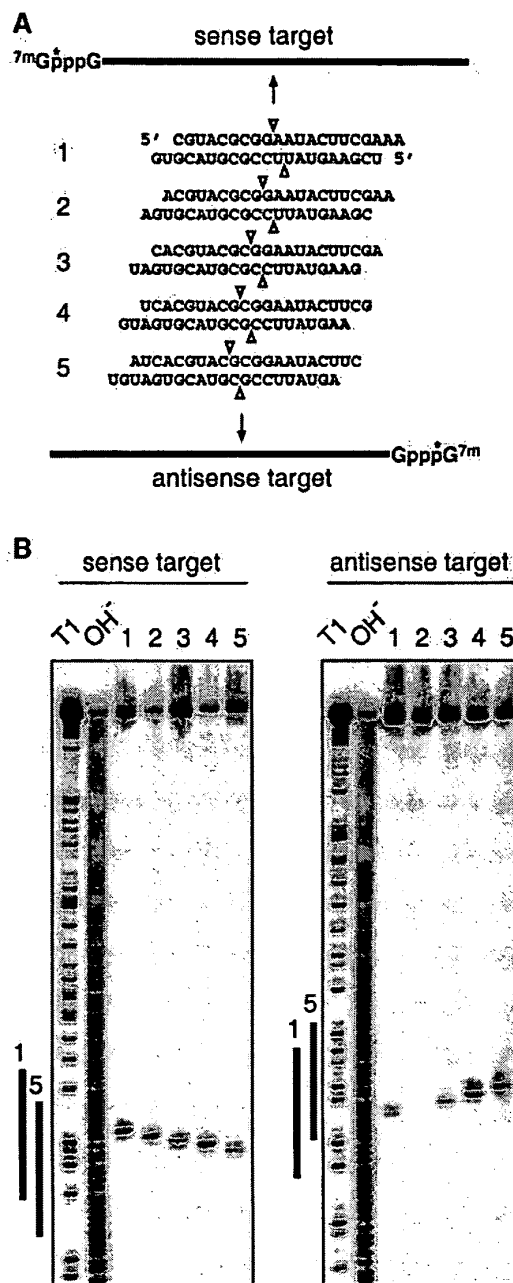


Fig. 5. Mapping of sense and antisense target RNA cleavage by 21 nt siRNA duplexes with 2 nt 3' overhangs. (A) Representation of ^{32}P (asterisk) cap-labelled sense and antisense target RNAs and siRNA duplexes. The position of sense and antisense target RNA cleavage is indicated by triangles on top and below the siRNA duplexes, respectively. (B) Mapping of target RNA cleavage sites. After 2 h incubation of 10 nM target RNA with 100 nM siRNA duplex in *D.melanogaster* embryo lysate, the 5' cap-labelled substrate and the 5' cleavage products were resolved on 6% sequencing gels. Length markers were generated by partial RNase T1 digestion (T1) and partial alkaline hydrolysis (OH) of the target RNAs. The bold lines to the left of the images indicate the region covered by the siRNA strands 1 and 5 of the same orientation as the target.

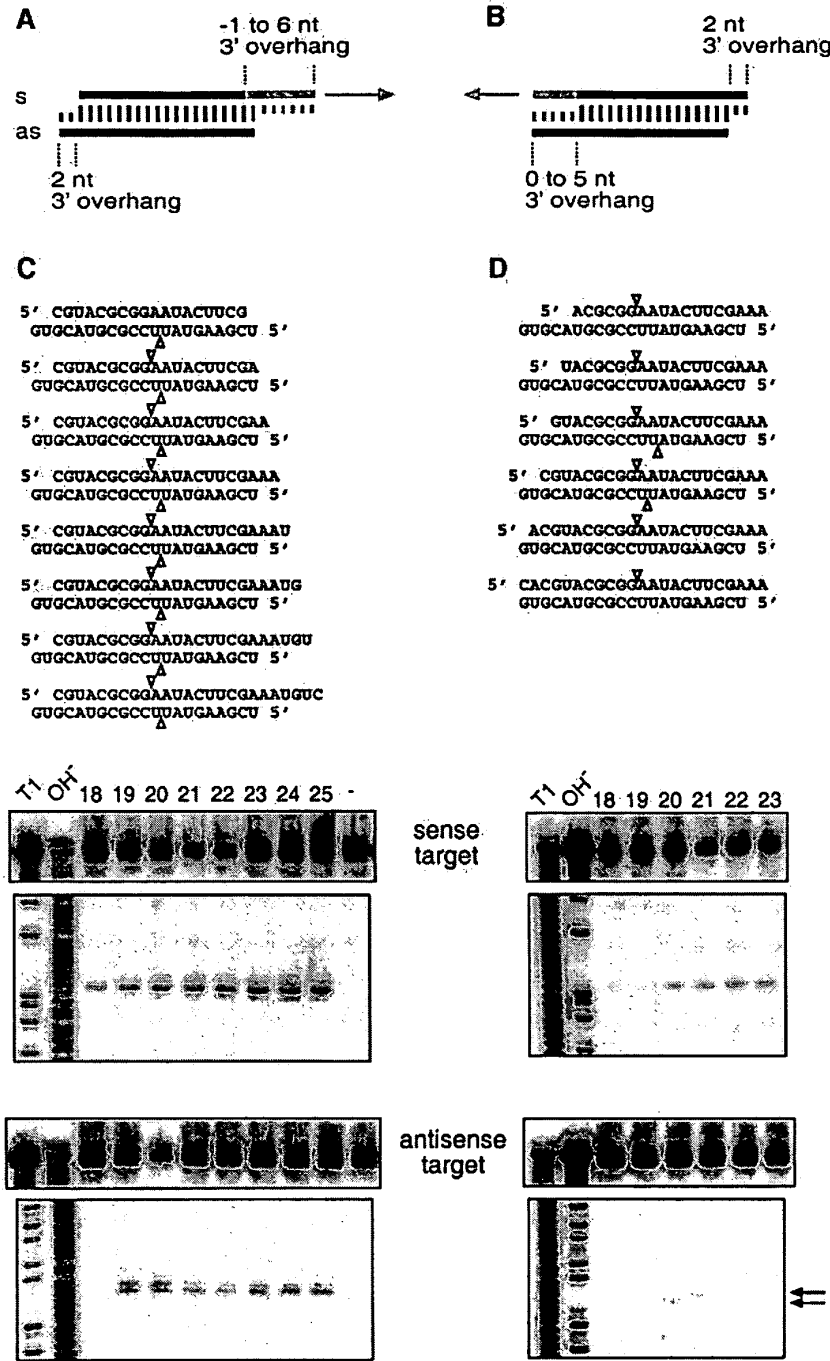


Fig. 6. The 5' end of a guide siRNA defines the position of target RNA cleavage. (A and B) Representation of the experimental strategy. The antisense siRNA was the same in all siRNA duplexes, but the sense strand was varied between 18 and 25 nt by changing the 3' end (A) or 18 and 23 nt by changing the 5' end (B). The position of sense and antisense target RNA cleavage is indicated by triangles on top and below the siRNA duplexes, respectively. (C and D) Analysis of target RNA cleavage using cap-labelled sense (top) or antisense (bottom) target RNAs. The residual amount of targeted substrate and the cap-labelled 5' cleavage products are shown. The sequences of the siRNA duplexes are indicated and the length of the sense siRNA strands is marked on top. The control lane, marked with a dash in (C), shows target RNA incubated in absence of siRNAs. Markers were as described in Figure 5. The arrows in (D), bottom, indicate the target RNA cleavage sites that differ by 1 nt.

and B. A 21 nt antisense siRNA, which was kept invariant for this study, was paired with sense siRNAs that were

modified in length at either of their 5' or 3' ends. The position of sense and antisense target RNA cleavage was

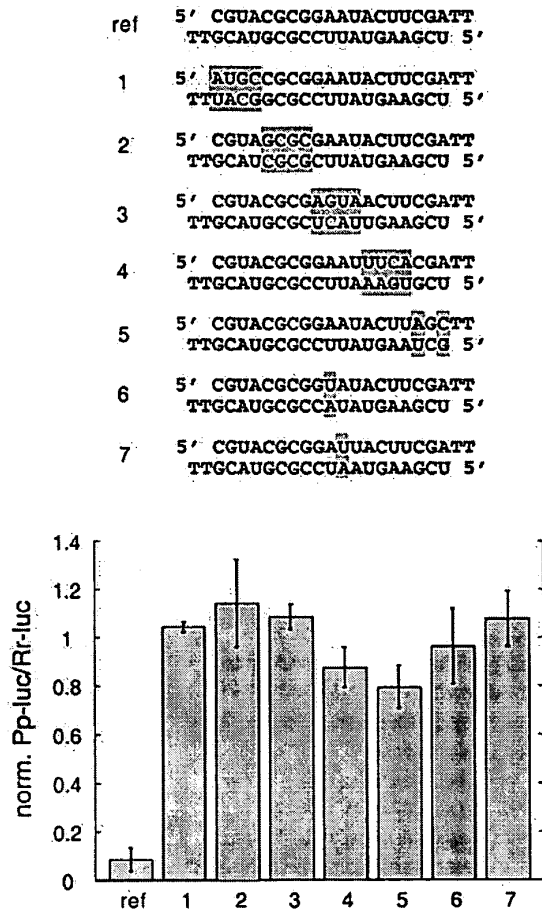


Fig. 8. Sequence specificity of target recognition. The sequences of the mismatched siRNA duplexes are shown, modified sequence segments or single nucleotides are shaded in grey. The reference duplex (ref) and the siRNA duplexes 1–7 contain 2'-deoxythymidine 2 nt overhangs. The silencing efficiency of the thymidine-modified reference duplex was comparable to the wild-type sequence (Figure 7). Normalized interference ratios were determined as described in Figure 1.

efficiency comparable to a 500 bp dsRNA, given that comparable quantities of total RNA are used.

The siRNA user guide

Efficiently silencing siRNA duplexes are composed of 21 nt sense and 21 nt antisense siRNAs and must be selected to form a 19 bp double helix with 2 nt 3'-overhanging ends. 2'-deoxy substitutions of the 2 nt 3'-overhanging ribonucleotides do not affect RNAi, but help to reduce the costs of RNA synthesis and may enhance RNase resistance of siRNA duplexes. More extensive 2'-deoxy or 2'-O-methyl modifications reduce the ability of siRNAs to mediate RNAi, probably by interfering with protein association for siRNP assembly.

Target recognition is a highly sequence-specific process, mediated by the siRNA complementary to the target. The 3'-most nucleotide of the guide siRNA does not contribute to the specificity of target recognition, while the penultimate nucleotide of the 3' overhang affects target

RNA cleavage and a mismatch reduces RNAi 2- to 4-fold. The 5' end of the guide siRNA also appears more permissive for mismatched target RNA recognition when compared with the 3' end. Nucleotides in the centre of the siRNA, located opposite to the target RNA cleavage site, are important specificity determinants and even single nucleotide changes reduce RNAi to undetectable levels. This suggests that siRNA duplexes may be able to discriminate mutant or polymorphic alleles in gene targeting experiments, which may become an important feature for future therapeutic developments.

Sense and antisense siRNAs, when associated with the protein components of the endonuclease complex or its commitment complex, were suggested to play distinct roles; the relative orientation of the siRNA duplex in this complex defines which strand can be used for target recognition (Elbashir *et al.*, 2001b). Synthetic siRNA duplexes with an equal number of overhanging nucleotides have dyad symmetry with respect to the double-helical structure, but not with respect to sequence. The association of siRNA duplexes with the RNAi proteins in the *D.melanogaster* lysate leads to the formation of two asymmetric complexes. In such hypothetical complexes, the chiral environment is distinct for sense and antisense siRNA, hence their function. The prediction obviously does not apply to palindromic siRNA sequences or to RNAi proteins that could associate as homodimers. To minimize sequence effects that may affect the ratio of sense- and antisense-targeting siRNPs, we suggest using siRNA sequences with identical 3'-overhanging sequences. We recommend adjusting the sequence of the overhang of the sense siRNA to that of the antisense 3' overhang because the sense siRNA does not have a target in typical knock-down experiments. Asymmetry in the reconstitution of sense- and antisense-cleaving siRNPs could be, partially, responsible for the variation in RNAi efficiency observed for various 21 nt siRNA duplexes with 2 nt 3' overhangs used in this study (Figure 1). Alternatively, the nucleotide sequence at the target site and/or the accessibility of the target RNA structure may be responsible for the variation in efficiency observed for these siRNA duplexes. It should be noted that all siRNAs used in this study are derived from a short region of one gene. Thus, it is more likely that differences in siRNA efficiency are a consequence of the primary sequences of the siRNAs and the respective target sites, rather than the secondary or tertiary structure of the targeted RNA.

Natural siRNAs versus synthetic siRNAs

In *D.melanogaster*, siRNA duplexes are produced *in vitro* and *in vivo* from long dsRNAs (Hammond *et al.*, 2000; Yang *et al.*, 2000; Zamore *et al.*, 2000). About 45% of these short RNAs are precisely 21 nt long, 28% are 22 nt long and a few percent are shorter or longer RNAs (Elbashir *et al.*, 2001b). This length distribution correlates with our finding that 21 nt siRNA duplexes are the most efficient mediators of mRNA degradation. Beside the length, the paired structure and overhang are also important. This structural feature may explain why siRNA duplexes isolated from the dsRNA processing reaction under denaturing conditions were less potent for RNAi than longer dsRNAs that were processed to siRNAs during the targeting reaction (Zamore *et al.*, 2000). Presumably,

denaturation followed by renaturation favoured the formation of the thermodynamically more stable, blunt-ended, but less active, siRNA duplexes. Isolation of siRNAs under native conditions does not reduce siRNA activity (Nykänen *et al.*, 2001).

Production of siRNAs from long dsRNA requires the RNase III enzyme dicer (Bernstein *et al.*, 2001). Dicer is a bidentate RNase III, which also contains an ATP-dependent RNA helicase domain and a PAZ domain, presumably important for dsRNA unwinding and mediation of protein-protein interactions, respectively (Cerutti *et al.*, 2000; Bernstein *et al.*, 2001). Dicer is evolutionarily conserved in worms, flies, plants, fungi and mammals (Matsuda *et al.*, 2000), and has a second cellular function important for the development of these organisms (Ray *et al.*, 1996; Jacobsen *et al.*, 1999; Grishok *et al.*, 2001; Hutvagner *et al.*, 2001; Knight and Bass, 2001). At present, it is uncertain whether dicer activity in species other than *D.melanogaster* produces siRNAs of predominantly 21 nt in length. The estimates of siRNA size vary in the literature between 21 and 25 nt (Hamilton and Baulcombe, 1999; Hammond *et al.*, 2000; Hutvagner *et al.*, 2000; Parrish *et al.*, 2000; Yang *et al.*, 2000; Zamore *et al.*, 2000; Elbashir *et al.*, 2001b).

In a recent study of the effect of siRNA length in mammalian cells (primary mouse embryonic fibroblasts, 293 and HeLa cells), duplexes of 21–27 nt siRNAs with 2 nt 3' overhangs were directed against different co-transfected reporter genes (Caplen *et al.*, 2001). Duplexes of 22 and 23 nt siRNAs were found to be slightly more efficient in triggering sequence-specific gene silencing than 21 nt siRNA duplexes. In our hands, using the dual luciferase assay system in HeLa cells, 21 nt siRNA duplexes with 2 nt 3' overhang are 2- to 3-fold more efficient than 20 or 22–25 nt siRNA duplexes (data not shown), therefore recapitulating the results obtained from the *D.melanogaster* biochemical system. In contrast to the *D.melanogaster* system, siRNA duplexes >23 nt in length are still triggering some RNAi in HeLa cells and also in *C.elegans* (Caplen *et al.*, 2001). However, it remains to be determined whether the RNA strands finally incorporated into the active endonuclease complex are of the initially provided length. It is possible that exonucleases present in *C.elegans* and mammalian cells trim longer siRNAs to their optimum length and that these exonucleases are absent from *D.melanogaster* lysate.

The functional anatomy of long dsRNAs as a trigger for RNAi was analysed previously in *C.elegans* (Parrish *et al.*, 2000). Activation of RNAi by injection of long dsRNA requires at least two steps: dsRNA processing by dicer RNase III and siRNP or RISC formation. Substitution of one of the strands of the long dsRNA by DNA abolished RNAi and even the substitution of C by dC or U by dT in only one of the strands caused a substantial decrease in RNAi. Because introduction of 2'-fluoro modifications into long RNA had no effect, it was suggested that an A-form double helical structure was important for triggering RNAi (Parrish *et al.*, 2000). We have been able to substitute eight ribose residues of a siRNA duplex by 2'-deoxyribose residues without substantial reduction of RNAi, although it should be noted the 2'-deoxy modifications were clustered at the 3' end of the siRNAs, including the 2 nt 3' overhangs. It is possible that the four

2'-deoxy modifications, which are located in the paired region at the end of the helix, do not affect the overall A-form helical structure and do not strongly compromise RISC formation. Complete modification of one or both siRNA strands by 2'-deoxyribose, however, abolished RNAi. Interestingly, substitution by 2'-O-methylribose, which adopts the ribose sugar pucker, also abolished RNAi, probably because methylation of the 2'-hydroxyls blocked hydrogen bond formation or introduced steric hindrance.

It was recently demonstrated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA function and that ATP is used to maintain the 5'-phosphates of the siRNAs (Nykänen *et al.*, 2001). However, 5'-phosphorylation of fully 2'-deoxy- or 2'-O-methyl-modified siRNA strands was not able to restore RNAi (data not shown). Unmodified siRNA duplexes with free 5'-hydroxyls and 2 nt 3' overhangs are readily phosphorylated in *D.melanogaster* embryo lysate (Nykänen *et al.*, 2001). In this respect, it should be noted that our reported RNAi efficiencies were determined by pre-incubating the siRNA duplexes for 15 min in *D.melanogaster* lysate before adding target and control mRNAs, thus providing sufficient time for 5'-phosphorylation of siRNA duplexes to occur. Comparison of the RNAi efficiencies of 5'-phosphorylated and 5'-non-phosphorylated siRNAs (for duplexes shown in Figures 1E, F and 2C) did not reveal any sizeable differences (data not shown).

Conclusions

We have performed an extensive analysis of the length, sequence and structure of siRNA duplexes in *D.melanogaster* embryo lysate. Duplexes of 21 nt siRNAs with 2 nt 3' overhangs were shown to be the most efficient triggers of RNAi-based mRNA degradation. The target recognition is a highly sequence-specific process, although not all positions of a guide siRNA contribute equally to specificity. These results are important for the design of efficient siRNAs in order to silence genes in *D.melanogaster* and provide a basis for similar studies in other organisms.

Materials and methods

RNA preparation and RNAi assay

Chemical RNA synthesis, annealing and luciferase-based RNAi assays were performed as described previously (Tuschl *et al.*, 1999; Zamore *et al.*, 2000; Elbashir *et al.*, 2001b). Synthetic RNAs were gel purified after deprotection. The formation of siRNA duplexes was verified by agarose gel electrophoresis using 4% NuSieve GTG agarose (BMA, Rockland, ME) in 0.5× TBE buffer. All siRNA duplexes were directed against firefly luciferase and the luciferase mRNA sequence was derived from pGEM-luc (DDBJ/EMBL/GenBank accession No. X65316) as described (Tuschl *et al.*, 1999). The siRNA duplexes were incubated in a *D.melanogaster* RNAi/translation reaction for 15 min prior to addition of mRNAs. Translation-based RNAi assays were performed at least in triplicate.

For mapping of sense target RNA cleavage, a 177 nt transcript was generated, corresponding to the firefly luciferase sequence between positions 113 and 273 relative to the start codon, followed by the 17 nt complement of the SP6 promoter sequence (Elbashir *et al.*, 2001b). For mapping of antisense target RNA cleavage, a 166 nt transcript was produced from a template, which was amplified from plasmid sequence by PCR using the 5' primer TAATACGACTCACTATAGAGCCCATATCGTTTCATA (T7 promoter underlined) and 3' primer AGAG-

GATGGAACCGCTGG. The target sequence corresponds to the complement of the firefly luciferase sequence between positions 50 and 215 relative to the start codon. Guanlyl transferase labelling was performed as described previously (Zamore *et al.*, 2000). For mapping of target RNA cleavage, 100 nM siRNA duplex was incubated with 5–10 nM target RNA in *D.melanogaster* embryo lysate under standard conditions (Zamore *et al.*, 2000) for 2 h at 25°C. The reaction was stopped by the addition of 8 vols of proteinase K buffer [200 mM Tris-HCl pH 7.5, 25 mM EDTA, 300 mM NaCl, 2% (w/v) SDS]. Proteinase K (dissolved in water; Merck) was added to a final concentration of 0.6 mg/ml. The reactions were then incubated for 15 min at 65°C, extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with 3 vols of ethanol. Samples were loaded on 6% sequencing gels. Length standards were generated by partial RNase T1 digestion and partial base hydrolysis of the cap-labelled sense or antisense target RNAs.

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EXHIBIT 3 of
EXHIBIT B

Tolerance for mutations and chemical modifications in a siRNA

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ABSTRACT

Short interfering RNA (siRNA), the active agent of RNA interference, shows promise of becoming a valuable tool in both basic and clinical research. We explore the tolerance to mutations and chemical modifications in various parts of the two 21-nt strands of a siRNA targeting the blood clotting initiator Tissue Factor. The mutations were G/C transversions. The chemical modifications were 2'-O-methylation, 2'-O-allylation and phosphorothioates. We found that siRNA generally tolerated mutations in the 5' end, while the 3' end exhibited low tolerance. This observation may facilitate the design of siRNA for specific targeting of transcripts containing single nucleotide polymorphisms. We further demonstrate that in our system the single antisense strand of the wild-type siRNA is almost as effective as the siRNA duplex, while the corresponding methylated M2+4 version of the antisense had reduced activity. Most of the chemically modified versions tested had near-wild-type initial activity, while the long-term activity was increased for certain siRNA species. Our results may improve the design of siRNAs for *in vivo* experiments.

INTRODUCTION

RNA interference (RNAi), the process of depleting RNA targets by the use of double-stranded RNA (dsRNA), was first reported in 1998 (1). Since the demonstration of the efficacy of short interfering RNA (siRNA) in mammalian cells (2–4), this new tool has been used to successfully target various infectious agents like HIV, poliovirus and RSV (5–9). In some cases, newly constructed vectors were used to produce short hairpin RNA (shRNA) that is processed to siRNA intracellularly (10–14).

The mechanism of RNAi is not well understood (15–22). The triggering long dsRNA or aberrant RNA is assumed to be processed by the RNase III-like enzyme Dicer to approximately 21–23 nt siRNA, which is then incorporated into the RNA-induced silencing complex (RISC). This step can be bypassed by transfection of chemically synthesised siRNA.

Dicer has also been implicated in processing of the siRNA-related short temporal RNA let-7, shown in *Caenorhabditis elegans* to be involved in control of larval development, and widely conserved in other species, among them humans (23–25).

The physiological function of RNAi is assumed to be defence against viral infections. This has been convincingly demonstrated in plants, where some viruses have evolved counter-measures against RNAi (26,27). An insect virus has recently been shown to both activate RNA silencing and express a suppressor protein in *Drosophila* cells (28). In *C.elegans*, mutations in RNAi genes have resulted in the activation of transposons (29,30), arguing for their involvement in the defence against these genomic parasites.

The tolerance of the effect of siRNA for mutations is still unclear. Boutla *et al.* (31) reported that a mutated siRNA with a single centrally located mismatch relative to the mRNA target sequence retained substantial activity in *Drosophila*. In contrast, Elbashir *et al.* (32) found that a single mismatch was deleterious to activity in an *in vitro* *Drosophila* embryo lysate assay. In previous work (33) we tried to reconcile these conflicting results by depicting the RNAi process *in vivo* as a dynamic one where several factors influenced the final outcome, among them siRNA target position, siRNA concentration, mRNA concentration, mRNA synthesis rate and siRNA's inherent cleavage activity, an activity that can be reduced gradually by mismatch mutations.

In the present work we explore how various mutations and chemical modifications alter the efficacy and duration of our most effective siRNA (hTF167i) targeting the human Tissue Factor (hTF) mRNA. The objectives were, firstly, to find regions less tolerant in their siRNA effect for single mutations, thus possibly facilitating the design of siRNA for specific targeting of transcripts containing single nucleotide polymorphisms. Secondly, we wished to improve the long-term activity of our siRNA by stabilising the RNA strands against nucleases through introducing phosphorothioates and modifications of the 2'-OH.

We find that hTF167i has a general tolerance to mutations, with less tolerance for mutations at 3' end of the siRNA. Furthermore, with the exception of certain allyl-modifications, the backbone modifications did not reduce the activity of the siRNA to a significant degree. Extensive use of phosphorothioate modifications resulted in cytotoxicity. The 2'-O-methylation modifications, on the other hand, showed promise

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

as they resulted in increased persistence of activity with no toxicity to cells.

MATERIALS AND METHODS

SiRNA preparation

RNAs (21 nt) were chemically synthesised using phosphoramidites (Glen Research and PerSeptive Biosystems). Deprotected and desilylated synthetic oligoribonucleotides were purified by reverse-phase HPLC. Ribonucleotides were annealed at 10 μ M in 200 μ l 10 mM Tris-HCl pH 7.5 by boiling and gradual cooling in a water bath. Successful annealing was confirmed by non-denaturing (15%) polyacrylamide gel electrophoresis.

Cell culture and transfections

The human keratinocyte cell line HaCaT was cultured in serum-free keratinocyte medium (Gibco BRL) supplemented with 2.5 ng/ml epidermal growth factor and 25 μ g/ml bovine pituitary extract. The cell line was regularly passaged at sub-confluence and plated 1 or 2 days before transfection. HaCaT cells in 6-well plates were transfected at low confluency (<40%) with 1.0 ml 100 nM siRNA in serum-free medium, using Lipofectamine 2000. For complexation, 10 μ M stock solution of siRNA was diluted with 10 $\times$ vol. of serum-free medium and mixed with an equal volume of medium-diluted Lipofectamine 2000, at a v/w ratio of liposome to siRNA of 5:2. Batch dilutions of liposomes were performed for each 6-well plate and allowed to pre-incubate at room temperature for 5–7 min before addition to the medium-diluted siRNA. Complexes were replaced with full medium 5 h after initiation of transfection. For standard assays of activity, cells were harvested the day after transfection. For longer incubations and time-course experiments, medium was replaced every second day after transfection.

Northern analysis

Polyadenylated mRNA was isolated using Dynabeads oligo(dT)₂₅ (Dyna). Isolated mRNA was fractionated by electrophoresis for 16–18 h on 1.3% agarose/formaldehyde (0.8 M) gels and blotted on to nylon membranes (MagnaCharge, Micron Separations). Membranes were hybridised with random-primed Tissue Factor (TF) (position 61–1217 in cDNA) and GAPDH (1.2 kb) cDNA probes in PerfectHyb hybridisation buffer (Sigma).

RESULTS

Mutational scanning of siRNA targeting hTF167

We have previously demonstrated that one and two central mutations in siRNA targeting position 167 in hTF did not abolish its ability to deplete endogenous TF mRNA levels (33). We decided to map this siRNA more systematically in order to determine whether mutations were tolerated equally well within the whole siRNA. To simplify analysis, only GC pairs were mutated by inversion of the pairs. A total of eight different single-mutant siRNAs were designed and named according to the position (starting from the 5' of the sense strand) of the mutation (s1, s2, s3, s4, s7, s11, s13, s16) (Fig. 1).

wt	5' -gogcuucaggcacuacaaaua-3' 3' -gccgcgaaguccgugauguuu-5'
s1	5' - g ogcuucaggcacuacaaaua-3' 3' -gccgcgaaguccgugauguuu-5'
s2	5' -gggcuucaggcacuacaaaua-3' 3' -gccgcgaaguccgugauguuu-5'
s3	5' -gccc <u>u</u> ucaggcacuacaaaua-3' 3' -gccgcgaaguccgugauguuu-5'
s4	5' -gogcuucaggcacuacaaaua-3' 3' -gccgcgaaguccgugauguuu-5'
s7	5' -gogcuu g aggcacuacaaaua-3' 3' -gccgcgaaguccgugauguuu-5'
s10	5' -gogcuucaggcacuacaaaua-3' 3' -gccgcgaaguccgugauguuu-5'
s11	5' -gogcuucagg g cacuacaaaua-3' 3' -gccgcgaaguccgugauguuu-5'
s13	5' -gogcuucaggcacuacaaaua-3' 3' -gccgcgaaguccgugauguuu-5'
s16	5' -gogcuucaggcacuacaaaua-3' 3' -gccgcgaaguccgugauguuu-5'
ds7/10	5' -gogcuu g aggcacuacaaaua-3' 3' -gccgcgaaguccgugauguuu-5'
ds10/11	5' -gogcuucagg g cacuacaaaua-3' 3' -gccgcgaaguccgugauguuu-5'
ds10/13	5' -gogcuucaggcacuacaaaua-3' 3' -gccgcgaaguccgugauguuu-5'
ds10/16	5' -gogcuucaggcacuacaaaua-3' 3' -gccgcgaaguccgugauguuu-5'

Figure 1. Mutated and wild-type (wt) versions of the siRNA hTF167i. The sequence of the sense strand of wild-type siRNA corresponds to position 167–187 in hTF (Acc.No. M16553). Single (s1, s2, s3, s4, s7, s10, s11, s13, s16) and double (ds7/10, ds10/11, ds10/13, ds10/16) mutants are all named according to the position of the mutation, counted from the 5' end of the sense strand. All mutations (in bold) are GC inversions relative to the wild-type.

The previously described centrally located single-mutant M1 (33), was included in this analysis and renamed s10.

These mutant siRNAs were analysed for their ability to deplete endogenous TF mRNA in HaCaT cells (Fig. 2). The wild-type siRNA, and the mutant s10, included as positive controls, depleted TF mRNA to 10 and 20% residual levels, as reported previously (33). The activities of the other mutants could be categorised into three groups depending on their position along the siRNA. Mutations in the extreme 5' end of the siRNA were well tolerated, exhibiting essentially the same activity as the wild-type. Mutations localised towards the 3' end, and up to the approximate midpoint of the siRNA (s4, s7, s10, s11), were slightly impaired in their activity, resulting in depletion of mRNA to 20–30% residual levels. Both of the mutations in the 3' half of the siRNA (s13, s16), however, exhibited severely impaired activity, suggesting a bias in the tolerance for mutations in the reaction(s) involving siRNA. The activities of four double mutants, in which the central position (s10) was mutated in conjunction with one additional position (s7, s11, s13, s16), were also analysed. The bias in mutation tolerance was also evident for these double mutants, as the rank order of their activity mirrored that of the activity

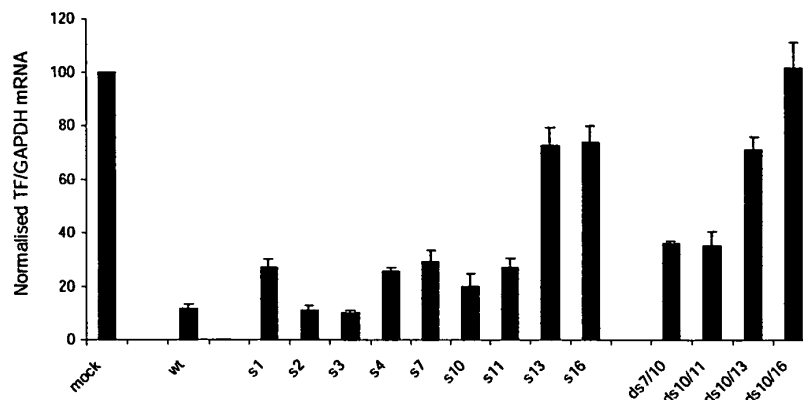


Figure 2. Activity of mutant siRNAs against endogenous hTF mRNA. HaCaT cells were harvested for mRNA isolation 24 h post-transfection. TF expression was normalised to that of GAPDH. Normalised expression in mock-transfected cells was set as 100%. Data are averages + s.d. of at least three independent experiments.

of the single mutants of the variant position. This observation strengthens the proposition that the differential activity of mutants is due to an intrinsic bias in the tolerance for target mismatches along the sequence of the siRNA.

Chemical modification of siRNAs for increased persistence of silencing

The effect of siRNA in human cells is transient and typically decreases during a few days in cell culture (18,33). The ability to extend the period of effective silencing would be of importance for the possible use of siRNA in therapy. To increase the intracellular stability of siRNA, we introduced a gradual increase of various chemical modifications to both ends of the siRNA. We have previously used phosphorothioate linkages and 2'-O-modifications in the form of methylation and allylation to stabilise hammerhead ribozymes (34), and decided to try a similar strategy for siRNAs. In all modified siRNA species, the same modifications were introduced in both strands. It has been reported that siRNA with a general 2'-O-methylation in either strand have no activity (32). We therefore started with less extensive modification. Initially, siRNAs with one modification in each of the extreme 5' and 3' ends of their strands (P1+1, M1+1, A1+1, Fig. 3) were synthesised. The 5' end of the siRNAs might be more sensitive to modification since it must be phosphorylated in order to be active *in vivo* (22). We therefore included siRNAs with modifications only in the non-basepairing 3' overhangs (P0+2, M0+2 and A0+2, Fig. 3), which were known to tolerate various types of modifications (2,3,32,33).

Northern analysis of transfected HaCaT cells demonstrated essentially undiminished activity of all the chemically modified siRNAs, with the exception of the siRNA with allylation at both ends (Fig. 3). Allyl-modification in the 3' end only had no effect on activity. The presence of a bulky substituent in the 2'-hydroxyl of the 5' terminal nucleotide might interfere with the *in vivo* phosphorylation of the siRNA necessary for its activity (22). Further modification of the siRNA by methylation resulted in gradual attenuation of activity. One day after transfection with siRNAs carrying 4+4, 4+6, 6+6 or 6+8

methyl groups in their ends, the cells exhibited residual reporter gene expression levels of 28, 42, 68 and 75%, respectively.

We next wanted to know if any of these modifications increased the persistence of siRNA-mediated silencing. The level of endogenous TF mRNA recovers gradually 3–5 days after transfection with wild-type siRNA targeting hTF167 (33). Transfecting HaCaT cells with unmodified and chemically modified siRNAs in parallel did not result in any significant differences in their silencing activities at 3 and 5 days post-transfection until a rather high degree of modification was used. Allylated siRNAs were not tested in this experiment, since they showed reduced effectiveness even with only one substituent in the 5' end (Fig. 3). SiRNAs methylated in the 2'-OH in up to 2+4 positions showed well conserved activity at 24 h [16–18% residual levels compared with 11% in cells transfected with the wild-type (Fig. 3)]. The most extensively phosphorothioated siRNA proved to be cytotoxic, resulting in ~70% cell death compared with mock-transfected cells (measured as the expression level of the control mRNA GAPDH). This siRNA species was therefore not included in the further analysis. The remaining siRNA species were evaluated for increased persistence of silencing by analysing TF mRNA expression 5 days after a single transfection of 100 nM siRNA. At this point, TF expression in cells transfected with wild-type siRNA had recovered almost completely (80% residual expression compared with mock-transfected cells) (data not shown). In cells transfected with the most extensively 2'-O-methylated siRNA (M2+4), however, strong silencing was still evident (32% residual expression). The less extensively modified siRNA species (P2+2, M2+2), although less effective than M2+4, consistently resulted in lower TF expression 5 days post-transfection (55–60%) than the wild-type. Fewer modifications (P1+1, P0+2, M1+1, M0+2, A0+2) did not improve the silencing effect 5 days post-transfection. Time-course experiments demonstrated that the wild-type siRNA was still the most effective 3 days post-transfection, when silencing was relatively unimpaired. However, silencing drops off at a

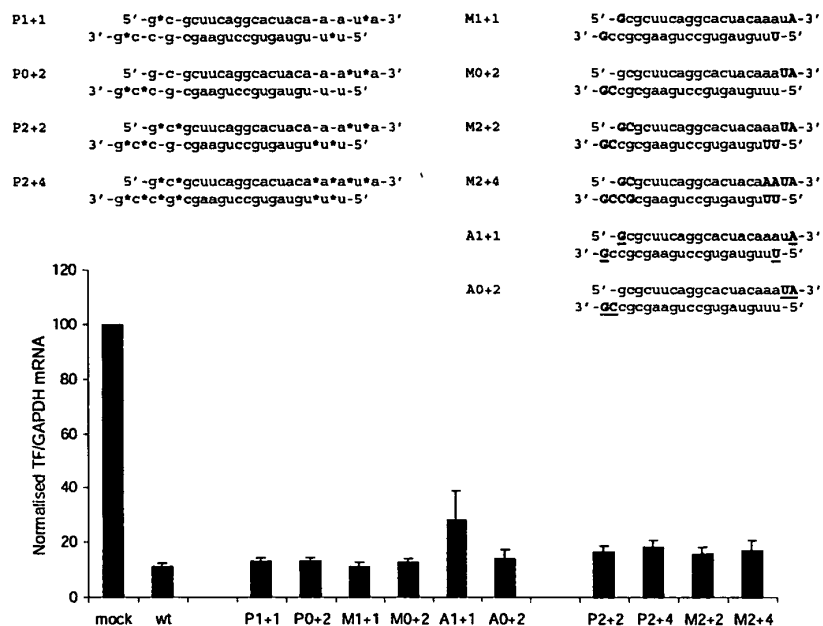


Figure 3. Activity of chemically modified versions of the siRNA hTF167i. Non-modified ribonucleotides are in lower case. Phosphorothioate linkages are indicated by asterisks (*), while 2'-O-methylated and 2'-O-allylated ribonucleotides are in normal and underlined bold upper case, respectively. Expression of TF and GAPDH mRNA was determined 24 h post-transfection of HaCaT cells. Experiments were performed and analysed as in Figure 2. Data are averages + s.d. of at least three independent experiments.

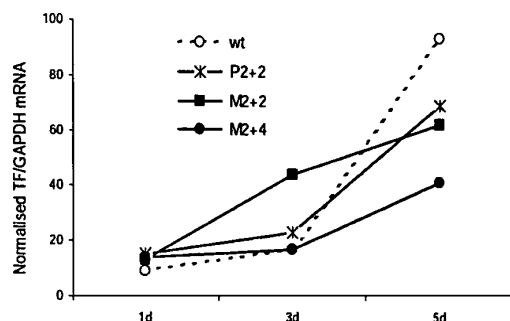


Figure 4. Persistence of TF silencing by chemically modified siRNAs in HaCaT cells harvested 1, 3 and 5 days after a single transfection of 100 nM siRNA. Medium was replaced every second day. Data are from one representative out of three independent experiments.

much higher rate thereafter for the unmodified siRNA (Fig. 4), possibly due to a faster depletion of the intracellular siRNA pool.

Comparison of the effect of duplex and single-stranded antisense siRNA in whole cells

The antisense strand of siRNA is incorporated into RISC in HeLa cell extracts and supports RISC-specific target RNA cleavage, although at lower efficiency than the siRNA duplex (35,36). The antisense RNA was shown to silence transgene expression in a dose-dependent manner (36). We investigated whether antisense siRNA could silence endogenous gene

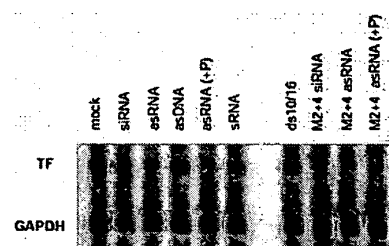


Figure 5. Levels of TF and control mRNA (GAPDH) in cells 24 h after transfection with various siRNA derivatives (see Figs 1 and 3). Cells were transfected with 100 nM siRNA duplex or 200 nM single-stranded RNA or DNA. RNA samples phosphorylated prior to transfection are indicated with '(+P)'. Phosphorylation was by polynucleotide kinase (New England Biolabs), followed by phenol/chloroform extraction and desalting on G25 Sephadex Quick-Spin columns (Roche).

expression with an efficiency comparable to duplex siRNA at moderate concentrations of the antisense oligo. HaCaT cells were transfected in parallel with 100 nM siRNA or 200 nM of the antisense strand, with or without pre-phosphorylation of the oligo (Fig. 5). In our quantitative assay the antisense RNA was as efficient as the siRNA duplex in depleting endogenous TF mRNA. A clear cleavage product was seen for both agents. The observation that antisense RNA exhibits almost full activity most likely reflects the high efficiency and excess capacity of this particular siRNA species. For the slightly less efficient chemically modified siRNA M2+4, on the other hand, there is a clear difference in depletion rate between the duplex

siRNA and antisense siRNA (Fig. 5), suggesting that higher concentrations are required for maximum effect of this antisense RNA. Phosphorylation prior to transfection did not improve the efficacy of either antisense strand, consistent with recent observations that single-stranded RNA can be phosphorylated *in vivo* (36).

DISCUSSION

This work has demonstrated tolerance of a highly effective siRNA, hTF167i, for a wide range of mutational and chemical modifications, as well as for removal of the sense strand from the duplex. Although allyl-modification of both terminal nucleotides resulted in some loss of activity, a total of six modifications in the form of either methylation of the sugar moiety or thiolation of the backbone were well tolerated, causing only a marginal reduction in the maximal silencing observed with the unmodified siRNA. Toxicity was, however, observed with longer stretches of phosphorothioates, but not with the same level of methyl-modification. SiRNA with 2+4 2'-O-methylated terminal nucleotides (M2+4) demonstrated both conserved initial activity and its increased duration in time-course experiments. These active end-methylated siRNAs are in possible contrast to the observations by Elbashir *et al.* (32) that fully 2'-O-methylated siRNAs are inactive. While full substitution of either strand with 2'-deoxynucleotides destroys siRNA activity (31–33), up to four deoxynucleotide modifications in the 3' ends were found to support good activity in an *in vitro* cleavage assay (32). Taken together, the above data suggest the existence of different degrees of tolerance for chemical modification of siRNAs.

We find that single-stranded antisense siRNA can be highly effective in depleting endogenous gene expression at relatively moderate concentrations. Although antisense siRNA is still less effective than the corresponding siRNA duplex, this observation should help reduce the cost of selecting the best siRNA site, as the initial screening can be accomplished through the synthesis of the antisense strand only.

We have demonstrated a lower tolerance to mutations in the 3' end of our most active siRNA. This bias cannot be due to differences in siRNA duplex stability as all mutations were G-C inversions and thus to a first approximation energetically equal. The stability of the duplex between a mutated siRNA antisense strand and the mRNA should be more severely affected by a central than a peripheral target mismatch. Consistent with this, we observe that 5' end mutations have a negligible effect on activity compared with more centrally located mutations. The observed bias might be linked to the proposed existence of a 'ruler' region in the siRNA which is primarily used by the RISC complex to 'measure' the target mRNA for cleavage (32). It was demonstrated that the 5' end of the antisense strand of siRNA sets the ruler for target RNA cleavage. This is likely to occur by a physical interaction of RISC with this region of the siRNA, which should therefore be more sensitive to mismatches with the target RNA. The universality of this observation is currently being investigated, using siRNAs targeting other sites in TF. The identification of a region of generally increased sensitivity to the effect of mismatches with the target mRNA, would be of great importance for the potential use of siRNAs to specifically

target transcripts of disease-associated alleles in various dominant-negative disorders.

Highly diverging effects of mutations on siRNA activity have been reported. Jacque *et al.* (6) found that a single mismatch in siRNA targeting the HIV LTR resulted in partial loss of activity, while another siRNA targeting the HIV VIF exhibited almost full activity. Four mutations, however, abolished activity completely. Complete abolishment of activity has been reported by Gitlin *et al.* (8), Klahre *et al.* (37) and Garrus *et al.* (38), for siRNAs with 5, 6 and 7 mutations, respectively. Kisielow *et al.* (39) reported that a siRNA resulting in essentially complete knockdown of the expression of its target gene, was unable to inhibit the expression of a transgene with two non-contiguous mutations in the recognition sequence of the siRNA. The positions of these mutations correspond to our ds10/13 double mutant, which also exhibited low activity in our assay. A central double mutation, reported by Boutla *et al.* (31) and ourselves (33) to support partial activity, led to severe loss of activity for Yu *et al.* (40) and Wilda *et al.* (41), the latter using a siRNA with only 17 base pairs. On the other hand, abolishment of *in vivo* activity by a single mutation has been reported. Brummelkamp *et al.* (10), using a shRNA assumed to be processed to siRNA by Dicer (13), achieved inactivation by single mutations in either the second or ninth position from the putative 5' end of the shRNA. Gitlin *et al.* (8), argued the case for single mutation inactivation more strongly by isolating siRNA-resistant poliovirus strains containing single mutations in the target site on the genomic RNA, corresponding to the sixth or ninth nucleotide of the siRNA, counted from the 5' end of the sense strand. On balance, different siRNAs seem to be inactivated by mutations to different degrees, the outcome being at least in part target-sequence dependent.

In previous work (33) we established that the siRNA effect depends at least in some instances on the siRNA target position, mirroring earlier observations for ribozymes and antisense oligos (34,42,43). Further support for siRNA position effects has now emerged from several different sources. Several examples of inactive siRNAs in mammalian cells have recently been described (11,44,45), while a weak positional effect in *Drosophila* lysates can be inferred from published data (32). In *C.elegans*, Simmer *et al.* (46) managed to activate dozens of previously inactive dsRNA stretches, using the RNAi-sensitive rrf-3 negative strain, and in some cases created RNAi knockout phenotypes from genes that had hitherto not been responsive to dsRNA. Finally, Yang *et al.* (47) recently reported some inactive chemically synthesised and *in vitro* transcribed siRNA, as well as inactive shRNA. The authors demonstrated that the new technique of esiRNA, in which an overlapping set of siRNAs are produced *in vitro* by partial digestion with *Escherichia coli* RNase III, can be superior even to dsRNA. Processing of dsRNA by Dicer starts from a fixed end and proceeds in a sequential manner (3,48,49), producing a largely non-overlapping set of siRNAs. Cleavage of a dsRNA by *E.coli* RNase III will thus create a larger and more complete set of siRNAs than the non-overlapping set produced by Dicer from dsRNA. The higher activity of the esiRNA supports the argument that different siRNAs have different activities. Otherwise any set of siRNA from a dsRNA would have the same activity.

The inactivation of siRNA by mismatches has implications for the proposed function of RNAi as a defence system against retro-transposons and viruses. It is unclear why a viral defence mechanism should allow escape of a virus by a single mismatch. A differentiated population of siRNA with widely differing activities seems more likely. Some siRNAs with an intermediate activity can thus be more vulnerable to siRNA:target mismatch, while intrinsically stronger siRNAs have excess capacity and tolerate a single mismatch, as clearly exemplified by the tolerance exhibited to chemical and mutational modifications of hTF167i in the present study. This tolerance in highly active siRNAs should make viral escape more difficult, and our model is therefore consistent with both the published data and the proposed biological role of RNAi as a viral defence.

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EXHIBIT 4 of
EXHIBIT B

siRNA function in RNAi: A chemical modification analysis

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ABSTRACT

Various chemical modifications were created in short-interfering RNAs (siRNAs) to determine the biochemical properties required for RNA interference (RNAi). Remarkably, modifications at the 2'-position of pentose sugars in siRNAs showed the 2'-OHs were not required for RNAi, indicating that RNAi machinery does not require the 2'-OH for recognition of siRNAs and catalytic ribonuclease activity of RNA-induced silencing complexes (RISCs) does not involve the 2'-OH of guide antisense RNA. In addition, 2' modifications predicted to stabilize siRNA increased the persistence of RNAi as compared with wild-type siRNAs. RNAi was also induced with chemical modifications that stabilized interactions between A–U base pairs, demonstrating that these types of modifications may enhance mRNA targeting efficiency in allele-specific RNAi. Modifications altering the structure of the A-form major groove of antisense siRNA–mRNA duplexes abolished RNAi, suggesting that the major groove of these duplexes was required for recognition by activated RISC*. Comparative analysis of the stability and RNAi activities of chemically modified single-stranded antisense RNA and duplex siRNA suggested that some catalytic mechanism(s) other than siRNA stability were linked to RNAi efficiency. Modified or mismatched ribonucleotides incorporated at internal positions in the 5' or 3' half of the siRNA duplex, as defined by the antisense strand, indicated that the integrity of the 5' and not the 3' half of the siRNA structure was important for RNAi, highlighting the asymmetric nature of siRNA recognition for initiation of unwinding. Collectively, this study defines the mechanisms of RNAi in human cells and provides new rules for designing effective and stable siRNAs for RNAi-mediated gene-silencing applications.

Keywords: RNAi; siRNA; human; nucleotide modification; GFP

INTRODUCTION

The evolutionarily conserved phenomenon RNA interference (RNAi), the process by which specific mRNAs are targeted for degradation by complementary short-interfering RNAs (siRNAs), has increasingly become a powerful tool for genetic analysis and is likely to become a potent therapeutic approach for gene silencing (for review, see Hammond et al. 2001; McManus and Sharp 2002). Consequently, understanding the mechanism of RNAi has become critical for developing the most effective RNAi methodologies for both laboratory and clinical applications. The general mechanism of RNAi involves the cleavage of double-stranded RNA (dsRNA) to short 21–23-nt siRNAs. This processing event is catalyzed by Dicer, a highly conserved, dsRNA-specific endonuclease that is a member of the RNase III family (Hammond et al. 2000; Zamore et al.

2000; Bernstein et al. 2001; Hamilton et al. 2002; Provost et al. 2002; Zhang et al. 2002). Processing by Dicer results in siRNA duplexes that have 5'-phosphate and 3'-hydroxyl termini, and subsequently, these siRNAs are recognized by the RNA-induced silencing complex (RISC; Hammond et al. 2000). Active RISC complexes (RISC*) promote the unwinding of the siRNA through an ATP-dependent process, and the unwound antisense strand guides RISC* to the complementary mRNA (Nykanen et al. 2001). The targeted mRNA is then cleaved by RISC* at a single site that is defined with regard to where the 5'-end of the antisense strand is bound to the mRNA target sequence (Hammond et al. 2000; Elbashir et al. 2001b). For RNAi-mediated mRNA cleavage and degradation to be successful, 5'-phosphorylation of the antisense strand must occur, and the double helix of the antisense-target mRNA duplex must be in the A form (Chiu and Rana 2002).

One highlighted difference between mammalian RNAi and RNAi in other eukaryotes is the lack of an amplification system for long-term persistence of RNAi in mammalian cells. For example, in *Drosophila*, ~35 molecules of dsRNA can silence ~1000 copies of the targeted mRNA per cell and can persist over the course of many generations (Kennerdell

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and Carthew 1998; Zamore 2001). In mammalian cells, RNAi only persists effectively for an average of ~66 h before the siRNA is likely diluted out over the course of several cell divisions (Chiu and Rana 2002). The amplification that is seen in flies and other lower eukaryotes can potentially be attributed to three factors. One is that the conversion of long trigger dsRNA to smaller 21–23-nt siRNAs by Dicer adds a degree of RNAi amplification, whereas in mammalian cells long trigger dsRNA invokes the interferon response that activates the protein kinase PKR (Stark et al. 1998). This suggests that only siRNA transfections successfully trigger RNAi in mammalian cells without other side effects, and thus, no amplification would take place through the processing of longer RNAs. A second factor in amplification is the presence of RNA-dependent RNA polymerase (RdRP), which has been found in plants, worms, fungi, and flies (Cogoni and Macino 1999; Dalmay et al. 2000; Sijen et al. 2001). RdRP has been postulated to amplify target mRNA, through a random, degradative PCR model (Lipardi et al. 2001; Nishikura 2001; Sijen et al. 2001), into dsRNA, which can be targeted by Dicer. However, no RdRP homologs have been found in mammalian cells, and the 3'-OH that is required for RdRP-dependent degradative PCR is not required for RNAi in mammalian cells (Chiu and Rana 2002; Schwarz et al. 2002; Stein et al. 2003), indicating that PCR-based amplification likely does not occur in mammals. A third factor in amplification may be the high enzymatic turnover rate of RISC* during the targeting and cleavage of mRNA (Hutvagner and Zamore 2002), which may add a degree of amplification to RNAi induction in all eukaryotes, including mammals. However, as the persistence of RNAi occurs for only a short period of time, finding methods for increasing the longevity of siRNAs in human cells will be fundamental for applying RNAi to laboratory and therapeutic applications.

To address this issue of siRNA stability for prolonging the duration of dsRNA-mediated gene silencing and to further dissect the mechanism of RNAi in human cells, various chemically modified nucleotides predicted to affect siRNA stability were incorporated into siRNAs to study whether specific modifications increased or decreased the efficacy and persistence of RNAi in vivo. The most important of these modifications was to the 2'-OH of the ribonucleotide that distinguishes RNA from DNA and is required for the nucleophilic attack occurring during the hydrolysis of the RNA backbone, the reaction catalyzed by degradative RNases. Our results showed that the 2'-OH was not required for RNAi, indicating that structural rather than chemical properties of siRNA-mRNA duplexes were the key to inducing RNAi and that RISC* did not require the 2'-OH for ribonuclease activity. 2'-modified siRNAs also increased the persistence of RNAi in human cells. Modifications that stabilized base-pairing interactions were also incorporated into the antisense strand of siRNAs and were able to initiate RNAi, signifying that this class of chemical

modifications could be used to increase the targeting efficiency of siRNAs for mRNA target sequences and for allele-specific inhibition of gene expression.

Other chemical modifications affected the formation of the major groove of the A-form helix of the antisense-siRNA-target-mRNA duplex, and potentially disrupted H-bonds or sterically hindered protein contacts, most probably preventing the RISC* complex from stably interacting with the dsRNA duplex. These modifications completely abolished RNAi, demonstrating that an intact major groove in the A-form helix and stable RNA-protein interactions were required for RNAi in human cells. Finally, previous observations of psorelan cross-linked siRNAs implied that unwinding of siRNA occurred from the 5'-end of the antisense strand and that complete unwinding may not be necessary for effective RNAi (Chiu and Rana 2002). By using mismatched or chemically modified nucleotides on either the 3' or 5' half of the antisense strand within the siRNA duplex, we have shown here that RNAi depended on the integrity of the 5', and not the 3', half of the siRNA duplex, as defined by the antisense strand. Altogether, these results gave insight into the essential biochemical properties of functional siRNAs and how specific changes in the siRNA structure can affect the efficacy of RNAi. Furthermore, these studies present new methodologies for improving the stability and utility of siRNAs for future RNAi applications.

RESULTS

2'-OH is not required for siRNA to enter the RNAi pathway

Previous results showed that RNAi effects typically peaked between 42 and 54 h posttransfection, and targeted gene expression started to be restored by 66 h posttransfection (Chiu and Rana 2002). To determine if the duration of RNAi could be prolonged by increasing the half-life of siRNAs, various chemical modifications were made to nucleotides that affected siRNA stability. These modified siRNAs were then tested in an improved dual fluorescence reporter assay based on the original assay developed previously (Chiu and Rana 2002). Briefly, GFP and RFP were constitutively expressed from pEGFP-C1 and pDsRed2-N1, respectively. EGFP mRNA was targeted for RNAi using a 21-nt siRNA targeted to nucleotides 238–258 of the EGFP mRNA (Fig. 1A). The fluorescence intensity ratio of target (GFP) to control (RFP) fluorophores was determined in the presence of siRNA duplexes and normalized to that observed in the mock-treated cells. The sequence of EGFP siRNA and EGFP mRNA, the specific mRNA cleavage site, plus the structures of the chemically modified nucleotides are diagrammed in Figure 1. As outlined previously, the cleavage site was defined precisely 11 nt downstream of the target position complementary to the 3'-most nucleotide of

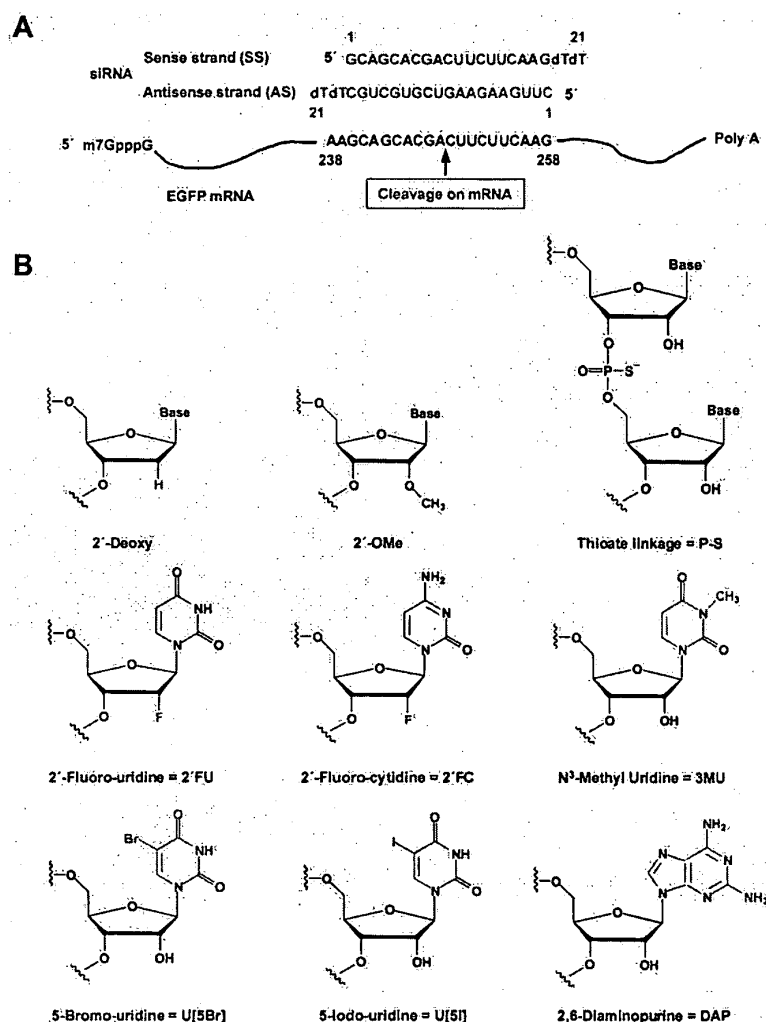


FIGURE 1. Structures of EGFP siRNA and chemical modifications. (A) Graphical representation of dsRNAs used for targeting EGFP mRNA. EGFP was encoded by the pEGFP-C1 reporter plasmid. siRNAs were synthesized with 2-nt deoxythymidine overhangs at the 3'-end. The position of the first nucleotide of the mRNA target site is indicated relative to the start codon of EGFP mRNA. The sequence of the antisense strand of siRNA is exactly complementary to the mRNA target site. (B) Structure and nomenclature of chemical modifications.

the antisense guide siRNA (Elbashir et al. 2001a). The specific chemical modifications, the particular siRNA strand(s) where modifications were made, and the effect of the chemically modified siRNA on RNAi activity are summarized in Table 1. The RNAi activity of siRNAs was evaluated with eight different siRNA concentrations (ranging from 1 to 200 nM). Each experiment was completed in duplicate and repeated twice.

The effects of modifying the 2'-OH of nucleotides on RNAi were studied by replacing uridine and cytidine in the antisense strand of siRNA with 2'-fluoro-uridine (2'-FU)

and 2'-fluoro-cytidine (2'-FC), respectively, which have a fluoro group at the 2'-position in place of the 2'-OH (Fig. 1B). Where these modified 2'-FU, 2'-FC nucleotides reside in the siRNA sequence are highlighted in red in Figure 2A. Addition of a 2'-fluoro group should increase the stability of the siRNA by making the siRNAs less recognizable to RNases, thereby providing siRNAs protection from degradation (see below). When measured in the dual fluorescence assay, 2'-FU, 2'-FC siRNAs, modified only in the sense strand, only in the antisense strand, or in both strands, all showed decreased EGFP fluorescence when normalized to non-targeted RFP fluorescence that was comparable to the normalized decrease seen with wild-type siRNAs (Fig. 2; Table 1, rows 1–4). These results indicated that the 2'-OH was not required for RNAi and that nucleotides modified with 2'-fluoro groups could be used in siRNA constructs to successfully induce RNAi-mediated gene silencing.

To support the conclusion that the 2'-OH was not required for RNAi, adenine and guanine deoxynucleotides that inherently have 2'-H in place of the 2'-OH (Fig. 1B) were incorporated into the sense, antisense, or both strands of 2'-FU, 2'-FC-modified EGFP siRNAs to determine their effect on RNAi (Fig. 2A; green nucleotides). When 2'-FU, 2'-FC nucleotides were incorporated into the EGFP siRNA antisense strand with guanine and adenine deoxynucleotides at positions 9, 10, and 13, which base pair with nucleotides lining the cleavage site (Fig. 2A), EGFP RNAi effects were almost indistinguishable from wild-type levels (Fig. 2B; Table 1, row 5). This same antisense construct base-paired to

2'-FU, 2'-FC-modified sense strands also showed considerable EGFP silencing at ~64% (Table 1, row 6). In addition, siRNAs that had the entire antisense strand replaced with 2'-FU, 2'-FC, dATP, and dGTP nucleotides still showed moderate levels of RNAi activity at ~42%, or ~44% if the sense strand was also modified with 2'-FU, 2'-FC (Table 1, rows 7,8). All together, these results demonstrated that a 2'-OH group was not required for RNAi-mediated degradation and, even more specifically, was not required for nucleotides base-paired with nucleotides lining the mRNA cleavage site. There was, however, a limit on the extent to

TABLE 1. RNA interference mediated by chemically modified siRNAs

Row no.	EGFP siRNA	Sense strand	Antisense strand	RNAi activity (%)	RNAi activity (+ or -)
1	DS (WT)	Unmodified	Unmodified	93 ± 0.70	++++
2	SS/AS-2'-FU, FC	Unmodified	2'-FU, FC	83 ± 3.48	++++
3	SS-2'-FU, FC/AS	2'-FU, FC	Unmodified	92 ± 0.98	++++
4	DS-2'-FU, FC	2'-FU, FC	2'-FU, FC	83 ± 0.01	++++
5	SS/AS-2'-FU, FC + (9, 10, 13) dA, dG	Unmodified	2'-FU, FC + (9, 10, 13) dA, dG	85 ± 2.10	++++
6	SS-2'-FU, FC/AS-2'-FU, FC + (9, 10, 13) dA, dG	2'-FU, FC	2'-FU, FC + (9, 10, 13) dA, dG	64 ± 2.89	+++
7	SS/AS-2'-FU, FC + dA, dG	Unmodified	2'-FU, FC + dA, dG	42 ± 1.66	++
8	SS-2'-FU, FC/AS-2'-FU, FC + dA, dG	2'-FU, FC	2'-FU, FC + dA, dG	44 ± 0.60	++
9	SS/AS-Deoxy	Unmodified	Deoxy	0 ± 5.97	-
10	SS-Deoxy/AS	Deoxy	Unmodified	38 ± 2.95	+
11	DS-Deoxy	Deoxy	Deoxy	0 ± 0.01	-
12	SS/AS-2'-OMe	Unmodified	2'-OMe	16 ± 4.41	-
13	SS-2'-OMe/AS	2'-OMe	Unmodified	25 ± 1.75	+
14	DS-2'-OMe	2'-OMe	2'-OMe	0 ± 0.01	-
15	SS/AS-P-S	Unmodified	P-S	42 ± 6.03	++
16	SS-P-S/AS	P-S	Unmodified	62 ± 0.07	+++
17	DS-P-S	P-S	P-S	47 ± 0.03	++
18	SS/AS-2'-FU, FC + P-S	Unmodified	2'-FU, FC + P-S	22 ± 0.03	+
19	SS/AS-U[5Br]	Unmodified	U[5Br]	70 ± 1.88	+++
20	SS/AS-U[5I]	Unmodified	U[5I]	59 ± 11.2	+++
21	SS/AS-DAP	Unmodified	DAP	51 ± 0.57	++
22	SS-2'-FU, FC/AS-U[5Br]	2'-FU, FC	U[5Br]	31 ± 1.88	+
23	SS-2'-FU, FC, FC/AS-U[5I]	2'-FU, FC	U[5I]	42 ± 5.02	++
24	SS-2'-FU, FC/AS-DAP	2'-FU, FC	DAP	35 ± 7.69	+
25	SS/AS-3MU	Unmodified	3MU	0 ± 6.65	-
26	SS/AS-(11) 3MU	Unmodified	(11) 3MU	0 ± 1.71	-
27	SS/AS-(1, 2) mm	Unmodified	(1, 2) mm	35 ± 5.69	+
28	SS/AS-(18, 19) mm	Unmodified	(18, 19) mm	77 ± 2.00	+++
29	SS/AS-2'-FU, FC + (1-13) dA, dG	Unmodified	2'-FU, FC + (1-13) dA, dG	43 ± 0.09	++
30	SS-2'-FU, FC/AS-2'-FU, FC + (1-13) dA, dG	2'-FU, FC	2'-FU, FC + (1-13) dA, dG	45 ± 2.23	++
31	SS/AS-2'-FU, FC + (9-19) dA, dG	Unmodified	2'-FU, FC + (9-19) dA, dG	91 ± 0.36	++++
32	SS-2'-FU, FC/AS-2'-FU, FC + (9-19) dA, dG	2'-FU, FC	2'-FU, FC + (9-19) dA, dG	64 ± 0.42	+++

Summary of the specific chemical modifications analyzed, the particular siRNA strand(s) modified, and the effect of the chemically modified siRNA on RNAi activity in HeLa cells. RNAi activity was quantified by the dual fluorescence assay and is presented as the inhibition efficiency of target gene (EGFP) expression when cells were treated with 50 nM modified siRNAs. For comparison, the RNAi activity of unmodified, or wild-type, duplex siRNA (DS) normalized to 93% was designated (++++). Modified siRNAs assigned (+++++) showed >80% RNAi activity, (++++) showed 60%–80%, (++) showed 40%–60%, and (+) showed 20%–40%. Modified siRNAs showing <20% RNAi activity were considered nonfunctional (-) in the RNAi pathway. Each experiment measuring RNAi activity of siRNAs was completed in duplicate and repeated twice.

which deoxynucleotides could substitute for ribonucleotides because replacing the entire siRNA sense strand with deoxynucleotides decreased EGFP gene silencing to ~38% inhibition, and replacing either the antisense strand or both strands entirely with deoxynucleotides completely abolished EGFP RNAi (Fig. 2B; Table 1, rows 9–11). Nonetheless, these results collectively showed that nucleotides with either 2'-F or 2'-H groups can selectively replace ribonucleotides within the siRNA sequence to effectively induce RNAi.

An interesting result was seen by modifying the 2'-OH to a bulky methyl group to create 2'-OMe nucleotides that were incorporated into sense, antisense, or both strands of EGFP siRNAs (Fig. 1B). This modification was hypothesized to improve RNAi efficacy because 2'-OMe groups are thought to increase RNA stability by inducing an altered RNA conformation that is more resistant to nucleases (Cummins et al. 1995). This modification is also thought to

increase RNA affinity for RNA targets and improve hybridization kinetics (Majlessi et al. 1998). Despite these potential benefits, 2'-OMe nucleotides incorporated into either the sense or antisense strand greatly diminished EGFP gene silencing to ~25% or ~16%, respectively, whereas double-stranded 2'-OMe-modified siRNAs completely abolished RNAi (Table 1, rows 12–14). These results indicated that the methyl group, as a bulky group, may severely limit the interactions between siRNAs, target mRNAs, and the RNAi machinery required for successfully mediating RNAi. It is worth noting that because the bulkiness of the methyl group would likely be the cause of decreased RNAi activity rather than the actual lack of the 2'-OH specifically, these studies still supported the conclusion that the 2'-OH was not required for RNAi.

In a final analysis of modifications that may potentially increase siRNA stability without disrupting RNAi potency,

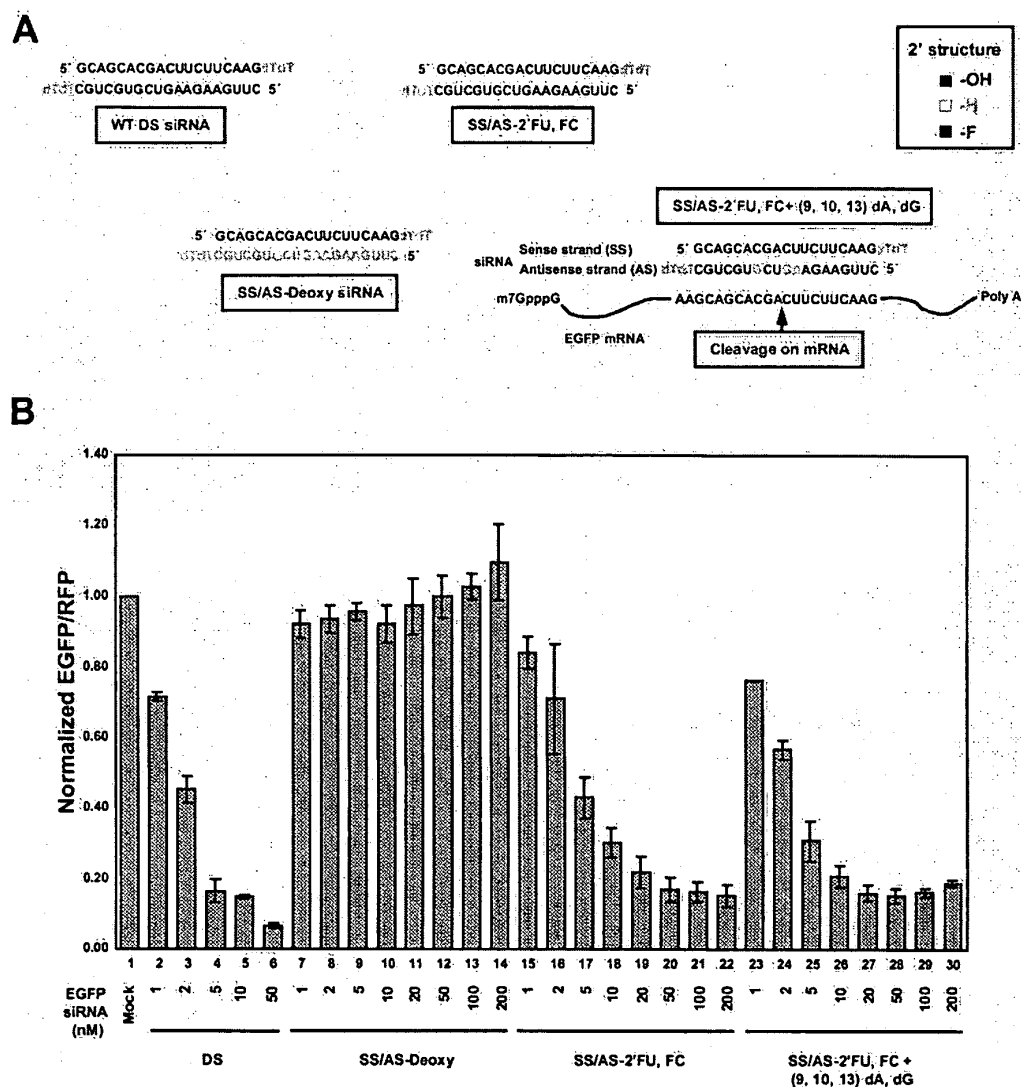


FIGURE 2. siRNA 2'-OH is not required to guide mRNA cleavage. (A) Sequence and structure of siRNA duplexes with modification at the 2'-position of the sugar unit. Nucleotides with 2'-hydroxyl groups (-OH) are black. Nucleotides with 2'-deoxy groups (-H) are cyan. Nucleotides with 2'-fluoro groups (-F) are red. The cleavage site on the target mRNA is also shown (red arrow). (B) Ratios of normalized GFP to RFP fluorescence intensity in lysates from modified siRNA-treated HeLa cells. The fluorescence intensity ratio of target (GFP) to control (RFP) fluorophores was determined in the presence of EGFP siRNA duplexes with modifications at the 2'-position of the sugar unit. Normalized ratios at <1.0 indicate specific RNA interference effects. For comparison, results from unmodified duplex siRNA-treated cells are included.

a thioate linkage (P-S) was integrated into the backbone of the EGFP siRNA strand(s). P-S linkages were previously used in antisense methodology for increasing resistance to ribonucleases (for review, see Stein 1996) and, therefore, were postulated to enhance the stability of siRNAs. Incorporating the P-S linkages into the double-stranded siRNA sense strand led to moderate levels of RNAi activity (62% inhibition), whereas P-S linkages in either the antisense or both strands of the siRNAs led to just less than ~50% RNAi-

induced inhibition (Table 1, rows 15–17). These results implied that the P-S modifications did not prohibit RNAi-mediated degradation and only moderately affected the efficiency of RNAi. Interestingly, incorporating 2'-FU, 2'-FC modifications into the antisense strand in addition to the added P-S linkages showed lower levels of EGFP gene silencing (Table 1, row 18), indicating that there was a synergistic effect that decreased but did not inhibit RNAi-mediated degradation when both the 2'-F groups and the

P-S linkages were incorporated into siRNAs.

Stability of modified siRNAs and the persistence of their RNAi activity in vitro and in vivo

As the above experiments showed that siRNAs modified with stabilizing 2'-FU, 2'-FC groups could effectively mediate RNAi to levels comparable to wild type, it was necessary to show that these modifications did in fact enhance siRNA stability. To measure the stability of siRNA in cell extracts, unmodified or 2'-FU, 2'-FC-modified EGFP antisense strand siRNAs 5'-labeled with [γ - ^{32} P]ATP were annealed with sense strand siRNAs to form duplex siRNAs, which were then incubated in HeLa cell extracts. At various time points, siRNAs were extracted, analyzed on a 20% polyacrylamide gel containing 7 M urea, and visualized by phosphorimager analysis. Smaller siRNA degradation products were visualized in this analysis (data not shown), indicating that the loss of intact siRNA observed during these experiments was not caused by dephosphorylation of siRNAs. The top panel (a) of Figure 3A shows the stability of the various 2'-FU, 2'-FC-modified siRNAs as compared with wild-type siRNAs over time. Wild-type double-stranded siRNAs showed a steady loss of intact siRNAs over the course of the experiment, with only ~7% of the original concentration of intact siRNAs remaining after 1 h in extract (Fig. 3A[a], dark blue line). Intact modified or unmodified single-stranded antisense siRNAs were quickly lost over the time course and were virtually undetectable by 30 min in extract (Fig. 3A[a], black and red lines). This result showed that single-stranded modified siRNA was as susceptible to degradation as wild-type siRNA, indicating that single-stranded siRNAs, modified or unmodified, are inherently less stable than duplex siRNA. Double-stranded siRNAs with 2'-FU, 2'-FC modifications in either the antisense strand or both strands remained predominantly intact over the course of the experiment with ~68% or ~81%, respec-

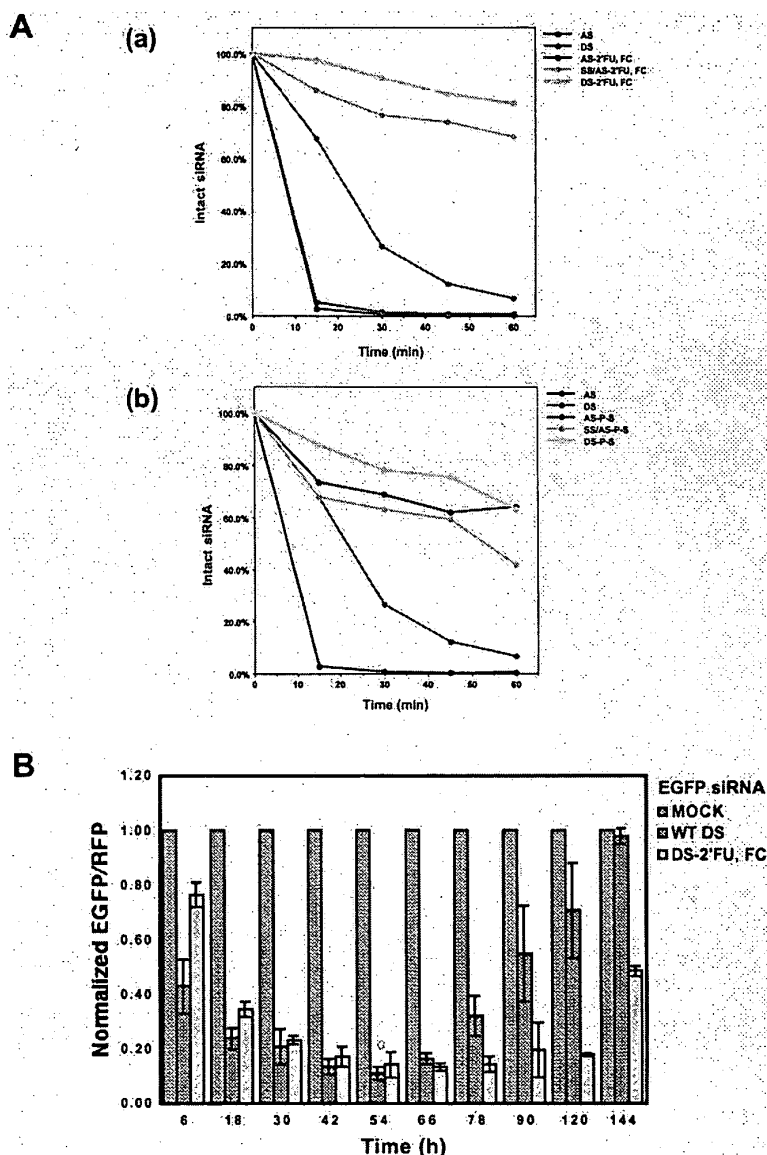


FIGURE 3. Extending the half-life of siRNA duplexes prolongs the persistence of RNA interference in vivo. (A) Comparing the stability of unmodified siRNAs with siRNAs containing 2'-fluoro-uridine and 2'-fluoro-cytidine (2'-FU, 2'-FC) modifications (a) and thioate linkage (P-S) modifications (b). Unmodified or modified EGFP antisense strand siRNAs (AS) were 5'-labeled with [γ - ^{32} P]ATP by T4 polynucleotide kinases. Duplex siRNAs were formed by annealing equal molar ratios of sense-strand (SS) siRNAs with the 5'- ^{32} P-labeled antisense strand. To analyze siRNA stability in HeLa cell extract, 50 pmole of siRNA was incubated with 500 μ g of HeLa cell extract in 50 μ L of reaction mixture containing 20 mM HEPES (pH 7.9), 100 mM KCl, 10 mM NaCl, 2 mM MgCl₂, and 10% glycerol. At various time points, siRNAs were extracted and analyzed on 20% polyacrylamide gels containing 7 M urea followed by phosphorimage analysis (Fugit). (B) Kinetics of RNAi effects of duplex siRNA with 2'-fluoro-uridine and 2'-fluoro-cytidine modification in HeLa cells over a 144-h time course. The fluorescence intensity ratio of target (GFP) to control (RFP) protein was determined in the presence of unmodified dsRNA (blue bars) and duplex siRNA with 2'-fluoro-uridine and -cytidine modifications (DS-2'-FU, 2'-FC, cyan bar) and normalized to the ratio observed in the presence of mock-treated cells (red bars). Normalized ratios at <1.0 indicated specific RNA interference.

tively, of the original siRNA population remaining intact throughout the duration of the experiment (Fig. 3A[a], green and light blue lines). These results indicated that the 2'-FU, 2'-FC modifications did, indeed, increase the stability of the siRNAs upon exposure to extract and that having these modifications in both strands provided the siRNAs with the most stability.

In a similar experiment, the stability of P-S-modified EGFP siRNAs was evaluated. Unmodified, double-stranded antisense siRNAs showed about the same rate of siRNA loss as described in the above experiment (Fig. 3A[b], dark blue lines). However, P-S-modified single-stranded antisense siRNAs demonstrated a markedly increased rate of stability over the course of the experiment, showing ~63% of the original siRNAs remaining intact after 1 h in extract as compared with 0% intact for single-stranded unmodified antisense siRNAs (Fig. 3A[b], black and red lines). The stability of double-stranded siRNAs with P-S modifications in both strands was comparable to the stability seen with the modified single-stranded antisense strand, with ~63% of the original siRNA population remaining intact after 1 h (Fig. 3A[b], light blue lines). Double-stranded siRNAs with P-S modifications in only the antisense strand showed weaker but still significant stability with ~42% of the original siRNA population remaining intact through 1 h in extract (Fig. 3A[b], green lines). These results showed that the P-S modifications increased the stability of the siRNAs and, most notably, increased the stability of both single- and double-stranded siRNAs.

These *in vitro* results indicated that siRNA stability is prolonged by these different modifications; however, it is important to note that these experiments address the general stability of siRNA in the context of endonucleases present in whole-cell extracts. Therefore, these experiments cannot distinguish whether the endonucleases affecting siRNAs in the *in vitro* assay would necessarily affect the stability of these various siRNAs *in vivo*. To address whether increased stability seen with modified siRNAs prolonged the duration of RNAi *in vivo*, RNAi, induced by unmodified and 2'-FU, 2'-FC-modified double-stranded EGFP siRNAs, was assayed in the dual fluorescence reporter assay over a period of 144 h. To visualize RNAi effects over an even longer period of time, HeLa cells were transfected with modified or unmodified siRNA and, 36 h later, transfected with dual fluorescence reporter plasmids; RNAi activity persisted but was tapering by 168 h (data not shown). Also, growth of cells containing modified siRNAs was comparable to cells containing wild-type siRNA, indicating that modified siRNAs were not affecting cell division (data not shown). Although 2'-FU, 2'-FC-modified EGFP siRNAs were slower to show RNAi effects by 6–18 h, maximal RNAi effects occurred by 42 h posttransfection for both modified and unmodified siRNAs. The maximal activity for both siRNAs was also in the same range, with both showing ~85%–90% inhibition of GFP expression. However, the

RNAi effects observed over the period of 66–120 h revealed that the effect of modified siRNAs was much more persistent than that of unmodified siRNAs. By 120 h posttransfection, the effect of modified siRNAs still remained at ~80% inhibition of GFP expression but the effect of unmodified siRNAs had dropped to less than ~40% inhibition. Similarly, prolonged RNAi activity was observed with 2'-FU, 2'-FC-modified siRNAs targeting endogenous human Cyclin T1 mRNA when compared with wild-type siRNAs targeting Cyclin T1 (see Discussion; Y.L. Chiu and T.M. Rana, unpubl.). Altogether, these results strongly indicated that there was a direct link between the duration of the RNAi effects and siRNA stability in human cells. Furthermore, these results showed conclusively that siRNAs stabilized by chemical modifications, like the 2'-FU, 2'-FC modifications, can be used to effectively induce and significantly prolong RNAi-mediated gene silencing *in vivo*.

Modified siRNAs that stabilize A–U base-pair interactions can induce RNAi

In addition to incorporating modifications that affected the stability of siRNAs, nucleotides chemically modified to strengthen the base-pair interactions between two complementary bases were analyzed. In theory, increasing the stability of base-pair interactions may increase the targeting efficiency of siRNAs to target mRNA sequences. Increasing targeting efficiency may then induce more robust RNAi effects with siRNAs that are weaker at binding to their target sequence or have mismatched sequences, and thus, are not showing a high degree of RNAi. This type of approach may also be used to significantly inhibit expression of one allele over another when both alleles are present in the same cell. To bolster base-pairing interactions, 5-bromo-uridine (U[5Br]), 5-iodo-uridine (U[5I]), or 2,6-diaminopurine (DAP; Fig. 1B), which are modified nucleotides known to increase the association constant between A–U base pairs (Saenger 1984), were incorporated into siRNAs and tested in the dual fluorescence report assay. Double-stranded siRNAs having U[5Br], U[5I], or DAP modifications incorporated into the antisense strand were capable of inducing RNAi activity at levels of ~70% for U[5Br], ~59% for U[5I], and ~51% for DAP (Fig. 4; Table 1, rows 19–21). Interestingly, when 2'-FU, 2'-FC stabilizing modifications in the sense strand were combined with these modifications in the antisense strand, gene silencing was not as efficient as wild type in inducing RNAi. EGFP gene silencing was 31% for the 2'-FU, 2'-FC-modified sense siRNA base-paired with U[5Br]-modified, ~42% for U[5I]-modified, or ~35% for DAP-modified antisense siRNAs (Fig. 4; Table 1, rows 22–24). These results indicated that enhancing the interactions between base pairs through these siRNA modifications was a viable option for increasing mRNA targeting efficiency, but that there was a limit to how stable the base-pairing

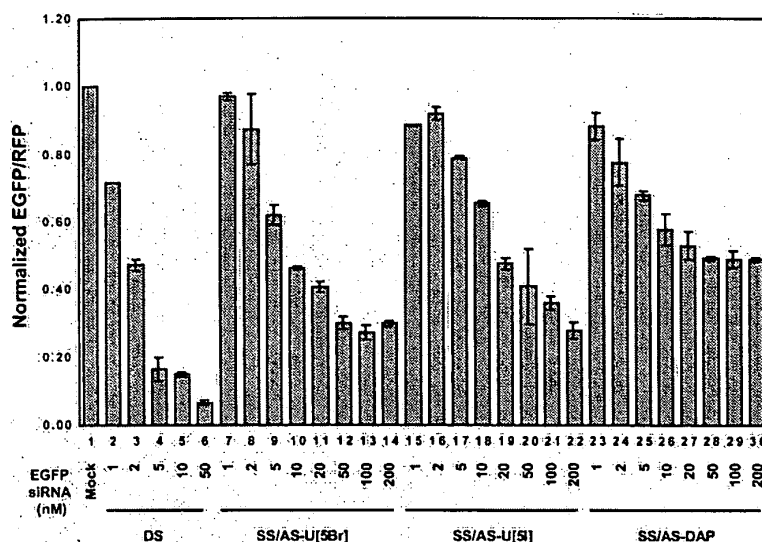


FIGURE 4. RNA interference mediated by siRNAs harboring modifications that stabilize base-pairing interactions. 5-Bromo-uridine (U[5Br]) or 5-iodo-uridine (U[5I]) replaced uridine or 2,6-diaminopurine (DAP) replaced adenine in siRNAs to stabilize base-pairing interactions. The activity of siRNAs with base modifications was quantified by the dual fluorescence assay. For comparison, results from unmodified duplex siRNA (DS, lanes 2–6)-treated cells are included.

interactions can be made before they interfere with siRNA unwinding (see Discussion).

The major groove of the A-form helix is required for RNAi

Previously, the A-form helix was shown to be required for the mechanism of RNAi, as 2-nt bulges that distort A-form helices between antisense siRNAs and target mRNAs abolished RNAi (Chiu and Rana 2002). To test whether the major groove of the A-form helix was required for RNAi, siRNAs were modified with *N*³-methyl uridine (3MU) nucleotides that remove an H-bond donor at *N*³-H. Structurally, the bulky *N*³-methyl group would jut into the major groove of the A-form helix, potentially introducing a steric clash between base pairs. In addition, the presence of 3MU in the major groove may also introduce a steric clash between RNA and RNA-interacting proteins (Saenger 1984). Therefore, both steric hindrance and the loss of an H-bond donor by the addition of the *N*³-methyl group should destabilize RNA–protein interactions in the major groove. 3MU-modified EGFP siRNAs introduced into HeLa cells completely abolished RNAi (Table 1, row 25). RNAi was also abolished if only one 3MU modification was introduced specifically at U11 of the antisense strand, which is one of the nucleotides that base pairs with A248 of the target EGFP mRNA cleavage site (Fig. 1A; Table 1, row 26). These results indicated that disrupting the functional

groups of the major groove of the A-form helix formed by the antisense strand and its target mRNA specifically at the cleavage site inhibited RNAi. These data also indicated that the major groove was required for mediating RNAi and for RNA–RISC interactions that subsequently lead to mRNA cleavage.

Structural integrity of the 5' half of siRNA duplex, as defined by the antisense strand, is important for mediating RNAi

Previous data using psoralen photochemistry suggested that complete unwinding of the siRNA duplex was not required for RNAi *in vivo* because psoralen cross-linked siRNAs did not completely abolish gene silencing (Chiu and Rana 2002). In describing these results, it was proposed that a single cross-linking event occurring near the 3'-end of the antisense strand still allowed for the initial unwinding of duplex siRNAs from the 5'-end, freeing enough of the nucleotides in the antisense strand to hybridize to the target mRNA and induce RNAi, even if unwinding was not complete. If this were the case, then unwinding of siRNAs must start from the 5'-end of the antisense strand, a conclusion supported by the fact that blocking either the 3'-end of the antisense siRNA strand or the 5'-end of the sense siRNA strand had no significant effect on RNAi activity (Chiu and Rana 2002).

If this 5'-to-3' unwinding model was correct, sequences within the 3' half of the siRNA duplex as defined by the antisense siRNA strand should be changeable without significantly interfering with RNAi. To test this hypothesis, EGFP siRNAs with mismatched base pairs at either the internal 5' (nt 1, 2) or 3' (nt 18, 19) positions within the duplex were introduced into the antisense strand (Fig. 5A, purple box, purple nucleotides). siRNAs with mismatches near the 5' half of the duplex structure showed only ~35% inhibition in the dual fluorescence reporter assay, whereas mismatches at the 3' half retained a significant level of gene silencing at ~77% (Fig. 5B; Table 1, rows 27–28). These results strongly indicated that the integrity at the 5' half of the duplex, as defined by the antisense strand, was functionally more important than that at the 3' half.

To explore this idea even further, 2'-FU, 2'-FC plus dATPs, dGTPs were incorporated within internal positions within the antisense-strand siRNAs predominantly at the 5' (nt 1–13) or predominantly within the 3' half (nt 9–19; Fig. 5A, red and cyan nucleotides). In the dual fluorescence

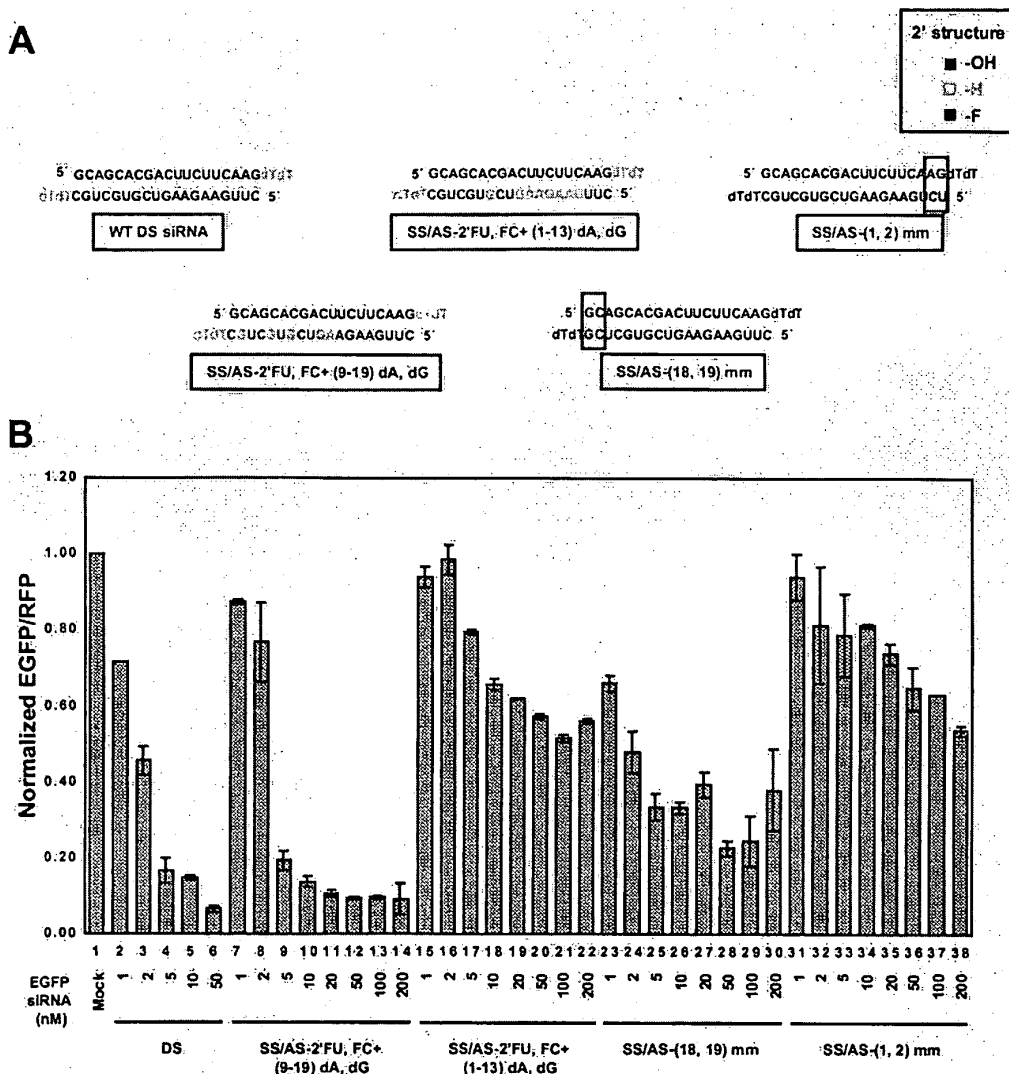


FIGURE 5. Structural integrity of the 5' half of siRNA duplexes, as defined by the antisense strand, was functionally more important than at the 3' half. (A) Graphical description of asymmetric requirement of duplex siRNA structure. The structure of unmodified (WT DS) siRNA duplexes, siRNAs with 2'-fluoro-uridine and 2'-fluoro-cytidine, 2'-deoxy modification at the 3' half (SS/AS-2'-FU, 2'-FC + [9-19] dA, dG) or 5' half (SS/AS-2'-FU, 2'-FC + [1-13] dA, dG) of the antisense strand, and siRNA duplexes with mismatches within the antisense 3' half (SS/AS-[18,19] mm) or 5' half (SS/AS-[1,2] mm) of the siRNA duplex are shown here. (B) Results from cells treated with duplex siRNAs with asymmetrically modified siRNA duplexes. For comparison, results from unmodified duplex siRNAs (DS, lanes 2-6)-treated cells are included.

reporter assay, predominantly 5'-modified antisense (AS-2'-FU, 2'-FC + [1-13] dA, dG) EGFP siRNAs were only moderately effective, inducing RNAi at ~43%, or at 45% if the sense strand was also modified to 2'-FU, 2'-FC (Fig. 5B; Table 1, rows 29,30). However, predominantly 3'-modified and 5'-unmodified antisense (AS-2'-FU, 2'-FC + [9-19] dA, dG) siRNAs significantly induced RNAi activity at ~91%, or at 64% if the sense strand was also modified to 2'-FU, 2'-FC (Fig. 5B; Table 1, rows 31,32). These contrasting results indicated that sequence structure within the 5'

region of the antisense strand was more sensitive to modification than in the 3' region. All together, these data indicated that recognition of siRNA duplexes by an as-yet-unidentified RNA helicase occurs asymmetrically with the structure of the antisense 5'-end of the duplex preferentially distinguished from the 3'-end during the initiation of unwinding.

Modified siRNAs enter into the RNAi pathway in vitro

Although the dual fluorescence reporter assay did detect changes in EGFP gene expression with the modified siRNAs

created herein, it was possible that gene silencing was being induced by a mechanism other than RNAi-mediated degradative pathways. To test whether the targeted mRNA was, indeed, being cleaved upon exposure to modified siRNAs, an *in vitro* RNAi assay was performed to measure the cleavage of a ^{32} P-cap-labeled mRNA target upon incubation with modified siRNAs and HeLa cytoplasmic extract. Cleavage products were resolved on an 8% polyacrylamide-7 M urea gel. Mock-treated mRNAs did not show an observable cleavage product (Fig. 6, lane 1), but wild-type and all modified siRNAs that displayed gene silencing effects *in vivo* showed clearly visible cleavage products *in vitro* (Fig. 6, lanes 2,8–11,14–17). Furthermore, modified siRNAs that did not show any marked gene-silencing effects *in vivo* did not show any distinct cleavage products in the *in vitro* assay (Fig. 6, lanes 1,6,7,12,13), implying that the cleavage events observed were specifically dependent on functional siRNAs. These *in vitro* results provided a strong correlation between the *in vivo* gene silencing observed with the modified siRNAs and target mRNA degradation, indicating that the modified siRNAs were distinctly targeting mRNAs for cleavage and subsequent degradation through the *in vivo* RNAi pathway.

DISCUSSION

RNAi has moved toward the forefront of reverse genetic analysis in human cells for characterizing loss of gene function phenotypes and establishing connections between gene structure and function. In light of its versatility, understanding the detailed mechanism behind the RNAi phenomenon and developing methods to extend the limits of its current capabilities is crucial for implementing this methodology even further into the laboratory and therapeutic realms. By introducing various chemical modifications into siRNAs and measuring their effects on RNAi, this study revealed new insights into the mechanism of RNAi and outlined new approaches for increasing the efficacy of RNAi *in vivo* in human cells for use in future applications.

From these data, a more complete picture of the stepwise process of RNAi can be envisioned and is depicted in Figure

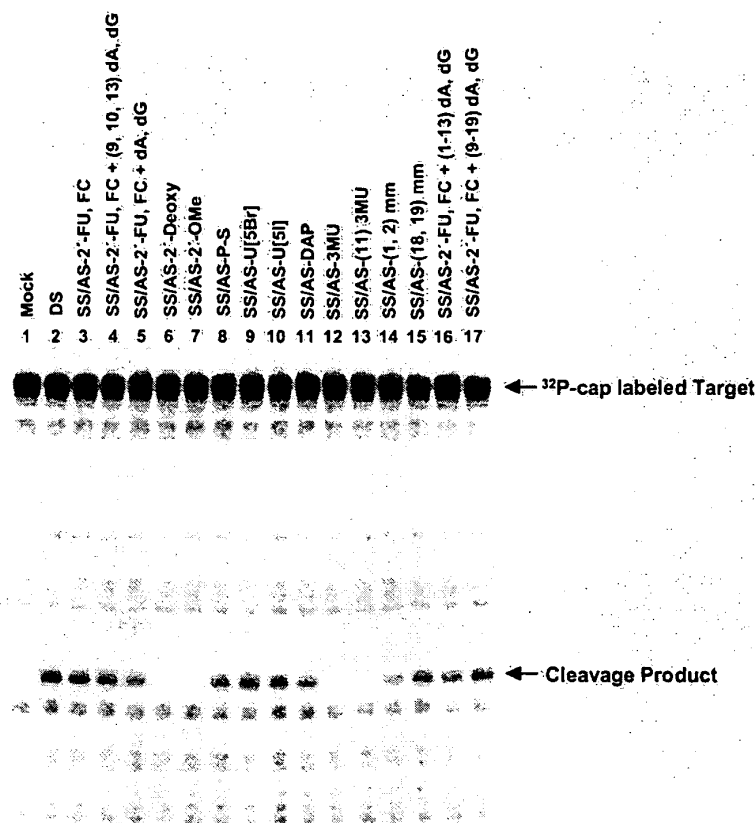


FIGURE 6. For analysis of siRNA-mediated target mRNA cleavage *in vitro*, 10 nM cap-labeled target EGFP mRNA was incubated with 100 nM siRNA and HeLa cytoplasmic extracts, as described in Materials and Methods. Reaction products were resolved on an 8% polyacrylamide-7 M urea gel. Arrows indicate the capped target EGFP mRNA and the 5' cleavage product, which were expected to be 124 nt and 55 nt, respectively. The identity of the cleavage product was assigned according to the RNase T1 partial digestion and a molecular weight marker of RNA (data not shown). The 3' fragment is unlabeled, and therefore, invisible.

7. In the first step of RNAi induction, the 5'-ends of the siRNA duplex are phosphorylated, resulting in the formation of an siRNA-RISC complex. The data presented here showing the asymmetric nature of unwinding then indicate an ATP-dependent event during which siRNA is unwound from the 5'-end of the antisense strand and RISC is activated. Following RISC activation, the antisense strand of the unwound siRNA guides the siRNA-RISC* complex to the target mRNA. The guide antisense strand base pairs with the target mRNA, forming an A-form helix, and the RISC* protein complex recognizes the major groove of the A-form

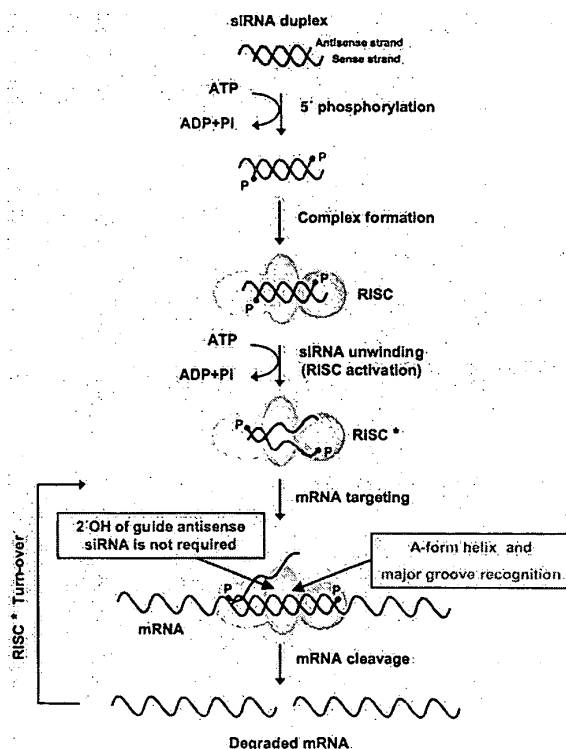


FIGURE 7. Model for RNAi in human cells highlighting the requirement of the A-form helix and major groove for mRNA cleavage with the steps not requiring the RNA 2'-OH of the guide antisense siRNA. See text for details.

helix, an event that occurs independently of the RNA 2'-OH of the guide antisense siRNA. In the final step of this process, the target mRNA is cleaved by RISC*, which is another event that occurs independently of the 2'-OH of the guide antisense siRNA. RISC* is then recycled to catalyze another cleavage event.

The requirement for the A-form helix supercedes the requirement for the 2'-OH in RNAi

Several important mechanistic findings were presented here that not only more clearly defined the mechanism of the RNAi pathway, but will also increase the utility of RNAi in various applications. Our results showing that the 2'-OH was not essential for RNAi have several important implications for the structural and catalytic elements required for the RNAi pathway. Remarkable functional implications were that the RNAi machinery does not require the 2'-OH for recognition of siRNAs, and the catalytic ribonuclease activity of RISC does not involve 2'-OH groups of the guide antisense RNA. Another consequence of this discovery was that a variety of chemical groups, including fluoro or deoxy groups, could substitute for the 2'-OH in siRNAs, indicat-

ing that no distinguishing chemical specificity was required for RNAi at the 2'-position. These findings would imply that other properties of the siRNA-mRNA duplexes, such as core structural elements, were essential for siRNA. If helical structure was the key to RNAi induction, then the A-form helix that forms between siRNAs and the target mRNA would, indeed, be required for RNAi, as was previously shown (Chiu and Rana 2002). Furthermore, the 2'-fluoro or combined 2'-fluoro, deoxy-modified antisense siRNAs lacking the 2'-OH would have to competently form an A-form helix to induce RNAi as shown here. This will likely turn out to be the case because 2'-fluoro-modified RNA-RNA hybrids were previously reported to exhibit an A-form helical conformation (Cummins et al. 1995; Luy and Marino 2001), lending significant merit to the idea that helical structure strongly influences RNAi efficiency. Still another implication of these particular results was that alternate chemical groups at the 2'-position that allow the A-form helix to be retained but help siRNAs evade recognition by RNases can increase siRNA stability and prolong RNAi effects induced in vivo.

It was previously shown in *Caenorhabditis elegans* and *Drosophila* extracts that completely substituting one or both siRNA strands with deoxynucleotides abolished RNAi (Parish et al. 2000; Elbashir et al. 2001b), and those observations were consistent with the data presented here. The failure of true DNA-RNA hybrids to induce RNAi most plausibly relates to the argument that structure, and thus the A-form helix, was an essential determinant for RNAi induction. Based on circular dichroism spectra, DNA-RNA hybrids displayed characteristics that were intermediate between A- and B-form helices (Cummins et al. 1995). Following the contention that the A-form helix was an absolute requirement for RNAi induction, 2'-deoxy siRNA-mRNA target duplexes would not be recognized by the RNAi machinery because they would not be forming the proper A-form helical structure. Therefore, RNAi would not be induced by DNA-RNA hybrids, as has been observed. It is also worth mentioning that microRNAs (miRNAs) induce posttranscriptional gene silencing (PTGS) through the same pathway as RNAi but, ultimately, only inhibit translation machinery instead of inducing RNA degradation, the event that defines RNAi. The only observable difference between the two mechanisms is that RNAi requires the A-form helix, but miRNA-induced PTGS does not, as miRNAs often mismatch with their target mRNAs, forming a bulge that would distort the helical structure. This would indicate that the differences between the miRNA-induced silencing mechanism and siRNA-mediated RNAi may solely be attributable to differences in RNA-RNA helical structure, and further support a model in which helical structure was the sole determinant for whether RNAi was induced.

It was also previously reported that replacement of uridine with 2'-FU, corresponding to one-fourth of the bases of long dsRNAs, elicited RNAi effects in *C. elegans*, whereas

deoxycytidine incorporated into long dsRNAs diminished RNAi effects (Parrish et al. 2000). However, exactly where these modified nucleotides fell within the sequence structure of RNAi-inducing siRNAs and whether these modified nucleotides in the longer RNAs corresponded to the mRNA cleavage site or major groove after being processed to siRNAs was not clear. It has also been reported that siRNAs in which 3' overhangs and two of the 3'-end ribonucleotides were replaced with deoxyribonucleotides retained RNAi activity upon exposure to *Drosophila* extracts (Elbashir et al. 2001b). Presumably, replacing two of the 3'-end base-paired nucleotides with deoxynucleotides would not disrupt the overall A-form structure of the siRNA-mRNA duplex required for RNAi and would thereby allow RNAi induction.

Neither analyses in *C. elegans* nor in *Drosophila* extracts ascertained whether there was a distinct requirement for the 2'-OH for cleavage-site recognition and the cleavage event itself during RNAi induction. The results presented here demonstrated that exclusively using 2'-FU, 2'-FC modifications in siRNAs and selectively substituting in deoxyribonucleotides for nucleotides base-paired with the nucleotides lining the mRNA cleavage site, or even replacing the entire sequence of siRNA with a combination of 2'-fluoro- and 2'-deoxynucleotides, elicited RNAi induction. Therefore, it has now been definitively established that recognition of the mRNA-target cleavage site and subsequent cleavage did not require the 2'-OH of the antisense siRNA to induce RNAi. As a final point, the inhibitory RNAi effects seen with the bulky 2'-OMe modification, which was also shown previously with *Drosophila* (Elbashir et al. 2001b), did demonstrate that there were steric constraints on the types of 2' modifications that would be amenable for inducing RNAi. As 2'-OMe modifications probably did not disrupt the A-form helix of the siRNA-mRNA duplex (Cummins et al. 1995), the methyl group may be sterically interfering with protein-RNA interactions, thereby preventing RNAi. Nevertheless, steric constraints notwithstanding, this analysis conclusively showed that the nonessential nature of the 2'-position could very much be exploited for improving the efficacy of RNAi in a variety of applications.

Improving the efficacy of RNAi using chemical modifications

Modifications like the 2'-fluoro and P-S linkages both increased the half-life of siRNAs upon exposure to cytoplasmic extracts, and in vivo studies with 2'-FU, 2'-FC siRNAs showed that increasing the half-life of siRNAs did, in fact, prolong the effects of RNAi. In addition, we have observed similar prolonged RNAi activity with silencing lasting over 90 h using these same modifications in siRNAs targeting the endogenous target, human Cyclin T1 (Y.L. Chiu and T.M. Rana, unpubl.). These observations demonstrated that the siRNA modifications studied here can be used to effectively

silence endogenous human genes over prolonged periods of time. Our results also indicated that short-lived RNAi effects usually observed in human cells were caused at least in part by the degradation of siRNAs. Our findings that the stabilizing siRNA modifications still allowed for a substantial level of RNAi induction showed that these modifications will be invaluable for studying the phenotypic effects of prolonged gene silencing in cell culture or in increasing the long-term in vivo effects of siRNAs in clinical applications. Interestingly, the P-S-modified, single-stranded antisense strand did not show increased RNAi effects in the dual fluorescence reporter assay used here (data not shown), despite showing significantly increased stability (Fig. 3A[a]). Similarly, single-stranded antisense siRNAs modified with 2'-OMe or 2'-FU, 2'-FC did not cause RNAi efficiently (data not shown). This strongly indicated that siRNA stability was not the main reason that single-stranded antisense RNAi was not as effective in inducing RNAi as dsRNA. Nonetheless, creating P-S modifications in the siRNA backbone showed that stabilizing the siRNA backbone did not inhibit RNAi and signified that using chemical modifications that stabilized phosphate linkages was a viable option for prolonging RNAi effects.

Another option for increasing the efficacy of RNAi was uncovered by the analysis of modifications that should enhance base-pairing interactions between antisense siRNA and targeted mRNA. DAP is a naturally occurring nucleobase that sometimes replaces adenine in phages like the cyanophage S-2L (Kirnos et al. 1977). Incorporation of DAP into RNA strands promotes the formation of three H-bonds between DAP and uridine, increasing the stability of interactions seen between A-U base pairs (Bailey and Waring 1998). U[5Br] and U[5I] have also been shown to have higher association constants when base-paired to A residues than to unmodified uridine (Saenger 1984). When any of these modifications were incorporated into siRNAs, RNAi was still quite efficient, indicating that modifications that stabilize base-pairing interactions can be used in designing siRNAs for various applications. It was also notable that siRNAs with 2'-fluoro modifications introduced into sense strands and base-paired with the DAP, U[Br], or U[5I] antisense strands had decreased RNAi efficiency. 2'-fluoro modifications have been shown to significantly increase the melting temperature between base pairs (Cummins et al. 1995). Consequently, the stabilizing effect on base-pairing interactions when both the 2'-fluoro and DAP, U[Br], or U[5I] modifications were present may have actually hindered the unwinding of the siRNA duplex. These results indicated that the lower rates of siRNA unwinding account for the observed decrease in RNAi activity.

Despite some minor limitations on how much the base-pair interactions can be stabilized, an application for using these types of modifications would be to increase the targeting efficiency of one mRNA sequence over another closely homologous but not identical sequence. Precedent

for this type of sequence discrimination was set by Haaima et al. (1997), who showed that DAP improved the ability of an oligomer to discriminate against mismatches. Translated to an RNAi application, these modifications may be useful for specifically targeting a mutant mRNA in a population of both mutant and wild-type mRNAs to recover a recessive wild-type phenotype. These modifications may also be useful in increasing binding affinity between target mRNAs and siRNAs that appear to have weak gene-silencing effects.

Other structural determinants for RNAi induction

Another structural facet of the RNAi mechanism was uncovered using the 3MU modification, which showed that the major groove of the A-form helix was required for RNAi. This finding builds on previous data showing that the A-form helix was required for RNAi (Chiu and Rana 2002). Together, these results indicated that the specific structure of the A-form helical RNA that forms the major groove and contains the mRNA cleavage site was important for recognition by the RNAi machinery. Conceivably, RNA-RISC* contacts depend on the structural integrity of the major groove for precise interactions and, ultimately, to initiate cleavage of the target. By disrupting the major groove, RISC* may no longer be able to interact or only weakly interacts with the siRNA-mRNA target duplex, thereby preventing mRNA cleavage. Alternatively, RISC* might still be able to interact with the destabilized RNA helix but not recognize the cleavage site within the major groove as the catalytic site if the conformation of the RNA helix and more specifically the major groove were altered.

The other structural property of siRNAs defined by these analyses was the asymmetric nature of siRNA unwinding. Initiation of siRNA unwinding from the 5'-end was previously indicated from the ability of single cross-linked siRNAs to still induce RNAi (Chiu and Rana 2002). By using mismatched or modified nucleotides on either the 3' or 5' half of the antisense strand within the siRNA duplex, it was shown here that RNAi depended on the integrity of the 5', and not the 3', half of the siRNA duplex, as defined by the antisense strand. Previous studies from our laboratory and from other groups have observed that mutations or modifications at the 3'-end overhang of the antisense strands are well-tolerated (Chiu and Rana 2002; Martinez et al. 2002; Amarzguoui et al. 2003; Holen et al. 2003). However, it is important to note that these studies did not address the role of nucleotides and the structure of siRNA at internal sequence positions. Here, we have specifically demonstrated the significance of nucleotides within the RNA duplex and their role in defining the structure of siRNA required for RNAi in vivo. These results indicated that, like RISC*, the RNA helicase, which has not yet been identified, also recognizes structural properties of the siRNA duplex as opposed to specific sequences of the RNA strands. This recognition appears to be asymmetric, with the structure of

the antisense 5' half of siRNA duplexes favored over the 3' half, and is similar to how restriction enzymes can preferentially cleave the DNA backbone asymmetrically within a palindromic sequence. Further structural analysis of siRNAs to define what properties within the antisense 5' half of the duplex contribute to the asymmetric nature of the duplex should help elucidate the specific structural elements required for duplex recognition by the RNA helicase for siRNA unwinding.

Our results showing that the modified siRNAs displayed effective RNAi in vivo and in vitro was also significant as it confirmed that the observed gene silencing was mediated by the RNAi pathway. These results also indicated that using chemical modifications that allow for efficient RNAi induction should work in the design of any given siRNA to increase its stability and capacity to specifically induce RNAi in vivo. Using these chemical modifications should take the field of RNAi quite a large step forward beyond the limits presently imposed by unmodified siRNAs with respect to long-term RNAi induction and targeting efficiency. One can imagine numerous applications for all of the chemical modifications used in this analysis, from studying prolonged RNAi effects on multiple genes in human cell cultures to opening up the door for long-term RNAi efficacy in therapeutic realms for curing a variety of genetic diseases.

From the data presented here, the mechanism of RNAi has been further elucidated and the groundwork for incorporating RNAi successfully into therapeutic realms has been laid out. We hope that future studies using the insight garnered here will not only help direct studies for further dissection of the mechanism of RNAi but will lead to new discoveries about gene function and facilitate the introduction of RNAi into vital clinical applications.

MATERIALS AND METHODS

siRNA preparation

The sequences of EGFP target-specific siRNA duplexes were designed as previously described (Chiu and Rana 2002). The 21-nt RNAs were chemically synthesized as 2'-bis(acetoxyethoxy)-methyl ether-protected oligonucleotides by Dharmacon. Synthetic oligonucleotides were deprotected, annealed, and purified; successful duplex formation was confirmed by 20% nondenaturing polyacrylamide gel electrophoresis (Chiu and Rana 2002).

Culture and transfection of cells

HeLa cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). Cells were regularly passaged at subconfluence and plated 16 h before transfection at 70% confluency. Lipofectamine (Invitrogen)-mediated transient cotransfections of reporter plasmids and siRNAs were performed in duplicate 6-well plates. A transfection mixture containing 0.16 µg of pEGFP-C1

and 0.33 μ g of pDsRed2-N1 reporter plasmids (Clontech), various amounts of siRNA (1.0–200 nM), and 10 μ L of lipofectamine in 1 mL of serum-reduced OPTI-MEM (Invitrogen) was added to each well. Cells were incubated in the transfection mixture for 6 h and further cultured in antibiotic-free DMEM. Cells were treated under the same conditions without siRNA for mock experiments. At various time intervals, the transfected cells were washed twice with phosphate-buffered saline (PBS, Invitrogen), flash-frozen in liquid nitrogen, and stored at -80°C for reporter gene assays.

Improved dual fluorescence assay

In an improved dual fluorescence reporter assay, EGFP-C1 encoded enhanced green fluorescence protein (GFP) and DsRed2-N1 encoded red fluorescence protein DsRed2 (RFP), a DsRed variant that has been engineered for faster maturation and lower unspecific aggregation. The extinction coefficient of DsRed2 is $43,800\text{ M}^{-1}\text{ cm}^{-1}$, and the quantum yield is 0.55, a significant quantitative increase when compared with the DsRed1 vector used in the dual fluorescence assay (Chiu and Rana 2002). To quantify RNAi effects, cell lysates were prepared from siRNA duplex-treated cells at 42 h posttransfection, as described previously (Chiu and Rana 2002). Then 240 μ g of total cell lysate in 160 μ L of reporter lysis buffer was measured by fluorescence spectrophotometry (Photo Technology International). The slit widths were set at 4 nm for both excitation and emission. All experiments were carried out at room temperature. GFP fluorescence in cell lysates was detected by exciting at 488 nm and recording from 498 to 650 nm. The spectrum peak at 507 nm represents the fluorescence intensity of GFP. RFP fluorescence in the same cell lysates was detected by exciting at 568 nm and recording from 588 to 650 nm. The spectrum peak at 583 nm represents the fluorescence intensity of RFP. The fluorescence intensity ratio of target (GFP) to control (RFP) fluorophores was determined in the presence of siRNA duplexes and normalized to that observed in the mock-treated cells. Normalized ratios at <1.0 indicated specific interference.

Study of duplex siRNA stability in HeLa cell lysate

Unmodified or modified EGFP antisense strand siRNAs were 5'-labeled with [γ - ^{32}P]ATP (3000 Ci/mM; ICN) by T4 polynucleotide kinases (New England Biolabs) at 37°C for 1 h and chase-kinased by adding 1 mM ATP at 37°C for 15 min. Free ATP and kinases were removed by the QIAGEN nucleotide removal kit. RNA was then purified by 20% polyacrylamide gel containing 7 M urea. Duplex siRNAs were formed by annealing equal molar ratios of unmodified or modified sense-strand siRNAs with the 5'- ^{32}P -labeled antisense strand. Duplex formation was confirmed by 20% PAGE under native condition. We incubated 50 pmole of duplex siRNAs labeled at the 5'-end of the antisense strand with 500 μ g of HeLa whole-cell extract in a 50- μ L reaction mixture containing 20 mM HEPES (pH 7.9), 100 mM KCl, 10 mM NaCl, 2 mM MgCl_2 , and 10% glycerol. At various time points, 8- μ L aliquots were mixed with 16 μ L of loading buffer (0.01% bromophenol blue, 0.01% xylene cyanol, 98% formaldehyde, and 5 mM EDTA). The products were then denatured by heating at 95°C for 10 min and analyzed on 20% polyacrylamide gel containing 7 M urea followed by phosphorimager analysis (Fugi).

Preparation of HeLa cells cytoplasmic extract

Cytoplasm from HeLa cells was prepared following the Dignam protocol for isolation of HeLa cell nuclei (Dignam et al. 1983). The cytoplasmic fraction was dialyzed against cytoplasmic extract buffer (20 mM HEPES at pH 7.9, 100 mM KCl, 200 μ M EDTA, 500 μ M DTT, 500 μ M PMSF, 2 mM MgCl_2 , 10% glycerol). The extract can be stored frozen at -70°C after quick-freezing in liquid nitrogen. The protein concentration of HeLa cytoplasmic extract varied between 4 and 5 mg/mL as determined by a Biorad protein assay kit.

Preparation of cap-labeled target mRNA

For mapping of the target RNA cleavage, a 124-nt EGFP transcript, corresponding to nucleotides 195–297 relative to the start codon followed by the 21-nt complement of the SP6 promoter sequence, was amplified from template pEGFP-C1 by PCR using the 5' primer GCCTAATACGACTCACTATAGACCTACGGCGTGCA GTGC (T7 promoter underlined) and the 3' primer TTGATTTAG GTGACACTATAGATGGTGCGCTCCTG-GACGT (SP6 promoter underlined). His-tagged mammalian capping enzyme was expressed in *Escherichia coli* from a plasmid generously provided by Stewart Shuman (Molecular Biology Program, Sloan-Kettering Institute, New York) and purified to homogeneity. Guanylyltransferase labeling was performed by incubating 1 nmole of transcripts with 50 pmole of His-tagged mammalian capping enzyme in the 100- μ L capping reaction containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 2.5 mM MgCl_2 , 1 U/ μ L RNasin RNase inhibitor (Promega), and [α - ^{32}P]GTP at 37°C for 1 h. Reactions were chased for 30 min by supplementing GTP concentration to 100 μ M. Cap-labeled target mRNAs were resolved on 10% polyacrylamide-7 M urea gel and purified.

In vitro target mRNA cleavage assay

siRNA-mediated cleavage of target mRNA in human cytoplasmic extract was performed as described (Martinez et al. 2002) with some modifications. siRNA duplexes were preincubated in HeLa cytoplasmic extract at 37°C for 15 min prior to addition of the 124-nt cap-labeled target mRNA generated as described above. After addition of all components, the final concentrations were 500 nM siRNA, 50 nM target mRNA, 1 mM ATP, 0.2 mM GTP, 1 U/ μ L RNasin, 30 μ g/mL creatine kinase, 25 mM creatine phosphate, and 50% S100 extract. Incubation was continued for 1.5 h. Cleavage reactions were stopped by the addition of 8 volumes of proteinase K buffer (200 mM Tris-HCl at pH 7.5, 25 mM EDTA, 300 mM NaCl, and 2% [w/v] SDS). Proteinase K, dissolved in 50 mM Tris-HCl (pH 8.0), 5 mM CaCl_2 , and 50% glycerol, was added to a final concentration of 0.6 mg/mL. Reaction products were extracted with phenol/chloroform/isoamyl alcohol (25:24:1), chloroform, and precipitated with 3 volumes of ethanol. Samples were separated on 8% polyacrylamide-7 M urea gels.

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EXHIBIT 5 of
EXHIBIT B

Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs

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RNA interference (RNAi) holds considerable promise as a therapeutic approach to silence disease-causing genes, particularly those that encode so-called 'non-druggable' targets that are not amenable to conventional therapeutics such as small molecules, proteins, or monoclonal antibodies. The main obstacle to achieving *in vivo* gene silencing by RNAi technologies is delivery. Here we show that chemically modified short interfering RNAs (siRNAs) can silence an endogenous gene encoding apolipoprotein B (apoB) after intravenous injection in mice. Administration of chemically modified siRNAs resulted in silencing of the apoB messenger RNA in liver and jejunum, decreased plasma levels of apoB protein, and reduced total cholesterol. We also show that these siRNAs can silence human apoB in a transgenic mouse model. In our *in vivo* study, the mechanism of action for the siRNAs was proven to occur through RNAi-mediated mRNA degradation, and we determined that cleavage of the apoB mRNA occurred specifically at the predicted site. These findings demonstrate the therapeutic potential of siRNAs for the treatment of disease.

RNAi has been applied widely as a target validation tool in post-genomic research, and it represents a potential strategy for *in vivo* target validation and therapeutic product development¹. *In vivo* gene silencing with RNAi has been reported using both viral vector delivery² and high-pressure, high-volume intravenous (i.v.) injection of synthetic siRNAs³, but these approaches have limited if any clinical use. *In vivo* gene silencing has also been reported after local, direct administration (intravitreal, intranasal and intrathecal) of siRNAs to sequestered anatomical sites in models of choroidal neovascularization⁴, lung ischaemia-reperfusion injury⁵ and neuropathic pain⁶, respectively. These reported approaches demonstrate the potential for delivery to organs such as the eye, lungs and central nervous system. However, there are no published reports of systemic activity for siRNAs towards endogenous targets after conventional and clinically acceptable routes of administration. A critical requirement for achieving systemic RNAi *in vivo* is the introduction of 'drug-like' properties, such as stability, cellular delivery and tissue bioavailability, into synthetic siRNAs.

Conferring drug-like properties on siRNAs

In exploring the potential of synthetic siRNAs to silence endogenous target genes, we found that chemically stabilized and cholesterol-conjugated siRNAs⁷ have markedly improved pharmacological properties *in vitro* and *in vivo*. Chemically stabilized siRNAs with partial phosphorothioate backbone and 2'-O-methyl sugar modifications on the sense and antisense strands showed significantly enhanced resistance towards degradation by exo- and endonucleases in serum and in tissue homogenates. The conjugation of cholesterol to the 3' end of the sense strand of a siRNA molecule by means of a pyrrolidine linker (thereby generating chol-siRNA) did not result in a significant loss of gene-silencing activity in cell culture. Furthermore, unlike unconjugated siRNAs, a chol-siRNA directed to luciferase (chol-luc-siRNA) showed reduction in luciferase activity in HeLa cells transiently expressing luciferase, with a half-maximal inhibitory concentration (IC₅₀) of about 200 nM in

the absence of transfection reagents or electroporation.

Binding of chol-siRNAs to human serum albumin (HSA) was determined by surface plasmon resonance measurement (data not shown). Unconjugated siRNAs demonstrated no measurable binding to HSA, whereas chol-siRNAs bound to HSA with an estimated dissociation constant (K_d) of 1 μ M. Presumably because of enhanced binding to serum proteins, chol-siRNAs administered to rats by i.v. injection showed improved *in vivo* pharmacokinetic properties as compared to unconjugated siRNAs. After i.v. injection in rats at 50 mg kg⁻¹, radioactively labelled chol-siRNAs had an elimination half life (two compartments), $t_{1/2}$ of 95 min and a corresponding plasma clearance (CL) of 0.5 ml min⁻¹, whereas unconjugated siRNAs had a $t_{1/2}$ of 6 min and CL of 17.6 ml min⁻¹. As measured by an RNase protection assay (RPA), chol-siRNAs showed broad tissue biodistribution 24 h after injection in mice. Although no detectable amounts of unconjugated siRNAs were observed in tissue samples, significant levels of chol-siRNAs were detected in liver, heart, kidney, adipose, and lung tissue samples. Together, these studies demonstrate that cholesterol conjugation significantly improves *in vivo* pharmacological properties of siRNAs.

Selection of apoB as an endogenous gene target

Apolipoprotein B is the essential protein for formation of low-density lipoproteins (LDL) in metabolism of dietary and endogenous cholesterol, and is the ligand for the LDL receptor⁸. Mouse apoB is a large protein of 4,515 amino acids and is expressed predominantly in liver and jejunum. apoB mRNA is subject to post-transcriptional editing, and the unedited and edited transcripts encode the full-length protein apoB-100, and a carboxy-terminal truncated isoform, apoB-48, respectively. In mice, editing of apoB mRNA occurs in both the liver and jejunum: apoB-48 is the predominant protein form in the jejunum and both apoB-48 and apoB-100 are expressed in the liver. Heterozygous knockout mice for apoB show a 20% decrease in cholesterol levels and are resistant to

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diet-induced hypercholesterolaemia⁹.

Serum levels of apoB, LDL and cholesterol correlate significantly with increased risk of coronary artery disease (CAD). A diminished number of functional LDL receptors on the cell surface, disrupting receptor-mediated removal of apoB-containing LDL from circulation, has been identified as the basis for familial hypercholesterolaemia (FH)¹⁰. Patients with homozygous and heterozygous FH have accelerated CAD leading to premature atherosclerosis and cardiac mortality. Conversely, patients with hypobetalipoproteinaemia have reduced levels of LDL and cholesterol and are at reduced risk for CAD¹¹. Accordingly, lowering of serum cholesterol and LDL levels is a predominant clinical strategy for management of CAD and is achieved by modification of dietary sources of cholesterol and/or inhibition of endogenous cholesterol synthesis with pharmacological therapies. Notwithstanding significant improvements in the management of CAD with these approaches, millions of patients remain at significant risk for CAD and its clinical sequelae—acute coronary syndromes such as myocardial infarction and cardiac mortality—due to advanced atherosclerosis from intractably high levels of cholesterol and LDL. Clearly, new therapeutic strategies are needed. Accordingly, apoB, a protein not amenable to inhibition by conventional small-molecule- or protein-based therapeutics, was selected as a potential clinical target for development of siRNA therapeutics.

Using conventional bioinformatics, 84 siRNAs specific for both human and mouse apoB mRNA were designed and synthesized (data not shown). These apoB-siRNAs were screened for their ability to reduce apoB mRNA and protein levels, as measured by polymerase chain reaction with reverse transcription (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively, in HepG2 liver cells after transfection at a concentration of 100 nM. Five apoB-siRNAs were identified that reduced both mRNA and protein levels by >70%. Because exonucleolytic degradation is the predominant mechanism for siRNA degradation in serum, two selected apoB-siRNAs (apoB-1-siRNA and apoB-2-siRNA) and one four-nucleotide mismatch control for apoB-1-siRNA (mismatch-siRNA) were stabilized at the 3' end of the sense and antisense strands by phosphorothioate backbone modifications and additional incorporation of two 2'-O-methyl nucleotides at the 3' end of the antisense strand. Chol-siRNAs were synthesized by linkage of cholesterol to the 3' end of the sense strand via a pyrrolidine linker. Chol-apoB-1-siRNA was significantly more stable than unconjugated apoB-1-siRNA in human serum: gel electrophoresis showed >50% intact chol-apoB-1-siRNA after a 1 h incubation at 37 °C compared with <5% intact unconjugated apoB-1-siRNA. Similar data were obtained for chol-apoB-2-siRNA, although this siRNA was less stable than chol-apoB-1-siRNA. Dose

response curves for the activity of conjugated and unconjugated apoB-specific and control siRNAs were measured in HepG2 cells using transfection. Two conjugated control siRNAs (chol-luc-siRNA and chol-mismatch-siRNA) showed no significant inhibition of apoB protein expression at concentrations as high as 30 nM. In contrast, three specific siRNAs (unconjugated apoB-1-siRNA, chol-apoB-1-siRNA and chol-apoB-2-siRNA) showed dose-dependent silencing of apoB protein expression based on apoB ELISA measurements—IC₅₀ values of 0.5 nM, 5 nM and 8 nM were calculated, respectively.

In vivo studies with modified siRNAs

To demonstrate the ability of chol-apoB-siRNAs to silence apoB expression *in vivo*, experiments were first performed in C57BL/6

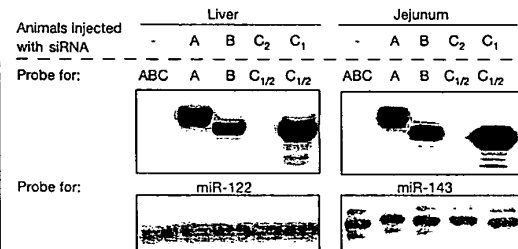


Figure 1 Biodistribution of siRNAs in liver and jejunum. An RPA was used to detect siRNAs in pooled liver and jejunum tissue lysates from animals injected with saline (–), chol-luc-siRNA (A), chol-mismatch-siRNA (B), unconjugated apoB-1-siRNA (C₁) or chol-apoB-1-siRNA (C₂). Detection by RPA of endogenous miRNAs in liver (miR-122) and jejunum (miR-143) served as an internal loading control.

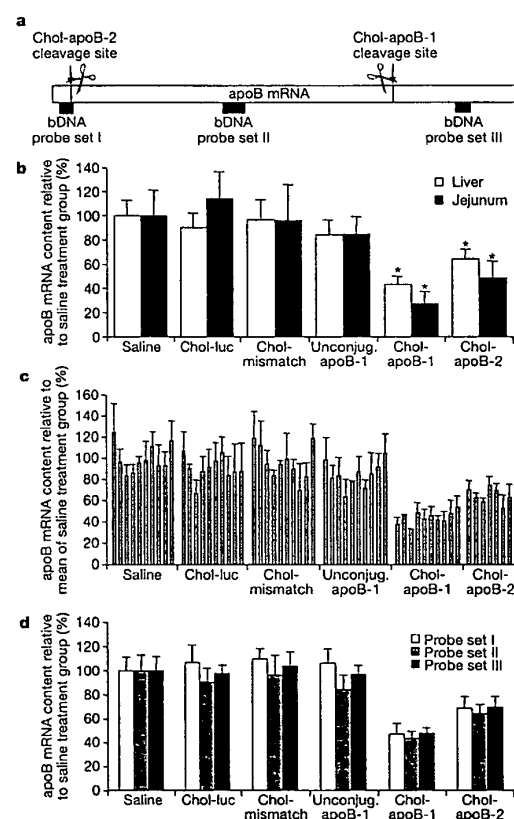


Figure 2 *In vivo* silencing of murine apoB mRNA by siRNAs in wild-type mice. Treatment groups comprised saline control ($n = 10$), chol-luc-siRNA control ($n = 10$), chol-mismatch-siRNA control ($n = 10$), unconjugated apoB-1-siRNA ($n = 10$), chol-apoB-1-siRNA ($n = 10$) and chol-apoB-2-siRNA ($n = 7$). bDNA measurements were performed with probe set II. Error bars represent the standard deviation (s.d.) of the mean. Statistical analysis was by analysis of variance (ANOVA) with Bonferroni post-hoc *t*-test, one-tailed. Asterisk, $P < 0.0001$ compared with saline control animals. **a**, Schematic representation of the apoB mRNA illustrating the binding regions of three bDNA probe sets in relation to the two siRNA cleavage sites. **b**, Effects of siRNA administration on mean apoB mRNA levels. **c**, apoB mRNA levels from individual mice treated with saline or siRNAs. Data are mean values from three liver samples from each individual animal. **d**, Effects of siRNA administration on the reduction of apoB mRNA measured by bDNA assays using three different probe sets.

mice fed a normal chow diet. siRNAs were administered by tail-vein injection with normal volume (0.2 ml) and normal pressure. Biodistribution of siRNAs was assessed by RPA of siRNAs in tissue samples from liver and jejunum obtained 24 h after the last injection. Significant levels of chol-luc-siRNA, chol-apoB-1-siRNA and chol-mismatch-siRNA were detected in liver and jejunum (100–200 ng g⁻¹ tissue for chol-apoB-1-siRNA), whereas levels of unconjugated apoB-1-siRNA were below our detection limit (Fig. 1). Levels of chol-apoB-2-siRNA were also detected but at levels approximately 10% of those observed for other chol-siRNAs.

The primary measure of RNAi-mediated effects is the reduction (that is, silencing) of the target mRNA. To measure silencing of apoB mRNA, we used a branched-DNA (bDNA) detection method and bDNA probes (Fig. 2a) to quantify apoB mRNA levels in liver and jejunum, two organs where apoB is known to be expressed. As shown in Fig. 2b, mice treated with chol-apoB-1-siRNA and chol-apoB-2-siRNA showed statistically significant reductions (mean $\pm$ s.d.; 57 $\pm$ 6% and 36 $\pm$ 8%, respectively) in apoB mRNA levels in liver samples as compared with saline control (P < 0.0001). In jejunum tissue samples, mice injected with chol-apoB-1-siRNA and chol-apoB-2-siRNA showed an even more substantial reduction in apoB mRNA levels of 73 $\pm$ 10% and 51 $\pm$ 13%, respectively, as compared with saline control (P < 0.0001). Individual animal results for apoB mRNA reduction in the liver are shown in Fig. 2c and demonstrate the consistent and robust effect observed for specific chol-siRNAs as compared with other treatment groups. Similar results were observed for apoB mRNA reduction in the jejunum from individual animals (data not shown). Owing to the extended length of the apoB mRNA, two additional probes at the distal ends of the apoB open reading frame (ORF) were designed. As measured with the three divergent probe sets, identical levels of apoB mRNA reduction were detected for animals treated with chol-apoB-1-siRNA and chol-apoB-2-siRNA (Fig. 2d). These data suggest a uniform and rapid degradation of apoB mRNA after treatment with chol-apoB-siRNAs, and argue against the potential existence of truncated amino-terminal apoB protein fragments translated from incompletely degraded siRNA-cleavage products, as has been reported for ribozyme-mediated cleavage of apoB mRNA¹².

Silencing of the apoB mRNA would be expected to result in a corresponding reduction in apoB protein levels. An ELISA-based method specific for detection of apoB-100 protein was used to measure the effects of chol-apoB-siRNA treatment on plasma levels of apoB protein. In addition to the effects on apoB mRNA levels, treatment with chol-apoB-1-siRNA and chol-apoB-2-siRNA reduced plasma levels of apoB-100 protein 24 h after siRNA treatment by 68 $\pm$ 14% and 31 $\pm$ 18%, respectively, compared with

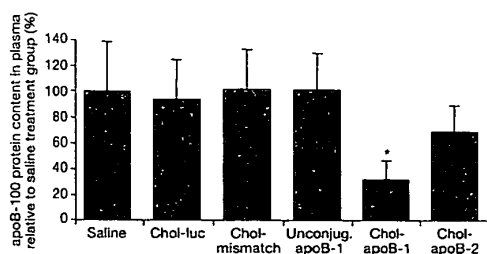


Figure 3 Effects of siRNA administration on apoB-100 protein levels. Average plasma levels of apoB-100 protein for the different treatment groups as measured by ELISA. Error bars represent the s.d. of the mean. Statistical analysis was by ANOVA with Bonferroni post-hoc *t*-test, one-tailed. Asterisk, P < 0.0001 compared with saline control animals.

levels in saline-treated control animals (Fig. 3). These results achieved statistical significance (P < 0.0001) for the group treated with the more potent and stable chol-apoB-1-siRNA. As the LF3 antibody used in this study recognizes only apoB-100, and not apoB-48, the observed apoB-100 reduction may underestimate the full effect of chol-apoB-1-siRNA at the protein level.

To confirm the physiological relevance of apoB mRNA silencing on lipoprotein metabolism, we characterized the effect of siRNA treatment and the resulting reduction of apoB protein levels on lipoprotein profiles and cholesterol levels. Using an NMR-based method, complete lipoprotein profiles were generated and concentrations of chylomicrons, very-low-density lipoprotein (VLDL), LDL and high-density lipoprotein (HDL) particles were calculated (Fig. 4a). As expected, HDL represented the predominant lipoprotein fraction in mouse plasma. Similar to results observed in heterozygous knockout mice for apoB⁹, treatment with chol-apoB-1-siRNA resulted in a 25% reduction in HDL particle

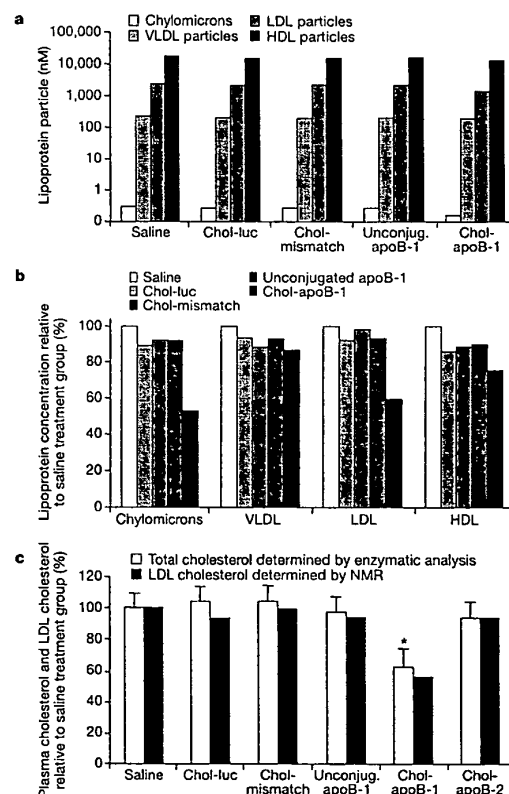


Figure 4 Therapeutic reduction of lipoprotein and cholesterol levels after siRNA treatment. **a**, Lipoprotein profile of pooled plasma samples from treatment groups determined by NMR analysis. **b**, Relative reduction of lipoprotein classes for the siRNA treatment groups normalized against the average levels of saline control group. **c**, Effects of siRNA administration on plasma cholesterol and LDL cholesterol. Plasma cholesterol was determined by enzymatic assay and LDL cholesterol calculated from NMR measurements. Error bars represent the s.d. of the mean. Statistical analysis was by ANOVA with Bonferroni post-hoc *t*-test, one-tailed. Asterisk, P < 0.0001 compared with saline and chol-mismatch-siRNA control animals. NMR data are based on single measurements of pooled plasma from treatment groups.

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concentration (Fig. 4b). Furthermore, treatment of mice with chol-apoB-1 siRNA resulted in an almost 50% reduction of chylomicron levels and an approximately 40% reduction in LDL levels, whereas VLDL levels were not altered. Treatment with either of the control siRNAs did not change the lipoprotein profile significantly. In addition to reductions in lipoprotein concentrations, *in vivo* silencing of apoB by chol-apoB-1-siRNA led to a significant reduction ($37 \pm 11\%$; $P < 0.0001$) of total plasma cholesterol as compared with saline control animals (Fig. 4c). Treatment with the less potent chol-apoB-2-siRNA failed to show significant reductions in cholesterol, consistent with the reduced activity of this chol-siRNA on apoB mRNA and protein levels. Treatment with chol-apoB-1-siRNA also resulted in a 44% decrease in LDL-associated cholesterol, consistent with the effects observed on apoB protein levels. In aggregate, the effects on cholesterol reduction and lipoprotein profiles would be considered highly clinically significant in patients with hypercholesterolaemia, and actually exceed the level of cholesterol reduction observed in heterozygous apoB knockout mice⁹.

To extend our findings of *in vivo* silencing by chol-apoB-siRNAs in normal mice, we performed an additional study in a human apoB transgenic mouse model¹³. These mice express human apoB-100 in liver and have elevated levels of apoB as compared with normal mice; when fed a high-fat diet, these mice develop severe atherosclerosis¹⁴. In our experiments, we administered saline, chol-mismatch-siRNA and chol-apoB-1-siRNA to apoB transgenic mice fed a normal chow diet. As shown in Fig. 5, chol-apoB-1-siRNA brought about a significant reduction of endogenous murine apoB expressed in both liver and jejunum tissue samples ($P < 0.0001$, relative to saline and chol-mismatch-siRNA treatment). Relative to the saline control, levels of murine apoB mRNA were reduced by $57 \pm 10\%$ in liver and $42 \pm 12\%$ in jejunum. In addition, chol-apoB-1-siRNA, which was selected in part owing to its sequence identity to both human and mouse apoB, showed significant silencing of the human transgene expressed in the liver, where human apoB mRNA was silenced by $60 \pm 10\%$ ($P < 0.0001$). In contrast to these effects, chol-mismatch-siRNA showed no effect on mouse or human apoB mRNA levels. These results confirm the effect of specific chol-siRNAs on apoB silencing in a different mouse model. Moreover, this specific chol-siRNA was shown to silence a transgenic human mRNA *in vivo*.

An important consideration for siRNA-mediated inhibition of gene expression is whether the observed effects are specific and not due to nonspecific "off target" effects¹⁵ and potential interferon responses¹⁶, which have been reported with siRNAs *in vitro* and other oligonucleotide-based approaches *in vivo*. In our experiments, the effects of apoB-specific, cholesterol-conjugated siRNAs were seen with two divergent siRNAs targeting separate sequence regions of the apoB mRNA. Furthermore, the *in vivo* silencing of

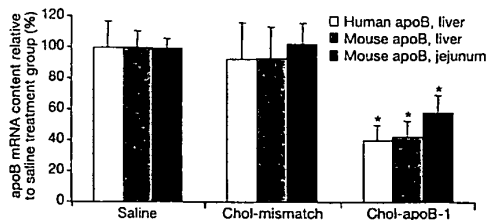


Figure 5 *In vivo* silencing of murine and human apoB mRNA in mice transgenic for human apoB. Reduction of human and mouse apoB mRNA levels in mice transgenic for human apoB that received saline ($n = 8$), chol-mismatch-siRNA ($n = 8$) and chol-apoB-1-siRNA ($n = 8$). Statistical analysis was by ANOVA with Bonferroni post-hoc *t*-test, one-tailed. Asterisk, $P < 0.0001$ compared with saline and chol-mismatch-siRNA control animals. Error bars illustrate s.d. of the mean.

apoB by these siRNAs was specific as neither an irrelevant siRNA (chol-luc-siRNA) nor a mismatch control siRNA (chol-mismatch-siRNA)—although present at comparable concentrations in liver and jejunum—mediated a significant reduction in apoB mRNA, plasma apoB protein levels, or total cholesterol. Finally, the silencing of apoB mRNA by chol-apoB-siRNAs in liver as measured by bDNA assay and normalization to GAPDH mRNA was also demonstrated with normalization to three other liver mRNAs, including factor VII, glucose-6-phosphatase and VEGF (Supplementary Fig. 1).

Determination of *in vivo* mechanism of action

To prove that the *in vivo* activity was due to siRNA-directed cleavage, we characterized specific mRNA cleavage products using a modified 5'-RACE (rapid amplification of cDNA ends) technique previously used to demonstrate microRNA (miRNA)-directed mRNA cleavage in plants¹⁷ and mouse embryos¹⁸. As it relates to the specific cleavage of apoB mRNA by apoB-1-siRNAs, total RNA from mice in the different treatment groups was isolated, and then PCR was used to reveal fragments of the predicted length in animals receiving chol-apoB-1-siRNA treatment (Fig. 6a). Identity of the PCR products was confirmed by direct sequencing of the excised bands, which demonstrated that cleavage occurred at the predicted position for the siRNA duplex. Indeed, sequencing revealed cleavage after position 10,061 of the apoB ORF, exactly ten nucleotides downstream of the 5' end of the siRNA antisense strand. Specific cleavage fragments were detected in both liver and jejunum of animals receiving chol-apoB-1-siRNA treatment (Fig. 6b). No fragments were detected in tissues of animals receiving control siRNAs (chol-luc-siRNA or chol-mismatch-siRNA) or saline. As expected, in this 5'-RACE experiment of apoB mRNA cleavage mediated by chol-apoB-1-siRNA, no fragments were detected in tissues from animals receiving the alternative apoB-specific siRNA (chol-apoB-2-siRNA). Notably, a low level of specific cleavage product was detected in the jejunum of animals receiving the unconjugated apoB-1-siRNA despite no evidence for significant knockdown of total apoB mRNA levels by this siRNA. This indicates

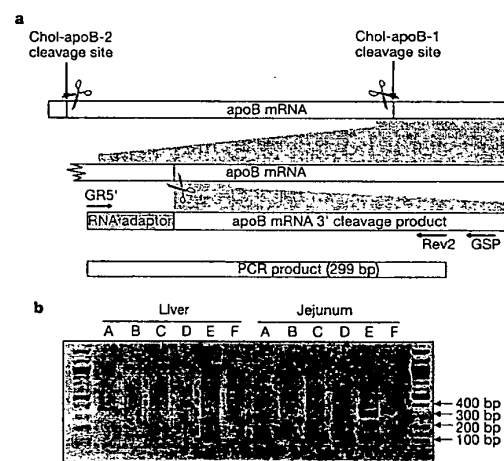


Figure 6 siRNA-mediated cleavage of apoB mRNA *in vivo*. a, Schematic representation of the apoB mRNA illustrating siRNA cleavage sites and RACE strategy to detect cleavage product. Cleaved mRNA ligated to an RNA adaptor was reverse transcribed using primer GSP. b, Agarose gel of 5'-RACE-PCR amplification, using the primer pair GRS' and Rev2, showing specific cleavage products in liver and jejunum. Treatment groups are: A, saline; B, chol-luc-siRNA; C, chol-mismatch-siRNA; D, apoB-1-siRNA; E, chol-apoB-1-siRNA; F, chol-apoB-2-siRNA.

that some unconjugated apoB-1-siRNA is able to enter epithelial cells of the jejunum after systemic administration despite lacking cholesterol conjugation. Together, these data demonstrate that inhibition of apoB was achieved by an RNAi mechanism of action. To our knowledge, this is the first demonstration of silencing of an endogenous gene in mammals by a mechanism of RNAi-mediated degradation of the target mRNA.

Discussion

Our findings demonstrate that RNAi can be used to silence endogenous genes involved in the cause or pathway of human disease with a clinically acceptable formulation and route of administration by means of systemic delivery. In our study, we have shown that the mechanism of action for chemically modified siRNAs was by RNAi-mediated degradation of the target mRNA. Chol-apoB-siRNAs, but not unconjugated apoB-siRNAs, showed biological activity, demonstrating an important role for cholesterol conjugation of siRNAs to achieve systemic *in vivo* activity, and suggesting the opportunity to further optimize systemic activity through chemical conjugation strategies. Indeed, further optimization is warranted to achieve improved *in vivo* potency for chol-siRNAs at doses and dose regimens that are clinically acceptable. Nevertheless, these findings hold promise for the development of a new class of therapeutics that harnesses the RNAi mechanism. Of particular interest is the use of RNAi therapeutics to silence genes (such as the apoB gene) or mutated or variant alleles whose proteins are refractory to the discovery of traditional small molecules or biotherapeutic drugs. □

Methods

Synthesis of siRNAs

The siRNAs used in this study consisted of a 21-nucleotide sense strand and a 23-nucleotide antisense strand resulting in a two-nucleotide overhang at the 3' end of the antisense strand. apoB-1-siRNA (ORF position 10049–10071): sense 5'-GUCAUACACUGAAUACCAA*U-3', antisense 5'-AUUGGUUUUACAGUGAUGAC*a*U-3'; chol-apoB-1-siRNA: sense 5'-GUCAUACACUGAAUACCAAU*chol-3', antisense 5'-AUUGGUUUUACAGUGAUGAC*a*U-3'; chol-mismatch-siRNA: sense 5'-GUCAUACACUGAAUACCAAU*chol-3', antisense 5'-AUUGGUUUUACAGUGAUGAC*a*U-3'; chol-apoB-2-siRNA (ORF position 327–349): sense 5'-AGGUGUAUGGCUUACAACCCUG*chol-3', antisense 5'-CAGGGUUAAGCCAUACACU*c*U-3'; chol-LUC-siRNA: sense 5'-GAACUGUGUGAGAGGUCCU*chol-3', antisense 5'-AGGACUCUCACACACAGUUc*g*U-3'. The lower-case letters represent 2'-O-methyl-modified nucleotides; asterisks represent phosphorothioate linkages.

RNA oligonucleotides were synthesized using commercially available 5'-O-(4,4'-dimethoxytrityl)-3'-O-(2-cyanoethyl)-N,N-diisopropyl phosphoramidite monomers of uridine (U), 4-N-benzoylcytidine (C^{Bz}), 6-N-benzoyladenine (A^{Bz}) and 2-N-isobutyrylguanosine (G^{ibu}) with 2'-O-t-butylmethylsilyl protected phosphoramidites and the corresponding 2'-O-methyl phosphoramidites according to standard solid phase oligonucleotide synthesis protocols¹⁸. After cleavage and de-protection, RNA oligonucleotides were purified by anion-exchange high-performance liquid chromatography and characterized by ES mass spectrometry and capillary gel electrophoresis. RNA with phosphorothioate backbone at a given position was achieved by oxidation of phosphite with Besuag reagent²⁰ during oligonucleotide synthesis. Chol-siRNAs were synthesized using the same protocols as above except that the RNA synthesis started from a controlled-pore glass solid support carrying a cholesterol-aminocaproic acid-pyrrolidine linker (V.Kc, K.G.R. and M.M., unpublished data). For this support, the first nucleotide linkage was achieved using a phosphorothioate linkage to provide additional 3'-exonuclease stability. To generate siRNAs from RNA single strands, equimolar amounts of complementary sense and antisense strands were mixed and annealed, and siRNAs were further characterized by native gel electrophoresis.

In vitro activity and stability assays

To determine *in vitro* activity of siRNAs, HepG2 cells were transfected with siRNAs using oligofectamine (Invitrogen) and siRNA concentrations ranging from 0.1, 0.3, 1, 3, 10 to 30 nM. apoB protein concentration was determined from cell culture supernatant by a sandwich ELISA capturing apoB with a polyclonal goat anti-human apoB antibody (Chemicon International). apoB detection was performed with a horseradish peroxidase-conjugated goat anti-human apoB-100 polyclonal antibody (Academy Bio-Medical Company). The remaining apoB protein content was calculated as the ratio of apoB protein concentration in the supernatant of cells treated with the apoB-specific siRNA duplex to the apoB concentration in the supernatant of cells treated with an unspecific control siRNA duplex. Mouse serum (Sigma-Aldrich Chemie GmbH) was used for stability assays. Double-stranded siRNAs (5 µM) were incubated in 95% serum, and the mixture was incubated at 37 °C for various lengths of time (for example, 0, 15 or 30 min, or 1, 2, 4, 8, 16 or 24 h). siRNAs were isolated by hot phenol extraction in the presence of

sodium dodecyl sulphate followed by ethanol precipitation. Re-suspended RNA samples were run on a denaturing 14% polyacrylamide gel containing 20% formamide for 2 h at 45 mA. RNA bands were visualized by staining with the 'Stains-All' reagent (Sigma-Aldrich Chemie GmbH) according to the manufacturer's instructions.

In vivo silencing experiments

C57BL/6 mice received, on three consecutive days, tail vein injections of saline or different siRNAs. All siRNAs were administered at doses of 50 mg kg⁻¹ in approximately 0.2 ml per injection. Measurements of apoB mRNA, apoB protein levels, lipoprotein concentrations and plasma cholesterol content were performed 24 h after the last *i.v.* injection. Experiments were carried out in a blinded fashion. The same experimental design was used for experiments with the human apoB transgenic mice (1004-T hemizygotes, Taconic).

In vivo bioanalytical methods

An RPA, using radiolabelled probes complementary to the antisense strands, was used to detect siRNAs in pooled liver and jejunum tissue lysates from animals treated with saline or siRNAs. RPA for endogenous miRNAs was used as a loading control for jejunum (miR-143, sequence 5'-UGAGAUGAAGCACUGUAGCUCA-3') and liver (miR-122, 5'-UGGAGUGUGACAUGGUGUUUG-3').

The QuantiGene assay (Genospectra) was used to quantify the reduction of mouse apoB mRNA in liver and jejunum tissue after siRNA treatment. Small uniform tissue samples were collected 24 h after the last injection. Lysates from three tissue samples per animal were directly used for apoB and GAPDH mRNA quantification, and the ratio of apoB and GAPDH mRNA was calculated and expressed as a group average relative to the saline control group. Specific probes for detection of apoB mRNA levels were designed to the following regions of the apoB mRNA ORF: probe set I 83–385; probe set II 5,045–5,673; probe set III 12,004–12,411. Furthermore, apoB mRNA reduction in liver was quantified from purified (RNeasy mRNA isolation kit, Qiagen), pooled mRNA for each treatment group. As well as GAPDH, factor VII, glucose-6-phosphatase and VEGF mRNAs were also used for normalization.

ELISA was used to quantify the reduction of apoB-100 protein levels in mouse plasma after siRNA treatment. apoB-100 from plasma samples of individual animals was detected using the primary antibody LF3 against mouse apoB-100 (gift of S. Young; see ref. ²¹). Levels were normalized to plasma volume and expressed as group averages relative to the saline control group.

Total cholesterol levels in the plasma were measured using the Cholesterol detection kit (Diasys). For NMR determination of the plasma lipoprotein profile a Bruker DRX 600 with cryoprobe head was used (LipoFIT Analytic GmbH). Single measurements of 500 µl mouse plasma (pooled from ten animals per treatment group) were performed. The lipoprotein subclass distribution was calculated from the NMR data by using computer algorithms that are based on human blood standards²². The particle number for lipoprotein classes was calculated based on the correlation of known particle size and composition with the experimentally determined NMR signal intensity. On the basis of this correlation, the cholesterol content in the LDL fraction was computed. The cholesterol values calculated from NMR data were confirmed by the presence of comparable levels of total cholesterol in plasma and HDL-cholesterol as determined by enzymatic assays.

5'-RACE analysis

Total RNA (5 µg) from pooled liver and jejunum samples from animals treated with different siRNAs was ligated to a GeneRacer adaptor (Invitrogen) without prior treatment. Ligated RNA was reverse transcribed using a gene-specific primer (GSP: 5'-CTCTCTGTTGCAGTAGAGTGACGCT-3'). To detect cleavage products, PCR was performed using primers complementary to the RNA adaptor (GRS: 5'-CTCTACAGGAGCTGGAGCAGAGGACACTA-3') and apoB mRNA (Rev2: 5'-ACGGCTGCAGCTGGGAGCAGTGGAGCTTGGCAGTTGTTTC-3'). Amplification products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The identity of specific PCR products was confirmed by sequencing of the excised bands.

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Supplementary Information accompanies the paper on www.nature.com/nature.

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Competing interests statement The authors declare competing financial interests: details accompany the paper on Nature's website (<http://www.nature.com/nature>).

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EXHIBIT C

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Klaus GIESE, et al.	)	Confirmation No: 6369
	)	
Application Serial No.: 10/633,630	)	Group Art Unit: 1635
	)	
Filed: August 5, 2003	)	Examiner: Kimberly Chong
	)	
For: INTERFERING RNA MOLECULES	)	

United States Patent and Trademark Office
Randolph Building
401 Dulany Street
Alexandria, Virginia 22314

Declaration under 37 C.F.R. § 1.132

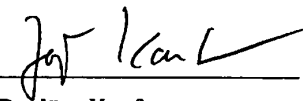
I, Dr. Jörg Kaufmann, declare and say:

1. I am an inventor of the captioned application..
2. I make this declaration to explain how to calculate the number of possible ways of arranging 2'- modified and unmodified nucleotides on antisense and sense strands of a double stranded RNA molecule where each strand is 23 nucleotides long.
3. In a double stranded RNA molecule of this type, any nucleotide on either strand can be either modified or unmodified. In other words, there are two possibilities for each position. Thus, on a single strand that is n nucleotides long, there are 2^n possible ways of arranging modified and unmodified nucleotides on the strand. For example, each strand might be completely unmodified, might have from 1 to 22 modifications arranged in any possible combination of positions in the strand, or might be modified on every nucleotide.
4. On a strand containing 23 nucleotides, there are therefore 2^{23} possible ways of arranging modified and unmodified nucleotides along the strand.

5. In a double stranded molecule where each strand is 23 nucleotides long, there are 2^{23} possible ways of arranging modified and unmodified nucleotides on each strand, and the arrangements on each strand are independent. Accordingly, in such a double stranded molecule there are $2^{23} \times 2^{23} = 2^{46} = 7.0 \times 10^{13}$ possible arrangements.

6. All statements made herein of my knowledge are true and all statements made on information and belief are believed to be true; and further, these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any document or any registration resulting therefrom.

Date: Feb. 08 / 2008



Dr. Jörg Kaufmann

EXHIBIT D

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Klaus GIESE, et al.	)	Confirmation No: 6369
	)	
Application Serial No.: 10/633,630	)	Group Art Unit: 1635
	)	
Filed: August 5, 2003	)	Examiner: Kimberly Chong
	)	
For: INTERFERING RNA MOLECULES	)	

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Declaration under 37 C.F.R. § 1.132

I, Dr. Klaus Giese, declare and say:

1. I received a B.S. degree in Biochemistry in 1986 and a Ph.D. degree in 1990, both from the Free University of Berlin, Germany. From 1990 to 1991 I was a postdoctoral researcher and scientist at the Max-Planck-Institute in Berlin in the laboratory of Prof. Wittmann. From 1991 to 1994 I was a postdoctoral researcher and scientist at the University of California, San Francisco in the laboratory of Professor Grosschedl. From 1994 to 1998 I had positions of increasing responsibility (Scientist II, Principle Scientist, Senior Scientist) at Chiron Corporation. Since 1999 I have been Chief Scientific Office of Atugen AG, now known as Silence Therapeutics AG. A copy of my *Curriculum Vitae* is attached as EXHIBIT 1.

2. I am an inventor of the captioned application.

3. The claimed invention is directed to double stranded nucleic acid molecules where each strand contains a stretch of ribonucleotides that is 15-23 ribonucleotides long. Each stretch is made up of alternating unmodified ribonucleotides and 2'-O-methyl ribonucleotides, linked by natural phosphodiester bonds. The strands are "staggered" such that a 2'-O-methyl ribonucleotide on one strand base pairs with an unmodified ribonucleotide on the other strand.

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The double stranded RNA molecules are highly resistant to degradation in serum while maintaining potent activity in gene silencing via an RNA interference (RNAi) mechanism. The molecules may be blunt-ended or have one or two overhangs.

5. The molecules having the structure recited in the claims of the captioned application provide surprisingly good results both with respect to stability against nuclease degradation and to RNAi activity (gene silencing).

6. For example, Figure 15 of the captioned application describes molecules having the structure recited in the claims, and shows that they not only are very stable in serum but are also very active at inducing RNAi. Thus, Figure 15 shows a double stranded molecule made up of two complementary oligoribonucleotide molecules, shown as PTENA V15 and PTENB V15. The gel data in Figure 15B show that these molecules are essentially unchanged after a 2 hour incubation in serum (see lane 15) whereas the equivalent unmodified molecule was completely digested after only 15 minutes in serum (lane AB). Figure 15D is an immunoblot showing the effect on PTEN expression of the modified and unmodified molecules. At 48h, both the modified and unmodified molecules achieve complete knock-down of PTEN expression (lanes 15 and AB, respectively). However, after 96h, PTEN expression was still low with the modified molecule (lane V15) but had increased to pre-treatment levels with the unmodified molecule (lane AB). These data demonstrate that a molecule as claimed in the captioned application is both surprisingly effective at initiating RNA interference but is also surprisingly stable.

7. This ability of the claimed molecules to be both active and stable is yet more surprising in light of the experiences of others such as those described in US Application 2005/0209179 ("McSwiggen II", attached hereto as EXHIBIT 2), which is a continuation-in-part application that claims priority from 2003/0190635, cited by the Examiner. McSwiggen II cites various methods for attempting to identify modified RNA molecules that are active yet stable, including:

(a) generating a plurality of unmodified siNA molecules, (b) screening 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, and (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). In one embodiment,

the method further comprises re-screening the chemically modified siNA molecules of step (c) under conditions suitable for isolating chemically modified siNA molecules that are active in mediating RNA interference against the target nucleic acid sequence.

See paragraph 193. Another method described by McSwiggen II involves the iterative method described in Figure 11:

Chemical modifications are introduced into the siNA construct based on educated design parameters (e.g. introducing 2'-modifications [sic], base modifications, backbone modifications, terminal cap modifications etc). The modified construct in [sic] 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

See paragraph 269. Both of these methods are iterative and require time-consuming trial and error preparation and testing of many modified molecules to obtain RNAi reagents that are both active and stable. McSwiggen II was filed in June 2004, which is later than the filing date of the captioned application, and shows that, even at this later date, McSwiggen required laborious trial and error methods to prepare and identify active, stable, RNAi agents.

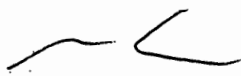
9. In sharp contrast, we have found that molecules having the structure recited in the instant claims are reliably active and stable to such an extent that we no longer prepare unmodified molecules to test for RNAi activity prior to preparing modified molecules. Rather, we directly design, synthesize, and test molecules having the "staggered" structures recited in the claims. Indeed, we have shown that the results seen with PTENA V15 and PTENB V15 are generally applicable to siRNA by successfully silencing over 100 different target mRNAs using dsRNA with the structure recited in the claims

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Serial No. 10/633,630

10. All statements made herein of my knowledge are true and all statements made on information and belief are believed to be true; and further, these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any document or any registration resulting therefrom.

Date: Feb 10, 2008



Dr. Klaus Giese

h

EXHIBIT 1 of
EXHIBIT D

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Issued/granted patents

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EXHIBIT 2 of
EXHIBIT D



US 20050209179A1

(19) **United States**(12) **Patent Application Publication**
McSwiggen et al.(10) **Pub. No.: US 2005/0209179 A1**(43) **Pub. Date: Sep. 22, 2005**(54) **RNA INTERFERENCE MEDIATED
TREATMENT OF ALZHEIMER'S DISEASE
USING SHORT INTERFERING NUCLEIC
ACID (SINA)**(75) **Inventors: James McSwiggen, Boulder, CO (US);
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CHICAGO, IL 60606 (US)**(73) **Assignee: Sirna Therapeutics, Inc., Boulder, CO**(21) **Appl. No.: 10/877,889**(22) **Filed: Jun. 25, 2004****Related U.S. Application Data**

(63) Continuation-in-part of application No. 10/607,933, filed on Jun. 27, 2003, which is a continuation-in-part of application No. 09/930,423, filed on Aug. 15, 2001, now abandoned, and which is a continuation-in-part of application No. PCT/US03/04710, filed on Feb. 18, 2003, which is a continuation-in-part of application No. 10/205,309, filed on Jul. 25, 2002. Continuation-in-part of application No. PCT/US04/16390, filed on May 24, 2004, which is a continuation-in-part of application No. 10/826,966, filed on Apr. 16, 2004, which is a continuation-in-part of application No. 10/757,803, filed on Jan. 14, 2004, which is a continuation-in-part of application No. 10/720,448, filed on Nov. 24, 2003, which is a continuation-in-part of application No. 10/693,059, filed on Oct. 23, 2003, which is a continuation-in-part of application No. 10/444,853, filed on May 23, 2003, which is a continuation-in-part of application No. PCT/US03/05346, filed on Feb. 20, 2003, and which is a continuation-in-part of application No. PCT/US03/05028, filed on Feb. 20, 2003.

Continuation-in-part of application No. PCT/US04/13456, filed on Apr. 30, 2004, which is a continuation-in-part of application No. 10/780,447, filed on Feb. 13, 2004, and which is a continuation-in-part of application No. 10/427,160, filed on Apr. 30, 2003, which is a continuation-in-part of application No. PCT/US02/15876, filed on May 17, 2002. Continuation-in-part of application No. 10/727,780, filed on Dec. 3, 2003.

(60) Provisional application No. 60/358,580, filed on Feb. 20, 2002. Provisional application No. 60/363,124, filed on Mar. 11, 2002. Provisional application No. 60/386,782, filed on Jun. 6, 2002. Provisional application No. 60/406,784, filed on Aug. 29, 2002. Provisional application No. 60/408,378, filed on Sep. 5, 2002. Provisional application No. 60/409,293, filed on Sep. 9, 2002. Provisional application No. 60/440,129, filed on Jan. 15, 2003. Provisional application No. 60/362,016, filed on Mar. 6, 2002. Provisional application No. 60/292,217, filed on May 18, 2001. Provisional application No. 60/306,883, filed on Jul. 20, 2001. Provisional application No. 60/311,865, filed on Aug. 13, 2001. Provisional application No. 60/543,480, filed on Feb. 10, 2004.

Publication Classification

(51) **Int. Cl.⁷ A61K 48/00; C07H 21/02**
(52) **U.S. Cl. 514/44; 536/23.1**

(57)

ABSTRACT

This invention relates to compounds, compositions, and methods useful for modulating beta-secretase (BACE), amyloid precursor protein (APP), PIN-1, presenilin 1 (PS-1) and/or presenilin 2 (PS-2) gene expression using short interfering nucleic acid (siNA) molecules. This invention also relates to compounds, compositions, and methods useful for modulating the expression and activity of other genes involved in pathways of BACE, APP, PIN-1, PS-1 and/or PS-2 gene expression and/or activity 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 (mRNA), and short hairpin RNA (shRNA) molecules and methods used to modulate the expression of BACE, APP, PIN-1, PS-1 and/or PS-2 genes.

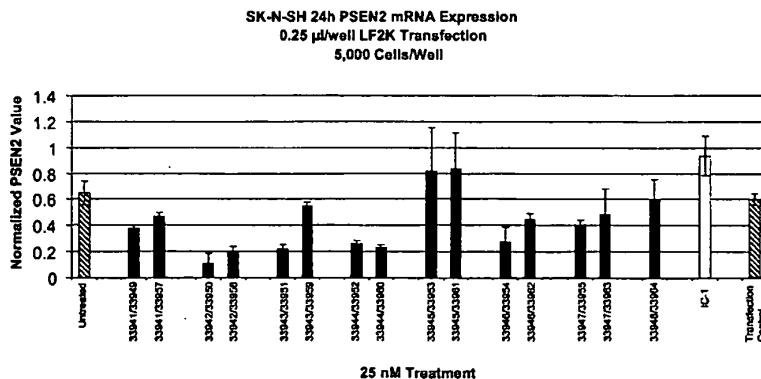
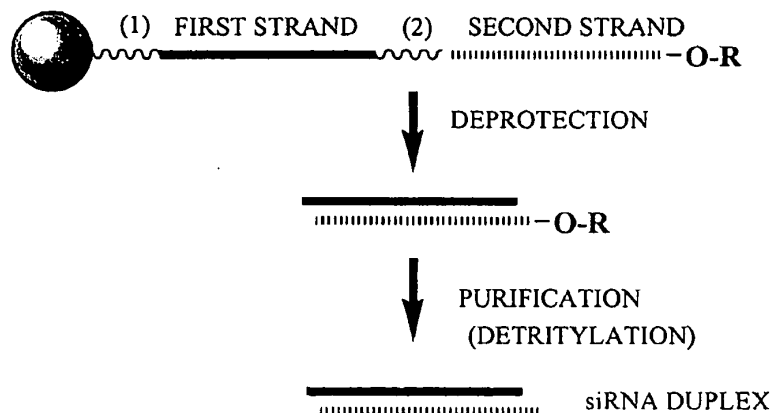


Figure 1

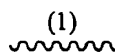


= SOLID SUPPORT

R = TERMINAL PROTECTING GROUP

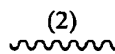
FOR EXAMPLE:

DIMETHOXYTRITYL (DMT)



(1) = CLEAVABLE LINKER

(FOR EXAMPLE: NUCLEOTIDE SUCCINATE OR
INVERTED DEOXYABASIC SUCCINATE)



(2) = CLEAVABLE LINKER

(FOR EXAMPLE: NUCLEOTIDE SUCCINATE OR
INVERTED DEOXYABASIC SUCCINATE)

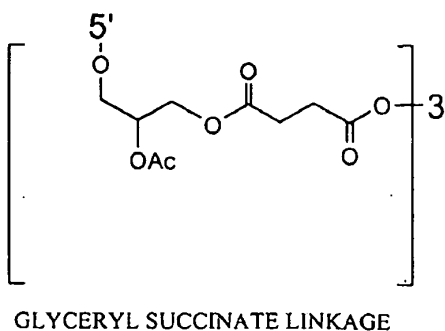
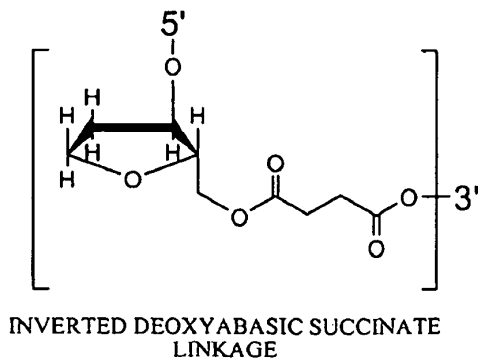


Figure 2

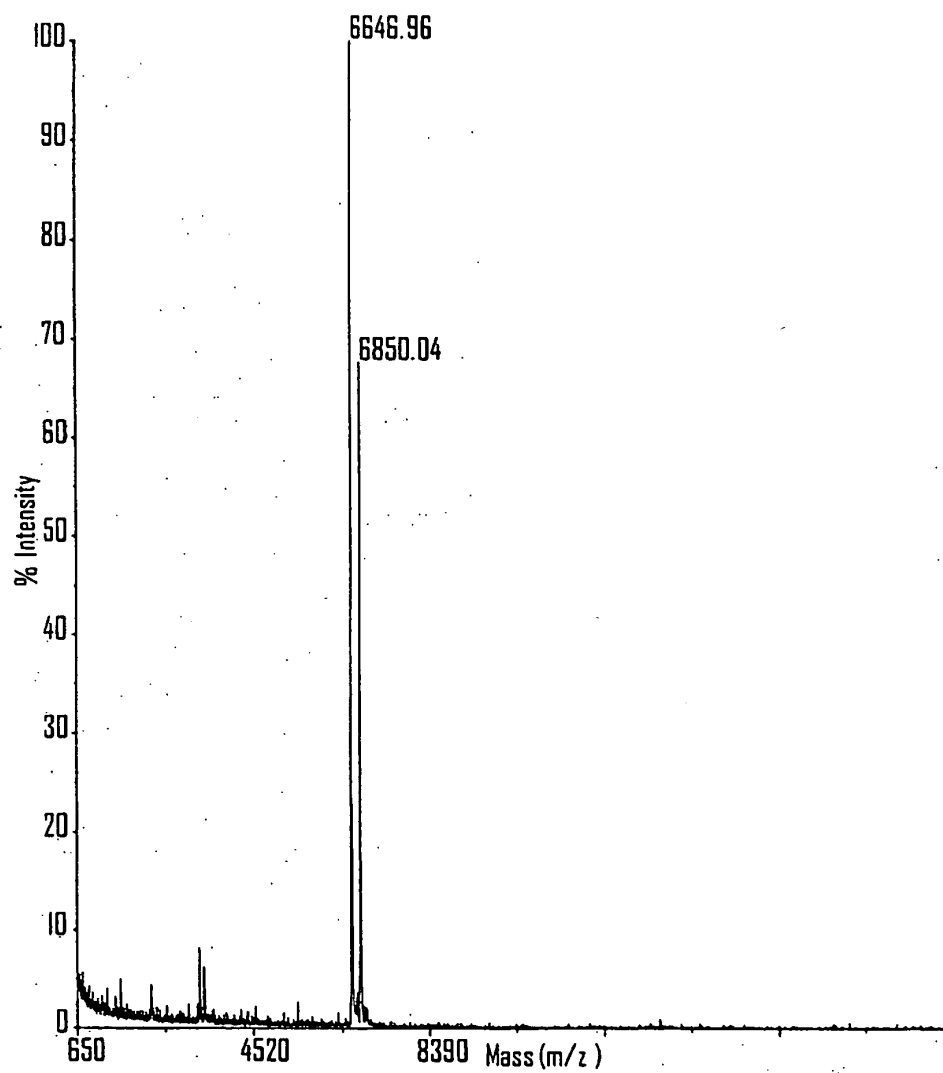


Figure 3

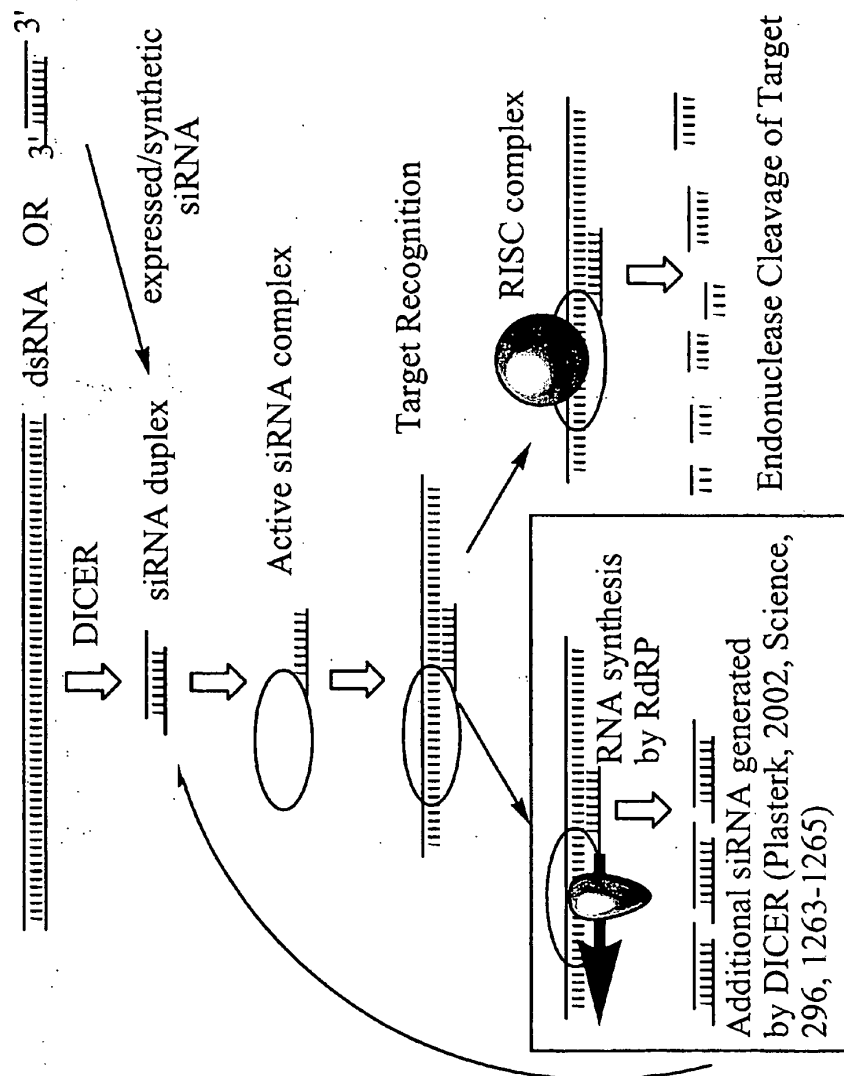
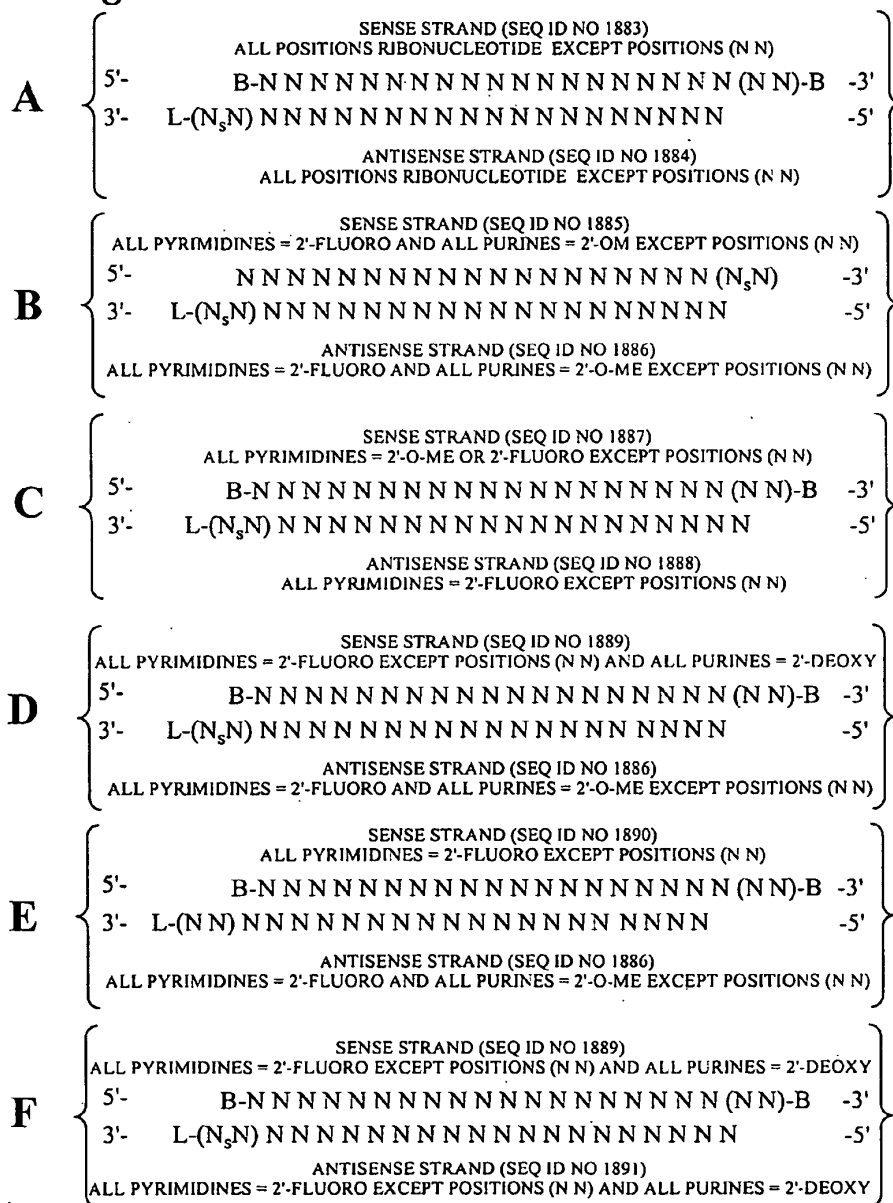
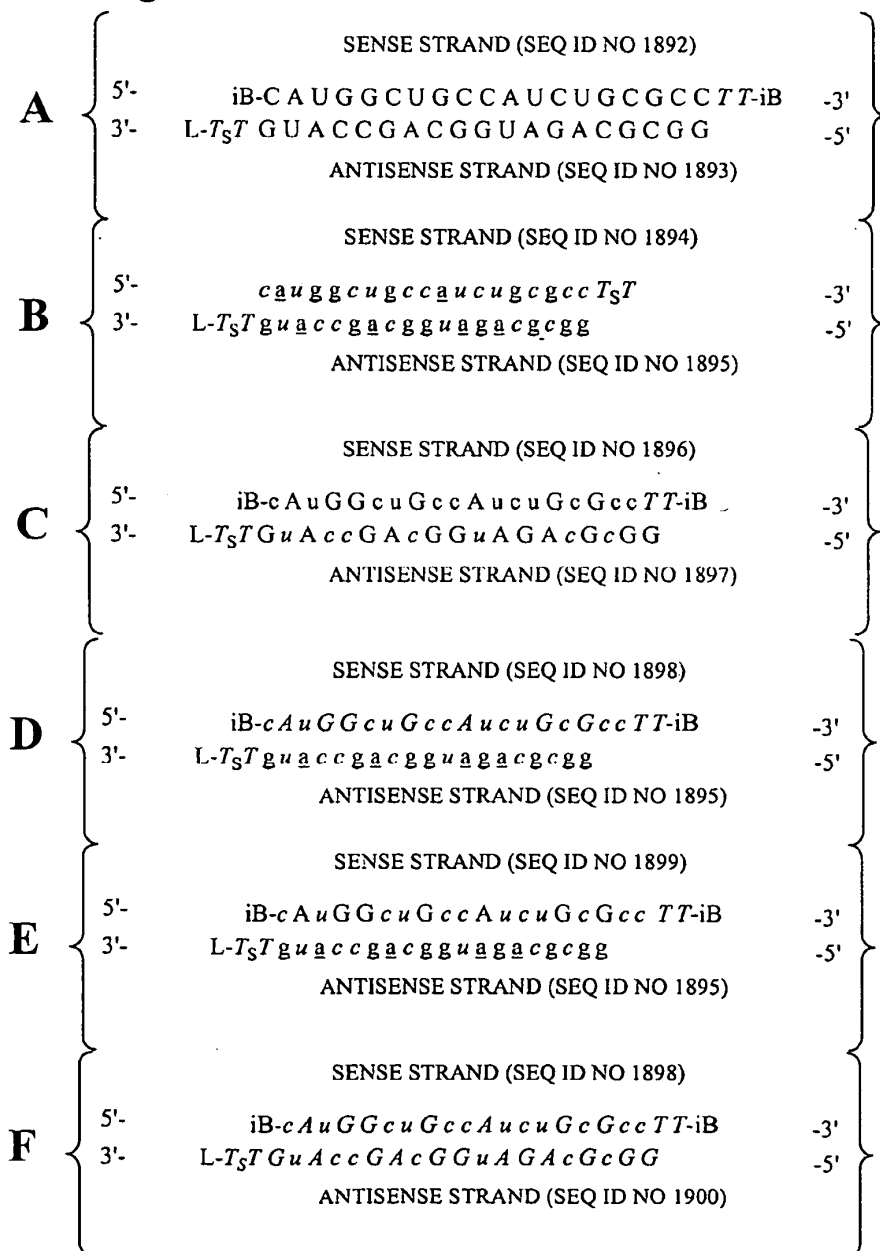


Figure 4



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 5

lower case = 2'-O-Methyl or 2'-deoxy-2'-fluoro

italic lower case = 2'-deoxy-2'-fluorounderline = 2'-O-methyl

ITALIC UPPER CASE = DEOXY

iB = INVERTED DEOXYABASIC

L = GLYCERYL MOIETY OR iB OPTIONALLY PRESENT

S = PHOSPHOROTHIOATE OR

PHOSPHORODITHIOATE OPTIONALLY PRESENT

Figure 6

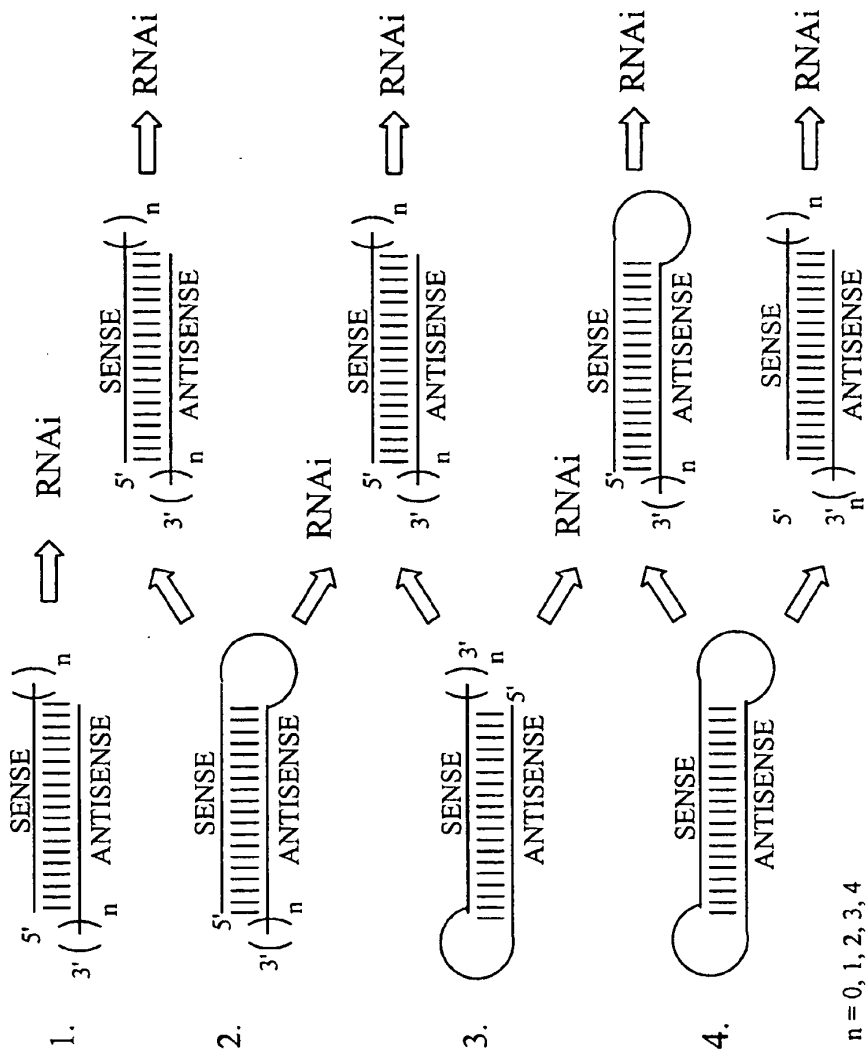


Figure 9: Target site Selection using siRNA

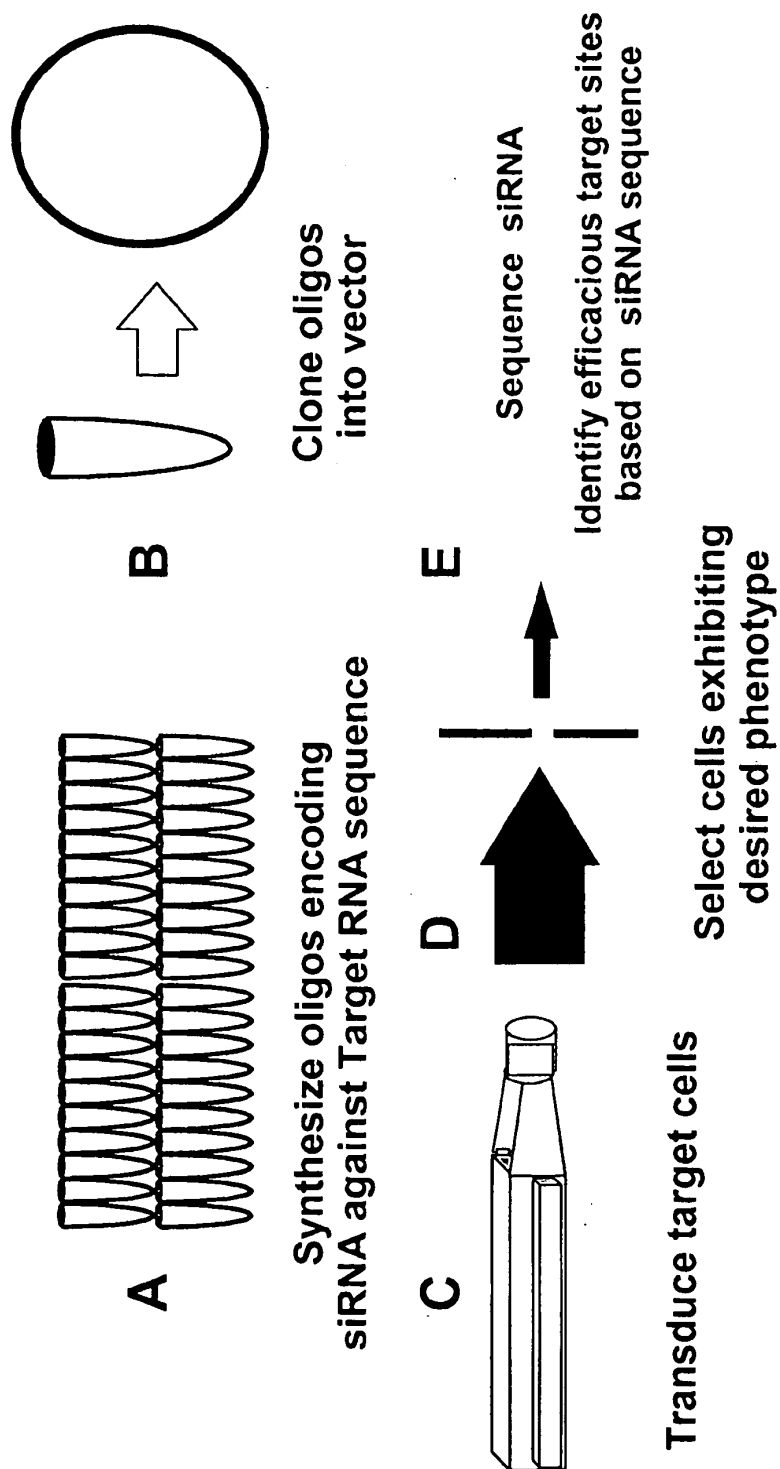
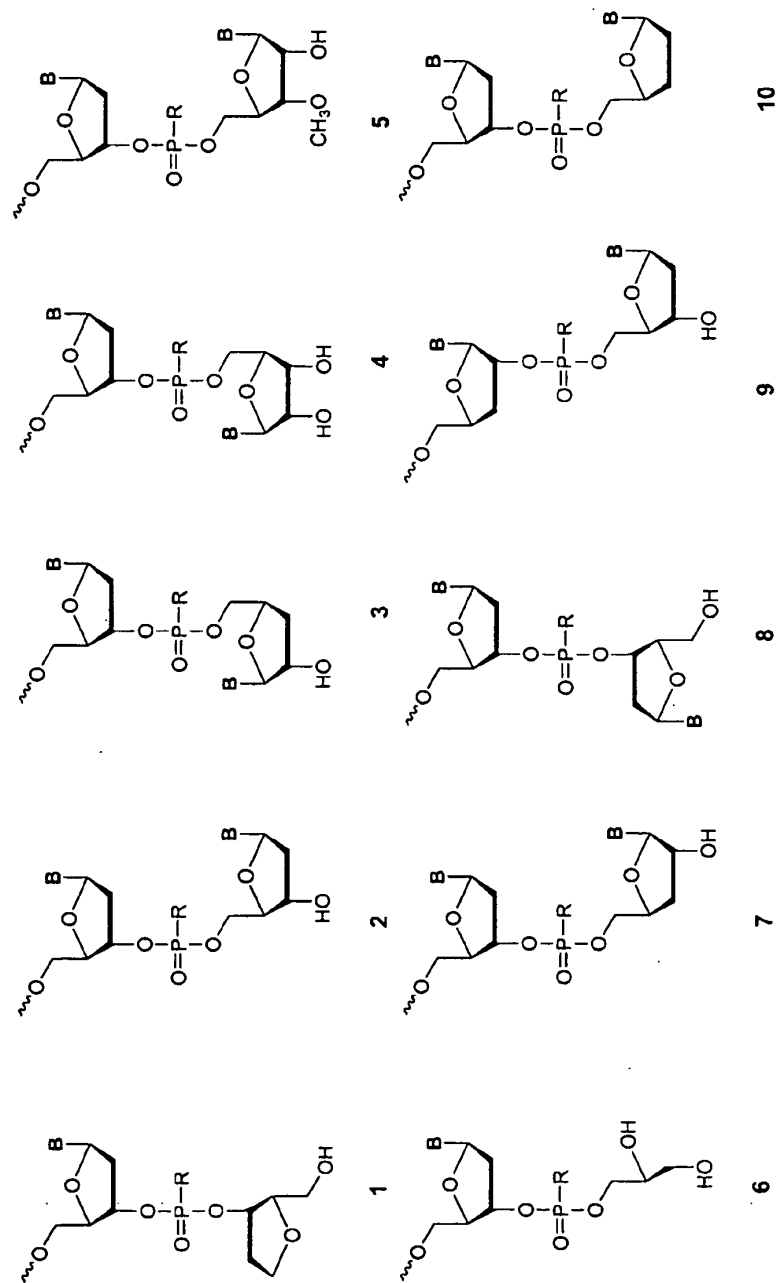


Figure 10



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 11: Modification Strategy

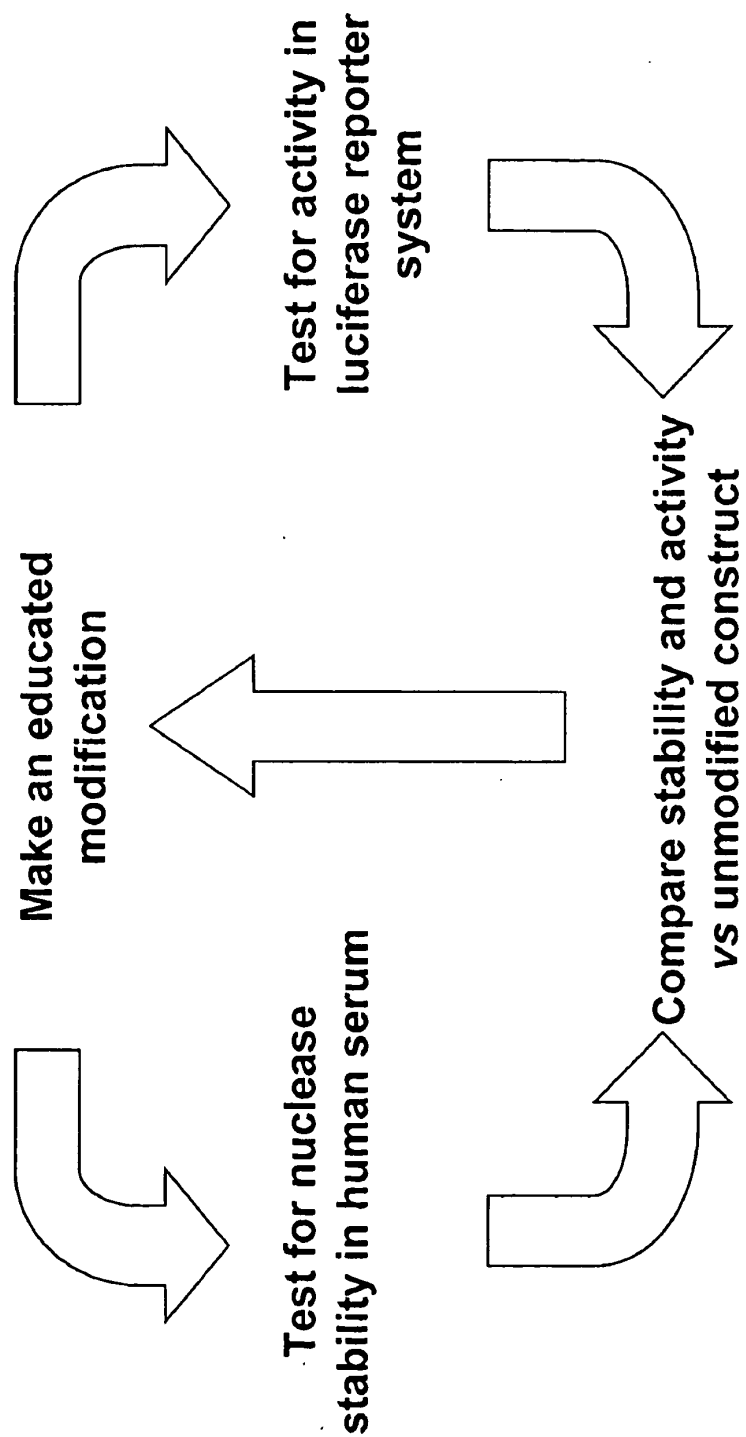
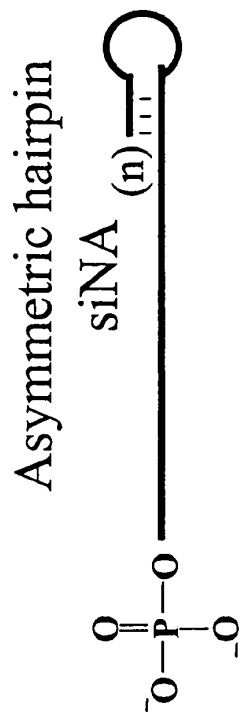
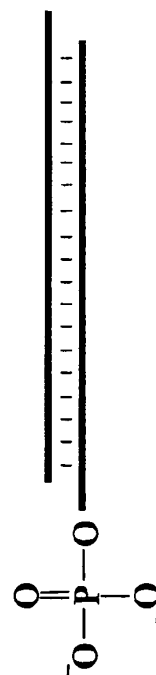


Figure 12: Phosphorylated siNA constructs



Phosphates can be modified
as described herein



Asymmetric duplex
siNA

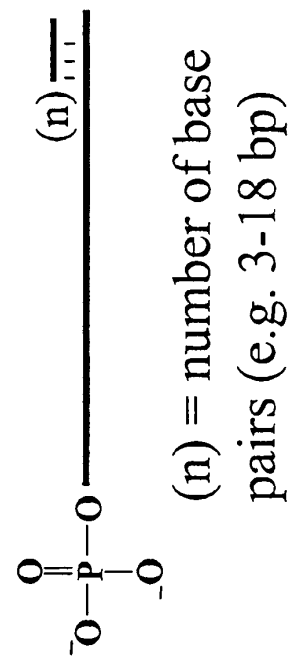


**Figure 14A: Duplex forming oligonucleotide constructs that utilize
Palindrome or repeat sequences**

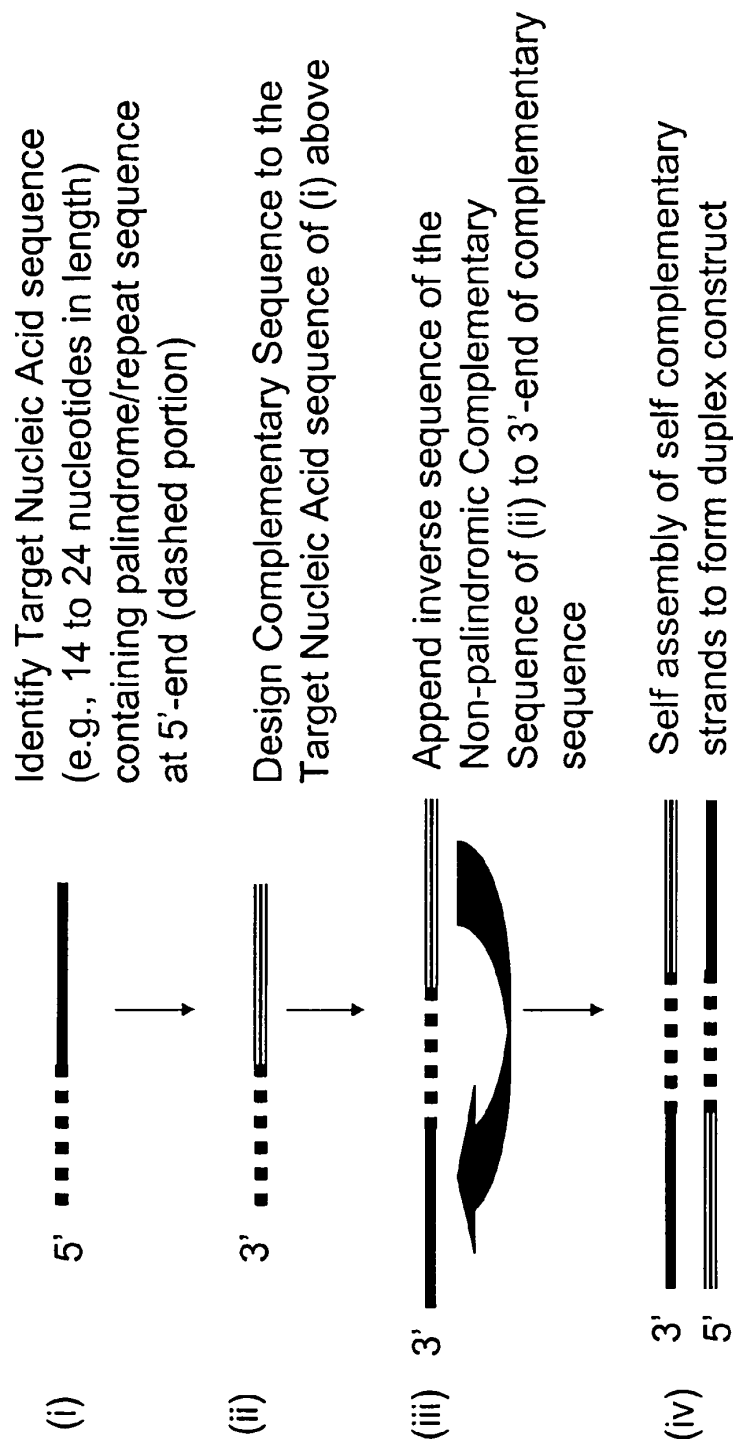


Figure 14B: Example of a duplex forming oligonucleotide sequence that utilizes a palindrome or repeat sequence

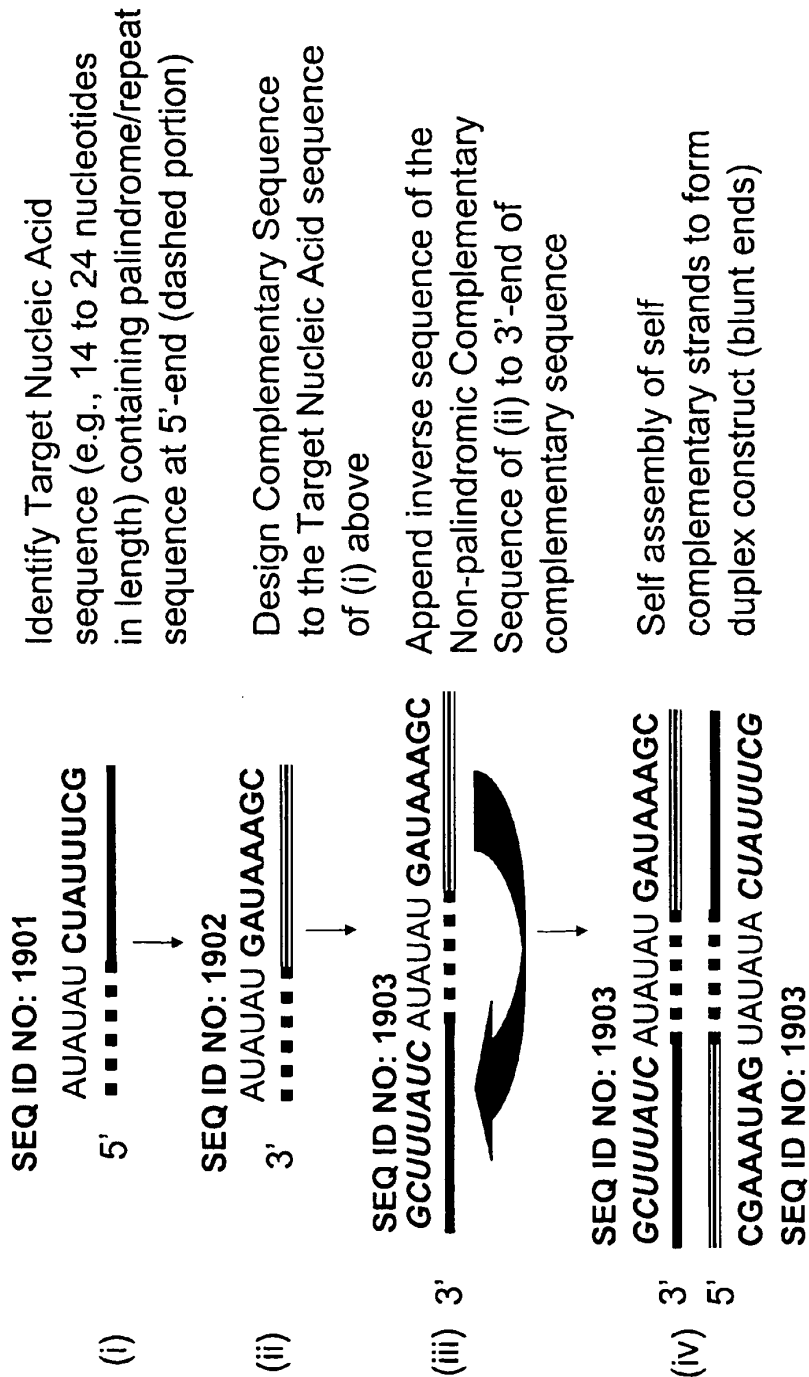


Figure 14C: Example of a duplex forming oligonucleotide sequence that utilizes a palindrome or repeat sequence, self assembly

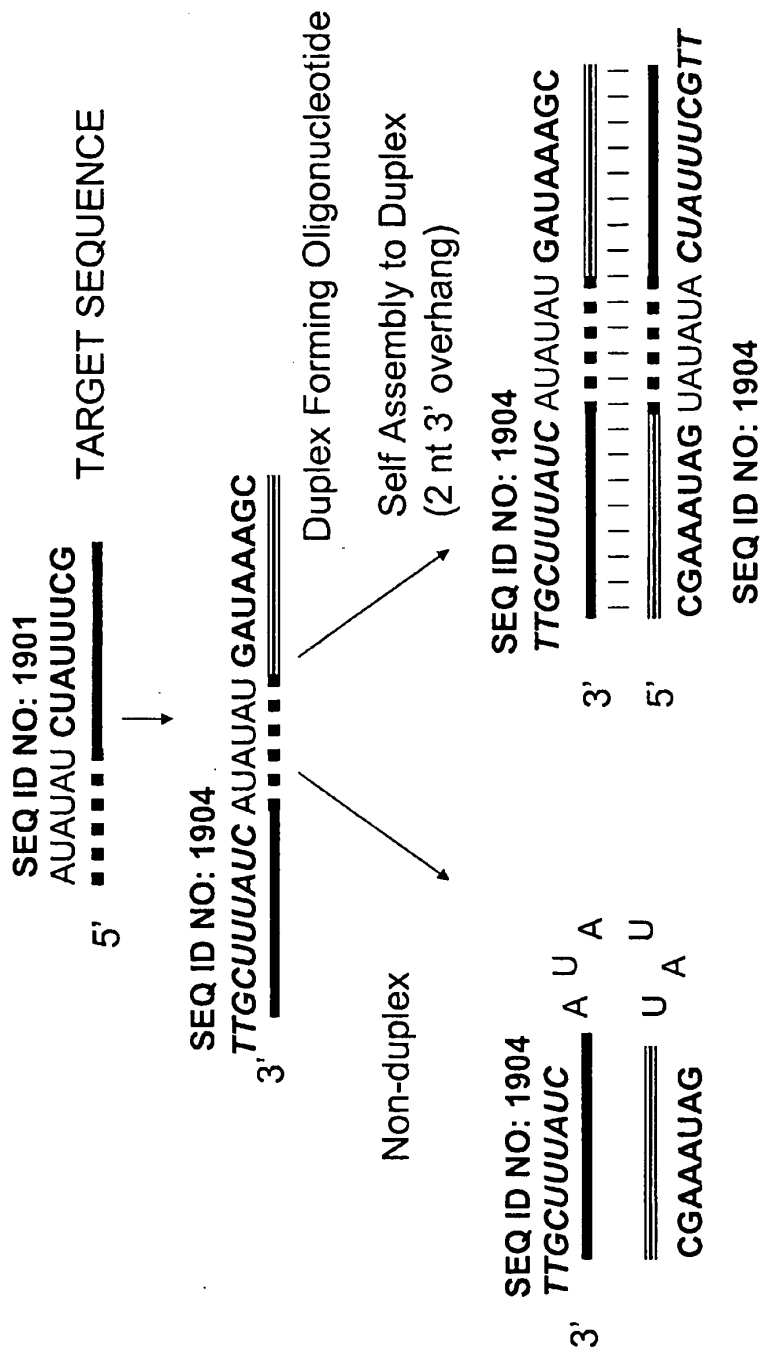


Figure 15: Duplex forming oligonucleotide constructs that utilize artificial palindrome or repeat sequences

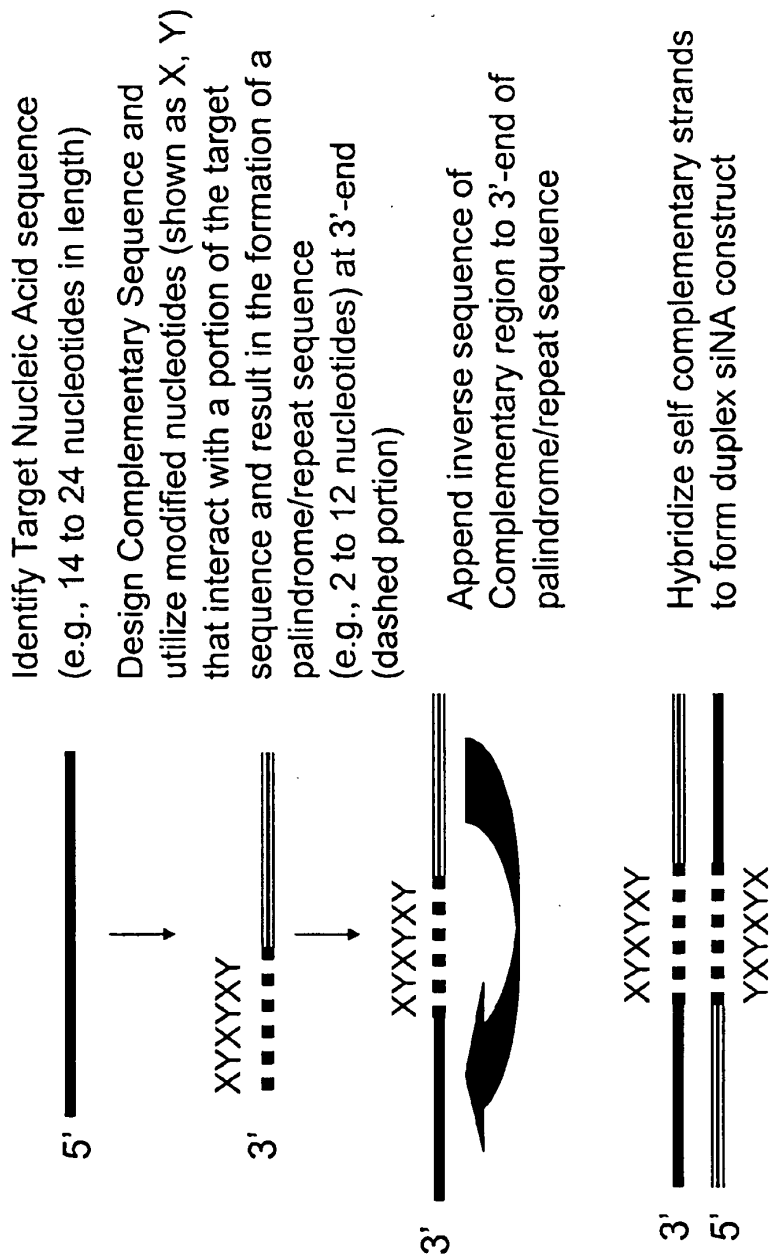


Figure 16: Examples of double stranded multifunctional siNA constructs with distinct complementary regions

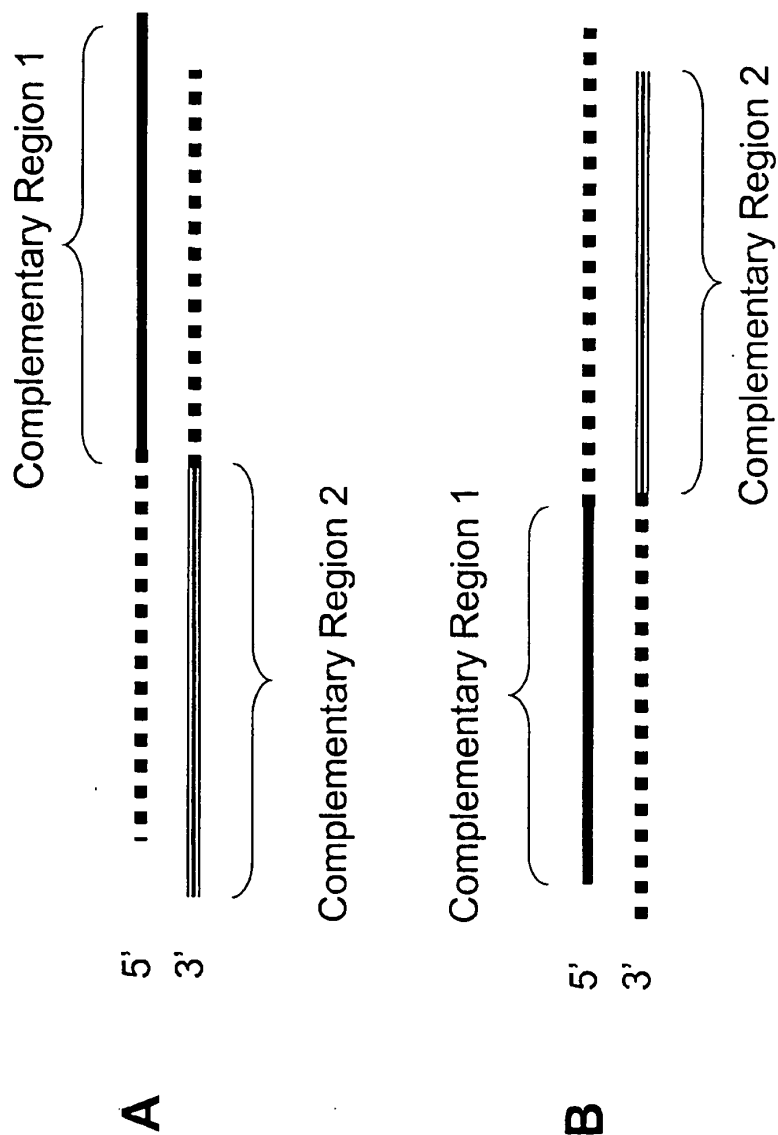


Figure 17: Examples of hairpin multifunctional siNA constructs with distinct complementary regions

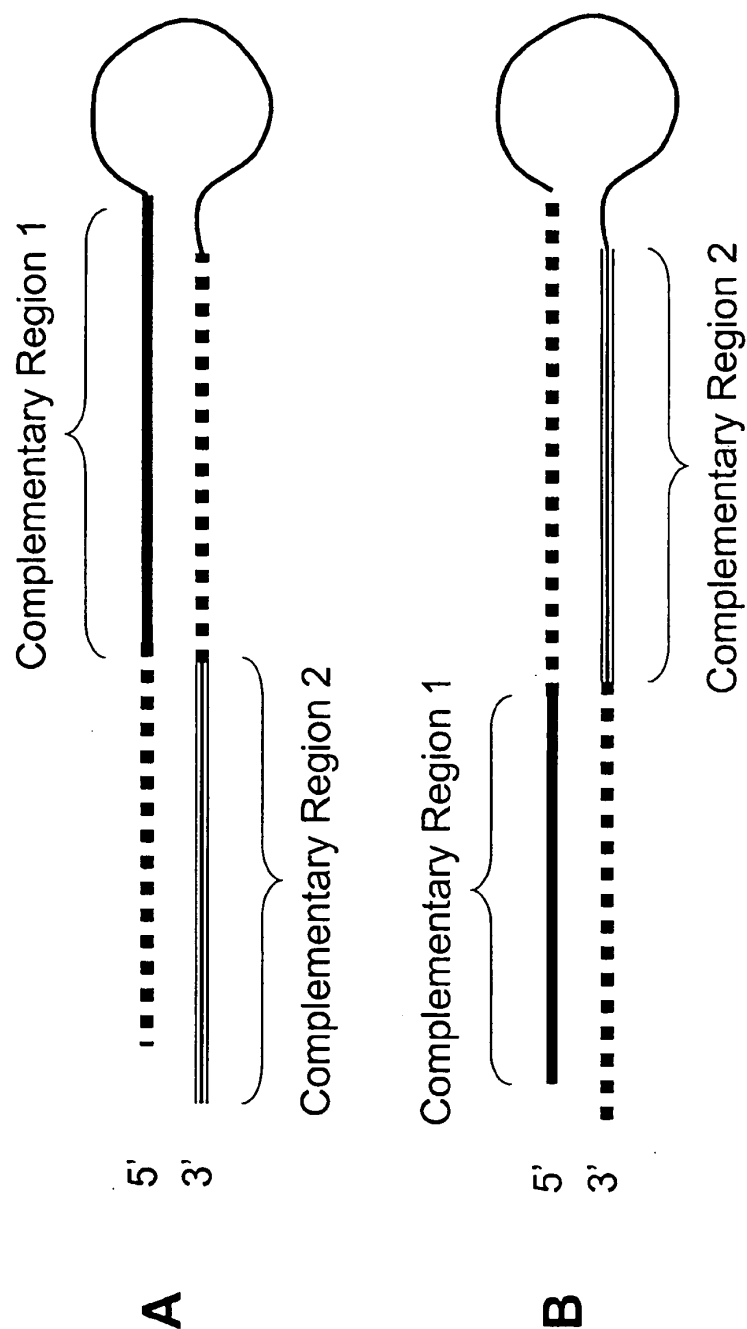


Figure 18: Examples of double stranded multifunctional siNA constructs with distinct complementary regions and a self complementary/palindrome region

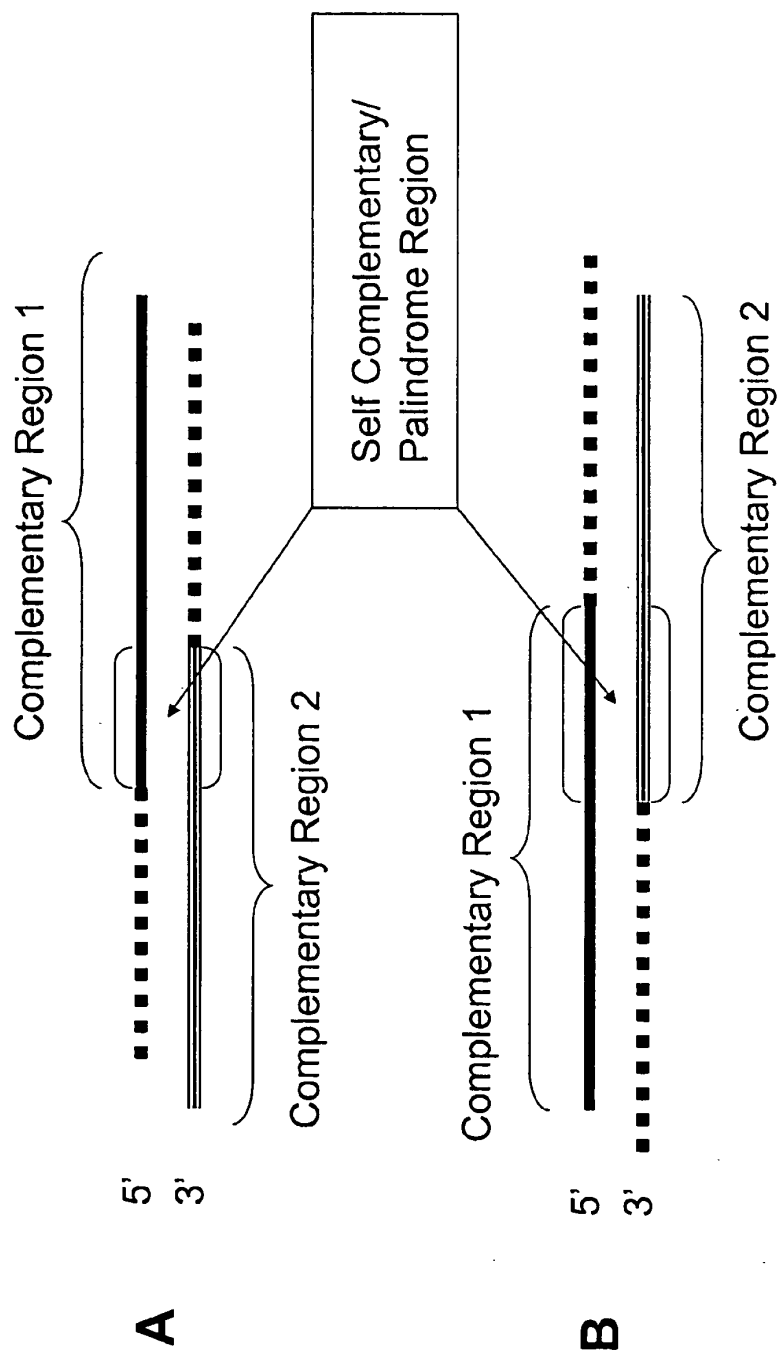


Figure 19: Examples of hairpin multifunctional siNA constructs with distinct complementary regions and a self complementary/palindrome region

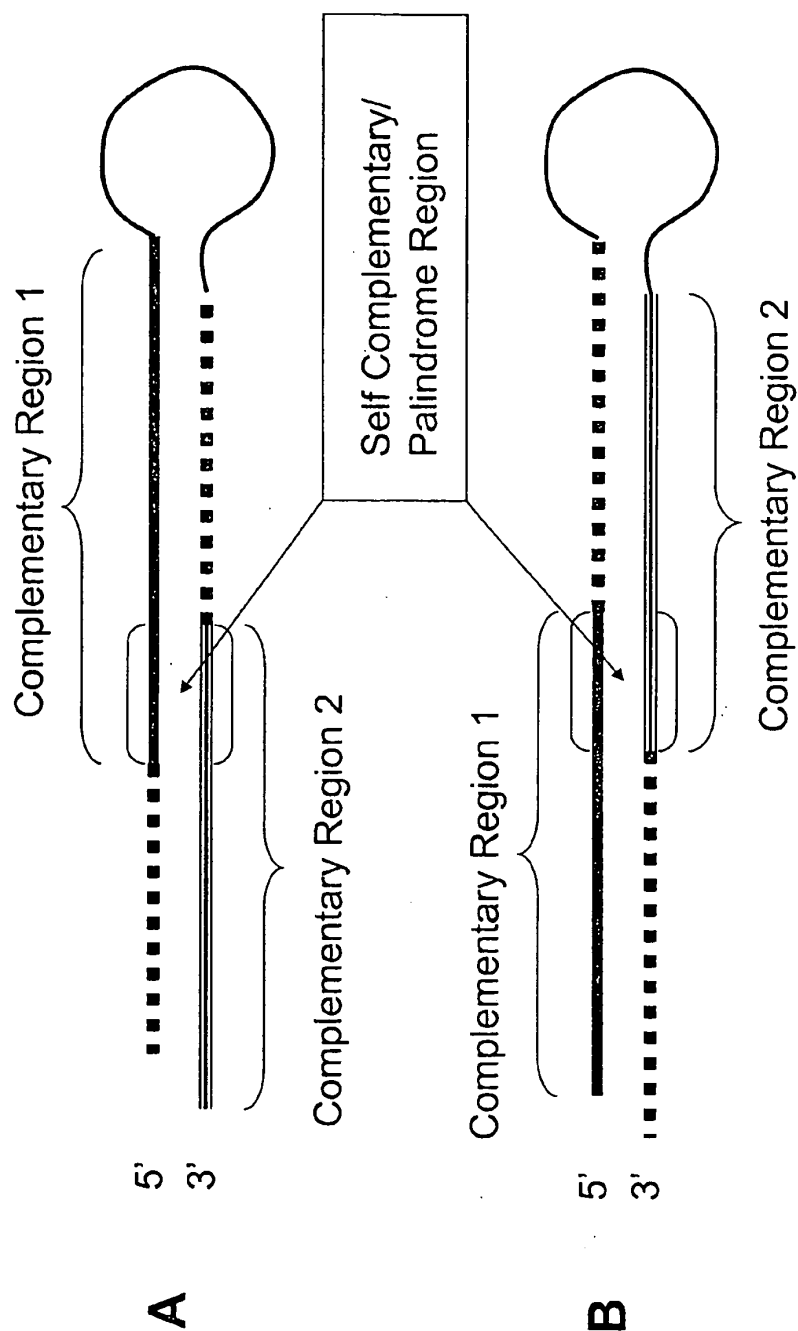


**Figure 20: Example of multifunctional siNA targeting two
Separate Target nucleic acid sequences**

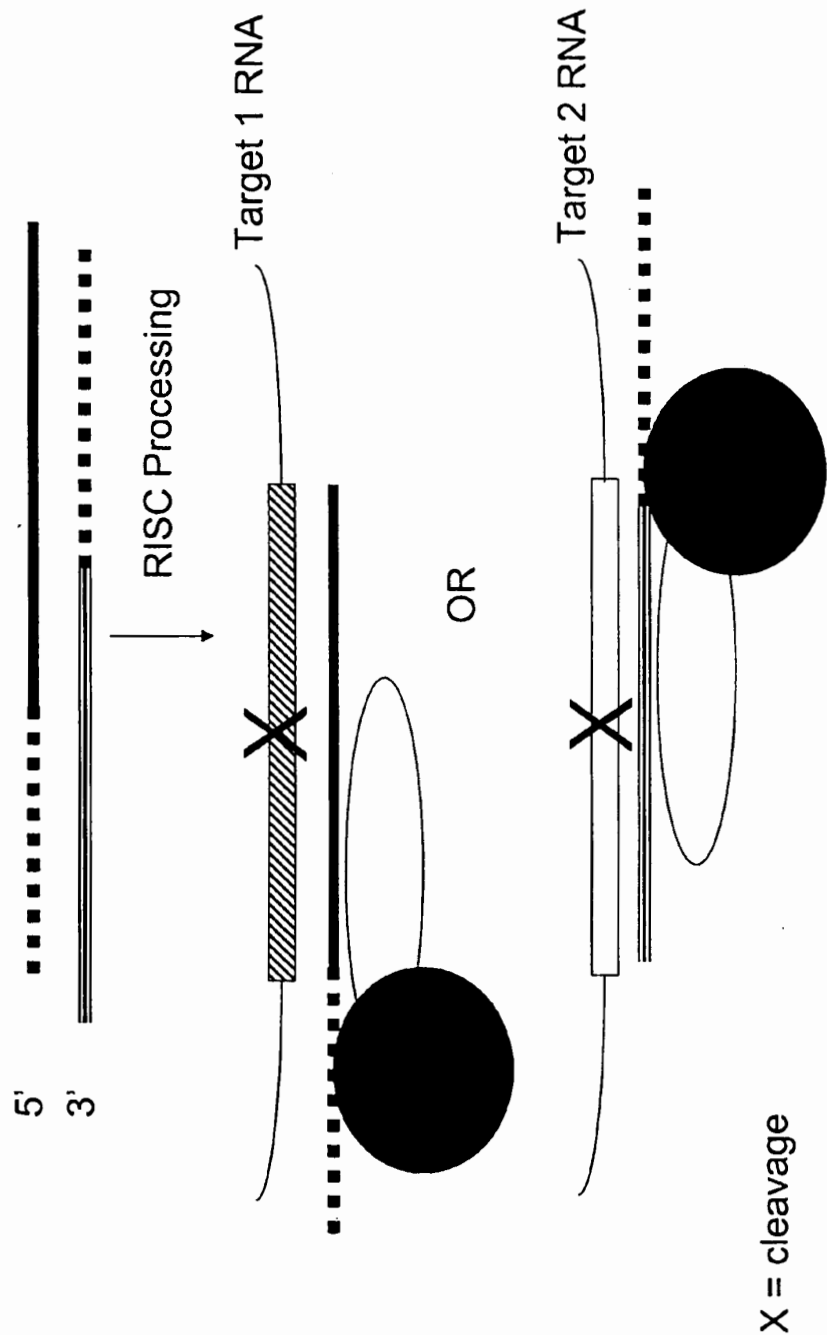


Figure 21: Example of multifunctional siNA targeting two regions within the same target nucleic acid sequence

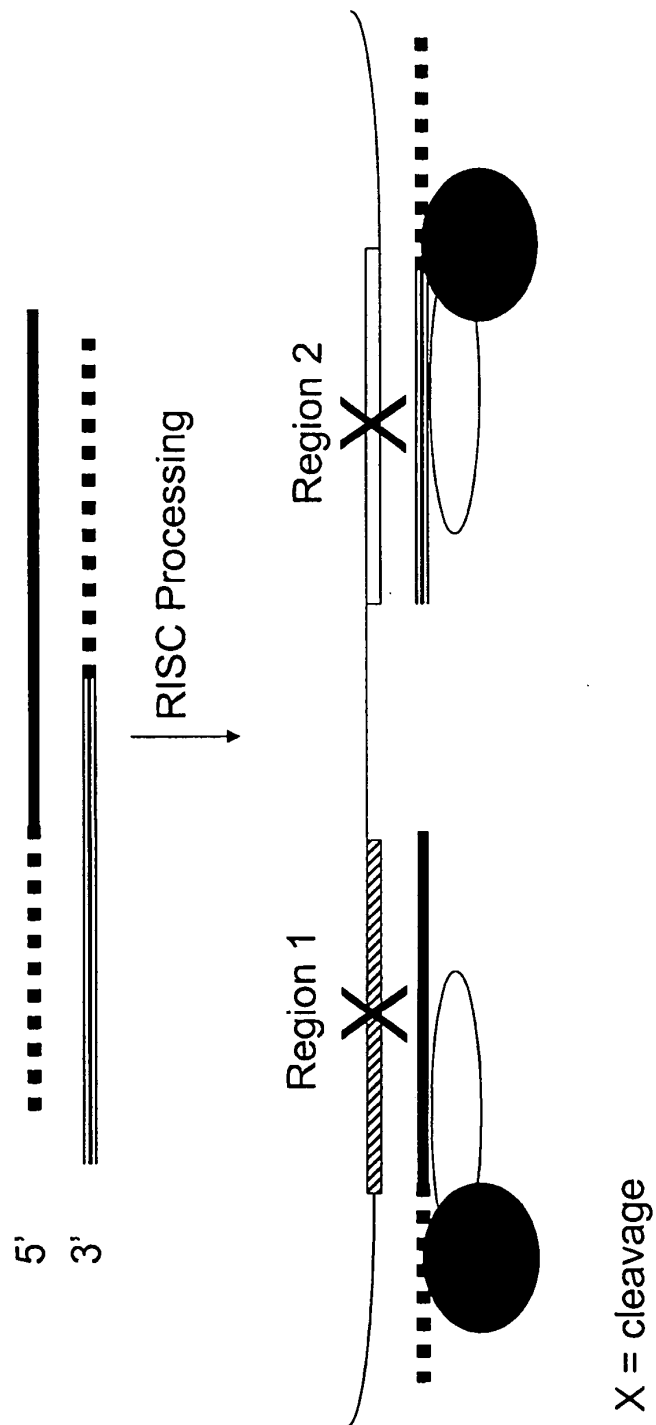


Figure 22: A549 24h BACE mRNA Expression

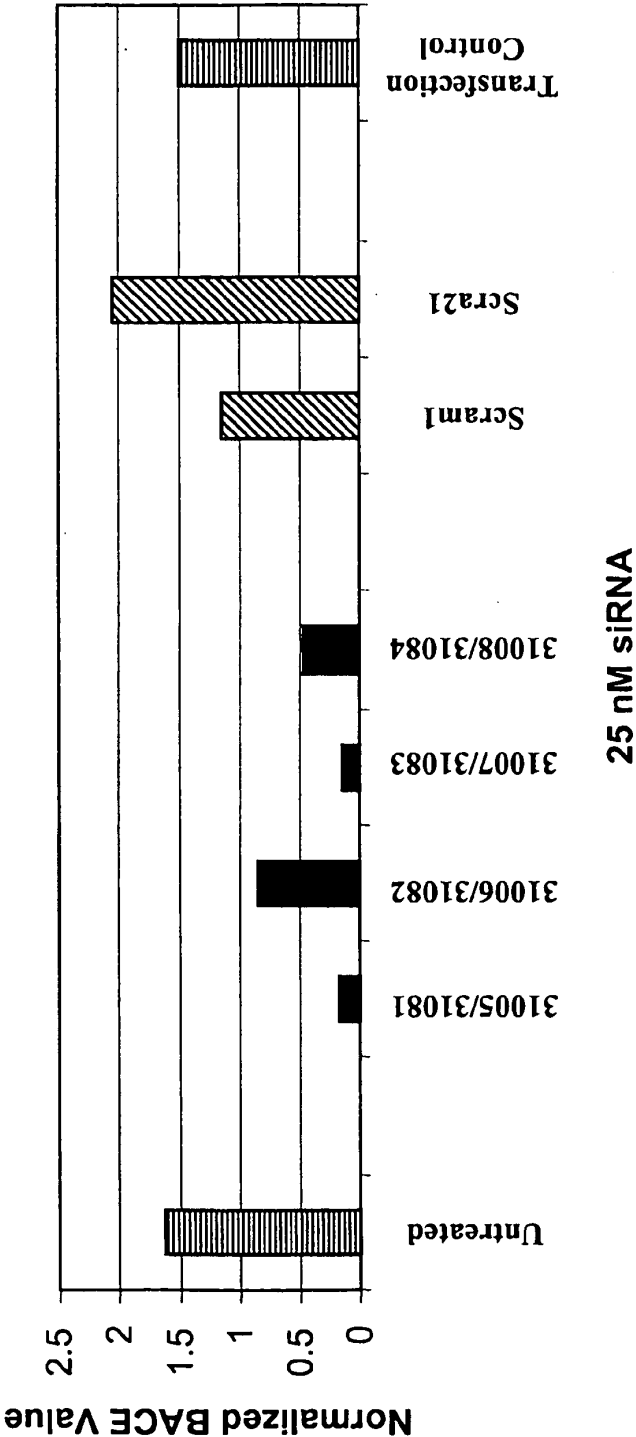


**Figure 23: A549 24h BACE mRNA Expression
using modified siNA**

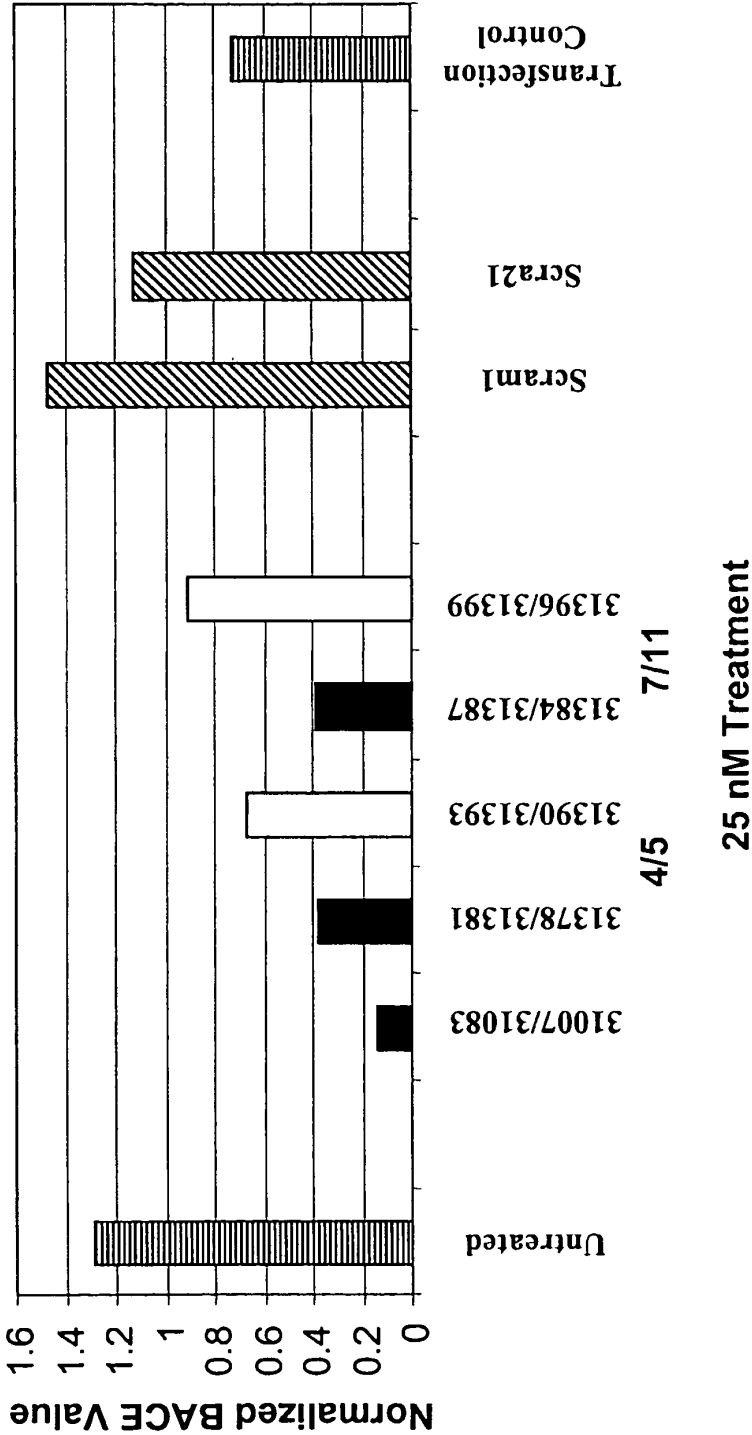


FIGURE 24

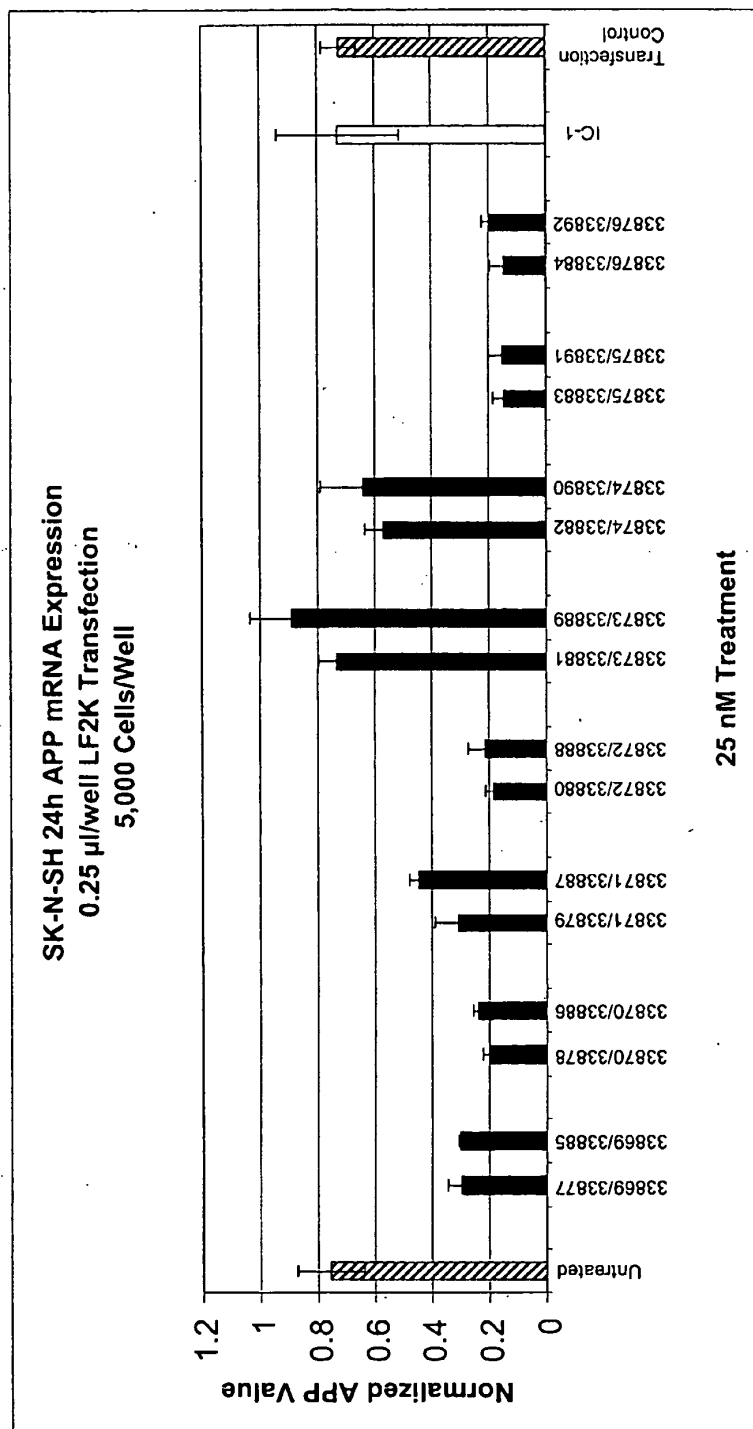


FIGURE 25

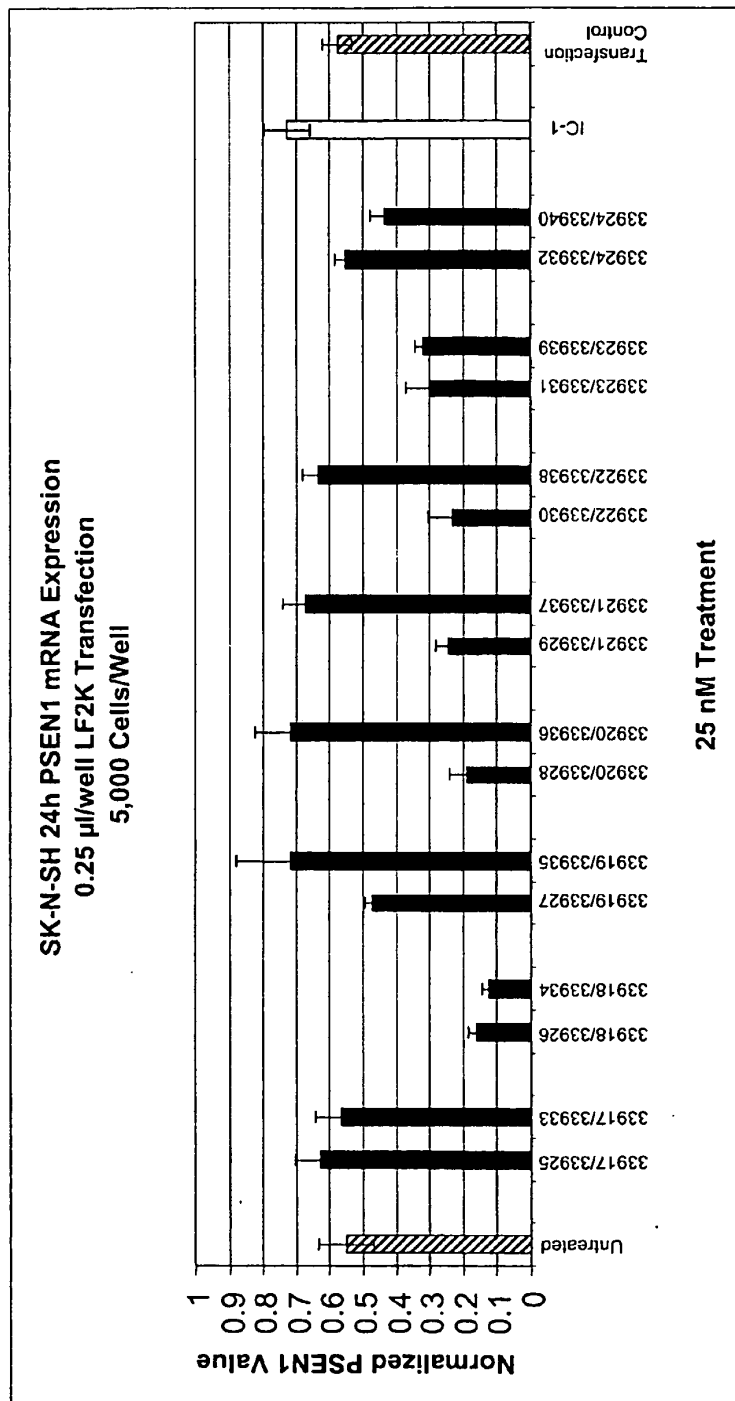
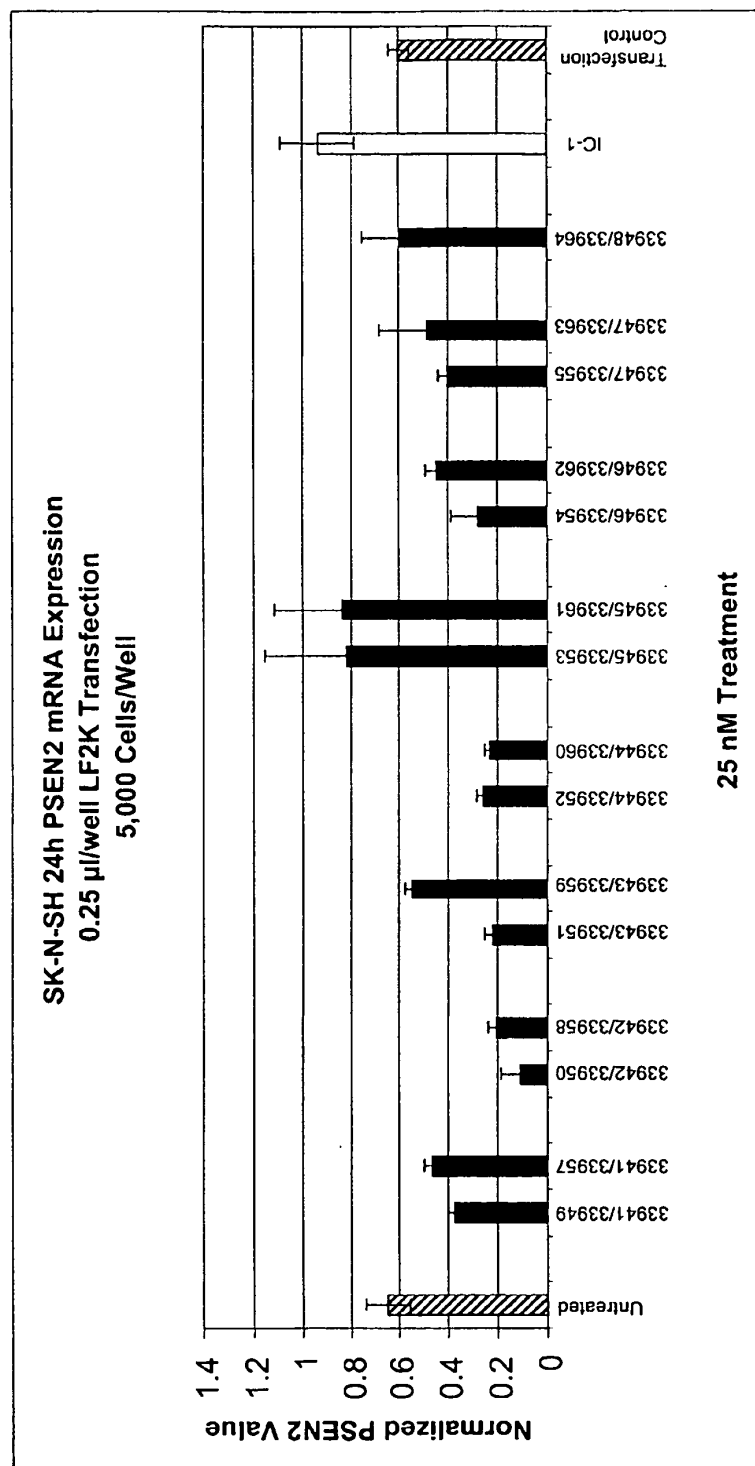


FIGURE 26



RNA INTERFERENCE MEDIATED TREATMENT OF ALZHEIMER'S DISEASE USING SHORT INTERFERING NUCLEIC ACID (SINA)

[0001] This application is a continuation-in-part of U.S. patent application Ser. No. 10/607,933, filed Jun. 27, 2003, which is a continuation-in-part of U.S. patent application Ser. No. 09/930,423, filed Aug. 15, 2001 and is also a continuation-in-part of International Patent Application No. PCT/US03/04710, filed Feb. 18, 2003, which is a continuation-in-part of U.S. patent application Ser. No. 10/205,309, filed Jul. 25, 2002. This application is also a continuation-in-part of International Patent Application No. PCT/US04/16390, filed May 24, 2004, which is a continuation-in-part of U.S. patent application Ser. No. 10/826,966, filed Apr. 16, 2004, which is continuation-in-part of U.S. patent application Ser. No. 10/757,803, filed Jan. 14, 2004, which is a continuation-in-part of U.S. patent application Ser. No. 10/720,448, filed Nov. 24, 2003, which is a continuation-in-part of U.S. patent application Ser. No. 10/693,059, filed Oct. 23, 2003, which is a continuation-in-part of U.S. patent application Ser. No. 10/444,853, filed May 23, 2003, which is a continuation-in-part of International Patent Application No. PCT/US03/05346, filed Feb. 20, 2003, and a continuation-in-part of International Patent Application No. PCT/US03/05028, filed Feb. 20, 2003, both of which claim the benefit of U.S. Provisional Application No. 60/358,580, filed Feb. 20, 2002, U.S. Provisional Application No. 60/363,124, filed Mar. 11, 2002, U.S. Provisional Application No. 60/386,782, filed Jun. 6, 2002, U.S. Provisional Application No. 60/406,784, filed Aug. 29, 2002, U.S. Provisional Application No. 60/408,378, filed Sep. 5, 2002, U.S. Provisional Application No. 60/409,293, filed Sep. 9, 2002, and U.S. Provisional Application No. 60/440,129, filed Jan. 15, 2003. This application is also a continuation-in-part of International Patent Application No. PCT/US04/13456, filed Apr. 30, 2004, which is a continuation of patent application Ser. No. 10/780,447, filed Feb. 13, 2004, which is a continuation-in-part of U.S. patent application Ser. No. 10/427,160, filed Apr. 30, 2003, which is a continuation-in-part of International Patent Application No. PCT/US02/15876, filed May 17, 2002, which claims the benefit of U.S. Provisional Application No. 60/362,016, filed Mar. 6, 2002, and U.S. Provisional Application No. 60/292,217, filed May 18, 2001. This application is also a continuation-in-part of U.S. patent application Ser. No. 10/727,780, filed Dec. 3, 2003. This application also claims the benefit of U.S. Provisional Application No. 60/543,480, filed Feb. 10, 2004. The instant application claims the benefit of all the listed applications, which are hereby incorporated by reference herein in their entireties, including the drawings.

FIELD OF THE INVENTION

[0002] The present invention relates to compounds, compositions, and methods for the study, diagnosis, and treatment of traits, diseases and conditions associated with Alzheimer's disease. The present invention is also directed to compounds, compositions, and methods relating to traits, diseases and conditions that respond to the modulation of expression and/or activity of genes involved in beta-secretase (BACE), amyloid precursor protein (APP), PIN-1, presenilin 1 (PS-1) and/or presenilin 2 (PS-2) gene expression pathways or other cellular processes that mediate the maintenance or development of such traits, diseases and conditions. Specifically, the invention relates to small

nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (mRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA interference (RNAi) against beta-secretase (BACE), amyloid precursor protein (APP), PIN-1, presenilin 1 (PS-1) and/or presenilin 2 (PS-2) gene expression. Such small nucleic acid molecules are useful, for example, in providing compositions for treatment of traits, diseases and conditions that can respond to modulation of beta-secretase (BACE), amyloid precursor protein (APP), PIN-1, presenilin 1 (PS-1) and/or presenilin 2 (PS-2) gene expression in a subject, such as Alzheimer's disease or dementia.

BACKGROUND OF THE INVENTION

[0003] 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.

[0004] 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; Sharp, 1999, *Genes & Dev.*, 13:139-141; 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 U.S. Pat. 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).

[0005] 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; Bernstein 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).

[0006] 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).

[0007] 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.

[0008] 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.

[0009] 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. Zemicka-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. Plactinck 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 antiviral 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.

[0010] 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., U.S. Pat. No. 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.

[0011] McSwiggen et al., International PCT Publication No. WO 01/16312, describes nucleic acid mediated inhibition of BACE, PS-1, and PS-2 expression.

SUMMARY OF THE INVENTION

[0012] This invention relates to compounds, compositions, and methods useful for modulating the expression of genes associated with the maintenance or development of Alzheimer's disease and/or dementia, for example, beta-secretase (BACE), amyloid precursor protein (APP), PIN-1, presenilin 1 (PS-1) and/or presenilin 2 (PS-2) gene expression using short interfering nucleic acid (siNA) molecules. This invention also relates to compounds, compositions, and methods useful for modulating the expression and activity of other genes involved in pathways of BACE, APP, PIN-1, PS-1 and/or PS-2 gene expression and/or activity 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 BACE, APP, PIN-1, PS-1 and/or PS-2 genes or other genes associated with the maintenance or development of Alzheimer's disease and/or dementia.

[0013] A siNA of the invention can be unmodified or chemically-modified. A siNA of the instant invention can be chemically synthesized, expressed from a vector or enzymatically synthesized. The instant invention also features various chemically-modified synthetic short interfering nucleic acid (siNA) molecules capable of modulating BACE, APP, PIN-1, PS-1 and/or PS-2 gene expression or activity in cells by RNA interference (RNAi). The use of chemically-modified siNA improves various properties of native siNA molecules through increased resistance to nuclease degradation in vivo and/or through improved cellular uptake. Further, contrary to earlier published studies, siNA having multiple chemical modifications retains its RNAi activity. The siNA molecules of the instant invention provide useful reagents and methods for a variety of therapeutic, diagnostic, target validation, genomic discovery, genetic engineering, and pharmacogenomic applications.

[0014] In one embodiment, the invention features one or more siNA molecules and methods that independently or in combination modulate the expression of BACE, APP, PIN-1, PS-1 and/or PS-2 genes encoding proteins, such as proteins comprising BACE, APP, PIN-1, PS-1 and/or PS-2 associated with the maintenance and/or development of Alzheimer's disease and other neurodegenerative disorders or conditions such as dementia and stroke/cardiovascular accident (CVA), such as genes encoding sequences comprising those sequences referred to by GenBank Accession Nos. shown in Table I, referred to herein generally as BACE, APP, PIN-1, PS-1 and/or PS-2. The description below of the various aspects and embodiments of the invention is provided with

reference to exemplary BACE gene referred to herein as BACE. However, the various aspects and embodiments are also directed to other BACE genes, such as BACE homolog genes, transcript variants and polymorphisms (e.g., single nucleotide polymorphism, (SNPs)) associated with certain BACE genes. As such, the various aspects and embodiments are also directed to other genes which express other BACE related proteins or other proteins associated with Alzheimer's disease, such as APP, PIN-1, PS-1 and/or PS-2, including mutant genes and splice variants thereof. The various aspects and embodiments are also directed to other genes that are involved in BACE, APP, PIN-1, PS-1 and/or PS-2 mediated pathways of signal transduction or gene expression that are involved, for example, in the progression, development, or maintenance of disease (e.g., Alzheimer's disease). These additional genes can be analyzed for target sites using the methods described for BACE 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.

[0015] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a BACE gene, wherein said siNA molecule comprises about 18 to about 21 base pairs.

[0016] In one embodiment, the invention features a double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of BACE RNA via RNA interference (RNAi), wherein the double stranded siNA molecule comprises a first and a second strand, each strand of the siNA molecule is about 18 to about 23 nucleotides in length, the first strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the BACE RNA for the siNA molecule to direct cleavage of the BACE RNA via RNA interference, and the second strand of said siNA molecule comprises nucleotide sequence that is complementary to the first strand.

[0017] In one embodiment, the invention features a chemically synthesized double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of a BACE RNA via RNA interference (RNAi), wherein each strand of the siNA molecule is about 18 to about 23 nucleotides in length; and one strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the BACE RNA for the siNA molecule to direct cleavage of the BACE RNA via RNA interference.

[0018] In one embodiment, the invention features a siNA molecule that down-regulates expression of a BACE gene, for example, wherein the BACE gene comprises BACE encoding sequence. In one embodiment, the invention features a siNA molecule that down-regulates expression of a BACE gene, for example, wherein the BACE gene comprises BACE non-coding sequence or regulatory elements involved in BACE gene expression.

[0019] In one embodiment, a siNA of the invention is used to inhibit the expression of BACE genes or a BACE 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, for example mismatches and/or wobble base pairs, that can provide additional target sequences. In instances where mismatches are identified, non-canonical base pairs (for example, 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 sequences for differing BACE targets that share sequence homology. As such, 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 the nucleotide sequence that is conserved between the homologous genes. In this approach, a single siNA can be used to inhibit expression of more than one gene instead of using more than one siNA molecule to target the different genes.

[0020] In one embodiment, the invention features a siNA molecule having RNAi activity against BACE RNA, wherein the siNA molecule comprises a sequence complementary to any RNA having BACE encoding sequence, such as those sequences having GenBank Accession Nos. shown in Table I. In another embodiment, the invention features a siNA molecule having RNAi activity against BACE RNA, wherein the siNA molecule comprises a sequence complementary to an RNA having variant BACE encoding sequence, for example other mutant BACE genes not shown in Table I but known in the art to be associated with the maintenance and/or development of Alzheimer's disease and/or dementia. 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 BACE gene and thereby mediate silencing of BACE gene expression, for example, wherein the siNA mediates regulation of BACE gene expression by cellular processes that modulate the chromatin structure or methylation patterns of the BACE gene and prevent transcription of the BACE gene.

[0021] In one embodiment, siNA molecules of the invention are used to down regulate or inhibit the expression of BACE proteins arising from BACE haplotype polymorphisms that are associated with a disease or condition, (e.g., Alzheimer's disease and other neurodegenerative disorders or conditions such as dementia and stroke/cardiovascular accident (CVA)). Analysis of BACE genes, or BACE protein or RNA levels can be used to identify subjects with such polymorphisms or those subjects who are at risk of developing traits, conditions, or diseases described herein. These subjects are amenable to treatment, for example, treatment with siNA molecules of the invention and any other composition useful in treating diseases related to BACE gene expression. As such, analysis of BACE protein or RNA levels can be used to determine treatment type and the course of therapy in treating a subject. Monitoring of BACE 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 BACE proteins associated with a trait, condition, or disease.

[0022] 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 BACE protein. The siNA

further comprises a sense strand, wherein said sense strand comprises a nucleotide sequence of a BACE gene or a portion thereof.

[0023] In another embodiment, a siNA molecule comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence encoding a BACE protein or a portion thereof. The siNA molecule further comprises a sense region, wherein said sense region comprises a nucleotide sequence of a BACE gene or a portion thereof.

[0024] In another embodiment, the invention features a siNA molecule comprising a nucleotide sequence in the antisense region of the siNA molecule that is complementary to a nucleotide sequence or portion of sequence of a BACE 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 BACE gene sequence or a portion thereof.

[0025] In one embodiment, the antisense region of BACE siNA constructs comprises a sequence complementary to sequence having any of SEQ ID NOs. 399-723, 1471-1478, 1591-1598, 1607-1614, 1623-1630, 1639-1646, 1655-1662, 1687, or 1689. In one embodiment, the antisense region of BACE constructs comprises sequence having any of SEQ ID NOs. 724-1048, 1599-1606, 1615-1622, 1631-1638, 1647-1654, 1663-1686, 1688, 1690, 1884, 1886, 1888, 1891, 1893, 1895, 1897, or 1900. In another embodiment, the sense region of BACE constructs comprises sequence having any of SEQ ID NOs. 399-723, 1471-1478, 1591-1598, 1607-1614, 1623-1630, 1639-1646, 1655-1662, 1687, 1689, 1883, 1885, 1887, 1889, 1890, 1892, 1894, 1896, 1898, or 1899.

[0026] In one embodiment, the antisense region of APP siNA constructs comprises a sequence complementary to sequence having any of SEQ ID NOs. 1-199, 1463-1470, 1495-1502, 1511-1518, 1527-1534, 1543-1550, or 1559-1566. In one embodiment, the antisense region of APP constructs comprises sequence having any of SEQ ID NOs. 200-398, 1503-1510, 1519-1526, 1535-1542, 1551-1558, 1567-1590, 1884, 1886, 1888, or 1891. In another embodiment, the sense region of APP constructs comprises sequence having any of SEQ ID NOs. 1-199, 1463-1470, 1495-1502, 1511-1518, 1527-1534, 1543-1550, 1559-1566, 1883, 1885, 1887, 1889, or 1890.

[0027] In one embodiment, the antisense region of PSEN1 siNA constructs comprises a sequence complementary to sequence having any of SEQ ID NOs. 1049-1131, 1479-1486, 1691-1698, 1707-1714, 1723-1730, 1739-1746, 1755-1762. In one embodiment, the antisense region of PSEN1 constructs comprises sequence having any of SEQ ID NOs. 1132-1214, 1699-1706, 1715-1722, 1731-1738, 1747-1754, 1763-1786, 1884, 1886, 1888, or 1891. In another embodiment, the sense region of PSEN1 constructs comprises sequence having any of SEQ ID NOs. 1049-1131, 1479-1486, 1691-1698, 1707-1714, 1723-1730, 1739-1746, 1755-1762, 1883, 1885, 1887, 1889, or 1890.

[0028] In one embodiment, the antisense region of PSEN2 siNA constructs comprises a sequence complementary to sequence having any of SEQ ID NOs. 1215-1338, 1487-1494, 1787-1794, 1803-1810, 1819-1826, 1835-1842, 1851-1858. In one embodiment, the antisense region of PSEN2

constructs comprises sequence having any of SEQ ID NOs. 1339-1462, 1795-1802, 1811-1818, 1827-1834, 1843-1850, 1859-1882, 1884, 1886, 1888, or 1891. In another embodiment, the sense region of PSEN2 constructs comprises sequence having any of SEQ ID NOs. SEQ ID NOs. 1215-1338, 1487-1494, 1787-1794, 1803-1810, 1819-1826, 1835-1842, 1851-1858, 1883, 1885, 1887, 1889, or 1890.

[0029] In one embodiment, a siNA molecule of the invention comprises any of SEQ ID NOs. 1-1900. The sequences shown in SEQ ID NOs: 1-1900 are not limiting. A siNA molecule of the invention can comprise any contiguous BACE sequence (e.g., about 18 to about 25, or about 18, 19, 20, 21, 22, 23, 24, or 25 contiguous BACE nucleotides).

[0030] In yet another embodiment, the invention features a siNA molecule comprising a sequence, for example, the antisense sequence of the siNA construct, complementary to a sequence or portion of sequence comprising sequence represented by GenBank Accession Nos. shown in Table I. Chemical modifications in Tables III and IV and described herein can be applied to any siNA construct of the invention.

[0031] In one embodiment of the invention a siNA molecule comprises an antisense strand having about 18 to about 29 (e.g., about 18, 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 BACE protein, and wherein said siNA further comprises a sense strand having about 18 to about 29 (e.g., about 18, 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 18 complementary nucleotides.

[0032] In another embodiment of the invention a siNA molecule of the invention comprises an antisense region having about 18 to about 29 (e.g., about 18, 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 BACE protein, and wherein said siNA further comprises a sense region having about 18 to about 29 (e.g., about 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29) nucleotides, wherein said sense region and said antisense region comprise a linear molecule with at least about 19 complementary nucleotides.

[0033] In one embodiment, a siNA molecule of the invention has RNAi activity that modulates expression of RNA encoded by a BACE gene. Because BACE genes can share some degree of sequence homology with each other, siNA molecules can be designed to target a class of BACE genes or alternately specific BACE genes (e.g., polymorphic variants) by selecting sequences that are either shared amongst different BACE targets or alternatively that are unique for a specific BACE target. Therefore, in one embodiment, the siNA molecule can be designed to target conserved regions of BACE RNA sequences having homology among several BACE gene variants so as to target a class of BACE genes with one siNA molecule. Accordingly, in one embodiment, the siNA molecule of the invention modulates the expression of one or both BACE alleles in a subject. In another embodiment, the siNA molecule can be designed to target a sequence that is unique to a specific BACE RNA sequence (e.g., a single BACE allele or BACE single nucleotide polymorphism (SNP)) due to the high degree of specificity that the siNA molecule requires to mediate RNAi activity.

[0034] 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 duplex nucleic acid molecules containing about 18 base pairs between oligonucleotides comprising about 18 to about 25 (e.g., about 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides. In yet another embodiment, siNA molecules of the invention comprise duplex nucleic acid molecules 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 18 base pairs and 3'-terminal mononucleotide, dinucleotide, or trinucleotide overhangs.

[0035] In one embodiment, the invention features one or more chemically-modified siNA constructs having specificity for BACE expressing nucleic acid molecules, such as RNA encoding a BACE protein. In one embodiment, the invention features a RNA based siNA molecule (e.g., a siNA comprising 2'-OH nucleotides) having specificity for BACE expressing nucleic acid molecules that includes one or more chemical modifications described herein. 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, (e.g., RNA based 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.

[0036] 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 about 5% to about 100% modified nucleotides (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides). The actual percentage of modified nucleotides present in a given siNA molecule will depend 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.

[0037] One aspect of the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a BACE gene. In one embodiment, the 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 18 to about 29 (e.g., about 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29) nucleotides, wherein each strand comprises about 18 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 BACE gene, and the second strand of the double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence of the BACE gene or a portion thereof.

[0038] In another embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a BACE gene comprising an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of the BACE 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 BACE gene or a portion thereof. In one embodiment, the antisense region and the sense region each comprise about 18 to about 23 (e.g. about 18, 19, 20, 21, 22, or 23) nucleotides, wherein the antisense region comprises about 18 nucleotides that are complementary to nucleotides of the sense region.

[0039] In another embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a BACE 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 BACE gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region.

[0040] In one embodiment, a siNA molecule of the invention comprises blunt ends, i.e., ends that do not include any overhanging nucleotides. For example, a siNA molecule comprising modifications described herein (e.g., comprising nucleotides having Formulae I-VII or siNA constructs comprising "Stab 00"-"Stab 25" (Table IV) or any combination thereof (see Table IV)) and/or any length described herein can comprise blunt ends or ends with no overhanging nucleotides.

[0041] 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 one embodiment, the 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 embodiment, the 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, the 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, 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, for example, mismatches, bulges, loops, or wobble base pairs to modulate the activity of the siNA molecule to mediate RNA interference.

[0042] By "blunt ends" is meant symmetric termini or termini of a double stranded siNA molecule having no overhanging nucleotides. The two strands of a double stranded siNA molecule align with each other without overhanging nucleotides at the termini. For example, a blunt ended siNA construct comprises terminal nucleotides that are complementary between the sense and antisense regions of the siNA molecule.

[0043] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a BACE gene, wherein the siNA molecule 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 a non-nucleotide linker.

[0044] In one embodiment, the invention features double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a BACE gene, wherein the siNA molecule comprises about 18 to about 21 base pairs, and wherein each strand of the siNA molecule comprises one or more chemical modifications. In another embodiment, one of the strands of the double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence of a BACE gene or a portion thereof, and the second strand of the double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of the BACE gene. In another embodiment, one of the strands of the double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence of a BACE gene or portion thereof, and the second strand of the double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or portion thereof of the BACE gene. In another embodiment, each strand of the siNA molecule comprises about 18 to about 23 nucleotides, and each strand comprises at least about 18 nucleotides that are complementary to the nucleotides of the other strand. The BACE gene can comprise, for example, sequences referred to in Table I.

[0045] In one embodiment, a siNA molecule of the invention comprises no ribonucleotides. In another embodiment, a siNA molecule of the invention comprises ribonucleotides.

[0046] In one embodiment, a siNA molecule of the invention comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence of a BACE gene or a portion thereof, and the siNA further comprises a sense region comprising a nucleotide sequence substantially similar to the nucleotide sequence of the BACE gene or a portion thereof. In another embodiment, the

antisense region and the sense region each comprise about 18 to about 23 nucleotides and the antisense region comprises at least about 18 nucleotides that are complementary to nucleotides of the sense region. The BACE gene can comprise, for example, sequences referred to in Table I.

[0047] 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 of RNA encoded by a BACE gene, or a portion thereof, and the sense region comprises a nucleotide sequence that is complementary to the antisense region. In one embodiment, the siNA molecule 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. In another embodiment, the sense region is connected to the antisense region via a linker molecule, such as a nucleotide or non-nucleotide linker. The BACE gene can comprise, for example, sequences referred to in Table I.

[0048] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a BACE 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 BACE gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region, and wherein the siNA molecule has one or more modified pyrimidine and/or purine nucleotides. In one embodiment, the pyrimidine nucleotides in the sense region are 2'-O-methyl pyrimidine nucleotides or 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 in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides. In another embodiment, the pyrimidine nucleotides in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-deoxy purine nucleotides. In one embodiment, the pyrimidine nucleotides in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the antisense region are 2'-O-methyl or 2'-deoxy purine nucleotides. In another embodiment of any of the above-described siNA molecules, any nucleotides present in a non-complementary region of the sense strand (e.g. overhang region) are 2'-deoxy nucleotides.

[0049] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a BACE gene, wherein the siNA molecule 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, and wherein the fragment comprising 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 fragment. In one embodiment, the terminal cap moiety is an inverted deoxy abasic moiety or glyceryl moiety. In one embodiment, each of the two fragments of the siNA molecule comprise about 21 nucleotides.

[0050] In one embodiment, the invention features a siNA molecule comprising at least one modified nucleotide, wherein the modified nucleotide is a 2'-deoxy-2'-fluoro nucleotide. The siNA can be, for example, of length between about 12 and about 36 nucleotides. In one embodiment, all pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides. In one 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 one embodiment, all uridine nucleotides present in the siNA are 2'-deoxy-2'-fluoro uridine nucleotides. In one embodiment, all cytidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro cytidine nucleotides. In one embodiment, all adenosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro adenosine nucleotides. In one 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 one 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.

[0051] 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 one embodiment, all pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides. In one 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 one embodiment, all uridine nucleotides present in the siNA are 2'-deoxy-2'-fluoro uridine nucleotides. In one embodiment, all cytidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro cytidine nucleotides. In one embodiment, all adenosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro adenosine nucleotides. In one 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 one 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.

[0052] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a BACE 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 BACE gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region, and wherein the purine nucleotides present in the antisense region comprise 2'-deoxy-purine nucleotides. In an alternative embodiment, the purine nucleotides present in the antisense region comprise 2'-O-methyl purine nucleotides. In either of the above embodiments, the

antisense region can comprise a phosphorothioate internucleotide linkage at the 3' end of the antisense region. Alternatively, in either of the above embodiments, the antisense region can comprise 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.

[0053] In one embodiment, the antisense region of a siNA molecule of the invention comprises sequence complementary to a portion of a BACE transcript having sequence unique to a particular BACE disease related allele, such as sequence comprising a single nucleotide polymorphism (SNP) associated with the disease 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 against the disease, condition, or trait related allele.

[0054] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a BACE gene, wherein the siNA molecule 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 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 a 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 BACE gene. In another embodiment, about 21 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the BACE gene. In any of the above embodiments, the 5'-end of the fragment comprising said antisense region can optionally include a phosphate group.

[0055] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a BACE RNA sequence (e.g., wherein said target RNA sequence is encoded by a BACE gene involved in the BACE pathway), 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, such as 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.

[0056] In one embodiment, the invention features a chemically synthesized double stranded RNA molecule that directs

cleavage of a BACE 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 BACE RNA for the RNA molecule to direct cleavage of the BACE 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 nucleotides, 2'-O-methoxyethyl nucleotides etc.

[0057] In one embodiment, the invention features a medicament comprising a siNA molecule of the invention.

[0058] In one embodiment, the invention features an active ingredient comprising a siNA molecule of the invention.

[0059] In one embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siNA) molecule to down-regulate expression of a BACE 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., about 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, or 28 or more) nucleotides long.

[0060] In one embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a BACE 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 BACE 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.

[0061] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a BACE 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 BACE RNA or a portion thereof, wherein 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.

[0062] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a BACE 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 BACE RNA that encodes a protein or 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, each strand of the siNA molecule comprises about 18 to about 29 or more (e.g., about 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29 or

more) nucleotides, wherein each strand comprises at least about 18 nucleotides that are complementary to the nucleotides of the other strand. In one embodiment, the siNA molecule 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 one 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 a further 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'-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 still another 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'-deoxy purine nucleotides. In another embodiment, the antisense strand comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides and one or more 2'-O-methyl purine nucleotides. In another 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 a further embodiment the sense strand comprises a 3'-end and a 5'-end, wherein a terminal cap moiety (e.g., an inverted deoxy abasic moiety or inverted deoxy nucleotide moiety such as inverted thymidine) is present at the 5'-end, the 3'-end, or both of the 5' and 3' ends of the sense strand. In another 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.

[0063] In any of the above-described embodiments of a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a BACE gene, wherein a majority of the pyrimidine nucleotides present in the double-stranded siNA molecule comprises a sugar modification, each of the two strands of the siNA molecule can comprise about 21 nucleotides. In one embodiment, about 21 nucleotides of each strand of the siNA molecule are base-paired to the complementary nucleotides of the other strand of the siNA molecule. In another 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, wherein 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 another embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule is a 2'-deoxy-pyrimidine, such as 2'-deoxy-thymidine. In one 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 BACE RNA or a portion thereof. In one embodiment,

about 21 nucleotides of the antisense strand are base-paired to the nucleotide sequence of the BACE RNA or a portion thereof.

[0064] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a BACE 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 BACE 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 the 5'-end of the antisense strand optionally includes a phosphate group.

[0065] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a BACE 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 BACE 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 the nucleotide sequence or a portion thereof of the antisense strand is complementary to a nucleotide sequence of the untranslated region or a portion thereof of the BACE RNA.

[0066] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits expression of a BACE 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 BACE RNA or a portion thereof, wherein the other strand is a sense strand which comprises nucleotide sequence that is complementary to a nucleotide sequence of the antisense strand, 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 BACE RNA or a portion thereof that is present in the BACE RNA.

[0067] In one embodiment, the invention features a composition comprising a siNA molecule of the invention in a pharmaceutically acceptable carrier or diluent.

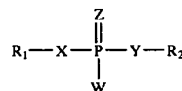
[0068] In a non-limiting example, the introduction of chemically-modified nucleotides into nucleic acid molecules provides a powerful tool in overcoming potential limitations of in vivo stability and bioavailability inherent to native RNA molecules that are delivered exogenously. For example, the use 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.

[0069] 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.

[0070] 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 a RNA or DNA sequence encoding BACE 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.

[0071] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against BACE 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:

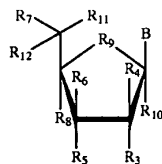


[0072] wherein each R1 and R2 is independently any nucleotide, non-nucleotide, or polynucleotide which can be naturally-occurring or chemically-modified, each X and Y is independently O, S, N, alkyl, or substituted alkyl, each Z and W is independently O,

S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, aralkyl, or acetyl 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).

[0073] 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.

[0074] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against BACE 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:

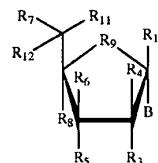


[0075] wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO₂, NO₂, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkyl-

lamino, substituted silyl, or group having Formula I or II; R9 is O, S, CH2, S=O, CHF, or CF2, 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.

[0076] 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 another 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.

[0077] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against BACE 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:



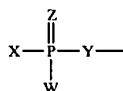
[0078] wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO₂, NO₂, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or group having Formula I or II; R9 is O, S, CH2, S=O, CHF, or CF2, 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.

[0079] 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 another 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.

[0080] 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.

[0081] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against BACE inside a cell or reconstituted in vitro system, wherein the chemical modification comprises a 5'-terminal phosphate group having Formula IV:



[0082] 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, alkylhalo, or acetyl; and wherein W, X, Y and Z are not all O.

[0083] 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.

[0084] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against BACE inside a cell or reconstituted in vitro system, wherein the chemical modification comprises one or more phosphorothioate 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 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.

[0085] 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.

[0086] 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.

[0087] 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.

[0088] 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.

[0089] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule having about 1 to about 5 or more (specifically about 1, 2, 3, 4, 5 or more) phosphorothioate internucleotide linkages in each strand of the siNA molecule.

[0090] 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.

[0091] 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.

[0092] 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 one embodiment, a linear hairpin siNA molecule of the invention comprises a loop portion comprising a non-nucleotide linker.

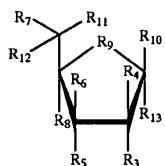
[0093] In another embodiment, a siNA molecule of the invention comprises an asymmetric hairpin structure, wherein the siNA is about 25 to about 50 (e.g., about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides in length having about 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 one 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.

[0094] 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 and 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 asymmetric 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).

[0095] 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 18, 19, 20, 21, 22, or 23) 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.

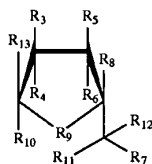
[0096] In another embodiment, a circular siNA molecule of the invention contains two loop motifs, wherein one or both loop portions of the siNA molecule is biodegradable. For example, a circular siNA molecule of the invention is designed such that degradation of the loop portions of the siNA molecule in vivo can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

[0097] 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:



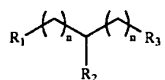
[0098] wherein each R3, R4, R5, R6, R7, R8, R10, R11, R12, and R13 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or group having Formula I or II; R9 is O, S, CH2, S=O, CHF, or CF2.

[0099] 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:



[0100] wherein each R3, R4, R5, R6, R7, R8, R10, R11, R12, and R13 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, 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, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or group having Formula I or II; R9 is O, S, CH2, S=O, CHF, or CF2, and either R2, R3, R8 or R13 serve as points of attachment to the siNA molecule of the invention.

[0101] 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:



[0102] wherein each n is independently an integer from 1 to 12, each R1, R2 and R3 is independently H, OH, alkyl,

substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, 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, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, or a group having Formula I, and R1, R2 or R3 serves as points of attachment to the siNA molecule of the invention.

[0103] In another embodiment, the invention features a compound having Formula VII, wherein R1 and R2 are hydroxyl (OH) groups, n=1, and R3 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 FIG. 10).

[0104] In another embodiment, a chemically modified nucleoside or non-nucleoside (e.g. 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, chemically modified nucleoside or non-nucleoside (e.g., 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 one embodiment, the chemically modified nucleoside or non-nucleoside (e.g., a moiety having Formula V, VI or VII) is present at the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. In one embodiment, the chemically modified nucleoside or non-nucleoside (e.g., a moiety having Formula V, VI or VII) is present at the terminal position of the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. In one embodiment, the chemically modified nucleoside or non-nucleoside (e.g., a moiety having Formula V, VI or VII) is present at the two terminal positions of the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. In one embodiment, the chemically modified nucleoside or non-nucleoside (e.g., a moiety having Formula V, VI or VII) is present at the penultimate position of the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. 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.

[0105] In another embodiment, a siNA molecule of the invention comprises an abasic residue having Formula V or VI, wherein the abasic residue having Formula VI 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.

[0106] 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.

[0107] In another embodiment, a siNA molecule of the invention comprises one or more (e.g., about 1, 2, 3, 4, 5, 6,

[0109] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising a sense region, wherein 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 wherein 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.

[0110] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising a sense region, wherein 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 wherein 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).

[0111] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising a sense region, wherein 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), wherein 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), and wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

[0113] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising an antisense region, wherein 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), 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), and wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said antisense region are 2'-deoxy nucleotides.

[0114] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising an antisense region, wherein 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'-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).

[0115] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising an antisense region, wherein 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).

[0116] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention capable of mediating RNA interference (RNAi) against BACE inside a cell or reconstituted in vitro system comprising a sense region, wherein 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 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 an antisense region, wherein 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 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). The sense region and/or the antisense region can have a terminal cap modification, such as any modification described herein or shown in FIG. 10, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense and/or antisense sequence. The sense and/or antisense region can optionally further comprise a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides. The overhang nucleotides can further comprise one or more (e.g., about 1, 2, 3, 4 or more) phosphorothioate, phosphonoacetate, and/or thiophosphonoacetate internucleotide linkages. Non-limiting examples of these chemically-modified siNAs are shown in FIGS. 4 and 5 and Tables III and IV herein. In any of these described embodiments, the purine nucleotides present in the sense region are alternatively 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 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). Also, in any of these embodiments, one or more purine nucleotides present in the sense region are alternatively purine ribonucleotides (e.g., wherein all purine nucleotides are purine ribonucleotides or alternately a plurality of purine nucleotides are purine ribonucleotides) and 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). Additionally, in any of these embodiments, one or more purine nucleotides present in the sense region and/or present in the antisense region are alternatively 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).

[0117] 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.

[0118] In one embodiment, the sense strand of a double stranded siNA molecule of the invention comprises a terminal cap moiety, (see for example FIG. 10) such as an inverted deoxyribose moiety, at the 3'-end, 5'-end, or both 3' and 5'-ends of the sense strand.

[0119] In one embodiment, the invention features a chemically-modified short interfering nucleic acid molecule (siNA) capable of mediating RNA interference (RNAi) against BACE 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., U.S. Ser. No. 10/427,160, filed Apr. 30, 2003, incorporated by reference herein in its entirety, including the drawings. 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. Ser. No. 10/201,394, filed Jul. 22, 2002 incorporated by reference herein. 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.

[0120] 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 about 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 generally known 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.)

[0121] 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; Jsche 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 Ferenz and Verdine, *J. Am. Chem. Soc.* 1991, 113:4000, all hereby incorporated by reference herein. 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.

[0122] 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 oligonucleotide 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 oligonucleotide 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 presence 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.

[0123] 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 comprising 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 18 to about 29 (e.g., about 18, 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.

[0124] 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 comprising a single stranded polynucleotide having complementarity to a target nucleic acid sequence, 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 FIG. 10, 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.

[0125] In one embodiment, the invention features a method for modulating the expression of a BACE 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 BACE gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the BACE gene in the cell.

[0126] In one embodiment, the invention features a method for modulating the expression of a BACE 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 BACE gene and wherein the sense strand sequence of the siNA comprises a sequence identical or 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 BACE gene in the cell.

[0127] In another embodiment, the invention features a method for modulating the expression of more than one BACE 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 BACE genes; and (b) introducing the siNA molecules into a cell under conditions suitable to modulate the expression of the BACE genes in the cell.

[0128] In another embodiment, the invention features a method for modulating the expression of two or more BACE genes within a cell comprising: (a) synthesizing one or more siNA molecules of the invention, which can be chemically-modified, wherein the siNA strands comprise sequences complementary to RNA of the BACE genes and wherein the sense strand sequences of the siNAs comprise sequences identical or substantially similar to the sequences of the target RNAs; and (b) introducing the siNA molecules into a cell under conditions suitable to modulate the expression of the BACE genes in the cell.

[0129] In another embodiment, the invention features a method for modulating the expression of more than one BACE 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 BACE gene and wherein the sense strand sequence of the siNA comprises a sequence identical or substantially similar to the sequences of the target RNAs; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the BACE genes in the cell.

[0130] In one embodiment, siNA molecules of the invention are used as reagents in ex vivo applications. For example, siNA reagents are introduced 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 targeting 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. In one embodiment, the invention features a method of modulating the expression of a BACE 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 BACE 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 BACE 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 BACE gene in that organism.

[0131] In one embodiment, the invention features a method of modulating the expression of a BACE 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 BACE gene and wherein the sense strand sequence of the siNA comprises a sequence identical or 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 BACE 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 BACE gene in that organism.

[0132] In another embodiment, the invention features a method of modulating the expression of more than one BACE 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 BACE 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 BACE 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 BACE genes in that organism.

[0133] In one embodiment, the invention features a method of modulating the expression of a BACE gene in a subject or 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 BACE gene; and (b) introducing the siNA molecule into the subject or organism under conditions suitable to modulate the expression of the BACE gene in the subject or organism. The level of BACE protein or RNA can be determined using various methods well-known in the art.

[0134] In another embodiment, the invention features a method of modulating the expression of more than one BACE gene in a subject or 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 BACE genes; and (b) introducing the siNA molecules into the subject or organism under conditions suitable to modulate the expression of the BACE genes in the subject or organism. The level of BACE protein or RNA can be determined as is known in the art.

[0135] In one embodiment, the invention features a method for modulating the expression of a BACE 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 BACE gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the BACE gene in the cell.

[0136] In another embodiment, the invention features a method for modulating the expression of more than one BACE 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 BACE gene; and (b) contacting the cell in vitro or in vivo with the siNA molecule under conditions suitable to modulate the expression of the BACE genes in the cell.

[0137] In one embodiment, the invention features a method of modulating the expression of a BACE 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 BACE gene; and (b) contacting a cell of the tissue explant derived from a particular subject or organism with the siNA molecule under conditions suitable to modulate the expression of the BACE gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the subject or organism the tissue was derived from or into another subject or organism under conditions suitable to modulate the expression of the BACE gene in that subject or organism.

[0138] In another embodiment, the invention features a method of modulating the expression of more than one BACE 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 BACE gene; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular subject or organism under conditions suitable to modulate the expression of the BACE genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the subject or organism the tissue was derived from or into another subject or organism under conditions suitable to modulate the expression of the BACE genes in that subject or organism.

[0139] In one embodiment, the invention features a method of modulating the expression of a BACE gene in a subject or 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 BACE gene; and (b) introducing the siNA molecule into the subject or organism under conditions suitable to modulate the expression of the BACE gene in the subject or organism.

[0140] In another embodiment, the invention features a method of modulating the expression of more than one BACE gene in a subject or 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 BACE gene; and (b) introducing the siNA molecules into the subject or organism under conditions suitable to modulate the expression of the BACE genes in the subject or organism.

[0141] In one embodiment, the invention features a method of modulating the expression of a BACE gene in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the BACE gene in the subject or organism.

[0142] In one embodiment, the invention features a method for treating Alzheimer's disease in a subject or

organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the BACE gene in the subject or organism.

[0143] In one embodiment, the invention features a method for treating neurodegenerative disorders or conditions, such as dementia, in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the BACE gene in the subject or organism.

[0144] In one embodiment, the invention features a method for treating stroke/cardiovascular accident in a subject or organism comprising contacting the subject or organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the BACE gene in the subject or organism.

[0145] In another embodiment, the invention features a method of modulating the expression of more than one BACE gene in a subject or organism comprising contacting the subject or organism with one or more siNA molecules of the invention under conditions suitable to modulate the expression of the BACE genes in the subject or organism.

[0146] The siNA molecules of the invention can be designed to down regulate or inhibit target (e.g., BACE) 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).

[0147] In another embodiment, the siNA molecules of the invention are used to target conserved sequences corresponding to a gene family or gene families such as BACE family genes. As such, siNA molecules targeting multiple BACE 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, Alzheimer's disease and other neurodegenerative disorders or conditions, such as dementia, and stroke/cardiovascular accident.

[0148] In one embodiment, siNA molecule(s) and/or methods of the invention are used to down regulate the expression of gene(s) that encode RNA referred to by Genbank Accession, for example, BACE genes encoding RNA sequence(s) referred to herein by Genbank Accession number, for example, Genbank Accession Nos. shown in Table I.

[0149] 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 18 to about 25 (e.g., about 18, 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.

[0150] 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 (e.g. 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 BACE 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 18 to about 25 (e.g., about 18, 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 BACE 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 BACE RNA sequence. The target BACE 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.

[0151] 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 18 to about 25 (e.g., about 18, 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.

[0152] 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.

[0153] 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.

[0154] 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 or condition in a subject comprising administering to the subject a composition of the invention under conditions suitable for the diagnosis of the disease or condition in the subject. In another embodiment, the invention features a method for treating or preventing a disease or condition in a subject, comprising administering to the subject a composition 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 yet another embodiment, the invention features a method for treating Alzheimer's disease and/or other neurodegenerative disorders, such as dementia and stroke/cardiovascular accident in a subject comprising administering to the subject a composition of the invention under conditions suitable for the treatment of Alzheimer's disease and/or other neurodegenerative disorders, such as dementia and stroke/cardiovascular accident in the subject.

[0155] In another embodiment, the invention features a method for validating a BACE 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 BACE target gene; (b) introducing the siNA molecule into a cell, tissue, subject or organism under conditions suitable for modulating expression of the BACE target gene in the cell, tissue, subject, or organism; and (c) determining the function of the gene by assaying for any phenotypic change in the cell, tissue, subject, or organism.

[0156] In another embodiment, the invention features a method for validating a BACE 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 BACE target gene; (b) introducing the siNA molecule into a biological system under conditions suitable for modulating expression of the BACE target gene in the biological system; and (c) determining the function of the gene by assaying for any phenotypic change in the biological system.

[0157] By "biological system" is meant, material, in a purified or unpurified form, from biological sources, including but not limited to human or animal, wherein the system comprises the components required for RNAi activity. The term "biological system" includes, for example, a cell, tissue, subject, or organism, or extract thereof. The term biological system also includes reconstituted RNAi systems that can be used in an in vitro setting.

[0158] 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.

[0159] 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 BACE target gene in a biological system, including, for example, in a cell, tissue, subject, 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 BACE target gene in a biological system, including, for example, in a cell, tissue, subject, or organism.

[0160] 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.

[0161] 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.

[0162] 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.

[0163] 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 as 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.

[0164] 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.

[0165] 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.

[0166] In another embodiment, the method of synthesis of siNA molecules of the invention comprises the teachings of Scaringe et al., U.S. Pat. Nos. 5,889,136; 6,008,400; and 6,111,086, incorporated by reference herein in their entirety.

[0167] In one embodiment, the invention features siNA constructs that mediate RNAi against BACE, 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.

[0168] 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.

[0169] In one embodiment, the invention features siNA constructs that mediate RNAi against BACE, 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.

[0170] In another embodiment, the invention features a method for generating siNA molecules with increased bind-

ing 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.

[0171] In one embodiment, the invention features siNA constructs that mediate RNAi against BACE, 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.

[0172] In one embodiment, the invention features siNA constructs that mediate RNAi against BACE, 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.

[0173] 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.

[0174] 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.

[0175] In one embodiment, the invention features siNA constructs that mediate RNAi against BACE, 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.

[0176] 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.

[0177] In one embodiment, the invention features chemically-modified siNA constructs that mediate RNAi against

BACE in a cell, 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.

[0178] In another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against BACE 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.

[0179] In yet another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against BACE 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.

[0180] In yet another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against BACE target DNA 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 DNA.

[0181] In one embodiment, the invention features siNA constructs that mediate RNAi against BACE, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the cellular uptake of the siNA construct.

[0182] In another embodiment, the invention features a method for generating siNA molecules against BACE 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.

[0183] In one embodiment, the invention features siNA constructs that mediate RNAi against BACE, 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. Ser. No. 10/201,394 incorporated by reference herein.

[0184] 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 aptam-

ers; 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.

[0185] 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 be recognized by cellular proteins that facilitate RNAi.

[0186] 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.

[0187] 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.

[0188] 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.

[0189] 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 one embodiment, the terminal cap moiety comprises an inverted abasic, inverted deoxy abasic, inverted nucleotide moiety, a group shown in FIG. 10, 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.

[0190] 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 one embodiment, each terminal cap moiety individually

comprises an inverted abasic, inverted deoxy abasic, inverted nucleotide moiety, a group shown in FIG. 10, 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.

[0191] 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 FIG. 10 (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 24/25" chemistries and variants thereof (see Table IV) wherein the 5'-end and 3'-end of the sense strand of the siNA do not comprise a hydroxyl group or phosphate group.

[0192] 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 activity. 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 24/25" chem-

istries and variants thereof (see Table IV) wherein the 5'-end and 3'-end of the sense strand of the siNA do not comprise a hydroxyl group or phosphate group.

[0193] In one embodiment, the invention features a method for screening siNA molecules that are active in mediating RNA interference against a target nucleic acid sequence comprising (a) generating a plurality of unmodified siNA molecules, (b) screening 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, and (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). In one embodiment, the method further comprises re-screening the chemically modified siNA molecules of step (c) under conditions suitable for isolating chemically modified siNA molecules that are active in mediating RNA interference against the target nucleic acid sequence.

[0194] In one embodiment, the invention features a method for screening chemically modified siNA molecules that are active in mediating RNA interference 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) screening 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.

[0195] 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 intercellular receptor. Interaction of the ligand with the receptor can result in a biochemical reaction, or can simply be a physical interaction or association.

[0196] 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.

[0197] 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.

[0198] 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).

[0199] 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., U.S. Pat. No. 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., U.S. Ser. No. 60/402,996). Such a kit can also include instructions to allow a user of the kit to practice the invention.

[0200] 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 FIGS. 4-6, and Tables II and III 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 embodiments, 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 linker molecules as is known in the art, or are alternately non-covalently linked by ionic interactions, hydrogen bonding, van der Waals interactions, hydrophobic interactions, 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 or methylation pattern 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).

[0201] In one embodiment, a siNA molecule of the invention is a duplex forming oligonucleotide "DFO", (see for example FIGS. 14-15 and Vaish et al., U.S. Ser. No. 10/727,780 filed Dec. 3, 2003 and International PCT Application No. US04/16390, filed May 24, 2004).

[0202] In one embodiment, a siNA molecule of the invention is a multifunctional siNA, (see for example FIGS. 16-21 and Jadhav et al., U.S. Ser. No. 60/543,480 filed Feb. 10, 2004 and International PCT Application No. US04/16390, filed May 24, 2004). The multifunctional siNA of the invention can comprise sequence targeting, for example, two regions of BACE RNA (see for example target sequences in Tables II and III).

[0203] 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 complementary 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, or about 19, 20, 21, or 22 nucleotides) and a loop region comprising about 4 to about 8 (e.g., about 4, 5, 6, 7, or 8) nucleotides, and a sense region 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) nucleotides that are complementary to the antisense region. The asymmetric hairpin siNA molecule can also comprise a 5'-terminal phosphate group that can be chemically modified. The loop portion of the asymmetric hairpin siNA molecule can comprise nucleotides, non-nucleotides, linker molecules, or conjugate molecules as described herein.

[0204] 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 complementary 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 (e.g. about 19, 20, 21, or 22) nucleotides and a sense region 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) nucleotides that are complementary to the antisense region.

[0205] 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.

[0206] 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, 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. In one embodiment, inhibition, down regulation, or reduction of gene expression is associated with post transcriptional silencing, such as RNAi mediated cleavage of a target nucleic acid molecule (e.g. RNA) or inhibition of translation. In one embodiment, inhibition, down regulation, or reduction of gene expression is associated with pretranscriptional silencing.

[0207] 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 (snoRNA), 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 a subject, 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. For a review, see for example Snyder and Gerstein, 2003, *Science*, 300, 258-260.

[0208] 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)-N-3-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 Ni-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.

[0209] By “BACE” or “beta secretase” as used herein is meant, BACE protein, peptide, or polypeptide having beta-secretase activity, such as that involved in generating beta-amyloid, for example, sequences encoded by BACE Genbank Accession Nos. shown in Table I. The term BACE also refers to nucleic acid sequences encoding any BACE protein, peptide, or polypeptide having BACE activity. The term “BACE” is also meant to include other BACE encoding sequence, such as BACE isoforms, mutant BACE genes, splice variants of BACE genes, and BACE gene polymorphisms.

[0210] By “APP” or “amyloid precursor protein” as used herein is meant any protein, peptide, or polypeptide that is processed to generate beta-amyloid. The term APP also refers to sequences that encode APP protein, for example, Genbank Accession Nos. shown in Table I. The term APP also refers to nucleic acid sequences encoding any APP protein, peptide, or polypeptide having APP activity. The term “APP” is also meant to include other APP encoding sequence, such as APP isoforms, mutant APP genes, splice variants of APP, and APP gene polymorphisms.

[0211] By “presenillin” or “PS”, i.e., “PS-1” or “PS-2”, or “PSEN”, i.e., “PSEN1” or “PSEN2”, as used herein is meant any protein, peptide, or polypeptide having gamma-secre-

tase activity, such as that involved in generating beta-amyloid. The term PS also refers to sequences that encode presenillin protein, for example, PS-1 or PS-2, (i.e., Genbank Accession Nos. shown in Table I). The term "PS", for example, "PS-1" or "PS-2", also refers to nucleic acid sequences encoding any PS protein, peptide, or polypeptide having PS activity. The term "PS", for example, "PS-1" or "PS-2", is also meant to include other PS encoding sequence, such as PS isoforms, mutant PS genes, splice variants of PS, and PS gene polymorphisms.

[0212] By "PIN-1" as used herein is meant any protein, peptide, or polypeptide having peptidyl-prolyl cis/trans isomerase activity, such as those involved in the development of Neurofibrillary Tangles. The term PIN-1 also refers to sequences that encode PIN-1 protein, i.e., Genbank Accession Nos. shown in Table I. The term PIN-1 also refers to nucleic acid sequences encoding any PIN-1 protein, peptide, or polypeptide having PIN-1 activity. The term "PIN-1" is also meant to include other PIN-1 encoding sequence, such as PIN-1 isoforms, mutant PIN-1 genes, splice variants of PIN-1, and PIN-1 gene polymorphisms.

[0213] Furthermore, as discussed previously, all embodiments, compositions, methods, and uses described herein using BACE as an exemplary gene are equally applicable to APP, PIN-1, and PS (i.e., PS-1, and PS-2) genes.

[0214] 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, different protein epitopes, different protein isoforms or completely divergent genes, such as a cytokine and its corresponding 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. 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.).

[0215] By "conserved sequence region" is meant, a nucleotide sequence of one or more regions in a polynucleotide does not vary significantly between generations or from one biological system, subject, or organism to another biological system, subject, or organism. The polynucleotide can include both coding and non-coding DNA and RNA.

[0216] 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.

[0217] 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.

[0218] 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 or RNA.

[0219] 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 oligonucleotide 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.

[0220] In one embodiment, siNA molecules of the invention that down regulate or reduce BACE gene expression are used for treating Alzheimer's disease in a subject or organism.

[0221] In one embodiment, the siNA molecules of the invention are used to treat neurodegenerative disorders or conditions, such as dementia, and stroke/cardiovascular accident in a subject or organism.

[0222] 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., about 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 II. Exemplary synthetic siNA molecules of the invention are shown in Table III and/or FIGS. 4-5.

[0223] 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.

[0224] 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 direct dermal application, transdermal application, or injection, with or without their incorporation in biopolymers. In particular embodiments, the nucleic acid molecules of the invention comprise sequences shown in Tables II-III and/or FIGS. 4-5. 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.

[0225] 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.

[0226] 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-ribofuranose 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.

[0227] 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.

[0228] 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.

[0229] 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.

[0230] 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.

[0231] 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).

[0232] 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.

[0233] The nucleic acid molecules of the instant invention, individually, or in combination or in conjunction with other drugs, can be used to for preventing or treating Alzheimer's disease and other neurodegenerative disorders or conditions, such as dementia and stroke/cardiovascular accident in a subject or organism.

[0234] For example, 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.

[0235] In a further embodiment, the siNA molecules can be used in combination with other known treatments to prevent or treat Alzheimer's disease and other neurodegenerative disorders or conditions, such as dementia and stroke/cardiovascular accident in a subject or organism. For example, the described molecules could be used in combination with one or more known compounds, treatments, or procedures to prevent or treat Alzheimer's disease and other neurodegenerative disorders or conditions, such as dementia and stroke/cardiovascular accident in a subject or organism as are known in the art.

[0236] In one embodiment, the invention features an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the invention, in a manner which allows expression of the siNA molecule. For example, the vector can contain sequence(s) encoding both strands of a siNA molecule comprising a duplex. The vector can also contain sequence(s) encoding a single nucleic acid molecule that is self-complementary and thus forms a siNA molecule. Non-limiting examples of such expression vectors are described in Paul et al., 2002, *Nature Biotechnology*, 19, 505; Miyagishi and Taira, 2002, *Nature Biotechnology*, 19, 497; Lee et al., 2002, *Nature Biotechnology*, 19, 500; and Novina et al., 2002, *Nature Medicine*, advance online publication doi: 10.1038/nm725.

[0237] In another embodiment, the invention features a mammalian cell, for example, a human cell, including an expression vector of the invention.

[0238] In yet another embodiment, the expression vector of the invention comprises a sequence for a siNA molecule having complementarity to a RNA molecule referred to by a Genbank Accession numbers, for example Genbank Accession Nos. shown in Table I.

[0239] In one embodiment, an expression vector of the invention comprises a nucleic acid sequence encoding two or more siNA molecules, which can be the same or different.

[0240] In another aspect of the invention, siNA molecules that interact with target RNA molecules and down-regulate gene encoding target RNA molecules (for example target RNA molecules referred to by Genbank Accession numbers herein) 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. Such vectors can be repeatedly administered as necessary. Once expressed, the siNA molecules bind and down-regulate gene function or expression via RNA interference (RNAi). Delivery of siNA expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from a subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell.

[0241] By "vectors" is meant any nucleic acid- and/or viral-based technique used to deliver a desired nucleic acid.

[0242] 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

[0243] FIG. 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.

[0244] FIG. 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.

[0245] FIG. 3 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 directly 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.

[0246] FIG. 4A-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.

[0247] FIG. 4A: 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.

[0248] FIG. 4B: 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.

[0249] FIG. 4C: 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.

[0250] FIG. 4D: 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 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.

[0251] FIG. 4E: 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.

[0252] FIG. 4F: 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 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 phosphorothio-

ate 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 FIG. 4A-F, the modified internucleotide linkage is optional.

[0253] FIG. 5A-F shows non-limiting examples of specific chemically-modified siNA sequences of the invention. A-F applies the chemical modifications described in FIG. 4A-F to a BACE siNA sequence. Such chemical modifications can be applied to any BACE sequence and/or BACE polymorphism sequence.

[0254] FIG. 6 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, preferably about 2 nucleotides. 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.

[0255] FIG. 7A-C is a diagrammatic representation of a scheme utilized in generating an expression cassette to generate siNA hairpin constructs.

[0256] FIG. 7A: A DNA oligomer is synthesized with a 5'-restriction site (R1) sequence followed by a region having sequence identical (sense region of siNA) to a predetermined BACE target sequence, wherein the sense region comprises, for example, about 19, 20, 21, or 22 nucleotides (N) in length, which is followed by a loop sequence of defined sequence (X), comprising, for example, about 3 to about 10 nucleotides.

[0257] FIG. 7B: The synthetic construct is then extended by DNA polymerase to generate a hairpin structure having self-complementary sequence that will result in a siNA transcript having specificity for a BACE target sequence and having self-complementary sense and antisense regions.

[0258] FIG. 7C: The construct is heated (for example to about 95° C.) to linearize the sequence, thus allowing

extension of a complementary second DNA strand using a primer to the 3'-restriction sequence of the first strand. The double-stranded DNA is then inserted into an appropriate vector for expression in cells. The construct can be designed such that a 3'-terminal nucleotide overhang results from the transcription, for example, by engineering restriction sites and/or utilizing a poly-U termination region as described in Paul et al., 2002, *Nature Biotechnology*, 29, 505-508.

[0259] FIG. 8A-C is a diagrammatic representation of a scheme utilized in generating an expression cassette to generate double-stranded siNA constructs.

[0260] FIG. 8A: A DNA oligomer is synthesized with a 5'-restriction (R1) site sequence followed by a region having sequence identical (sense region of siNA) to a predetermined BACE target sequence, wherein the sense region comprises, for example, about 19, 20, 21, or 22 nucleotides (N) in length, and which is followed by a 3'-restriction site (R2) which is adjacent to a loop sequence of defined sequence (X).

[0261] FIG. 8B: The synthetic construct is then extended by DNA polymerase to generate a hairpin structure having self-complementary sequence.

[0262] FIG. 8C: The construct is processed by restriction enzymes specific to R1 and R2 to generate a double-stranded DNA which is then inserted into an appropriate vector for expression in cells. The transcription cassette is designed such that a U6 promoter region flanks each side of the dsDNA which generates the separate sense and antisense strands of the siNA. Poly T termination sequences can be added to the constructs to generate U overhangs in the resulting transcript.

[0263] FIG. 9A-E 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.

[0264] FIG. 9A: 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.

[0265] FIGS. 9B&C: (FIG. 9B) The sequences are pooled and are inserted into vectors such that (FIG. 9C) transfection of a vector into cells results in the expression of the siNA.

[0266] FIG. 9D: Cells are sorted based on phenotypic change that is associated with modulation of the target nucleic acid sequence.

[0267] FIG. 9E: The siNA is isolated from the sorted cells and is sequenced to identify efficacious target sites within the target nucleic acid sequence.

[0268] FIG. 10 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.

[0269] FIG. 11 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 is 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.

[0270] FIG. 12 shows non-limiting examples of phosphorylated siNA molecules of the invention, including linear and duplex constructs and asymmetric derivatives thereof.

[0271] FIG. 13 shows non-limiting examples of chemically modified terminal phosphate groups of the invention.

[0272] FIG. 14A shows a non-limiting example of methodology used to design self complementary DFO constructs utilizing palindromic and/or repeat nucleic acid sequences that are identified 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 complementary DFO molecule comprising sequence complementary to the nucleic acid target. (iv) The DFO molecule can self-assemble to form a double stranded oligonucleotide. FIG. 14B shows a non-limiting representative example of a duplex forming oligonucleotide sequence. FIG. 14C shows a non-limiting example of the self assembly schematic of a representative duplex forming oligonucleotide sequence. FIG. 14D 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.

[0273] FIG. 15 shows a non-limiting example of the design of self complementary DFO constructs utilizing palindromic 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 palindromic/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 XXXYXY 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 complementary DFO comprising sequence complementary to the nucleic acid target. The DFO can self-assemble to form a double stranded oligonucleotide.

[0274] FIG. 16 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. FIG. 16A 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. FIG. 16B 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 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.

[0275] FIG. 17 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. FIG. 17A 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 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. FIG. 17B 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 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 FIG. 16.

[0276] FIG. 18 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 bifunctional siNA constructs that can mediate RNA interference against differing target nucleic acid sequences. FIG. 18A 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, 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. FIG. 18B 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 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.

[0277] FIG. 19 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 bifunctional siNA constructs that can mediate RNA interference against differing target nucleic acid sequences. FIG. 19A 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 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. FIG. 19B 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 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 FIG. 18.

[0278] FIG. 20 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 interference 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.

[0279] FIG. 21 shows a non-limiting example of how multifunctional siNA molecules of the invention can target two separate target nucleic acid sequences 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 interference 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.

[0280] FIG. 22 shows a non-limiting example of reduction of BACE mRNA levels in A549 cells after treatment with siNA molecules targeting BACE mRNA. A549 cells

were transfected with 0.25 μ g/well of lipid complexed with 25 nM siNA. A screen of siNA constructs comprising ribonucleotides and 3'-terminal dithymidine caps was compared to untreated cells, scrambled siNA control constructs (Scram 1 and Scram 2), and the cells transfected with lipid alone (transfection control). As shown in the Figure, all of the siNA constructs show significant reduction of BACE RNA expression.

[0281] FIG. 23 shows a non-limiting example of reduction of BACE mRNA levels in A549 cells (5,000 cells/well) 24 hours after treatment with siNA molecules targeting BACE mRNA. A549 cells were transfected with 0.25 μ g/well of lipid complexed with 25 nM siNA. A lead siNA construct (31007/31083) chosen from the screen described in FIG. 22 was further modified using chemical modifications described in Table IV herein. Chemically modified constructs having Stab 4/5 chemistry (31378/31381) and Stab 7/11 chemistry (31384/31387) (solid bars; see Tables III and IV) were tested for efficacy compared to matched chemistry inverted controls (open bars; sequences shown in Table III). The original lead siNA construct (31007/31083) and the Stab 4/5 and Stab 7/11 constructs were compared to untreated cells, scrambled siNA control constructs (Scram1 and Scram2), and cells transfected with lipid alone (transfection control). As shown in the figure, the original lead construct and the Stab 4/5 and Stab 7/11 modified siNA constructs all show significant reduction of BACE RNA expression.

[0282] FIG. 24 shows a non-limiting example of reduction of APP mRNA in SK-N-SH cells mediated by chemically modified siNAs that target APP mRNA. SK-N-SH cells were transfected with 0.25 μ g/well of lipid complexed with 25 nM siNA. Active siNA constructs comprising various stabilization chemistries (solid bars; see Tables III and IV) were compared to untreated cells, matched chemistry irrelevant siNA control constructs (IC1), and cells transfected with lipid alone (transfection control). As shown in the figure, the siNA constructs significantly reduce APP RNA expression.

[0283] FIG. 25 shows a non-limiting example of reduction of PSEN1 mRNA in SK-N-SH cells mediated by chemically modified siNAs that target PSEN1 mRNA. SK-N-SH cells were transfected with 0.25 μ g/well of lipid complexed with 25 nM siNA. Active siNA constructs comprising various stabilization chemistries (solid bars; see Tables III and IV) were compared to untreated cells, matched chemistry irrelevant siNA control constructs (IC1), and cells transfected with lipid alone (transfection control). As shown in the figure, the siNA constructs significantly reduce PSEN1 RNA expression.

[0284] FIG. 26 shows a non-limiting example of reduction of PSEN2 mRNA in SK-N-SH cells mediated by chemically modified siNAs that target PSEN2 mRNA. SK-N-SH cells were transfected with 0.25 μ g/well of lipid complexed with 25 nM siNA. Active siNA constructs comprising various stabilization chemistries (solid bars; see Tables III and IV) were compared to untreated cells, matched chemistry irrelevant siNA control constructs (IC1), and cells transfected with lipid alone (transfection control). As shown in the figure, the siNA constructs significantly reduce PSEN2 RNA expression.

DETAILED DESCRIPTION OF THE INVENTION

[0285] Mechanism of Action of Nucleic Acid Molecules of the Invention

[0286] 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 limiting only to siRNA 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.

[0287] RNA interference refers to the process of sequence specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (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.

[0288] 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 (siRNAs) 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 through 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.

[0289] 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.

[0290] Synthesis of Nucleic Acid Molecules

[0291] 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 mol-

ecules of the instant invention are chemically synthesized, and others can similarly be synthesized.

[0292] 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. All of these references are incorporated herein by reference. 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 μ mol 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 μ mol scale can be performed on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, Calif.) with minimal modification to the cycle. A 33-fold excess (60 μ L of 0.11 M=6.6 μ mol) of 2'-O-methyl phosphoramidite and a 105-fold excess of S-ethyl tetrazole (60 μ L of 0.25 M=15 μ mol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 22-fold excess (40 μ L of 0.11 M=4.4 μ mol) of deoxy phosphoramidite and a 70-fold excess of S-ethyl tetrazole (40 μ L of 0.25 M=10 μ mol) 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 calorimetric 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 I₂, 49 mM pyridine, 9% water in THF (PerSeptive Biosystems, Inc.). 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.

[0293] 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.

[0294] The method of synthesis used for RNA including certain siNA molecules of the invention follows the proce-

dures 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 μ mol 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 μ mol scale can be done on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, Calif.) with minimal modification to the cycle. A 33-fold excess (60 μ L of 0.11 M=6.6 μ mol) of 2'-O-methyl phosphoramidite and a 75-fold excess of S-ethyl tetrazole (60 μ L of 0.25 M=15 μ mol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 66-fold excess (120 μ L of 0.11 M=13.2 μ mol) of alkylsilyl (ribo) protected phosphoramidite and a 150-fold excess of S-ethyl tetrazole (120 μ L of 0.25 M=30 μ mol) 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 calorimetric 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 I₂, 49 mM pyridine, 9% water in THF (PerSeptive Biosystems, Inc.). 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.

[0295] 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 min. 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 μ L of a solution of 1.5 mL N-methylpyrrolidinone, 750 μ L 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 NH₄HCO₃.

[0296] 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 .

[0297] 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.

[0298] 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.

[0299] 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.

[0300] 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.

[0301] 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.

[0302] 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.

[0303] 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.

[0304] Optimizing Activity of the Nucleic Acid Molecule of the Invention.

[0305] 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.

[0306] 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., U.S. Ser. No. 60/082, 404 which was filed on Apr. 20, 1998; Karpeisky et al., 1998, *Tetrahedron Lett.*, 39, 1131; Eamshaw 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; all of the references are hereby incorporated in their totality by reference herein). 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, and are incorporated by reference herein. 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 in cells is not significantly inhibited.

[0307] 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.

[0308] 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 (incorporated by reference herein)) have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability, as described above.

[0309] 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).

[0310] 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.

[0311] 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.

[0312] The term "biodegradable" as used herein, refers to degradation in a biological system, for example, enzymatic degradation or chemical degradation.

[0313] 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.

[0314] The term "phospholipid" as used herein, refers to a hydrophobic molecule comprising at least one phosphorus group. For example, a phospholipid can comprise a phos-

phorus-containing group and saturated or unsaturated alkyl group, optionally substituted with OH, COOH, oxo, amine, or substituted or unsubstituted aryl groups.

[0315] 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.

[0316] 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.

[0317] Use of the nucleic acid-based molecules of the invention will lead to better treatments 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.

[0318] 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.

[0319] 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, incorporated by reference herein). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and may help in delivery and/or localization within a cell. The cap may be present at the 5'-terminus (5'-cap) or at the 3'-terminal (3'-cap) or may be present on both termini. In non-limiting examples, the 5'-cap includes, but is not limited to, glyceryl, inverted deoxy abasic residue (moiety); 4',5'-methylene nucleotide; 1-(beta-D-erythrofuransyl) nucleotide, 4'-thio nucleotide; carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; threo-pentofuransyl 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.

[0320] 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-erythrofuransyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate; 3-aminopropyl phosphate; 6-aminoethyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; threo-pentofuransyl 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; incorporated by reference herein).

[0321] 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.

[0322] An "alkyl" group refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably, it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkyl group can be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO₂ or N(CH₃)₂, amino, or SH. The term also includes alkenyl groups that are unsaturated hydrocarbon groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has 1 to 12 carbons. More preferably, it is a lower alkenyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkenyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO₂, halogen, N(CH₃)₂, amino, or SH. The term "alkyl" also includes alkynyl groups that have an unsaturated hydrocarbon group containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has 1 to 12 carbons. More preferably, it is a lower alkynyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkynyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO₂ or N(CH₃)₂, amino or SH.

[0323] Such alkyl groups can also include aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester groups. An "aryl" group refers to an aromatic group that has at least one ring having a conjugated pi electron system and includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which may be optionally substituted. 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 1 to 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.

[0324] 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*, all are hereby incorporated by reference herein). 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.

[0325] In one embodiment, the invention features modified siNA molecules, with phosphate backbone modifications comprising one or more phosphorothioate, 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.

[0326] 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.

[0327] 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.

[0328] 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.

[0329] In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant $2'\text{-NH}_2$ or $2'\text{-O—NH}_2$, 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, which are both incorporated by reference in their entireties.

[0330] 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.

[0331] Administration of Nucleic Acid Molecules

[0332] A siNA molecule of the invention can be adapted for use to prevent or treat a variety of neurodegenerative diseases, including Alzheimer's disease, dementia, stroke (CVA), or any other trait, disease or condition that is related to or will respond to the levels of BACE in a cell or tissue, alone or in combination with other therapies.

[0333] 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, all of which are incorporated herein by reference. 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 U.S. Pat. No. 6,447,796 and U.S. Patent Application Publication No. U.S. 2002130430), biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors (O'Hare and Normand, International PCT Publication No. WO 00/53722). 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.

[0334] In one embodiment, a siNA molecule of the invention is complexed with membrane disruptive agents such as

those described in U.S. Patent Application Publication No. 20010007666, incorporated by reference herein in its entirety including the drawings. 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. Pat. No. 6,235,310, incorporated by reference herein in its entirety including the drawings.

[0335] In one embodiment, a siNA molecule of the invention is complexed with delivery systems as described in U.S. Patent Application Publication No. 2003077829 and International PCT Publication Nos. WO 00/03683 and WO 02/087541, all incorporated by reference herein in their entirety including the drawings.

[0336] 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, *Pharmaceutical 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 Klivanov, 2002, *PNAS USA*, 99, 14640-14645; and Sagara, U.S. Pat. No. 6,586,524, incorporated by reference herein.

[0337] In one embodiment, a siNA molecule of the invention comprises a bioconjugate, for example a nucleic acid conjugate as described in Vargeese et al., U.S. Ser. No. 10/427,160, filed Apr. 30, 2003; U.S. Pat. No. 6,528,631; U.S. Pat. No. 6,335,434; U.S. Pat. No. 6,235,886; U.S. Pat. No. 6,153,737; U.S. Pat. No. 5,214,136; U.S. Pat. No. 5,138,045, all incorporated by reference herein.

[0338] 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 to 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 creams, gels, sprays, oils and other suitable compositions for topical, dermal, or transdermal administration as is known in the art.

[0339] 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.

[0340] A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, e.g., systemic or local 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.

[0341] In one embodiment, 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. Pharmacol.*, 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., U.S. Pat. No. 6,180,613 and Davidson, WO 04/013280, can be used to express nucleic acid molecules in the CNS.

[0342] 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 Broadus et al., 1998, *J. Neurosurg.*, 88(4), 734; Karle et al., 1997, *Eur. J. Pharmacol.*, 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.

[0343] 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., U.S. Pat. No. 6,180,613 and Davidson, WO 04/013280, can be used to express nucleic acid molecules in the CNS.

[0344] 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 polyvinylpyrrolidone). 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,N,N,N-tetramethyl-N,N,N,N-tetrapalmityl-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 Mannheim); and (4) Lipofectamine, 3:1 (M/M) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE (GIBCO BRL).

[0345] In one embodiment, siNA molecules of the invention are administered to a subject by systemic administration in a pharmaceutically acceptable composition or formulation. 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.

[0346] In one embodiment, siNA molecules of the invention are formulated or complexed with polyethylenimine (e.g., linear or branched PEI) and/or polycetylenimine 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, *AAIA PharmSci*, 3, 1-11; Furgeson et al., 2003, *Bioconjugate Chem.*, 14, 840-847; Kunath et al., 2002, *Pharmaceutical 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, U.S. Pat. No. 6,586,524, incorporated by reference herein.

[0347] By "pharmaceutically acceptable formulation" or "pharmaceutically acceptable composition" 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); biodegradable polymers, such as poly (DL-lactide-co-glycolide) microspheres for sustained release delivery (Emerich, D F et al, 1999, *Cell Transplant*, 8, 47-58); and loaded nanoparticles, such as those made of polybutylcyanoacrylate. 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.

[0348] 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.

[0349] 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), hereby incorporated by reference herein. 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.

[0350] 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.

[0351] 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.

[0352] 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 monostearate or glyceryl distearate can be employed.

[0353] 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.

[0354] 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.

[0355] 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.

[0356] 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.

[0357] 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.

[0358] 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.

[0359] 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.

[0360] 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.

[0361] 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.

[0362] 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.

[0363] 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.

[0364] 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.

[0365] Alternatively, certain siNA molecules of the instant invention can be expressed within cells from eukaryotic promoters (e.g., Izant and Weintraub, 1985, *Science*, 229, 345; McGarry and Lindquist, 1986, *Proc. Natl. Acad. Sci., USA* 83, 399; Scanlon et al., 1991, *Proc. Natl. Acad. Sci. USA*, 88, 10591-5; Kashani-Sabet et al., 1992, *Antisense Res. Dev.*, 2, 3-15; Dropulic et al., 1992, *J. Virol.*, 66, 1432-41; Weerasinghe et al., 1991, *J. Virol.*, 65, 5531-4; Ojwang et al., 1992, *Proc. Natl. Acad. Sci. USA*, 89, 10802-6; Chen et al., 1992, *Nucleic Acids Res.*, 20, 4581-9; Sarver et al., 1990 *Science*, 247, 1222-1225; Thompson et al., 1995, *Nucleic Acids Res.*, 23, 2259; Good et al., 1997, *Gene Therapy*, 4, 45. Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a enzymatic nucleic acid (Draper et al., PCT WO 93/23569, and Sullivan et al., PCT WO 94/02595; Ohkawa et al., 1992, *Nucleic Acids Symp. Ser.*, 27, 15-6; Taira et al., 1991, *Nucleic Acids Res.*, 19, 5125-30; Ventura et al., 1993, *Nucleic Acids Res.*, 21, 3249-55; Chowrira et al., 1994, *J. Biol. Chem.*, 269, 25856.

[0366] In another aspect of the invention, RNA molecules of the present invention can be expressed from transcription units (see for example Couture et al., 1996, *TIG.*, 12, 510) 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. In another embodiment, pol III based constructs are used to express nucleic acid molecules of the invention (see for example Thompson, U.S. Pats. Nos. 5,902,880 and 6,146,886). The recombinant vectors capable of expressing the siNA molecules can be delivered as described above, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of nucleic acid mol-

ecules. Such vectors can be repeatedly administered as necessary. Once expressed, the siNA molecule interacts with the target mRNA and generates an RNAi response. Delivery of siNA molecule expressing vectors can be systemic, such as by intravenous or intra-muscular administration, by administration to target cells ex-planted from a subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell (for a review see Couture et al., 1996, *TIG.*, 12, 510).

[0367] In one aspect the invention features an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the instant invention. The expression vector can encode one or both strands of a siNA duplex, or a single self-complementary strand that self hybridizes into a siNA duplex. The nucleic acid sequences encoding the siNA molecules of the instant invention can be operably linked in a manner that allows expression of the siNA molecule (see for example Paul et al., 2002, *Nature Biotechnology*, 19, 505; Miyagishi and Taira, 2002, *Nature Biotechnology*, 19, 497; Lee et al., 2002, *Nature Biotechnology*, 19, 500; and Novina et al., 2002, *Nature Medicine*, advance online publication doi:10.1038/nm725).

[0368] In another aspect, the invention features an expression vector comprising: a) a transcription initiation region (e.g., eukaryotic pol I, II or III initiation region); b) a transcription termination region (e.g., eukaryotic pol I, II or III termination region); and c) a nucleic acid sequence encoding at least one of the siNA molecules of the instant invention, wherein said sequence is operably linked to said initiation region and said termination region in a manner that allows expression and/or delivery of the siNA molecule. The vector can optionally include an open reading frame (ORF) for a protein operably linked on the 5' side or the 3'-side of the sequence encoding the siNA of the invention; and/or an intron (intervening sequences).

[0369] Transcription of the siNA molecule sequences can be driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters are expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type depends on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990, *Proc. Natl. Acad. Sci. USA*, 87, 6743-7; Gao and Huang 1993, *Nucleic Acids Res.*, 21, 2867-72; Lieber et al., 1993, *Methods Enzymol.*, 217, 47-66; Zhou et al., 1990, *Mol. Cell. Biol.*, 10, 4529-37). Several investigators have demonstrated that nucleic acid molecules expressed from such promoters can function in mammalian cells (e.g. Kashani-Sabet et al., 1992, *Antisense Res. Dev.*, 2, 3-15; Ojwang et al., 1992, *Proc. Natl. Acad. Sci. USA*, 89, 10802-6; Chen et al., 1992, *Nucleic Acids Res.*, 20, 4581-9; Yu et al., 1993, *Proc. Natl. Acad. Sci. USA*, 90, 6340-4; L'Huillier et al., 1992, *EMBO J.*, 11, 4411-8; Lisiewicz et al., 1993, *Proc. Natl. Acad. Sci. U S A*, 90, 8000-4; Thompson et al., 1995, *Nucleic Acids Res.*, 23, 2259; Sul-lenger & Cech, 1993, *Science*, 262, 1566). More specifically, transcription units such as the ones derived from genes encoding U6 small nuclear (snRNA), transfer RNA (tRNA) and adenovirus VA RNA are useful in generating high

concentrations of desired RNA molecules such as siNA in cells (Thompson et al., supra; Couture and Stinchcomb, 1996, supra; Noonberg et al., 1994, *Nucleic Acid Res.*, 22, 2830; Noonberg et al., U.S. Pat. No. 5,624,803; Good et al., 1997, *Gene Ther.*, 4, 45; Beigelman et al., International PCT Publication No. WO 96/18736. The above siNA transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, 1996, supra).

[0370] In another aspect the invention features an expression vector comprising a nucleic acid sequence encoding at least one of the siNA molecules of the invention in a manner that allows expression of that siNA molecule. The expression vector comprises in one embodiment; a) a transcription initiation region; b) a transcription termination region; and c) a nucleic acid sequence encoding at least one strand of the siNA molecule, wherein the sequence is operably linked to the initiation region and the termination region in a manner that allows expression and/or delivery of the siNA molecule.

[0371] In another embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an open reading frame; and d) a nucleic acid sequence encoding at least one strand of a siNA molecule, wherein the sequence is operably linked to the 3'-end of the open reading frame and wherein the sequence is operably linked to the initiation region, the open reading frame and the termination region in a manner that allows expression and/or delivery of the siNA molecule. In yet another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; and d) a nucleic acid sequence encoding at least one siNA molecule, wherein the sequence is operably linked to the initiation region, the intron and the termination region in a manner which allows expression and/or delivery of the nucleic acid molecule.

[0372] In another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) an open reading frame; and e) a nucleic acid sequence encoding at least one strand of a siNA molecule, wherein the sequence is operably linked to the 3'-end of the open reading frame and wherein the sequence is operably linked to the initiation region, the intron, the open reading frame and the termination region in a manner which allows expression and/or delivery of the siNA molecule.

[0373] BACE, APP, PIN-1 and PS Biology and Biochemistry

[0374] Alzheimer's disease is characterized by the progressive formation of insoluble plaques and vascular deposits in the brain consisting of the 4 kD amyloid β peptide ($A\beta$). These plaques are characterized by dystrophic neurites that show profound synaptic loss, neurofibrillary tangle formation, and gliosis. $A\beta$ arises from the proteolytic cleavage of the large type I transmembrane protein, β -amyloid precursor protein (APP) (Kang et al., 1987, *Nature*, 325, 733). Processing of APP to generate $A\beta$ requires two sites of cleavage by a β -secretase and a γ -secretase. β -secretase cleavage of APP results in the cytoplasmic release of a 100 kD soluble amino-terminal fragment, APPs β , leaving behind

a 12 kD transmembrane carboxy-terminal fragment, C99. Alternately, APP can be cleaved by a α -secretase to generate cytoplasmic APP α and transmembrane C83 fragments. Both remaining transmembrane fragments, C99 and C83, can be further cleaved by a γ -secretase, leading to the release and secretion of Alzheimer's related A β and a non-pathogenic peptide, p3, respectively (Vassar et al., 1999, *Science*, 286, 735-741). Early onset familial Alzheimer's disease is characterized by mutant APP protein with a Met to Leu substitution at position P1, characterized as the "Swedish" familial mutation (Mullan et al., 1992, *Nature Genet.*, 1, 345). This APP mutation is characterized by a dramatic enhancement in β -secretase cleavage (Citron et al., 1992, *Nature*, 360, 672).

[0375] The identification of β -secretase and γ -secretase constituents involved in the release of β -amyloid protein is of primary importance in the development of treatment strategies for Alzheimer's disease. Characterization of α -secretase is also important in this regard since α -secretase cleavage may compete with β -secretase cleavage resulting in changes in the relative amounts of non-pathogenic and pathogenic protein production. Involvement of the two metalloproteases, ADAM 10 and TACE, has been demonstrated in α -cleavage of AAP (Buxbaum et al., 1999, *J. Biol. Chem.*, 273, 27765, and Lammich et al., 1999, *Proc. Natl. Acad. Sci. U.S.A.*, 96, 3922). Studies of γ -secretase activity have demonstrated presenilin dependence (De Strooper et al., 1998, *Nature*, 391, 387, and De Strooper et al., 1999, *Nature*, 398, 518), and as such, presenilins have been proposed as γ -secretase even though presenilin does not present proteolytic activity (Wolfe et al., 1999, *Nature*, 398, 513).

[0376] Studies have shown β -secretase cleavage of AAP by the transmembrane aspartic protease beta site APP cleaving enzyme, BACE (Vassar et al., supra). While other potential candidates for β -secretase have been proposed (for review see Evin et al., 1999, *Proc. Natl. Acad. Sci. U.S.A.*, 96, 3922), none have demonstrated the full range of characteristics expected from this enzyme. Studies have shown that BACE expression and localization are as expected for β -secretase, that BACE overexpression in cells results in increased β -secretase cleavage of APP and Swedish APP, that isolated BACE demonstrates site specific proteolytic activity on APP derived peptide substrates, and that antisense mediated endogenous BACE inhibition results in dramatically reduced β -secretase activity (Vassar et al., supra).

[0377] Current treatment strategies for Alzheimer's disease rely on either the prevention or the alleviation of symptoms and/or the slowing down of disease progression. Two drugs approved in the treatment of Alzheimer's, donepezil (Aricept®) and tacrine (Cognex®), both cholinomimetics, attempt to slow the loss of cognitive ability by increasing the amount of acetylcholine available to the brain. Antioxidant therapy through the use of antioxidant compounds such as alpha-tocopherol (vitamin E), melatonin, and selegiline (Eldepryl®) attempt to slow disease progression by minimizing free radical damage. Estrogen replacement therapy is thought to incur a possible preventative benefit in the development of Alzheimer's disease based on limited data. The use of anti-inflammatory drugs may be associated with a reduced risk of Alzheimer's as well. Calcium channel blockers such as Nimodipine® are considered to have a potential benefit in treating Alzheimer's

disease due to protection of nerve cells from calcium overload, thereby prolonging nerve cell survival. Nootropic compounds, such as acetyl-L-carnitine (Alcar®) and insulin, have been proposed to have some benefit in treating Alzheimer's due to enhancement of cognitive and memory function based on cellular metabolism.

[0378] Whereby the above treatment strategies can all improve quality of life in Alzheimer's patients, there exists an unmet need in the comprehensive treatment and prevention of this disease. As such, there exists the need for therapeutics effective in reversing the physiological changes associated with Alzheimer's disease, specifically, therapeutics that can eliminate and/or reverse the deposition of amyloid β peptide. The use of compounds, such as small nucleic acid molecules (e.g., 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)), to modulate the expression of proteases that are instrumental in the release of amyloid β peptide, namely β -secretase (BACE), γ -secretase (presenilin), and the amyloid precursor protein (APP), is of therapeutic significance.

EXAMPLES

[0379] 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

[0380] 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.

[0381] 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.

[0382] 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 FIG. 1) or an equivalent cleavable linker. A non-limiting example of linker coupling conditions that can be used includes a hindered base such as diisopropylethy-

amine (DIPA) and/or DMAP in the presence of an activator reagent such as Bromotripyrrolidinophosphoniumhexafluorophosphate (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 50 mM NaOAc or 1.5M $\text{NH}_4\text{H}_2\text{CO}_3$.

[0383] Purification of the siNA duplex can be readily accomplished using solid phase extraction, for example, using a Waters C18 SepPak 1 g cartridge conditioned with 1 column volume (CV) of acetonitrile, 2 CV H_2O , and 2 CV 50 mM NaOAc. The sample is loaded and then washed with 1 CV H_2O or 50 mM NaOAc. Failure sequences are eluted with 1 CV 14% ACN (Aqueous with 50 mM NaOAc and 50 mM 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.

[0384] FIG. 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 construct 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

Identification of Potential siNA Target Sites in Any RNA Sequence

[0385] 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 or condition 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 to determine efficient reduction in target gene expression.

Example 3

Selection of siNA Molecule Target Sites in a RNA

[0386] The following non-limiting steps can be used to carry out the selection of siNAs targeting a given gene sequence or transcript.

[0387] 1. 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.

[0388] 2. 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.

[0389] 3. 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.

[0390] 4. 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.

[0391] 5. 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.

[0392] 6. 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 whenever better sequences are available. CCC is searched in the target strand because that will place GGG in the antisense strand.

[0393] 7. 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.

[0394] 8. 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 II and III). 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.

[0395] 9. 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.

[0396] 10. Other design considerations can be used when selecting target nucleic acid sequences, see, for example, Reynolds et al., 2004, *Nature Biotechnology Advanced Online Publication*, 1 Feb. 2004, doi:10.1038/nbt936 and Ui-Tei et al., 2004, *Nucleic Acids Research*, 32, doi:10.1093/nar/gkh247.

[0397] In an alternate approach, a pool of siNA constructs specific to a BACE target sequence is used to screen for target sites in cells expressing BACE RNA, such as cultured A549 cells, 7PA2 cells, Chinese hamster ovary (CHO) cells, or APPsw (Swedish type amyloid precursor protein expressing) cells. The general strategy used in this approach is shown in FIG. 9. A non-limiting example of such is a pool comprising sequences having any of SEQ ID NOS 1-1900. Cells expressing BACE (e.g., A549 cells) are transfected with the pool of siNA constructs and cells that demonstrate a phenotype associated with BACE inhibition are sorted. The pool of siNA constructs can be expressed from transcription cassettes inserted into appropriate vectors (see for example FIG. 7 and FIG. 8). The siNA from cells demonstrating a positive phenotypic change (e.g., decreased proliferation, decreased BACE mRNA levels or decreased BACE protein expression), are sequenced to determine the most suitable target site(s) within the target BACE RNA sequence.

Example 4

BACE Targeted siNA Design

[0398] siNA target sites were chosen by analyzing sequences of the BACE RNA target and optionally priori-

tizing 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 3, or alternately by using an in vitro siNA system as described in Example 6 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.

[0399] 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 quantify 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 FIG. 11).

Example 5

Chemical Synthesis and Purification of siNA

[0400] 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 complementary to the target sequence. Generally, siNA constructs can be synthesized using solid phase oligonucleotide synthesis methods as described herein (see for example Usman et al., U.S. Pat. Nos. 5,804,683; 5,831,071; 5,998,203; 6,117,657; 6,353,098; 6,362,323; 6,437,117; 6,469,158; Scaringe et al., U.S. Pat. Nos. 6,111,086; 6,008,400; 6,111,086 all incorporated by reference herein in their entirety).

[0401] In a non-limiting example, RNA oligonucleotides are synthesized in a stepwise fashion using the phosphoramidite chemistry as is known in the art. Standard phosphoramidite chemistry involves the use of nucleosides comprising any of 5'-O-dimethoxytrityl, 2'-O-tert-butyl dimethylsilyl,

3'-O-2-Cyanoethyl N,N-diisopropylphosphoramidite groups, and exocyclic amine protecting groups (e.g. N6-benzoyl adenosine, N4 acetyl cytidine, and N2-isobutyryl guanosine). Alternately, 2'-O-Silyl Ethers can be used in conjunction with acid-labile 2'-O-orthoester protecting groups in the synthesis of RNA as described by Scaringe supra. Differing 2' chemistries can require different protecting groups, for example 2'-deoxy-2'-amino nucleosides can utilize N-phthaloyl protection as described by Usman et al., U.S. Pat. No. 5,631,360, incorporated by reference herein in its entirety).

[0402] During solid phase synthesis, each nucleotide is added sequentially (3' to 5'-direction) to the solid support-bound oligonucleotide. The first nucleoside at the 3'-end of the chain is covalently attached to a solid support (e.g., controlled pore glass or polystyrene) using various linkers. The nucleotide precursor, a ribonucleoside phosphoramidite, and activator are combined resulting in the coupling of the second nucleoside phosphoramidite onto the 5'-end of the first nucleoside. The support is then washed and any unreacted 5'-hydroxyl groups are capped with a capping reagent such as acetic anhydride to yield inactive 5'-acetyl moieties. The trivalent phosphorus linkage is then oxidized to a more stable phosphate linkage. At the end of the nucleotide addition cycle, the 5'-O-protecting group is cleaved under suitable conditions (e.g., acidic conditions for trityl-based groups and Fluoride for silyl-based groups). The cycle is repeated for each subsequent nucleotide.

[0403] Modification of synthesis conditions can be used to optimize coupling efficiency, for example by using differing coupling times, differing reagent/phosphoramidite concentrations, differing contact times, differing solid supports and solid support linker chemistries depending on the particular chemical composition of the siNA to be synthesized. Deprotection and purification of the siNA can be performed as is generally described in Deprotection and purification of the siNA can be performed as is generally described in Usman et al., U.S. Pat. No. 5,831,071, U.S. Pat. No. 6,353,098, U.S. Pat. No. 6,437,117, and Bellon et al., U.S. Pat. No. 6,054,576, U.S. Pat. No. 6,162,909, U.S. Pat. No. 6,303,773, or Scaringe supra, incorporated by reference herein in their entirety. Additionally, deprotection conditions can be modified to provide the best possible yield and purity of siNA constructs. For example, applicant has observed that oligonucleotides comprising 2'-deoxy-2'-fluoro nucleotides can degrade under inappropriate deprotection conditions. Such oligonucleotides are deprotected using aqueous methylamine at about 35° C. for 30 minutes. If the 2'-deoxy-2'-fluoro containing oligonucleotide also comprises ribonucleotides, after deprotection with aqueous methylamine at about 35° C. for 30 minutes, TEA-HF is added and the reaction maintained at about 65° C. for an additional 15 minutes.

Example 6

RNAi in Vitro Assay to Assess siNA Activity

[0404] An in vitro assay that recapitulates RNAi in a cell-free system is used to evaluate siNA constructs targeting BACE RNA targets. The assay comprises the system described by Tuschl et al., 1999, *Genes and Development*, 13, 3191-3197 and Zamore et al., 2000, *Cell*, 101, 25-33 adapted for use with BACE target RNA. A *Drosophila*

extract derived from syncytial blastoderm is used to reconstitute RNAi activity in vitro. Target RNA is generated via in vitro transcription from an appropriate BACE expressing plasmid using T7 RNA polymerase or via chemical synthesis as described herein. Sense and antisense siNA strands (for example 20 uM each) are annealed by incubation in buffer (such as 100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) for 1 minute at 90° C. followed by 1 hour at 37° C., then diluted in lysis buffer (for example 100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate). Annealing can be monitored by gel electrophoresis on an agarose gel in TBE buffer and stained with ethidium bromide. The *Drosophila* lysate is prepared using zero to two-hour-old embryos from Oregon R flies collected on yeasted molasses agar that are dechorionated and lysed. The lysate is centrifuged and the supernatant isolated. The assay comprises a reaction mixture containing 50% lysate [vol/vol], RNA (10-50 pM final concentration), and 10% [vol/vol] lysis buffer containing siNA (10 nM final concentration). The reaction mixture also contains 10 mM creatine phosphate, 10 ug/ml creatine phosphokinase, 100 uM GTP, 100 uM UTP, 100 uM CTP, 500 uM ATP, 5 mM DTT, 0.1 U/uL RNasin (Promega), and 100 uM of each amino acid. The final concentration of potassium acetate is adjusted to 100 mM. The reactions are pre-assembled on ice and preincubated at 25° C. for 10 minutes before adding RNA, then incubated at 25° C. for an additional 60 minutes. Reactions are quenched with 4 volumes of 1.25xPassive Lysis Buffer (Promega). Target RNA cleavage is assayed by RT-PCR analysis or other methods known in the art and are compared to control reactions in which siNA is omitted from the reaction.

[0405] Alternately, internally-labeled target RNA for the assay is prepared by in vitro transcription in the presence of [α -³²P] CTP, passed over a G50 Sephadex column by spin chromatography and used as target RNA without further purification. Optionally, target RNA is 5'-³²P-end labeled using T4 polynucleotide kinase enzyme. Assays are performed as described above and target RNA and the specific RNA cleavage products generated by RNAi are visualized on an autoradiograph of a gel. The percentage of cleavage is determined by PHOSPHOR IMAGER® (autoradiography) quantitation of bands representing intact control RNA or RNA from control reactions without siNA and the cleavage products generated by the assay.

[0406] In one embodiment, this assay is used to determine target sites in the BACE RNA target for siNA mediated RNAi cleavage, wherein a plurality of siNA constructs are screened for RNAi mediated cleavage of the BACE RNA target, for example, by analyzing the assay reaction by electrophoresis of labeled target RNA, or by northern blotting, as well as by other methodology well known in the art.

Example 7

Nucleic Acid Inhibition of BACE Target RNA

[0407] siNA molecules targeted to the human BACE RNA are designed and synthesized as described above. These nucleic acid molecules can be tested for cleavage activity in vivo, for example, using the following procedure. The target sequences and the nucleotide location within the BACE RNA are given in Tables II and III.

[0408] Two formats are used to test the efficacy of siNAs targeting BACE. First, the reagents are tested in cell culture

using, for example, cultured A549 cells, 7PA2 cells, Chinese hamster ovary (CHO) cells, APPsw (Swedish type amyloid precursor protein expressing) cells, or SK-N-SH cells, to determine the extent of RNA and protein inhibition. siNA reagents (e.g.; see Tables II and III) are selected against the BACE target as described herein. RNA inhibition is measured after delivery of these reagents by a suitable transfection agent to, for example, A549 cells, 7PA2 cells, CHO cells, APPsw cells, or SK-N-SH cells. Relative amounts of target RNA are measured versus actin using real-time PCR monitoring of amplification (e.g., ABI 7700 TAQMAN®). A comparison is made to a mixture of oligonucleotide sequences made to unrelated targets or to a randomized siNA control with the same overall length and chemistry, but randomly substituted at each position. Primary and secondary lead reagents are chosen for the target and optimization performed. After an optimal transfection agent concentration is chosen, a RNA time-course of inhibition is performed with the lead siNA molecule. In addition, a cell-plating format can be used to determine RNA inhibition.

[0409] Delivery of siNA to Cells

[0410] Cells (e.g., A549 cells, 7PA2 cells, CHO cells, APPsw cells, or SK-N-SH cells) are seeded, for example, at 1×10^5 cells per well of a six-well dish in EGM-2 (BioWhittaker) the day before transfection. siNA (final concentration, for example 20 nM) and cationic lipid (e.g., final concentration 2 $\mu\text{g}/\text{ml}$) are complexed in EGM basal media (BioWhittaker) at 37° C. for 30 minutes in polystyrene tubes. Following vortexing, the complexed siNA is added to each well and incubated for the times indicated. For initial optimization experiments, cells are seeded, for example, at 1×10^5 in 96 well plates and siNA complex added as described. Efficiency of delivery of siNA to cells is determined using a fluorescent siNA complexed with lipid. Cells in 6-well dishes are incubated with siNA for 24 hours, rinsed with PBS and fixed in 2% paraformaldehyde for 15 minutes at room temperature. Uptake of siNA is visualized using a fluorescent microscope.

[0411] TAQMAN® (Real-Time PCR Monitoring of Amplification) and Lightcycler Quantification of mRNA

[0412] Total RNA is prepared from cells following siNA delivery, for example, using Qiagen RNA purification kits for 6-well or RNeasy extraction kits for 96-well assays. For TAQMAN® analysis (real-time PCR monitoring of amplification), dual-labeled probes are synthesized with the reporter dye, FAM or JOE, covalently linked at the 5'-end and the quencher dye TAMRA conjugated to the 3'-end. One-step RT-PCR amplifications are performed on, for example, an ABI PRISM 7700 Sequence Detector using 50 μl reactions consisting of 10 μl total RNA, 100 nM forward primer, 900 nM reverse primer, 100 nM probe, 1x TaqMan PCR reaction buffer (PE-Applied Biosystems), 5.5 mM MgCl_2 , 300 μM each dATP, dCTP, dGTP, and dTTP, 10U RNase Inhibitor (Promega), 1.25U AMPLITAQ GOLD® (DNA polymerase) (PE-Applied Biosystems) and 10U M-MLV Reverse Transcriptase (Promega). The thermal cycling conditions can consist of 30 minutes at 48° C., 10 minutes at 95° C., followed by 40 cycles of 15 seconds at 95° C. and 1 minute at 60° C. Quantitation of mRNA levels is determined relative to standards generated from serially diluted total cellular RNA (300, 100, 33, 11 ng/rxn) and normalizing to β -actin or GAPDH mRNA in parallel TAQ-

MAN® reactions (real-time PCR monitoring of amplification). For each gene of interest an upper and lower primer and a fluorescently labeled probe are designed. Real time incorporation of SYBR Green I dye into a specific PCR product can be measured in glass capillary tubes using a lightcycler. A standard curve is generated for each primer pair using control cRNA. Values are represented as relative expression to GAPDH in each sample.

[0413] Western Blotting

[0414] Nuclear extracts can be prepared using a standard micro preparation technique (see for example Andrews and Faller, 1991, *Nucleic Acids Research*, 19, 2499). Protein extracts from supernatants are prepared, for example using TCA precipitation. An equal volume of 20% TCA is added to the cell supernatant, incubated on ice for 1 hour and pelleted by centrifugation for 5 minutes. Pellets are washed in acetone, dried and resuspended in water. Cellular protein extracts are run on a 10% Bis-Tris NuPage (nuclear extracts) or 4-12% Tris-Glycine (supernatant extracts) polyacrylamide gel and transferred onto nitro-cellulose membranes. Non-specific binding can be blocked by incubation, for example, with 5% non-fat milk for 1 hour followed by primary antibody for 16 hour at 4° C. Following washes, the secondary antibody is applied, for example (1:10,000 dilution) for 1 hour at room temperature and the signal detected with SuperSignal reagent (Pierce).

Example 8

Models Useful to Evaluate the Down-Regulation of BACE Gene Expression

[0415] Cell Culture

[0416] Vassar et al., 1999, *Science*, 286, 735-741, describe a cell culture model for studying BACE inhibition. Specific antisense nucleic acid molecules targeting BACE mRNA were used for inhibition studies of endogenous BACE expression in 101 cells and APPsw (Swedish type amyloid precursor protein expressing) cells via lipid mediated transfection. Antisense treatment resulted in dramatic reduction of both BACE mRNA by Northern blot analysis, and APPsβsw ("Swedish" type β -secretase cleavage product) by ELISA, with maximum inhibition of both parameters at 75-80%. This model was also used to study the effect of BACE inhibition on amyloid β -peptide production in APPsw cells. Similarly, such a model can be used to screen siRNA molecules of the instant invention for efficacy and potency.

[0417] In several cell culture systems, cationic lipids have been shown to enhance the bioavailability of oligonucleotides to cells in culture (Bennet, et al., 1992, *Mol. Pharmacology*, 41, 1023-1033). In one embodiment, siNA molecules of the invention are complexed with cationic lipids for cell culture experiments. siNA and cationic lipid mixtures are prepared in serum-free DMEM immediately prior to addition to the cells. DMEM plus additives are warmed to room temperature (about 20-25° C.) and cationic lipid is added to the final desired concentration and the solution is vortexed briefly. siNA molecules are added to the final desired concentration and the solution is again vortexed briefly and incubated for 10 minutes at room temperature. In dose response experiments, the RNA/lipid complex is serially diluted into DMEM following the 10 minute incubation.

[0418] Animal Models

[0419] Evaluating the efficacy of anti-BACE agents in animal models is an important prerequisite to human clinical trials. Games et al., 1995, *Nature*, 373, 523-527, describe a transgenic mouse model in which mutant human familial type APP (Phe 717 instead of Val) is overexpressed. This model results in mice that progressively develop many of the pathological hallmarks of Alzheimer's disease, and as such, provides a model for testing therapeutic drugs, including siNA constructs of the instant invention.

Example 9**RNAi Mediated Inhibition of BACE, APP, PS1 or PS2 Expression in Cell Culture**

[0420] Inhibition of BACE, APP, PS1, or PS2 RNA Expression Using siNA Targeting BACE, APP, PS1, or PS2 RNA

[0421] siNA constructs (Table III) are tested for efficacy in reducing BACE, APP, PS1 or PS2 RNA Expression in A549 or SK-N-SH cells. Cells are plated approximately 24 hours before transfection in 96-well plates at 5,000-7,500 cells/well, 100 μ l/well, such that at the time of transfection cells are 70-90% confluent. For transfection, annealed siNAs are mixed with the transfection reagent (Lipofectamine 2000, Invitrogen) in a volume of 50 μ l/well and incubated for 20 minutes at room temperature. The siNA transfection mixtures are added to cells to give a final siNA concentration of 25 nM in a volume of 150 μ l. Each siNA transfection mixture is added to 3 wells for triplicate siNA treatments. Cells are incubated at 37° for 24 hours in the continued presence of the siNA transfection mixture. At 24 hours, RNA is prepared from each well of treated cells. The supernatants with the transfection mixtures are first removed and discarded, then the cells are lysed and RNA prepared from each well. Target gene expression following treatment is evaluated by RT-PCR for the target gene and for a control gene (36B4, an RNA polymerase subunit) for normalization. The triplicate data is averaged and the standard deviations determined for each treatment. Normalized data are graphed and the percent reduction of target mRNA by active siNAs in comparison to their respective inverted control siNAs is determined.

[0422] In a non-limiting example, using the method described above, siNA constructs were screened for activity (see FIG. 22) and compared to untreated cells, scrambled siNA control constructs (Scram1 and Scram2), and cells transfected with lipid alone (transfection control). As shown in FIG. 22, the siNA constructs show significant reduction of BACE RNA expression. Leads generated from such a screen are then further assayed. In a non-limiting example, siNA constructs comprising ribonucleotides and 3'-terminal dihydropyrimidine caps are assayed along with a chemically modified siNA construct comprising 2'-deoxy-2'-fluoro pyrimidine nucleotides and purine ribonucleotides, in which the sense strand of the siNA is further modified with 5' and 3'-terminal inverted deoxybasic caps and the antisense strand comprises a 3'-terminal phosphorothioate internucleotide linkage. Additional stabilization chemistries as described in Table IV are similarly assayed for activity. These siNA constructs are compared to appropriate matched chemistry inverted controls. In addition, the siNA constructs

are also compared to untreated cells, cells transfected with lipid and scrambled siNA constructs, and cells transfected with lipid alone (transfection control).

[0423] Using the method described above, a lead siNA construct (31007/31083) chosen from the screen described in FIG. 22 above was further modified using chemical modifications described in Table IV herein. Results are shown in FIG. 23. A549 cells were transfected with 0.25 μ g/well of lipid complexed with 25 nM siNA. Chemically modified constructs having Stab 4/5 chemistry (31378/31381) and Stab 7/11 chemistry (31384/31387) (solid bars; see Table IV) were tested for efficacy compared to matched chemistry inverted controls (open bars; sequences of the siNA constructs shown in Table III). The original lead siNA construct (31007/31083) and the Stab 4/5 and Stab 7/11 constructs were compared to untreated cells, scrambled siNA control constructs (Scram1 and Scram2), and cells transfected with lipid alone (transfection control). As shown in FIG. 23, the original lead construct and the Stab 4/5 and Stab 7/11 modified siNA constructs all show significant reduction of BACE RNA expression.

[0424] FIG. 24 shows a non-limiting example of the reduction of APP mRNA in SK-N-SH cells mediated by siNAs that target APP mRNA. SK-N-SH cells were transfected with 0.25 μ g/well of lipid complexed with 25 nM siNA. Active chemically modified siNA constructs (solid bars; see Tables III and IV) were compared to untreated cells, matched chemistry irrelevant siNA control constructs (IC-1), and cells transfected with lipid alone (transfection control). As shown in FIG. 24, the siNA constructs significantly reduce APP RNA expression compared with irrelevant siNA control and transfection control constructs.

[0425] FIG. 25 shows a non-limiting example of the reduction of PSEN1 mRNA in SK-N-SH cells mediated by siNAs that target PSEN1 mRNA. SK-N-SH cells were transfected with 0.25 μ g/well of lipid complexed with 25 nM siNA. Active chemically modified siNA constructs (solid bars; see Tables III and IV) were compared to untreated cells, matched chemistry irrelevant siNA control constructs (IC-1), and cells transfected with lipid alone (transfection control). As shown in FIG. 25, the siNA constructs significantly reduce PSEN1 RNA expression compared with irrelevant siNA control and transfection control constructs.

[0426] FIG. 26 shows a non-limiting example of the reduction of PSEN2 mRNA in SK-N-SH cells mediated by siNAs that target PSEN2 mRNA. SK-N-SH cells were transfected with 0.25 μ g/well of lipid complexed with 25 nM siNA. Active chemically modified siNA constructs (solid bars; see Tables III and IV) were compared to untreated cells, matched chemistry irrelevant siNA control constructs (IC-1), and cells transfected with lipid alone (transfection control). As shown in FIG. 26, the siNA constructs significantly reduce PSEN2 RNA expression compared with irrelevant siNA control and transfection control constructs.

Example 10**Indications**

[0427] Particular degenerative and disease states that can be associated with BACE, APP, PIN-1, PS-1 and/or PS-2 expression modulation include but are not limited to: Alzheimer's disease, dementia, stroke (CVA) and any other dis-

eases or conditions that are related to the levels of BACE, APP, PIN-1, PS-1 and/or PS-2 in a cell or tissue, alone or in combination with other therapies. The reduction of BACE, APP, PIN-1, PS-1 and/or PS-2 expression (specifically BACE, APP, PIN-1, PS-1 and/or PS-2 RNA levels) and thus reduction in the level of the respective protein relieves, to some extent, the symptoms of the disease or condition.

[0428] Those skilled in the art will recognize that other drug compounds and therapies may be readily combined with or used in conjunction with the nucleic acid molecules of the instant invention (e.g., siNA molecules) are hence within the scope of the instant invention.

Example 11

Diagnostic Uses

[0429] The siNA molecules of the invention can be used in a variety of diagnostic applications, such as in the identification of molecular targets (e.g., RNA) in a variety of applications, for example, in clinical, industrial, environmental, agricultural and/or research settings. Such diagnostic use of siNA molecules involves utilizing reconstituted RNAi systems, for example, using cellular lysates or partially purified cellular lysates. siNA molecules of this invention can be used as diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of endogenous or exogenous, for example viral, RNA in a cell. The close relationship between siNA activity and the structure of the target RNA allows the detection of mutations in any region of the molecule, which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple siNA molecules described in this invention, one can map nucleotide changes, which are important to RNA structure and function in vitro, as well as in cells and tissues. Cleavage of target RNAs with siNA molecules can be used to inhibit gene expression and define the role of specified gene products in the progression of disease or infection. In this manner, other genetic targets can be defined as important mediators of the disease. These experiments 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, siNA molecules coupled with known small molecule inhibitors, or intermittent treatment with combinations siNA molecules and/or other chemical or biological molecules). Other in vitro uses of siNA molecules of this invention are well known in the art, and include detection of the presence of mRNAs associated with a disease, infection, or related condition. Such RNA is detected by determining the presence of a cleavage product after treatment with a siNA using standard methodologies, for example, fluorescence resonance emission transfer (FRET).

[0430] In a specific example, siNA molecules that cleave only wild-type or mutant forms of the target RNA are used for the assay. The first siNA molecules (i.e., those that cleave only wild-type forms of target RNA) are used to identify wild-type RNA present in the sample and the second siNA molecules (i.e., those that cleave only mutant forms of target RNA) are used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA are cleaved by both siNA molecules to demonstrate the relative siNA efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The

cleavage products from the synthetic substrates also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus, each analysis requires two siNA molecules, two substrates and one unknown sample, which is combined into six reactions. The presence of cleavage products is determined using an RNase protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the phenotype (i.e., disease related or infection related) is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels is adequate and decreases the cost of the initial diagnosis. Higher mutant form to wild-type ratios are correlated with higher risk whether RNA levels are compared qualitatively or quantitatively.

[0431] All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

[0432] One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

[0433] It will be readily apparent to one skilled in the art that varying substitutions and modifications can be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims. The present invention teaches one skilled in the art to test various combinations and/or substitutions of chemical modifications described herein toward generating nucleic acid constructs with improved activity for mediating RNAi activity. Such improved activity can comprise improved stability, improved bioavailability, and/or improved activation of cellular responses mediating RNAi. Therefore, the specific embodiments described herein are not limiting and one skilled in the art can readily appreciate that specific combinations of the modifications described herein can be tested without undue experimentation toward identifying siNA molecules with improved RNAi activity.

[0434] The invention illustratively described herein suitably can be practiced in the absence of any element or elements, limitation or limitations that are not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such

terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments, optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the description and the appended claims.

[0435] In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

TABLE I

Accession Numbers
NM_012104
<i>Homo sapiens</i> beta-site APP-cleaving enzyme (BACE), transcript variant a, mRNA
gi 21040369 ref NM_012104.2 21040369]
NM_006222
<i>Homo sapiens</i> protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1-like (PIN1), mRNA
gi 5453899 ref NM_006222.1 5453899]
L76517
<i>Homo sapiens</i> (clone cc44) senilin 1 (PS1; S182) mRNA, complete cds
gi 1479973 gb L76517.1 HUMPS1.MRNA[1479973]
L43964
<i>Homo sapiens</i> (clone F-T03796) STM-2 mRNA, complete cds
gi 951202 gb L43964.1 HUMSTM2R[951202]
NM_138973
<i>Homo sapiens</i> beta-site APP-cleaving enzyme (BACE), transcript variant d, mRNA
gi 21040367 ref NM_138973.1 21040367]
NM_138972
<i>Homo sapiens</i> beta-site APP-cleaving enzyme (BACE), transcript variant b, mRNA
gi 21040365 ref NM_138972.1 21040365]
NM_138971
<i>Homo sapiens</i> beta-site APP-cleaving enzyme (BACE), transcript variant c, mRNA
gi 21040363 ref NM_138971.1 21040363]
<i>Homo sapiens</i> cDNA FLJ90568 fis, clone OVARC1001570, highly similar to <i>Homo sapiens</i> beta-site APP cleaving enzyme (BACE) mRNA
gi 22760888 dbj AK075049.1 22760888]
AF527782
<i>Homo sapiens</i> beta-site APP-cleaving enzyme (BACE) mRNA, partial cds, alternatively spliced
gi 22094870 gb AF527782.1 22094870]
AF324837
<i>Homo sapiens</i> beta-site APP cleaving enzyme mRNA, partial cds, alternatively spliced
gi 21449275 gb AF324837.1 21449275]
AF338817
<i>Homo sapiens</i> beta-site APP cleaving enzyme type C mRNA, complete cds
gi 13699247 gb AF338817.1 13699247]
AF338816
<i>Homo sapiens</i> beta-site APP cleaving enzyme type B mRNA, complete cds
gi 13699245 gb AF338816.1 13699245]
AB050438
<i>Homo sapiens</i> BACE mRNA for beta-site APP cleaving enzyme 1-432, complete cds

TABLE I-continued

Accession Numbers
gi 13568410 dbj AB050438.1 13568410]
AB050437
<i>Homo sapiens</i> BACE mRNA for beta-site APP cleaving enzyme 1-457, complete cds
gi 13568408 dbj AB050437.1 13568408]
AB050436
<i>Homo sapiens</i> BACE mRNA for beta-site APP cleaving enzyme 1-476, complete cds
gi 13568406 dbj AB050436.1 13568406]
AF190725
<i>Homo sapiens</i> beta-site APP cleaving enzyme (BACE) mRNA, complete cds
gi 6118538 gb AF190725.1 AF190725[6118538]
NM_007319
<i>Homo sapiens</i> presenilin 1 (Alzheimer disease 3) (PSEN1), transcript variant 1-374., mRNA
gi 7549814 ref NM_007319.1 7549814]
NM_138992
<i>Homo sapiens</i> beta-site APP-cleaving enzyme 2 (BACE2), transcript variant b, mRNA
gi 21040361 ref NM_138992.1 21040361]
NM_138991
<i>Homo sapiens</i> beta-site APP-cleaving enzyme 2 (BACE2), transcript variant c, mRNA
gi 21040359 ref NM_138991.1 21040359]
NM_012105
<i>Homo sapiens</i> beta-site APP-cleaving enzyme 2 (BACE2), transcript variant a, mRNA
gi 21040358 ref NM_012105.3 21040358]
AB066441
<i>Homo sapiens</i> APP mRNA for amyloid precursor protein, partial cds, D678N mutant
gi 16904654 dbj AB066441.1 16904654]
AB050438
<i>Homo sapiens</i> BACE mRNA for beta-site APP cleaving enzyme 1-432, complete cds
gi 13568410 dbj AB050438.1 13568410]
AB050437
<i>Homo sapiens</i> BACE mRNA for beta-site APP cleaving enzyme 1-457, complete cds
gi 13568408 dbj AB050437.1 13568408]
AB050436
<i>Homo sapiens</i> BACE mRNA for beta-site APP cleaving enzyme 1-476, complete cds
gi 13568406 dbj AB050436.1 13568406]
NM_012486
<i>Homo sapiens</i> presenilin 2 (Alzheimer disease 4) (PSEN2), transcript variant 2, mRNA
gi 7108359 ref NM_012486.1 7108359]
NM_000447
<i>Homo sapiens</i> presenilin 2 (Alzheimer disease 4) (PSEN2), transcript variant 1, mRNA
gi 4506164 ref NM_000447.1 4506164]
AF188277
<i>Homo sapiens</i> aspartyl protease (BACE2) mRNA, complete cds, alternatively spliced
gi 7025334 gb AF188277.1 AF188277[7025334]
AF188276
<i>Homo sapiens</i> aspartyl protease (BACE2) mRNA, complete cds, alternatively spliced
gi 7025332 gb AF188276.1 AF188276[7025332]
AF178532
<i>Homo sapiens</i> aspartyl protease (BACE2) mRNA, complete cds
gi 6851265 gb AF178532.1 AF178532[6851265]
D87675
<i>Homo sapiens</i> DNA for amyloid precursor protein, complete cds
gi 2429080 dbj D87675.1 2429080]
AF201468
<i>Homo sapiens</i> APP beta-secretase mRNA, complete cds
gi 6601444 gb AF201468.1 AF201468[6601444]

TABLE I-continued

Accession Numbers
AF190725 <i>Homo sapiens</i> beta-site APP cleaving enzyme (BACE) mRNA, complete cds gi 6118538 gb AF190725.1 AF190725[6118538] E14707 DNA encoding a mutated amyloid precursor protein gi 5709390 dbj E14707.11 pat JP1998001499 1[5709390] AF168956 <i>Homo sapiens</i> amyloid precursor protein homolog HSD-2 mRNA, complete cds gi 5702387 gb AF168956.1 AF168956[5702387] S60099 APPH = amyloid precursor protein homolog [human, placenta, mRNA, 3727 nt] gi 300168 bbm 300685 bbs 131198 gb S60099.1 S60099[300168] U50939 Human amyloid precursor protein-binding protein 1 mRNA, complete cds gi 1314559 gb U50939.1 HSU50939[1314559] NM_000484 <i>Homo sapiens</i> amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer disease) (APP), transcript variant 1, mRNA gi 41406053 ref NM_000484.2 [41406053] BC018937 <i>Homo sapiens</i> amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer disease), mRNA (cDNA clone IMAGE: 4126584) gi 39645179 gb BC018937.2 [39645179]

TABLE I-continued

Accession Numbers
NM_201413 <i>Homo sapiens</i> amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer disease) (APP), transcript variant 2, mRNA gi 41406054 ref NM_201413.1 [41406054] NM_201414 <i>Homo sapiens</i> amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer disease) (APP), transcript variant 3, mRNA gi 41406056 ref NM_201414.1 [41406056] BC065529 <i>Homo sapiens</i> amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer disease), transcript variant 2, mRNA (cDNA clone MGC: 75167 IMAGE: 6152423), complete cds gi 41350938 gb BC065529.1 [41350938] Y00264 Human mRNA for amyloid A4 precursor of Alzheimer's disease gi 28525 emb Y00264.1 HSAFPA4[28525] AF282245 <i>Homo sapiens</i> amyloid precursor protein 639 (APP639) mRNA, complete cds gi 33339673 gb AF282245.1 [33339673] X06989 <i>Homo sapiens</i> mRNA for amyloid A4 protein (APP gene) gi 28720 emb X06989.1 HSAPA4R[28720]

[0436]

TABLE II

APP, BACE, PSEN1, PSEN2 s1NA AND TARGET SEQUENCES							
APP NM_000484							
Pos	Seq	Seq ID	UPos	Upper seq	Seq ID	LPos	Lower seq
3	UUUCCUCGGCAGCGGUAGG	1	3	UUUCCUCGGCAGCGGUAGG	1	21	CCUACCGCUGCCGAGGAAA
21	GCGAGAGCACGCGGAGGAG	2	21	GCGAGAGCACGCGGAGGAG	2	39	CUCCUCCGCGUCUCUCGC
39	GCGUGCGCGGGGCCCCCGG	3	39	GCGUGCGCGGGGCCCCCGG	3	57	CCGGGGCCCCCGGCACGC
57	GGAGACGGCGGCGUGGCG	4	57	GGAGACGGCGGCGUGGCG	4	75	CGCCACCGCCCGCUCUCC
75	GGCGCGGGCAGAGCAAGGA	5	75	GGCGCGGGCAGAGCAAGGA	5	93	UCCUUGCUCUGCCCGGCC
93	ACGCGCGGGAUCCACUCG	6	93	ACGCGCGGGAUCCACUCG	6	111	CGAGUGGGAUCCCGCGCU
111	GCACAGCAGCGCACUCGGU	7	111	GCACAGCAGCGCACUCGGU	7	129	ACCGAGUGCGCUGCUGUC
129	UGCCCCGCGCAGGGUCGCG	8	129	UGCCCCGCGCAGGGUCGCG	8	147	CGCGACCCUGCGGGGCA
147	GAUGCUGCCCGGUUGGCA	9	147	GAUGCUGCCCGGUUGGCA	9	165	UGCCAAACCGGGCAGCAUC
165	ACUGCUCUCUGGCGCGCC	10	165	ACUGCUCUCUGGCGCGCC	10	183	GGCGGCAGCAGAGCAGU
183	CUGGACGGCUCGGGCGCUG	11	183	CUGGACGGCUCGGGCGCUG	11	201	CAGCGCCCGAGCCGUCCAG
201	GGAGGUACCCACUGAUGGU	12	201	GGAGGUACCCACUGAUGGU	12	219	ACCAUCAGUGGUACCUCC
219	UAAUGCUGGCCUGCUGGCU	13	219	UAAUGCUGGCCUGCUGGCU	13	237	AGCCAGCAGGCCAGCAUUA
237	UGAACCCAGAUUGCCAUG	14	237	UGAACCCAGAUUGCCAUG	14	255	CAUGGCAUUGGGGUUCA
255	GUUCUGGGCAGACUGAAC	15	255	GUUCUGGGCAGACUGAAC	15	273	GUUCAGUCUGCCACAGAAC

TABLE II-continued

APP, BACE, PSEN1, PSEN2 siRNA AND TARGET SEQUENCES									
273	CAUGCACAUGAAUGUCCAG	16	273	CAUGCACAUGAAUGUCCAG	16	291	CUGGACAUAUCAUGUGCAUG	215	
291	GAAUGGGAAGUGGGAUUA	17	291	GAAUGGGAAGUGGGAUUA	17	309	UGAAUCCACUCCCAUUC	216	
309	AGAUCCAUCAGGACCAAA	18	309	AGAUCCAUCAGGACCAAA	18	327	UUUGGUCCUGAUGGAUCU	217	
327	AACCUGCAUUGAUACCAAG	19	327	AACCUGCAUUGAUACCAAG	19	345	CUUGGUAUCAAUGCAGGUU	218	
345	GGAAGGCAUCCUGCAGUUA	20	345	GGAAGGCAUCCUGCAGUUA	20	363	AUACUGCAGGAUGCCUUC	219	
363	UUGCCAAGAAGUCUACCCU	21	363	UUGCCAAGAAGUCUACCCU	21	381	AGGGUAGACUUCUUGGCAA	220	
381	UGAACUGCAGAUACCAAU	22	381	UGAACUGCAGAUACCAAU	22	399	AUUGGUGAUCUGCAGUUA	221	
399	UGUGGUAAGAAGCAACCAA	23	399	UGUGGUAAGAAGCAACCAA	23	417	UUGGUUGGCUUCUACCACA	222	
417	ACCAGUGACCAUCCAGAAC	24	417	ACCAGUGACCAUCCAGAAC	24	435	GUUCUGGAUGGUCACUGGU	223	
435	CUGGUGCAAGCGGGCCGC	25	435	CUGGUGCAAGCGGGCCGC	25	453	GCGGCCCGCUUGCACCAG	224	
453	CAAGCAGUGCAAGACCAU	26	453	CAAGCAGUGCAAGACCAU	26	471	AUGGGUCUUGCACUGCUUG	225	
471	UCCCCACUUUGUAUUC	27	471	UCCCCACUUUGUAUUC	27	489	GGGAUACAAAGUGGGGA	226	
489	CUACCGCUGCUUAGUUGGU	28	489	CUACCGCUGCUUAGUUGGU	28	507	ACCAACUAAGCAGCGGUAG	227	
507	UGAGUUUGUAAGUGAGCC	29	507	UGAGUUUGUAAGUGAGCC	29	525	GGCAUCACUUAACAACUCA	228	
525	CCUUCUCGUUCCUGACAAG	30	525	CCUUCUCGUUCCUGACAAG	30	543	CUUGUCAGGAACGAGAAGG	229	
543	GUGCAAAUUCUACACCAG	31	543	GUGCAAAUUCUACACCAG	31	561	CUGGUGUAAGAAUUGCAC	230	
561	GGAGAGGAUGGAUGUUUGC	32	561	GGAGAGGAUGGAUGUUUGC	32	579	GCAAAUCCAUCCUCUCC	231	
579	CGAAACUCAUCUUCACUGG	33	579	CGAAACUCAUCUUCACUGG	33	597	CCAGUGAAGAGAGUUUCG	232	
597	GCACACCGUCGCCAAGAG	34	597	GCACACCGUCGCCAAGAG	34	615	CUCUUUGGCGACGGUGUC	233	
615	GACAUGCAGUGAGAAGAGU	35	615	GACAUGCAGUGAGAAGAGU	35	633	ACUCUUCUCACUGCAUGUC	234	
633	UACCAACUUGCAUGACUAC	36	633	UACCAACUUGCAUGACUAC	36	651	GUAGUCAUGCAAGUUGGUA	235	
651	CGGCAUGUUGCUGCCUUC	37	651	CGGCAUGUUGCUGCCUUC	37	669	GCAGGGCAGCAACAUGCCG	236	
669	CGGAAUUGACAAGUCCGA	38	669	CGGAAUUGACAAGUCCGA	38	687	UCGGAACUUGUCAAUUCCG	237	
687	AGGGGUAAGUUUGUGUGU	39	687	AGGGGUAAGUUUGUGUGU	39	705	ACACACAAACUCUACCCCU	238	
705	UUGCCCACUGGCUGAAGAA	40	705	UUGCCCACUGGCUGAAGAA	40	723	UUCUUCAGCCAGUGGGCAA	239	
723	AAGUGACAAUGUGGAUUCU	41	723	AAGUGACAAUGUGGAUUCU	41	741	AGAAUCCACAUUGUCACUU	240	
741	UGCUGAUGCGGAGGAGGAU	42	741	UGCUGAUGCGGAGGAGGAU	42	759	AUCCUCCUCCGCAUCAGCA	241	
759	UGACUCGGAUGUCUGGUGG	43	759	UGACUCGGAUGUCUGGUGG	43	777	CCACCAGACAUCGAGUCA	242	
777	GGCGGAGCAGACACAGAC	44	777	GGCGGAGCAGACACAGAC	44	795	GUCUGUGUCUGUCCGCC	243	
795	CUAUGCAGAUGGGAGUGAA	45	795	CUAUGCAGAUGGGAGUGAA	45	813	UUCACUCCCAUCUGCAUAG	244	
813	AGACAAAGUAGUAGAAGUA	46	813	AGACAAAGUAGUAGAAGUA	46	831	UACUUCUACUACUUUGUCU	245	
831	AGCAGAGGAGGAAGAAGUG	47	831	AGCAGAGGAGGAAGAAGUG	47	849	CACUUCUUCUCCUCUGCU	246	
849	GGCUGAGGUGGAAGAAGAA	48	849	GGCUGAGGUGGAAGAAGAA	48	867	UUCUUCUUCACCUCAGCC	247	
867	AGAAGCCGAUGAUGACGAG	49	867	AGAAGCCGAUGAUGACGAG	49	885	CUCGUAUCAUCGGCUUCU	248	
885	GGACGAUGAGGAUGGUGAU	50	885	GGACGAUGAGGAUGGUGAU	50	903	AUCACCAUCCUCAUCGUCC	249	
903	UGAGGUAGAGGAAGAGGCU	51	903	UGAGGUAGAGGAAGAGGCU	51	921	AGCCUUCUCCUCUACCUCA	250	
921	UGAGGAACCCUACGAAGAA	52	921	UGAGGAACCCUACGAAGAA	52	939	UUCUUCGUAGGGUCCUCA	251	

TABLE II-continued

APP, BACE, PSEN1, PSEN2 s1nA AND TARGET SEQUENCES									
939	AGCCACAGAGAGAACCACC	53	939	AGCCACAGAGAGAACCACC	53	957	GGUGGUUCUCUCUGUGGCU	252	
957	CAGCAUUGCCACCACCACC	54	957	CAGCAUUGCCACCACCACC	54	975	GGUGGUGGUGGCAAUGCUG	253	
975	CACCACCACCACAGAGUCU	55	975	CACCACCACCACAGAGUCU	55	993	AGACUCUGUGGUGGUGGUG	254	
993	UGUGGAAGAGGUGGUUCGA	56	993	UGUGGAAGAGGUGGUUCGA	56	1011	UCGAACCACCUCUCCACA	255	
1011	AGAGGUGUGCUCUGAACAA	57	1011	AGAGGUGUGCUCUGAACAA	57	1029	UUGUUCAGAGCACACCUCU	256	
1029	AGCCGAGACGGGCGCGUGC	58	1029	AGCCGAGACGGGCGCGUGC	58	1047	GCACGGCCCCGUCUCGGCU	257	
1047	CCGAGCAAUGAUCUCCCGC	59	1047	CCGAGCAAUGAUCUCCCGC	59	1065	GCGGAGAUCAUUGCUCGG	258	
1065	CUGGUACUUUGAUGUGACU	60	1065	CUGGUACUUUGAUGUGACU	60	1083	AGUCACAUCAAAGUACCAG	259	
1083	UGAAGGGAAGUGUGCCCCA	61	1083	UGAAGGGAAGUGUGCCCCA	61	1101	UGGGGCACACUCCCUUCA	260	
1101	AUUCUUUUACGGCGGAUGU	62	1101	AUUCUUUUACGGCGGAUGU	62	1119	ACAUCCGCCGUAAAAGAAU	261	
1119	UGGCGGCAACCGGAACAAC	63	1119	UGGCGGCAACCGGAACAAC	63	1137	GUUGUUCGGUUGGCCGCCA	262	
1137	CUUUGACACAGAAGAGUAC	64	1137	CUUUGACACAGAAGAGUAC	64	1155	GUACUCUUCUGUGUCAAG	263	
1155	CUGCAUGGCGCGUGUGUGGC	65	1155	CUGCAUGGCGCGUGUGUGGC	65	1173	GCCACACACGGCCAUGCAG	264	
1173	CAGCGCCAUGUCCCAAAGU	66	1173	CAGCGCCAUGUCCCAAAGU	66	1191	ACUUUGGGACAUUGGCGCUG	265	
1191	UUUACUCAAGACUACCCAG	67	1191	UUUACUCAAGACUACCCAG	67	1209	CUGGGUAGUCUUGAGUAAA	266	
1209	GGAACCUCUUGCCCGAGAU	68	1209	GGAACCUCUUGCCCGAGAU	68	1227	AUCUCGGGCAAGAGGUUCC	267	
1227	UCCUGUUAACUUCUACA	69	1227	UCCUGUUAACUUCUACA	69	1245	UGUAGGAAGUUUACAGGA	268	
1245	AACAGCAGCCAGUACCCCU	70	1245	AACAGCAGCCAGUACCCCU	70	1263	AGGGGUACUGGCUGCUGUU	269	
1263	UGAUGCCGUGACAAGUAU	71	1263	UGAUGCCGUGACAAGUAU	71	1281	AUACUUGUCAACGGCAUCA	270	
1281	UCUCGAGACACCUGGGGAU	72	1281	UCUCGAGACACCUGGGGAU	72	1299	AUCCCCAGGUGUCUCGAGA	271	
1299	UGAGAAUGAACAUGCCCAU	73	1299	UGAGAAUGAACAUGCCCAU	73	1317	AUGGGCAUGUUAUUCUCA	272	
1317	UUUCCAGAAAGCCAAGAG	74	1317	UUUCCAGAAAGCCAAGAG	74	1335	CUCUUUGGCUUUCUGGAAA	273	
1335	GAGGCUUGAGGCCAAGCAC	75	1335	GAGGCUUGAGGCCAAGCAC	75	1353	GUGC UUGGCCUCAAGCCUC	274	
1353	CCGAGAGAGAAUGUCCAG	76	1353	CCGAGAGAGAAUGUCCAG	76	1371	CUGGGACAUCUCUCUCGG	275	
1371	GGUCAUGAGAGAAUGGGAA	77	1371	GGUCAUGAGAGAAUGGGAA	77	1389	UUCCCAUUCUCUCAUGACC	276	
1389	AGAGGCAGAACGUAAGCA	78	1389	AGAGGCAGAACGUAAGCA	78	1407	UGCUUGACGUUCUGCCUCU	277	
1407	AAAGAACUUGCCUAAAGCU	79	1407	AAAGAACUUGCCUAAAGCU	79	1425	AGCUUUAGGCAAGUUCUUU	278	
1425	UGAUAAGAAGGCAGUUAUC	80	1425	UGAUAAGAAGGCAGUUAUC	80	1443	GAUAACUGCCUUCUUAUCA	279	
1443	CCAGCAUUUCCAGGAGAAA	81	1443	CCAGCAUUUCCAGGAGAAA	81	1461	UUUCUCCUGGAAAUGCUGG	280	
1461	AGUGGAAUCUUUGGAACAG	82	1461	AGUGGAAUCUUUGGAACAG	82	1479	CUGUUCCAAAGA UUCCACU	281	
1479	GGAAGCAGCCAACGAGAGA	83	1479	GGAAGCAGCCAACGAGAGA	83	1497	UCUCUCGUUGGCUUCUUC	282	
1497	ACAGCAGCUGGUGGAGACA	84	1497	ACAGGAGGUGGUGGAGACA	84	1515	UGUCUCCACCAGCUGCUGU	283	
1515	ACACAUGGCCAGAGUGGAA	85	1515	ACACAUGGCCAGAGUGGAA	85	1533	UUCCACUCUGGCCAUGUGU	284	
1533	AGCCAUGCUCUAAUGACCGC	86	1533	AGCCAUGCUCUAAUGACCGC	86	1551	GCGGUCAUUGAGCAUGGCU	285	
1551	CCGCCGCCUGGCCUGGAG	87	1551	CCGCCGCCUGGCCUGGAG	87	1569	CUCCAGGGCCAGGCGGCGG	286	
1569	GAACUACAUCACCGCUCUG	88	1569	GAACUACAUCACCGCUCUG	88	1587	CAGAGCGGUGAUGUAGUUC	287	
1587	GCAGGCUGUCCUCCUCGG	89	1587	GCAGGCUGUCCUCCUCGG	89	1605	CCGAGGAGGAACAGCCUGC	288	

TABLE II-continued

APP, BACE, PSEN1, PSEN2 s1nA AND TARGET SEQUENCES									
1605	GCCUCGUCACGUGUCAAU	90	1605	GCCUCGUCACGUGUCAAU	90	1623	AUUGAACACGUGACGAGGC	289	
1623	UAUGCUAAAGAAGUAUGUC	91	1623	UAUGCUAAAGAAGUAUGUC	91	1641	GACAUACUUCUUAGCAUA	290	
1641	CCGCGCAGAACAGAAGGAC	92	1641	CCGCGCAGAACAGAAGGAC	92	1659	GUCCUUCUGUUCUGCGCGG	291	
1659	CAGACAGCACACCCUAAAG	93	1659	CAGACAGCACACCCUAAAG	93	1677	CUUUGAGGGUGUGUGUCUG	292	
1677	GCAUUCGAGCAUGUGCGC	94	1677	GCAUUCGAGCAUGUGCGC	94	1695	GCGCACAUUCGAAAUGC	293	
1695	CAUGGUGGAUCCCAAGAAA	95	1695	CAUGGUGGAUCCCAAGAAA	95	1713	UUUCUUGGGAUCCACCAUG	294	
1713	AGCCGCUCAGAUCCGGUCC	96	1713	AGCCGCUCAGAUCCGGUCC	96	1731	GGACCGGAUCUGAGCGGCU	295	
1731	CCAGGUUAUGACACACCUC	97	1731	CCAGGUUAUGACACACCUC	97	1749	GAGGUGUGUCAUAACCUUG	296	
1749	CCGUGUGAUUUUAGAGCGC	98	1749	CCGUGUGAUUUUAGAGCGC	98	1767	GCGCUCAUAAAUACACGG	297	
1767	CAUGAAUCAGUCUCUCUCC	99	1767	CAUGAAUCAGUCUCUCUCC	99	1785	GGAGAGAGACUGAUUCAUG	298	
1785	CCUGCUCUACAACGUGCCU	100	1785	CCUGCUCUACAACGUGCCU	100	1803	AGGCACGUUGUAGAGCAGG	299	
1803	UGCAGUGGCCGAGGAGAUU	101	1803	UGCAGUGGCCGAGGAGAUU	101	1821	AAUUCUCCGCGCCACUGCA	300	
1821	UCAGGAUGAAGUUGAUGAG	102	1821	UCAGGAUGAAGUUGAUGAG	102	1839	CUCAUCAACUUAUCCUGA	301	
1839	GCUGCUUCAGAAAGAGCAA	103	1839	GCUGCUUCAGAAAGAGCAA	103	1857	UUGCUCUUUCUGAAGCAGC	302	
1857	AAACUAUUCAGAUACGUC	104	1857	AAACUAUUCAGAUACGUC	104	1875	GACGUCAUCUGAAUAGUUU	303	
1875	CUUGGCCAACAUUAUAGU	105	1875	CUUGGCCAACAUUAUAGU	105	1893	ACUAAUCAUGUUGGCCAAG	304	
1893	UGAACCAAGGAUCAGUUAC	106	1893	UGAACCAAGGAUCAGUUAC	106	1911	GUAAUCUGAUCCUUGGUUCA	305	
1911	CGGAAACGAUGCUCUCAUG	107	1911	CGGAAACGAUGCUCUCAUG	107	1929	CAUGAGAGCAUCGUUUCG	306	
1929	GCCAUUCUUGACCGAAACG	108	1929	GCCAUUCUUGACCGAAACG	108	1947	CGUUUCGGUCAAAAGUUGC	307	
1947	GAAACCACCGUGGAGCUC	109	1947	GAAACCACCGUGGAGCUC	109	1965	GAGCUCCACGGUGGUUUUC	308	
1965	CCUUCUCCGUGAAUGGAGAG	110	1965	CCUUCUCCGUGAAUGGAGAG	110	1983	CUCUCCAUUCACGGGAAGG	309	
1983	GUUCAGCCUGGACGAUCUC	111	1983	GUUCAGCCUGGACGAUCUC	111	2001	GAGAUUGUCCAGGCUGAAC	310	
2001	CCAGCCGUGGCAUUCUUUU	112	2001	CCAGCCGUGGCAUUCUUUU	112	2019	AAAAGAAUGCCACGGCUGG	311	
2019	UGGGGCUGACUCUGUGCCA	113	2019	UGGGGCUGACUCUGUGCCA	113	2037	UGGCACAGAGUCAGCCCCA	312	
2037	AGCCAACACAGAAAACGAA	114	2037	AGCCAACACAGAAAACGAA	114	2055	UUCGUUUUCUGUGUUGGCU	313	
2055	AGUUGAGCCUGUUGAUGCC	115	2055	AGUUGAGCCUGUUGAUGCC	115	2073	GGCAUCAACAGGCUCAACU	314	
2073	CCGCCUUGUCGCCGACCGA	116	2073	CCGCCUUGUCGCCGACCGA	116	2091	UCGGUCGGCAGCAGGGCGG	315	
2091	AGGACUGACCACUCGACCA	117	2091	AGGACUGACCACUCGACCA	117	2109	UGGUCGAGUGGUCAGUCCU	316	
2109	AGGUUCUGGGUUGACAAAU	118	2109	AGGUUCUGGGUUGACAAAU	118	2127	AUUUGUCAACCCAGAACCU	317	
2127	UAUCAAGACGGAGGAGAU	119	2127	UAUCAAGACGGAGGAGAU	119	2145	GAUCCUCCGUCUUGAUA	318	
2145	CUCUGAAGUGAAGAUUGAU	120	2145	CUCUGAAGUGAAGAUUGAU	120	2163	AUCCAUCUUCACUUCAGAG	319	
2163	UGCAGAAUUCGACAUGAC	121	2163	UGCAGAAUUCGACAUGAC	121	2181	GUCAUGUCGGAAUUCUGCA	320	
2181	CUCAGGAUAUGAAGUUAU	122	2181	CUCAGGAUAUGAAGUUAU	122	2199	AUGAACUUAUAUCCUGAG	321	
2199	UCAUCAAAAAUUGGUGUUC	123	2199	UCAUCAAAAAUUGGUGUUC	123	2217	GAACACCAAUUUUUGAUGA	322	
2217	CUUUGCAGAAGAUGUGGGU	124	2217	CUUUGCAGAAGAUGUGGGU	124	2235	ACCCACAUUCUUCGCAAAG	323	
2235	UUCAACAAAGGUGCAAUC	125	2235	UUCAACAAAGGUGCAAUC	125	2253	GAUUGCACCUCUUGUUGAA	324	
2253	CAUUGGACUCAUGGUGGGC	126	2253	CAUUGGACUCAUGGUGGGC	126	2271	GCCACCAUGAGUCCAAUG	325	

TABLE II-continued

APP, BACE, PSEN1, PSEN2 s1nA AND TARGET SEQUENCES											
2271	CGGUGUUGUCAUAGCGACA	127	2271	CGGUGUUGUCAUAGCGACA	127	2289	UGUCGCUAUGACAACACCG	326			
2289	AGUGAUCGUCAUACCCUUG	128	2289	AGUGAUCGUCAUACCCUUG	128	2307	CAAGGUGAUGACGAUCACU	327			
2307	GGUGAUGCUGAAGAAGAAA	129	2307	GGUGAUGCUGAAGAAGAAA	129	2325	UUUCUUCUUCAGCAUACCC	328			
2325	ACAGUACACAUCCAUAUUAU	130	2325	ACAGUACACAUCCAUAUUAU	130	2343	AUGAAUGGAUGUGUACUGU	329			
2343	UCAUGGUGUGGUGGAGGUU	131	2343	UCAUGGUGUGGUGGAGGUU	131	2361	AACCUCCACCACACCAUGA	330			
2361	UGACGCCGUGUACACCCCA	132	2361	UGACGCCGUGUACACCCCA	132	2379	UGGGGUGACAGCGGCGUCA	331			
2379	AGAGGAGCGCCACCUGUCC	133	2379	AGAGGAGCGCCACCUGUCC	133	2397	GGACAGGUGGCGCUCCUCU	332			
2397	CAAGAUGCAGCAGAACGGC	134	2397	CAAGAUGCAGCAGAACGGC	134	2415	GCCGUUCUGCUGCAUCUUG	333			
2415	CUACGAAAAUCCAACCUAC	135	2415	CUACGAAAAUCCAACCUAC	135	2433	GUAGGUUGGAUUUUCGUAG	334			
2433	CAAGUUCUUUGAGCAGAUG	136	2433	CAAGUUCUUUGAGCAGAUG	136	2451	CAUCUGCUCAAAGAACUUG	335			
2451	GCAGAACUAGACCCCGCC	137	2451	GCAGAACUAGACCCCGCC	137	2469	GGCGGGGUCUAGUUCUGC	336			
2469	CACAGCAGCCUCUGAAGUU	138	2469	CACAGCAGCCUCUGAAGUU	138	2487	AACUUCAGAGGCUGCUGUG	337			
2487	UGGACAGCAAAACCAUUGC	139	2487	UGGACAGCAAAACCAUUGC	139	2505	GCAAUGGUUUUGCUGUCCA	338			
2505	CUUCACUACCCAUCGGUGU	140	2505	CUUCACUACCCAUCGGUGU	140	2523	ACACCGAUGGGUAGUGAAG	339			
2523	UCCAUUUAUAGAAUAAUGU	141	2523	UCCAUUUAUAGAAUAAUGU	141	2541	ACAUAUUCUAUAAUUGGA	340			
2541	UGGGAAGAAACAAACCCGU	142	2541	UGGGAAGAAACAAACCCGU	142	2559	ACGGGUUUUGUUUCCCA	341			
2559	UUUAUUGAUUUACUAUUA	143	2559	UUUAUUGAUUUACUAUUA	143	2577	UAAUGAGUAAAUCAUAAA	342			
2577	AUCGCCUUUGACAGCUGU	144	2577	AUCGCCUUUGACAGCUGU	144	2595	ACAGCUGUCAAAAGCCGAU	343			
2595	UGCUGUAACACAAGUAGAU	145	2595	UGCUGUAACACAAGUAGAU	145	2613	AUCUACUUGUGUACAGCA	344			
2613	UGCCUGAACUUGAAUUAU	146	2613	UGCCUGAACUUGAAUUAU	146	2631	AUUAAUUCAGUUCAGGCA	345			
2631	UCCACACAUCAGUAAUGUA	147	2631	UCCACACAUCAGUAAUGUA	147	2649	UACAUUACUGAUGUGUGGA	346			
2649	AUUCUAUCUCUCUUUACAU	148	2649	AUUCUAUCUCUCUUUACAU	148	2667	AUGUAAAGAGAGAUAGAAU	347			
2667	UUUUGGUCUCUAUACUACA	149	2667	UUUUGGUCUCUAUACUACA	149	2685	UGUAGUAUAGAGACCAAAA	348			
2685	AUUUAUAAUGGGUUUUGUG	150	2685	AUUUAUAAUGGGUUUUGUG	150	2703	CACAAAACCCAUUAAUAAU	349			
2703	GUACUGUAAAGAAUUUAGC	151	2703	GUACUGUAAAGAAUUUAGC	151	2721	GCUAAAUUUUUACAGUAC	350			
2721	CUGUAUCAAAACUAGUGCAU	152	2721	CUGUAUCAAAACUAGUGCAU	152	2739	AUGCACUAGUUUGAUACAG	351			
2739	UGAAUAGAUUCUCCUGA	153	2739	UGAAUAGAUUCUCCUGA	153	2757	UCAGGAGAGAAUCUAUUA	352			
2757	AUUUAUUUACAUAGCCC	154	2757	AUUUAUUUACAUAGCCC	154	2775	GGGCUAUGUGAUAAAUAAU	353			
2775	CCUAGCCAGUUGUAUUAU	155	2775	CCUAGCCAGUUGUAUUAU	155	2793	AAUAUACAACUGGCUAAGG	354			
2793	UAUUCUUGUGGUUUGUGAC	156	2793	UAUUCUUGUGGUUUGUGAC	156	2811	GUCACAAACCACAAGAAUA	355			
2811	CCCAAUUAAUGUCCUACUUU	157	2811	CCCAAUUAAUGUCCUACUUU	157	2829	AAAGUAGGACUUAAUUGGG	356			
2829	UACAUUAGCUUUAAGAAUC	158	2829	UACAUUAGCUUUAAGAAUC	158	2847	GAUUCUUAAGCAUUAUGUA	357			
2847	CGAUGGGGGAUGCUUACUG	159	2847	CGAUGGGGGAUGCUUACUG	159	2865	CAUGAAGCAUCCCCAUCG	358			
2865	GUGAACUGGGAGUUCAGC	160	2865	GUGAACUGGGAGUUCAGC	160	2883	GCUGAACUCCACGUUCAC	359			
2883	CUGCUUCUCUUGCCUAAGU	161	2883	CUGCUUCUCUUGCCUAAGU	161	2901	ACUUAAGGCAAGAGAAGCAG	360			
2901	UAUUCUUUCCUGAUCACU	162	2901	UAUUCUUUCCUGAUCACU	162	2919	AGUGAUCAGGAAAGGAAUA	361			
2919	UAUGCAUUUAAAGUUAUA	163	2919	UAUGCAUUUAAAGUUAUA	163	2937	UUUAACUUUAAAUGCAUA	362			

TABLE II-continued

APP, BACE, PSEN1, PSEN2 siRNA AND TARGET SEQUENCES									
2937	ACAUUUUUAAGUAUUUCAG	164	2937	ACAUUUUUAAGUAUUUCAG	164	2955	CUGAAAUAUUUUUUUUGU	363	
2955	GAUGCUUUAGAGAGAUUUU	165	2955	GAUGCUUUAGAGAGAUUUU	165	2973	AAAUCUCUCUAAAGCAUC	364	
2973	UUUUUCCAUGACUGCAUUU	166	2973	UUUUUCCAUGACUGCAUUU	166	2991	AAUUGCAGUCAUGGAAAA	365	
2991	UUACUGUACAGAUUGCUGC	167	2991	UUACUGUACAGAUUGCUGC	167	3009	GCAGCAAUCUGUACAGUAA	366	
3009	CUUCUGCUAUUUUGUGAU	168	3009	CUUCUGCUAUUUUGUGAU	168	3027	AUCACAAUAUAGCAGAAG	367	
3027	UAUAGGAUUUAGAGGAUA	169	3027	UAUAGGAUUUAGAGGAUA	169	3045	UAUCCUCUUAUUUCCUAUA	368	
3045	ACACACGUUUUGUUUUCG	170	3045	ACACACGUUUUGUUUUCG	170	3063	CGAAGAAACAAACGUGUGU	369	
3063	GUGCCUGUUUAUGUGCAC	171	3063	GUGCCUGUUUAUGUGCAC	171	3081	GUGCACAUAAACAGGCAC	370	
3081	CACAUUAGGCAUUGAGACU	172	3081	CACAUUAGGCAUUGAGACU	172	3099	AGUCUCAUUGCCUAAUGUG	371	
3099	UUCAAGCUUUUUUUUUUU	173	3099	UUCAAGCUUUUUUUUUUU	173	3117	AAAAAAGAAAAGCUUGAA	372	
3117	UGUCCACGUAUCUUUGGGU	174	3117	UGUCCACGUAUCUUUGGGU	174	3135	ACCCAAAGAUACGUGGACA	373	
3135	UCUUUGAUAAAGAAAGAA	175	3135	UCUUUGAUAAAGAAAGAA	175	3153	UUCUUUUCUUUAUCAAAGA	374	
3153	AUCCUGUUCAUUGUAAGC	176	3153	AUCCUGUUCAUUGUAAGC	176	3171	GCUUACAAUGAACAGGGAU	375	
3171	CACUUUUACGGGCGGGUG	177	3171	CACUUUUACGGGCGGGUG	177	3189	CACCCGCCCGUAAAAGUG	376	
3189	GGGAGGGGUGCUCUGCUG	178	3189	GGGAGGGGUGCUCUGCUG	178	3207	CAGCAGGACCCCUCCCC	377	
3207	GGUCUUCAAUUACCAAGAA	179	3207	GGUCUUCAAUUACCAAGAA	179	3225	UUCUUGGUAAUUGAAGACC	378	
3225	AUUCUCCAAAACAUUUUC	180	3225	AUUCUCCAAAACAUUUUC	180	3243	GAAAAUUGUUUUGGAGAAU	379	
3243	CUGCAGGAUGAUUGUACAG	181	3243	CUGCAGGAUGAUUGUACAG	181	3261	CUGUACAAUUAUCCUGCAG	380	
3261	GAAUCAUUGCUUUGACAU	182	3261	GAAUCAUUGCUUUGACAU	182	3279	AUGUCAUAGCAAUGAUUC	381	
3279	UGAUCGCUUUCUACACUGU	183	3279	UGAUCGCUUUCUACACUGU	183	3297	ACAGUGUAGAAAGCGAUCA	382	
3297	UAUACAUAAAUAUUUA	184	3297	UAUACAUAAAUAUUUA	184	3315	UUAAUUUAUUUAUGUAAUA	383	
3315	AAUAAAAUAACCCGGGCA	185	3315	AAUAAAAUAACCCGGGCA	185	3333	UGCCCGGGGUUUUUUAUU	384	
3333	AAGACUUUCUUUGAAGGA	186	3333	AAGACUUUCUUUGAAGGA	186	3351	UCCUUCAAAGAAAGUCUU	385	
3351	AUGACUACAGACAUAAAU	187	3351	AUGACUACAGACAUAAAU	187	3369	AUUUAUUGUCUGUAGUCAU	386	
3369	UAAUCGAAGUAAUUUGGG	188	3369	UAAUCGAAGUAAUUUGGG	188	3387	CCCAAAAUUACUUCGAUUA	387	
3387	GUGGGGAGAGAGGCAGAU	189	3387	GUGGGGAGAGAGGCAGAU	189	3405	AUCUGCCUCUUCUCCCCAC	388	
3405	UUCAAUUUCUUUAACCAG	190	3405	UUCAAUUUCUUUAACCAG	190	3423	CUGGUUAAAGAAAAUUGAA	389	
3423	GUCUGAAGUUCAUUUAUG	191	3423	GUCUGAAGUUCAUUUAUG	191	3441	CAUAAAUGAAACUUCAGAC	390	
3441	GAUACAAAAGAAGAUAAA	192	3441	GAUACAAAAGAAGAUAAA	192	3459	UUUCAUUCUUUUUGUAUC	391	
3459	AAUGGAAGUGGCAUAUAA	193	3459	AAUGGAAGUGGCAUAUAA	193	3477	UUUAUUUGCCACUCCAUU	392	
3477	AGGGGAUGAGGAAGGCAUG	194	3477	AGGGGAUGAGGAAGGCAUG	194	3495	CAUGCCUCCUCAUCCCCU	393	
3495	GCCUGGACAAACCUUCUU	195	3495	GCCUGGACAAACCUUCUU	195	3513	AAGAAGGGUUUGUCCAGGC	394	
3513	UUUAAGAUUGUCUUCAAU	196	3513	UUUAAGAUUGUCUUCAAU	196	3531	AUUGAAGACACAUUUAAA	395	
3531	UUUGUAUAAAUGGUGUUU	197	3531	UUUGUAUAAAUGGUGUUU	197	3549	AAACACCAUUUUUACAAA	396	
3549	UUCAUGUAAAUAUACA	198	3549	UUCAUGUAAAUAUACA	198	3567	AUGUAUUUAUUUACAUGAA	397	
3567	UAAUACAUCUUGGAGGA	199	3567	UAAUACAUCUUGGAGGA	199	3577	UCCUCCAAGAAUGUAUUUA	398	

TABLE II-continued

APP, BACE, PSEN1, PSEN2 s1NA AND TARGET SEQUENCES								
BACE NM_012104								
Pos	Seq	Seq ID	UPos	Upper seq	Seq ID	LPos	Lower seq	Seq ID
1	CGCACUCGUCUCCAGCCCG	399	1	CGCACUCGUCUCCAGCCCG	399	19	CGGGCUGGGGACGAGUGCG	724
19	GCCCGGGAGCUGCGAGCCG	400	19	GCCCGGGAGCUGCGAGCCG	400	37	CGGCUCGCAGCUCUCCGGGC	725
37	GCGAGCUGGAUUAUGGUGG	401	37	GCGAGCUGGAUUAUGGUGG	401	55	CCACCAUAAUCCAGCUCGC	726
55	GCCUGAGCAGCCAACGCAG	402	55	GCCUGAGCAGCCAACGCAG	402	73	CUGCGUUGGCUCUCAGGC	727
73	GCCGCAGGAGCCCGAGCC	403	73	GCCGCAGGAGCCCGAGCC	403	91	GGUCUCCGGGCUCCUGCGGC	728
91	CCUUGCCCCUGCCCGCGCC	404	91	CCUUGCCCCUGCCCGCGCC	404	109	GGCGCGGGCAGGGGCAAGG	729
109	CGCCGCCCGCGGGGGGAC	405	109	CGCCGCCCGCGGGGGGAC	405	127	GUCCCCCGGCGGGCGCGG	730
127	CCAGGGAAGCCGCCACCGG	406	127	CCAGGGAAGCCGCCACCGG	406	145	CCGGUGGCGGCUUCCUUGG	731
145	GCCCGCCAUGCCCGCCCU	407	145	GCCCGCCAUGCCCGCCCU	407	163	AGGGCGGGCAUGGCGGGC	732
163	UCCAGCCCCCGCGGGAGC	408	163	UCCAGCCCCCGCGGGAGC	408	181	GCUCCCCGGGGGCUUGGA	733
181	CCCGCGCCCGUGCCAGG	409	181	CCCGCGCCCGUGCCAGG	409	199	CCUGGGCAGCGGGCGCGGG	734
199	GCUGCCCGCGCCGUGCCG	410	199	GCUGCCCGCGCCGUGCCG	410	217	CGGCACGGCGGCGGCCAGC	735
217	GAUGUAGCGGGCUCGGAU	411	217	GAUGUAGCGGGCUCGGAU	411	235	AUCCGGAGCCCGUACAUC	736
235	UCCAGCCUUCUCCUUGCU	412	235	UCCAGCCUUCUCCUUGCU	412	253	AGCAGGGGAGAGGCUUGGA	737
253	UCCCGUGCUCUGCGGAUCU	413	253	UCCCGUGCUCUGCGGAUCU	413	271	AGAUCGCGAGACACGGGA	738
271	UCCUUGACCGCUCUCCAC	414	271	UCCUUGACCGCUCUCCAC	414	289	GUGGAGAGCGGUCAGGGGA	739
289	CAGCCCGGACCGGGGGCU	415	289	CAGCCCGGACCGGGGGCU	415	307	AGCCCCCGGUCUCCGGGUG	740
307	UGGCCCAGGGCCUUCAGG	416	307	UGGCCCAGGGCCUUCAGG	416	325	CCUGCAGGGCCUUGGGCCA	741
325	GCCUGGCGUCCUGAUGCC	417	325	GCCUGGCGUCCUGAUGCC	417	343	GGCAUCAGGACGCCAGGGC	742
343	CCCCAAGCUCUCCUUG	418	343	CCCCAAGCUCUCCUUG	418	361	CAGGAGAGGGAGCUUGGGG	743
361	GAGAAGCCACCAGACCAC	419	361	GAGAAGCCACCAGACCAC	419	379	GUGGUGCUGGUGGCUUCUC	744
379	CCCAGACUUGGGGGCAGGC	420	379	CCCAGACUUGGGGGCAGGC	420	397	GCCUGCCCCCAAGUCUGGG	745
397	CGCCAGGGACGGACUGGG	421	397	CGCCAGGGACGGACUGGG	421	415	CCCACGUCUCCUUGGCG	746
415	GCCAGUGCGAGCCAGAGG	422	415	GCCAGUGCGAGCCAGAGG	422	433	CCUCUGGGCUCGCACUGGC	747
433	GGCCCCAAGGCCGGGGCCC	423	433	GGCCCCAAGGCCGGGGCCC	423	451	GGGCCCGGCCUUCGGGCC	748
451	CACCAUGGCCCAAGCCUG	424	451	CACCAUGGCCCAAGCCUG	424	469	CAGGGCUUGGGCCAUGGUG	749
469	GCCUUGGCUCCUGUGUGG	425	469	GCCUUGGCUCCUGUGUGG	425	487	CCACAGCAGGAGCCAGGGC	750
487	GAUGGGCGCGGAGUGCUG	426	487	GAUGGGCGCGGAGUGCUG	426	505	CAGCACUCCGCGCCCAUC	751
505	GCCUGCCACGGCACCCAG	427	505	GCCUGCCACGGCACCCAG	427	523	CUGGGUGCCGUGGGCAGGC	752
523	GCACGGCAUCCGGCUGCCC	428	523	GCACGGCAUCCGGCUGCCC	428	541	GGGACGCCGAUCCGUGGC	753
541	CCUCGCGACGGCCUGGGG	429	541	CCUCGCGACGGCCUGGGG	429	559	CCCCAGGCCGUGGCAGG	754
559	GGGCGCCCCUUGGGGUG	430	559	GGGCGCCCCUUGGGGUG	430	577	CAGCCCCAGGGGGGCGCCC	755
577	GCGGUGCCCCGGGAGACC	431	577	GCGGUGCCCCGGGAGACC	431	595	GGUCUCCCGGGGACGCCG	756
595	CGACGAAGAGCCGAGGAG	432	595	CGACGAAGAGCCGAGGAG	432	613	CUCCUCGGGCUUUCGUG	757

TABLE II-continued

APP, BACE, PSEN1, PSEN2 s1NA AND TARGET SEQUENCES											
613	GCCCCGCCGGAGGGGCAGC	433	613	GCCCCGCCGGAGGGGCAGC	433	631	GCUGCCCCUCCGGCCGGGC	758			
631	CUUUGUGGAGAUGGUGGAC	434	631	CUUUGUGGAGAUGGUGGAC	434	649	GUCCACCAUCCACAAAG	759			
649	CAACCUGAGGGGCAAGUCG	435	649	CAACCUGAGGGGCAAGUCG	435	667	CGACUUGCCCCUCAGGUUG	760			
667	GGGGCAGGGCUACUACGUG	436	667	GGGGCAGGGCUACUACGUG	436	685	CACGUAGUAGCCUGCCCC	761			
685	GGAGAUGACCGUGGGCAGC	437	685	GGAGAUGACCGUGGGCAGC	437	703	GCUGCCCACGGUCAUCC	762			
703	CCCCCGCAGACGCUCAAC	438	703	CCCCCGCAGACGCUCAAC	438	721	GUUGAGCGUCUGCGGGGG	763			
721	CAUCCUGGUGGAUACAGGC	439	721	CAUCCUGGUGGAUACAGGC	439	739	GCCUGUAUCCACCAGGAUG	764			
739	CAGCAGUAACUUUGCAGUG	440	739	CAGCAGUAACUUUGCAGUG	440	757	CACUGCAAAGUUACUGCUG	765			
757	GGGUGUGCCCCCACCCC	441	757	GGGUGUGCCCCCACCCC	441	775	GGGGUGGGGGCAGCACCC	766			
775	CUUCCUGCAUCGCUACUAC	442	775	CUUCCUGCAUCGCUACUAC	442	793	GUAGUAGCGAUGCAGGAAG	767			
793	CCAGAGGCAGCUGUCCAGC	443	793	CCAGAGGCAGCUGUCCAGC	443	811	GCUGGACAGCUGCCUCUGG	768			
811	CACAUACCGGACCUCCGG	444	811	CACAUACCGGACCUCCGG	444	829	CCGGAGGUCCCGUAUGUG	769			
829	GAAGGGUGUGUAUGUCCC	445	829	GAAGGGUGUGUAUGUCCC	445	847	GGGCACAUACACCCUUC	770			
847	CUACACCCAGGGCAAGUGG	446	847	CUACACCCAGGGCAAGUGG	446	865	CCACUUGCCCCUGGGUGUAG	771			
865	GGAAGGGGAGCUGGGCACC	447	865	GGAAGGGGAGCUGGGCACC	447	883	GGUGCCCAGCUCUCCUCC	772			
883	CGACCUUGGUAAGCAUCCCC	448	883	CGACCUUGGUAAGCAUCCCC	448	901	GGGGAUGC UUACCAGGUCG	773			
901	CCAUGGCCCCAACGUCACU	449	901	CCAUGGCCCCAACGUCACU	449	919	AGUGACGUUGGGGCCAUGG	774			
919	UGUGCGUGCCAACAUUGCU	450	919	UGUGCGUGCCAACAUUGCU	450	937	AGCAAUGUUGGCACGCACA	775			
937	UGCCAUCACUGAAUCAGAC	451	937	UGCCAUCACUGAAUCAGAC	451	955	GUCUGAUUCAGUGAUGGCA	776			
955	CAAGUUCUUAUCAACGGC	452	955	CAAGUUCUUAUCAACGGC	452	973	GCCGUUGAUGAAGAACUUG	777			
973	CUCCAACUGGGAAGGCAUC	453	973	CUCCAACUGGGAAGGCAUC	453	991	GAUGCCUCCAGUUGGAG	778			
991	CCUGGGGUGGCCUAUGCU	454	991	CCUGGGGUGGCCUAUGCU	454	1009	AGCAUAGGCCAGCCCCAGG	779			
1009	UGAGAUUGCCAGGCCUGAC	455	1009	UGAGAUUGCCAGGCCUGAC	455	1027	GUCAGGCCUGGCAAUUCA	780			
1027	CGACUCCUGGAGCCUUC	456	1027	CGACUCCUGGAGCCUUC	456	1045	GAAAGGCCUCCAGGGAGUCG	781			
1045	CUUUGACUCUCUGGUAAG	457	1045	CUUUGACUCUCUGGUAAG	457	1063	CUUUAACAGAGAGUCAAAG	782			
1063	GCAGACCCAGUUCCCAAC	458	1063	GCAGACCCAGUUCCCAAC	458	1081	GUUGGGAACUGGGUCUGC	783			
1081	CCUCUUCUCCUGCAGCUU	459	1081	CCUCUUCUCCUGCAGCUU	459	1099	AAGCUGCAGGGAGAAGAGG	784			
1099	UUGUGGUGUGGCUUCCCC	460	1099	UUGUGGUGUGGCUUCCCC	460	1117	GGGGAAGCCAGCACCAAA	785			
1117	CCUCAACCAGUCUGAAGUG	461	1117	CCUCAACCAGUCUGAAGUG	461	1135	CACUUCAGACUGGUUGAGG	786			
1135	GCUGGCCUCUGUCGAGGG	462	1135	GCUGGCCUCUGUCGAGGG	462	1153	CCUCCGACAGAGGCCAGC	787			
1153	GAGCAUGAUCAUUGGAGGU	463	1153	GAGCAUGAUCAUUGGAGGU	463	1171	ACCUCCAAUGAUCAUGCUC	788			
1171	UAUCGACCACUCGCUUAC	464	1171	UAUCGACCACUCGCUUAC	464	1189	GUACAGCGAGUGGUGGAUA	789			
1189	CACAGGCAGUCUCUGGUU	465	1189	CACAGGCAGUCUCUGGUU	465	1207	AUACCAGAGACUGCCUGUG	790			
1207	UACACCCAUCGGCGGGAG	466	1207	UACACCCAUCGGCGGGAG	466	1225	CUCCCGCCGAUGGGUGUA	791			
1225	GUGGUAAUUAUGAGGUCAUC	467	1225	GUGGUAAUUAUGAGGUCAUC	467	1243	GAUGACCUCAUAAUACCAC	792			
1243	CAUUGUGCGGGUGGAGAUC	468	1243	CAUUGUGCGGGUGGAGAUC	468	1261	GAUCCACCCCGCACAAUG	793			
1261	CAAUGGACAGGAUCUGAAA	469	1261	CAAUGGACAGGAUCUGAAA	469	1279	UUUCAGAUCCUGUCCAUUG	794			

TABLE II-continued

APP, BACE, PSEN1, PSEN2 siRNA AND TARGET SEQUENCES								
1279	AAUGGACUGCAAGGAGUAC	470	1279	AAUGGACUGCAAGGAGUAC	470	1297	GUACUCCUUGCAGUCCAUAU	795
1297	CAACUAUGACAAGAGCAUU	471	1297	CAACUAUGACAAGAGCAUU	471	1315	AAUGCUCUUGUCAUAGUUG	796
1315	UGUGGACAGUGGCACCACC	472	1315	UGUGGACAGUGGCACCACC	472	1333	GGUGGUGCCACUGUCCACA	797
1333	CAACCUUCGUUUUCCCCAAG	473	1333	CAACCUUCGUUUUCCCCAAG	473	1351	CUUGGGCAAACGAAGGUUG	798
1351	GAAAGUGUUUGAAGCUGCA	474	1351	GAAAGUGUUUGAAGCUGCA	474	1369	UGCAGCUUCAAACACUUUC	799
1369	AGUCAAAUCCAUAAGGCA	475	1369	AGUCAAAUCCAUAAGGCA	475	1387	UGCCUUGAUGGAUUUGACU	800
1387	AGCCUCCUCCACGGAGAAG	476	1387	AGCCUCCUCCACGGAGAAG	476	1405	CUUCUCCGUGGAGGAGGCU	801
1405	GUUCCUGAUGGUUUUCUGG	477	1405	GUUCCUGAUGGUUUUCUGG	477	1423	CCAGAAACCAUCAGGGAAC	802
1423	GCUAGGAGAGCAGCUGGUG	478	1423	GCUAGGAGAGCAGCUGGUG	478	1441	CACCAGCUGCUCUCCUAGC	803
1441	GUGCUGGCAAGCAGGCACC	479	1441	GUGCUGGCAAGCAGGCACC	479	1459	GGUGCCUGCUUGCCAGCAC	804
1459	CACCCUUGGAACAUAUUUC	480	1459	CACCCUUGGAACAUAUUUC	480	1477	GAAAUGUCCAAGGGGUG	805
1477	CCCAGUCAUCACUCUAC	481	1477	CCCAGUCAUCACUCUAC	481	1495	GUAGAGUGAGAUGACUGGG	806
1495	CCUAUUGGGUGAGGUUACC	482	1495	CCUAUUGGGUGAGGUUACC	482	1513	GGUAACCUCACCCAUUAGG	807
1513	CAACCAUCCUUCGCAUC	483	1513	CAACCAUCCUUCGCAUC	483	1531	GAUGCGGAAGGACUGGUUG	808
1531	CACCAUCCUUCGCGAGCAA	484	1531	CACCAUCCUUCGCGAGCAA	484	1549	UUGCUGCGGAAGGAUGGUG	809
1549	AUACCUUGCGCCAGUGGAA	485	1549	AUACCUUGCGCCAGUGGAA	485	1567	UUCCACUGGCCGCGAGUAU	810
1567	AGAUGUGGCCACGUCCCAA	486	1567	AGAUGUGGCCACGUCCCAA	486	1585	UUGGGACGUGGCCACAUCU	811
1585	AGACGACUGUUAACAAGUUU	487	1585	AGACGACUGUUAACAAGUUU	487	1603	AAACUUGUAACAGUCGUCU	812
1603	UGCCAUCUCACAGUCAUCC	488	1603	UGCCAUCUCACAGUCAUCC	488	1621	GGAUGACUGUGAGAUGGCA	813
1621	CACGGGCACUGUUAUGGGA	489	1621	CACGGGCACUGUUAUGGGA	489	1639	UCCCAUAACAGUGCCCGUG	814
1639	AGCUGUUAUCAUGGAGGGC	490	1639	AGCUGUUAUCAUGGAGGGC	490	1657	GCCCUCCAUGAUAACAGCU	815
1657	CUUCUACGUUGUCUUUGAU	491	1657	CUUCUACGUUGUCUUUGAU	491	1675	AUCAAAGACAACGUAAGAAG	816
1675	UCGGGCCCGAAAACGAAUU	492	1675	UCGGGCCCGAAAACGAAUU	492	1693	AAUUCGUUUUCGGCCCCGA	817
1693	UGGCUUUGCUGUCAGCGCU	493	1693	UGGCUUUGCUGUCAGCGCU	493	1711	AGCGCUGACAGCAAAGCCA	818
1711	UUGCCAUGUGCACGAUGAG	494	1711	UUGCCAUGUGCACGAUGAG	494	1729	CUCAUCGUGCACAUGGCCAA	819
1729	GUUCAGGACGGCAGCGGUG	495	1729	GUUCAGGACGGCAGCGGUG	495	1747	CACCGCUGCCGUCCUGAAC	820
1747	GGAAGGCCUUUUGUCACC	496	1747	GGAAGGCCUUUUGUCACC	496	1765	GGUGACAAAAGGGCCUUC	821
1765	CUUGGACAUGGAAGACUGU	497	1765	CUUGGACAUGGAAGACUGU	497	1783	ACAGUCUCCAUGUCCAAG	822
1783	UGGCUACAACAUAUCCACAG	498	1783	UGGCUACAACAUAUCCACAG	498	1801	CUGUGGAAUGUUGAAGCCA	823
1801	GACAGAUGAGUCAACCCUC	499	1801	GACAGAUGAGUCAACCCUC	499	1819	GAGGGUUGACUCAUCUGUC	824
1819	CAUGACCAUAGCCUAUGUC	500	1819	CAUGACCAUAGCCUAUGUC	500	1837	GACAUAGGCUAUGGUCAUG	825
1837	CAUGGCUGCCAUUCGCGCC	501	1837	CAUGGCUGCCAUUCGCGCC	501	1855	GGCGCAGAUGGCAGCCAUG	826
1855	CCUCUUAUGCUGCCACUC	502	1855	CCUCUUAUGCUGCCACUC	502	1873	GAGUGGCAGCAUGAAGAGG	827
1873	CUGCCUAUGGUGUGUCAG	503	1873	CUGCCUAUGGUGUGUCAG	503	1891	CUGACACACCAUGAGGCAG	828
1891	GUGGCGCUGCCUCCGUGC	504	1891	GUGGCGCUGCCUCCGUGC	504	1909	GCAGCGGAGGCAGCGCCAC	829
1909	CCUGCGCCAGCAGCAUGAU	505	1909	CCUGCGCCAGCAGCAUGAU	505	1927	AUCAUGCUGCUGGCGCAGG	830
1927	UGACUUUGCUGAUGACAUC	506	1927	UGACUUUGCUGAUGACAUC	506	1945	GAUGUCAUCAGCAAAGUCA	831

TABLE II-continued

APP, BACE, PSEN1, PSEN2 siRNA AND TARGET SEQUENCES					
1945 CUCCCUGCUGAAGUGAGGA	507	1945 CUCCCUGCUGAAGUGAGGA	507	1963 UCCUCACUUCAGCAGGGAG	832
1963 AGGCCCAUGGGCAGAAGAU	508	1963 AGGCCCAUGGGCAGAAGAU	508	1981 AUCUUCUGCCCAUGGGCCU	833
1981 UAGAGAUUCCCCUGGACCA	509	1981 UAGAGAUUCCCCUGGACCA	509	1999 UGGUCCAGGGGAAUCUCUA	834
1999 ACACCUCCUGGUUACACUU	510	1999 ACACCUCCUGGUUACACUU	510	2017 AAGUGAACACGGAGGUGU	835
2017 UUGGUCACAAGUAGGAGAC	511	2017 UUGGUCACAAGUAGGAGAC	511	2035 GUCUCCUACUUGUGACCAA	836
2035 CACAGAUGGCACCUUGUGGC	512	2035 CACAGAUGGCACCUUGUGGC	512	2053 GCCACAGGUGCCAUCUGUG	837
2053 CCAGAGCACCUCAGGACCC	513	2053 CCAGAGCACCUCAGGACCC	513	2071 GGGUCCUGAGGUGCUCUGG	838
2071 CUCCCCACCCACCAAUGC	514	2071 CUCCCCACCCACCAAUGC	514	2089 GCAUUUGUGGGUGGGGAG	839
2089 CCUCUGCCUUGAUGGAGAA	515	2089 CCUCUGCCUUGAUGGAGAA	515	2107 UUCUCCAUCAAGGCAGAGG	840
2107 AGGAAAAGGCUGGCAAGGU	516	2107 AGGAAAAGGCUGGCAAGGU	516	2125 ACCUUGCCAGCCUUUUCU	841
2125 UGGGUUCCAGGGACUGUAC	517	2125 UGGGUUCCAGGGACUGUAC	517	2143 GUACAGUCCUUGGAACCCA	842
2143 CCUGUAGGAAACAGAAAAG	518	2143 CCUGUAGGAAACAGAAAAG	518	2161 CUUUUCUGUUUCCUACAGG	843
2161 GAGAAGAAAGAAGCACUCU	519	2161 GAGAAGAAAGAAGCACUCU	519	2179 AGAGUGCUUCUUUCUUCUC	844
2179 UGCUGGCGGGAUACUCUU	520	2179 UGCUGGCGGGAUACUCUU	520	2197 AAGAGUAUUCGCCAGCA	845
2197 UGGUCACCUCAAAUUUAAG	521	2197 UGGUCACCUCAAAUUUAAG	521	2215 CUUAAAUUUGAGGUGACCA	846
2215 GUCGGGAAAUUCUGCUGCU	522	2215 GUCGGGAAAUUCUGCUGCU	522	2233 AGCAGCAGAAUUUCCGAC	847
2233 UUGAAACUUCAGCCCUGAA	523	2233 UUGAAACUUCAGCCCUGAA	523	2251 UUCAGGGCUGAAGUUUCA	848
2251 ACCUUUGUCCACCAUUCU	524	2251 ACCUUUGUCCACCAUUCU	524	2269 AGGAUUGGUGGACAAAGGU	849
2269 UUUAAAUUCUCCAAACCAA	525	2269 UUUAAAUUCUCCAAACCAA	525	2287 UUGGUUGGAGAAUUUAAA	850
2287 AAGUAUUCUUCUUUCUUA	526	2287 AAGUAUUCUUCUUUCUUA	526	2305 UAAGAAAAGAAAUACUU	851
2305 AGUUUCAGAAUACUGGCA	527	2305 AGUUUCAGAAUACUGGCA	527	2323 UGCCAGUACUUCUGAAACU	852
2323 AUCACACGCAGGUUACCU	528	2323 AUCACACGCAGGUUACCU	528	2341 AAGGUAACUGCGUGUGAU	853
2341 UGGCGUGUGUCCUGUGGU	529	2341 UGGCGUGUGUCCUGUGGU	529	2359 ACCACAGGGACACAGCCA	854
2359 UACCCUGGCAGAGAAGAGA	530	2359 UACCCUGGCAGAGAAGAGA	530	2377 UCUCUUCUCUGCCAGGUA	855
2377 ACCAAGCUUGUUUCCUGC	531	2377 ACCAAGCUUGUUUCCUGC	531	2395 GCAGGGAAACAAGCUUGGU	856
2395 CUGGCCAAAGUCAGUAGGA	532	2395 CUGGCCAAAGUCAGUAGGA	532	2413 UCCUACUGACUUUGGCCAG	857
2413 AGAGGAUGCACAGUUUGCU	533	2413 AGAGGAUGCACAGUUUGCU	533	2431 AGCAAACUGUGCAUCCUCU	858
2431 UAUUUGCUUUAGAGACAGG	534	2431 UAUUUGCUUUAGAGACAGG	534	2449 CCUGUCUCUAAAGCAAUA	859
2449 GGACUGUAUAAACAAGCCU	535	2449 GGACUGUAUAAACAAGCCU	535	2467 AGGCUUGUUUAUACAGUCC	860
2467 UAACAUUGGUGCAAAGAUU	536	2467 UAACAUUGGUGCAAAGAUU	536	2485 AAUCUUUGCACCAAUGUUA	861
2485 UGCCUCUUGAAUUAAAAAA	537	2485 UGCCUCUUGAAUUAAAAAA	537	2503 UUUUUUAAUUAAGAGGCA	862
2503 AAAAAACUAGAUGACUAU	538	2503 AAAAAACUAGAUGACUAU	538	2521 AUAGUCAUUCUAGUUUUU	863
2521 UUUUAUACAAUGGGGGCGG	539	2521 UUUUAUACAAUGGGGGCGG	539	2539 CCGCCCCAUUUGUAUAAA	864
2539 GCUGGAAAGAGGAGAAGGA	540	2539 GCUGGAAAGAGGAGAAGGA	540	2557 UCCUUCUCCUCUUUCCAGC	865
2557 AGAGGGAGUACAAGACAG	541	2557 AGAGGGAGUACAAGACAG	541	2575 CUGUCUUUGUACUCCUCU	866
2575 GGGAAUAGUGGGAUCAAG	542	2575 GGGAAUAGUGGGAUCAAG	542	2593 CUUUGAUCCACUAUUCUCC	867
2593 GCUAGGAAAGGCAGAAACA	543	2593 GCUAGGAAAGGCAGAAACA	543	2611 UGUUUCUGCCUUUCCUAGC	868

TABLE II-continued

APP, BACE, PSEN1, PSEN2 sRNA AND TARGET SEQUENCES									
2611	ACAACCACUCACCAGUCCU	544	2611	ACAACCACUCACCAGUCCU	544	2629	AGGACUGGUGAGUGGUUGU	869	
2629	UAGUUUAGACCUCAUCUC	545	2629	UAGUUUAGACCUCAUCUC	545	2647	GAGAUGAGGUCUAAAACUA	870	
2647	CCAAGAUAGCAUCCCAUCU	546	2647	CCAAGAUAGCAUCCCAUCU	546	2665	AGAUGGGAUGCUAUCUUGG	871	
2665	UCAGAAGAUGGGUGUUGUU	547	2665	UCAGAAGAUGGGUGUUGUU	547	2683	AACAACACCCAUUCUCUGA	872	
2683	UUUCAAGUUUUUUUUUCU	548	2683	UUUCAAGUUUUUUUUUCU	548	2701	AGAAAAGAAAACAUGAAA	873	
2701	UGUGGUUGCAGCCUGACCA	549	2701	UGUGGUUGCAGCCUGACCA	549	2719	UGGUCAGGCUGCAACCACA	874	
2719	AAAAGUGAGAUGGGAAGGG	550	2719	AAAAGUGAGAUGGGAAGGG	550	2737	CCCUUCCCAUCUCACUUUU	875	
2737	GCUUUAUCUAGCCAAGAGC	551	2737	GCUUUAUCUAGCCAAGAGC	551	2755	GCUCUUUGGCUAGAUAAAGC	876	
2755	CUCUUUUUAGCUCUCUUA	552	2755	CUCUUUUUAGCUCUCUUA	552	2773	UAAGAGAGCUAAAAAGAG	877	
2773	AAUAGAAGUGCCACUAAG	553	2773	AAUAGAAGUGCCACUAAG	553	2791	CUUAGUGGGCACUUCUUUU	878	
2791	GAAGUCCACUUAACACAU	554	2791	GAAGUCCACUUAACACAU	554	2809	AUGUGUUAAGUGGAACUUC	879	
2809	UGAAUUCUGCCAUUUUA	555	2809	UGAAUUCUGCCAUUUUA	555	2827	UUAAUAGGCGAGAAAUUA	880	
2827	AUUUCAUUGUCUAUCUG	556	2827	AUUUCAUUGUCUAUCUG	556	2845	CAGAUAGAGACAAUGAAAU	881	
2845	GAACCACCCUUUAUUCUAC	557	2845	GAACCACCCUUUAUUCUAC	557	2863	GUAGAAUAAAGGGUGGUUC	882	
2863	CAUAGAUAGGCAGCACUG	558	2863	CAUAGAUAGGCAGCACUG	558	2881	CAGUCUGCCUAUCAUAUG	883	
2881	GAAAUUAUCCUAAACCCCUA	559	2881	GAAAUUAUCCUAAACCCCUA	559	2899	UAGGGGGUUAGGAUUAUUC	884	
2899	AAGCUCCAGGUGCCCUUG	560	2899	AAGCUCCAGGUGCCCUUG	560	2917	CACAGGGCACUGGAGCUU	885	
2917	GGGAGAGCAACUGGACUUA	561	2917	GGGAGAGCAACUGGACUUA	561	2935	AUAGUCCAGUUGCUCUCCC	886	
2935	UAGCAGGGCUGGGCUCUGU	562	2935	UAGCAGGGCUGGGCUCUGU	562	2953	ACAGAGCCAGCCUCUGUA	887	
2953	UCUCCUGGUCUAAGGCUC	563	2953	UCUCCUGGUCUAAGGCUC	563	2971	GAGCCUAUGACCAGGAAGA	888	
2971	CACUCUUUCCCCAAUUCU	564	2971	CACUCUUUCCCCAAUUCU	564	2989	AGAUUUGGGGAAGAGUG	889	
2989	UUCUCUGGAGCUUUGCAG	565	2989	UUCUCUGGAGCUUUGCAG	565	3007	CUGCAAAGCUCCAGAGGAA	890	
3007	GCCAAGGUGCUAAAAGGAA	566	3007	GCCAAGGUGCUAAAAGGAA	566	3025	UUCUUUUAGCACCUUGGC	891	
3025	AUAGGUAGGAGACCUUUC	567	3025	AUAGGUAGGAGACCUUUC	567	3043	GAAGAGGUCUCCUACCUAU	892	
3043	CUAUCUAAUCCUUA AAAAGC	568	3043	CUAUCUAAUCCUUA AAAAGC	568	3061	GCUUUUUAGGAUUAAGAUAG	893	
3061	CAUAAUGUUGAACAUUCAU	569	3061	CAUAAUGUUGAACAUUCAU	569	3079	AUGAAUGUUAACAUAUUAUG	894	
3079	UUCAACAGCUGAUGCCCUA	570	3079	UUCAACAGCUGAUGCCCUA	570	3097	UAGGGCAUCAGCUGUUGAA	895	
3097	AUAACCCUGCCUGGAUUU	571	3097	AUAACCCUGCCUGGAUUU	571	3115	AAAUCCAGGCAGGGGUUAU	896	
3115	UCUCCUAUUAGGCUAUAA	572	3115	UCUCCUAUUAGGCUAUAA	572	3133	UUUAAGCCUAAUAGGAAGA	897	
3133	AGAAGUAGCAAGAUUUUA	573	3133	AGAAGUAGCAAGAUUUUA	573	3151	UAAAGAUCUUGCUACUUCU	898	
3151	ACAUAAUUCAGAGUGGUUU	574	3151	ACAUAAUUCAGAGUGGUUU	574	3169	AAACCACUCUGAAUUUAUGU	899	
3169	UCAUUGCCUUCUACCCUC	575	3169	UCAUUGCCUUCUACCCUC	575	3187	GAGGGUAGGAAGGCAUAGA	900	
3187	CUCUUAUGGCCCUCCAUAU	576	3187	CUCUUAUGGCCCUCCAUAU	576	3205	AAUGGAGGGGCCAUUAGAG	901	
3205	UUUUUUGACUAAAGCAUCA	577	3205	UUUUUUGACUAAAGCAUCA	577	3223	UGAUGC UUUAUGUCAAAUAA	902	
3223	ACACAGUGGCACUAGCAUU	578	3223	ACACAGUGGCACUAGCAUU	578	3241	AAUGCAGUGCCACUGUGU	903	
3241	UAUACCAAGAGUAUGAGAA	579	3241	UAUACCAAGAGUAUGAGAA	579	3259	UUCUCAUACUCUUGGUUAUA	904	
3259	AAUACAGUGCUUUAUGGCU	580	3259	AAUACAGUGCUUUAUGGCU	580	3277	AGCCAUA AAAGCACUGUAUU	905	

TABLE II-continued

APP, BACE, PSEN1, PSEN2 s1NA AND TARGET SEQUENCES									
3277	UCUAACAUUACUGCCUUA	581	3277	UCUAACAUUACUGCCUUA	581	3295	UGAAGGCAGUAAUGUUAGA	906	
3295	AGUAUCAAGGCUGCCUGGA	582	3295	AGUAUCAAGGCUGCCUGGA	582	3313	UCCAGGCAGCCUUGAUACU	907	
3313	AGAAAGGAUGGCAGCCUCA	583	3313	AGAAAGGAUGGCAGCCUCA	583	3331	UGAGGCUGCCAUCCUUUCU	908	
3331	AGGGCUUCCUUAUGUCCUC	584	3331	AGGGCUUCCUUAUGUCCUC	584	3349	GAGGACAUAAAGGAAGCCCU	909	
3349	CCACCACAAGAGCUCCUUG	585	3349	CCACCACAAGAGCUCCUUG	585	3367	CAAGGAGCUCUUGUGUGG	910	
3367	GAUGAAGGUAUCUUUUUC	586	3367	GAUGAAGGUAUCUUUUUC	586	3385	GAAAAGAUGACCUUCAUC	911	
3385	CCCCUUAUCCUGUUCUCCC	587	3385	CCCCUUAUCCUGUUCUCCC	587	3403	GGGAAGAACAGGAUAGGGG	912	
3403	CCUCCCCGCUCCUUAUGGU	588	3403	CCUCCCCGCUCCUUAUGGU	588	3421	ACCAUUAAGGAGCGGGAGG	913	
3421	UACGUGGGUACCCAGGCUG	589	3421	UACGUGGGUACCCAGGCUG	589	3439	CAGCCUGGGUACCCACGUA	914	
3439	GGUUCUUGGGCUAGGUAGU	590	3439	GGUUCUUGGGCUAGGUAGU	590	3457	ACUACCUAGCCCAAGAACC	915	
3457	UGGGGACCAAGUUAUUAAC	591	3457	UGGGGACCAAGUUAUUAAC	591	3475	GUAAUGAACUUGGUCCCCA	916	
3475	CCUCCCUAUCAGUUCUAGC	592	3475	CCUCCCUAUCAGUUCUAGC	592	3493	GCUAGAACUGAUAGGGAGG	917	
3493	CAUAGUAAACUACGGUACC	593	3493	CAUAGUAAACUACGGUACC	593	3511	GGUACCGUAGUUUACUAUG	918	
3511	CAGUGUUAUGGGGAAGAGC	594	3511	CAGUGUUAUGGGGAAGAGC	594	3529	GCUCUCCCCACUAACACUG	919	
3529	CUGGGUUUCCUAGUAUAC	595	3529	CUGGGUUUCCUAGUAUAC	595	3547	GUUAUACUAGGAAACCCAG	920	
3547	CCCACUGCAUCCUACUCCU	596	3547	CCCACUGCAUCCUACUCCU	596	3565	AGGAGUAGGAGCAGUGGG	921	
3565	UACCUGGUAACCCGCUGC	597	3565	UACCUGGUAACCCGCUGC	597	3583	GCACGGGUUGACCAGGUA	922	
3583	CUUCCAGGUUAGGGACCUG	598	3583	CUUCCAGGUUAGGGACCUG	598	3601	CAGGUCCCAUACUGGGAAG	923	
3601	GCUAAGUGUGGAAUUAACU	599	3601	GCUAAGUGUGGAAUUAACU	599	3619	AGGUAAUUCACACUUAGC	924	
3619	UGAUAAGGGAGAGGGAUUA	600	3619	UGAUAAGGGAGAGGGAUUA	600	3637	AUUUCCUCUCCCUUAUCA	925	
3637	UACAAGGAGGGCCUCUGGU	601	3637	UACAAGGAGGGCCUCUGGU	601	3655	ACCAGAGGCCCUCCUUGUA	926	
3655	UGUCCUGGCCUCAGCCAG	602	3655	UGUCCUGGCCUCAGCCAG	602	3673	CUGGCUGAGGCCAGGAACA	927	
3673	GCUGCCCAAGCCAUAAA	603	3673	GCUGCCCAAGCCAUAAA	603	3691	UUUAUGGCUUGUGGGCAGC	928	
3691	ACCAAUAAAACAAGAAUAC	604	3691	ACCAAUAAAACAAGAAUAC	604	3709	GUUUUCUUGUUUUUUGGU	929	
3709	CUGAGUCAGUUUUUAUCU	605	3709	CUGAGUCAGUUUUUAUCU	605	3727	AGAUAAAAAACUGACUCAG	930	
3727	UGGGUUCUCUUAUUCCEA	606	3727	UGGGUUCUCUUAUUCCEA	606	3745	UGGGAUAGAAGAGAACCCA	931	
3745	ACUGCACUUGGUGCUGCUU	607	3745	ACUGCACUUGGUGCUGCUU	607	3763	AAGCAGCACCAGUGCAGU	932	
3763	UUGGCUGACUGGGAACACC	608	3763	UUGGCUGACUGGGAACACC	608	3781	GGUGUUCUCCAGUCAGCCAA	933	
3781	CCCAUAACUACAGAGUCUG	609	3781	CCCAUAACUACAGAGUCUG	609	3799	CAGACUCUGUAGUUAUGGG	934	
3799	GACAGGAAGACUGGAGACU	610	3799	GACAGGAAGACUGGAGACU	610	3817	AGUCUCCAGUCUCCUGUC	935	
3817	UGUCCACUUCUAGCUCGGA	611	3817	UGUCCACUUCUAGCUCGGA	611	3835	UCCGAGCUAGAAGUGGACA	936	
3835	AACUUACUGUGUAAUAAA	612	3835	AACUUACUGUGUAAUAAA	612	3853	UUUAUUUACACAGUAAGUU	937	
3853	ACUUUCAGAACUGCUACCA	613	3853	ACUUUCAGAACUGCUACCA	613	3871	UGGUAGCAGUUCUGAAAGU	938	
3871	AUGAAGUGAAAAUGCCACA	614	3871	AUGAAGUGAAAAUGCCACA	614	3889	UGUGGCAUUUUCACUUCAU	939	
3889	AUUUUGCUUUUAAUUUCU	615	3889	AUUUUGCUUUUAAUUUCU	615	3907	AGAAAUUUAUAAAGCAAAU	940	
3907	UACCCAUGUUGGAAAAAAC	616	3907	UACCCAUGUUGGAAAAAAC	616	3925	GUUUUUCCCAACUAGGGUA	941	
3925	CUGGCUUUUUCCAGCCCU	617	3925	CUGGCUUUUUCCAGCCCU	617	3943	AGGGCUGGGAUAAAGCCAG	942	

TABLE II-continued

APP, BACE, PSEN1, PSEN2 s1nA AND TARGET SEQUENCES									
3943	UUUCCAGGGCAUAAAACUC	618	3943	UUUCCAGGGCAUAAAACUC	618	3961	GAGUUUUUUGCCUGGAAA	943	
3961	CAACCCCUUCGAUAGCAAG	619	3961	CAACCCCUUCGAUAGCAAG	619	3979	CUUGCUAUCGAAGGGUUG	944	
3979	GUCCCAUCAGCCUUAUUUU	620	3979	GUCCCAUCAGCCUUAUUUU	620	3997	AAUAAUAGGCUAUGGGAC	945	
3997	UUUUUUAAAGAAAACUUGC	621	3997	UUUUUUAAAGAAAACUUGC	621	4015	GCAAGUUUUCUUUAAAAA	946	
4015	CACUUGUUUUUCUUUUUAC	622	4015	CACUUGUUUUUCUUUUUAC	622	4033	GUAAAAAGAAAACAAGUG	947	
4033	CAGUUACUCCUCCUGGCC	623	4033	CAGUUACUCCUCCUGGCC	623	4051	GGCAGGAAGGAAGUACUG	948	
4051	CCCAAAAUUAUAAACUCUA	624	4051	CCCAAAAUUAUAAACUCUA	624	4069	UAGAGUUUAUAAUUUGGG	949	
4069	AAGUGUAAAAAAGUCUU	625	4069	AAGUGUAAAAAAGUCUU	625	4087	AAGACUUUUUUUACACUU	950	
4087	UAACAACAGCUUCUUGCUU	626	4087	UAACAACAGCUUCUUGCUU	626	4105	AAGCAAGAAGCUGUUGUUA	951	
4105	UGUAAAAUAUGUAUUUAUA	627	4105	UGUAAAAUAUGUAUUUAUA	627	4123	UAUAAUACAUUUUUUACA	952	
4123	ACAUCUGUAUUUUUAAAUU	628	4123	ACAUCUGUAUUUUUAAAUU	628	4141	AAUUUUAAAAUACAGAUGU	953	
4141	UCUGCUCUGAAAAAUGAC	629	4141	UCUGCUCUGAAAAAUGAC	629	4159	GUCAUUUUUCAGGAGCAGA	954	
4159	CUGUCCCAUUCUCCACUCA	630	4159	CUGUCCCAUUCUCCACUCA	630	4177	UGAGUGGAGAAUGGGACAG	955	
4177	ACUGCAUUUGGGGCCUUUC	631	4177	ACUGCAUUUGGGGCCUUUC	631	4195	GAAAGGCCCCAAAUGCAGU	956	
4195	CCCAUUGGUCUGCAUGUCU	632	4195	CCCAUUGGUCUGCAUGUCU	632	4213	AGACAUGCAGACCAUGGG	957	
4213	UUUUUAUCAUUGCAGGCCAG	633	4213	UUUUUAUCAUUGCAGGCCAG	633	4231	CUGGCCUGCAAUGAUAAAA	958	
4231	GUGGACAGAGGGAGAAGGG	634	4231	GUGGACAGAGGGAGAAGGG	634	4249	CCCUUCUCCUCUGUCCAC	959	
4249	GAGAACAGGGGUCGCCAAC	635	4249	GAGAACAGGGGUCGCCAAC	635	4267	GUUGGCGACCCUUGUUCUC	960	
4267	CACUUGUGUUGCUUUCUGA	636	4267	CACUUGUGUUGCUUUCUGA	636	4285	UCAGAAAGCAACACAAGUG	961	
4285	ACUGAUCCUGAACAGAAGA	637	4285	ACUGAUCCUGAACAGAAGA	637	4303	UUUCUUGUUCAGGAUCAGU	962	
4303	AGAGUAACACUGAGGCGCU	638	4303	AGAGUAACACUGAGGCGCU	638	4321	AGCGCCUCAGUGUUAUCUC	963	
4321	UCGCUCCCAUGCACAAUC	639	4321	UCGCUCCCAUGCACAAUC	639	4339	GAGUUGUGCAUGGGAGCGA	964	
4339	CUCCAAAACACUUAUCCUC	640	4339	CUCCAAAACACUUAUCCUC	640	4357	GAGGAUAAGUGUUUUGGAG	965	
4357	CCUGCAAGAGUGGGCUUUC	641	4357	CCUGCAAGAGUGGGCUUUC	641	4375	GAAAGCCCACUCUUGCAGG	966	
4375	CCAGGGUCUUUACUGGGAA	642	4375	CCAGGGUCUUUACUGGGAA	642	4393	UUCCCAAGUAAAGACCUGG	967	
4393	AGCAGUUAAGCCCCUCCU	643	4393	AGCAGUUAAGCCCCUCCU	643	4411	AGGAGGGGCUUACUGCU	968	
4411	UCACCCCUUCCUUUUUUCU	644	4411	UCACCCCUUCCUUUUUUCU	644	4429	AGAAAAAGGAAGGGGUGA	969	
4429	UUUCUUUACUCCUUUGGCU	645	4429	UUUCUUUACUCCUUUGGCU	645	4447	AGCCAAAGGAGUAAAGAAA	970	
4447	UUCAAAGGAUUUUGGAAAA	646	4447	UUCAAAGGAUUUUGGAAAA	646	4465	UUUCCAAAAUCCUUUGAA	971	
4465	AGAAACAUAUGCUUUACA	647	4465	AGAAACAUAUGCUUUACA	647	4483	UGUAAAGCAUAUUGUUUCU	972	
4483	ACUCAUUUUCAAUUUCUAA	648	4483	ACUCAUUUUCAAUUUCUAA	648	4501	UUAGAAAUUGAAAAUGAGU	973	
4501	AAUUUGCAGGGGAUACUGA	649	4501	AAUUUGCAGGGGAUACUGA	649	4519	UCAGUAUCCCCUGCAAUU	974	
4519	AAAAUACGGCAGGUGGCC	650	4519	AAAAUACGGCAGGUGGCC	650	4537	GGCCACCUGCCGUUUUUU	975	
4537	CUAAGGCUGCUGUAAAGUU	651	4537	CUAAGGCUGCUGUAAAGUU	651	4555	AACUUUACAGCAGCCUAG	976	
4555	UGAGGGGAGAGGAAUUCU	652	4555	UGAGGGGAGAGGAAUUCU	652	4573	AAGAUUCCUCUCCCUCA	977	
4573	UAAGAUAACAAGUAAAAA	653	4573	UAAGAUAACAAGUAAAAA	653	4591	UUUUUAUCUUGUAAUCUUA	978	
4591	AACGAAUCCCUAAACAAA	654	4591	AACGAAUCCCUAAACAAA	654	4609	UUUGUUUAGGGGAUUCGUU	979	

TABLE II-continued

APP, BACE, PSEN1, PSEN2 siRNA AND TARGET SEQUENCES									
4609	AAAGAACAUAAGAACUGGU	655	4609	AAAGAACAUAAGAACUGGU	658	627	ACCAGUUCUAUUGUUCUUU	980	
4627	UCUCCAUAUUUGCCACCUU	656	4627	UCUCCAUAUUUGCCACCUU	658	645	AAGGUGGCAAAAUGGAAGA	981	
4645	UCCUGUUAUGACAGCUA	657	4645	UCCUGUUAUGACAGCUA	658	663	UAGCUGUAUGAACAGGAA	982	
4663	ACUAAACUGGAGACAGUAA	658	4663	ACUAAACUGGAGACAGUAA	658	681	UUACUGUCUCCAGGUUAGU	983	
4681	ACAUUUAUAUAAACAAAGA	659	4681	ACAUUUAUAUAAACAAAGA	659	4699	UCUUUGGUUAAUGAAAUUGU	984	
4699	AAAGUGGGUACACUGACCU	660	4699	AAAGUGGGUACACUGACCU	660	717	AGGUCAGGUGACCCACUUU	985	
4717	UCUGAAGAGCUGAGUACUC	661	4717	UCUGAAGAGCUGAGUACUC	661	735	GAGUACUCAGCUCUUCAGA	986	
4735	CAGGCCACUCCAAUACCCC	662	4735	CAGGCCACUCCAAUACCCC	662	753	GGGUGAUUGGAGUGGCCUG	987	
4753	CUACAAGAUCCCAAGGAGG	663	4753	CUACAAGAUCCCAAGGAGG	662	771	CCUCCUUGGCAUCUUGUAG	988	
4771	GUCCCAGGAAGUCCAGCUC	664	4771	GUCCCAGGAAGUCCAGCUC	664	789	GAGCUGGACUCCUGGGAC	989	
4789	CCUUAACUGACGCUAGUC	665	4789	CCUUAACUGACGCUAGUC	665	807	GACUAGCGUCAGUUUAAAG	990	
4807	CAUUAACUGGCAAGUG	666	4807	CAUUAACUGGCAAGUG	666	825	CACUUGCCAGGUUUAUUG	991	
4825	GAGGCAAGAGAAUAGGGA	667	4825	GAGGCAAGAGAAUAGGGA	667	843	UCCUCAUUUCUUGCCUC	992	
4843	AAGAAUCCAUCUGUGAGGU	668	4843	AAGAAUCCAUCUGUGAGGU	668	861	ACCUCACAGAUGGAUUCUU	993	
4861	UGACAGGCAAGGAUGAAAG	669	4861	UGACAGGCAAGGAUGAAAG	668	879	CUUUAUCCUUGCCUGUCA	994	
4879	GACAAAGAAAGGAAAGAGU	670	4879	GACAAAGAAAGGAAAGAGU	670	897	ACUCUUUCCUUCUUGUC	995	
4897	UAUCAAGGAGAAAGGAG	671	4897	UAUCAAGGAGAAAGGAG	671	915	CUCCUUUCUGCCUUUGUA	996	
4915	GAUCAUUUAGUUGGUCUG	672	4915	GAUCAUUUAGUUGGUCUG	672	933	CAGACCCAAACUAAUAGU	997	
4933	GAAAGGAAAGUCUUUGCU	673	4933	GAAAGGAAAGUCUUUGCU	673	951	AGCAAAGACUUUCCUUC	998	
4951	UAUCCGACAUUACUGCUA	674	4951	UAUCCGACAUUACUGCUA	674	969	UAGCAGUACAUGCCGAUA	999	
4969	AGUACCUGUAAGCAUUUA	675	4969	AGUACCUGUAAGCAUUUA	675	987	UAAAAGCUGUACAGGUACU	1000	
4987	AGGUCCAGAAUGGAAAAA	676	4987	AGGUCCAGAAUGGAAAAA	676	005	UUUUUCCAUCUGGGACCU	1001	
5005	AAAAAUCAGCUAUUGGUAA	677	5005	AAAAAUCAGCUAUUGGUAA	677	023	UUACCAUAGCUGAUUUUU	1002	
5023	AUAUAUAAUUGUCCUUCC	678	5023	AUAUAUAAUUGUCCUUCC	678	041	GGAAAGGACAUUAUUAU	1003	
5041	CCUGGAGUCAGUUUUUUA	679	5041	CCUGGAGUCAGUUUUUUA	679	059	UAAAAAACUGACUCCAGG	1004	
5059	AAAAAGUUAACUCUUAGUU	680	5059	AAAAAGUUAACUCUUAGUU	680	077	AACUAAGAGUUAACUUUU	1005	
5077	UUUUAUCUGUUUAUUCUA	681	5077	UUUUAUCUGUUUAUUCUA	681	095	UAGAAUUAACAAGUAAAA	1006	
5095	AAAAGAGAAGGAGCUGAG	682	5095	AAAAGAGAAGGAGCUGAG	682	113	CUCAGCUCUCCUUCUUUU	1007	
5113	GGCAUCCUCCUGAGGAGU	683	5113	GGCAUCCUCCUGAGGAGU	683	131	ACUCCUACAGGGAAUGGCC	1008	
5131	UAAAGAUAAAAGGAUAGGA	684	5131	UAAAGAUAAAAGGAUAGGA	683	149	UCCUAUCCUUUAUCUUUA	1009	
5149	AAAAGAUCAAAGCUCUAA	685	5149	AAAAGAUCAAAGCUCUAA	685	167	UUAGAGCUUUGAAUCUUUU	1010	
5167	AUAGAGUCACAGCUUCCC	686	5167	AUAGAGUCACAGCUUCCC	686	185	GGGAAAGCUGUGACUCUAU	1011	
5185	CAGGUUAUAAACCUAAAAU	687	5185	CAGGUUAUAAACCUAAAAU	687	203	AUUUUAAGGUUUUAUACCUG	1012	
5203	UUUAGAAGUACAAUAGCA	688	5203	UUUAGAAGUACAAUAGCA	688	221	UGCUUUAUGUACUUCUUA	1013	
5221	AGAGSUGGAAAUGAUCUA	689	5221	AGAGSUGGAAAUGAUCUA	689	239	UAGAUAUUUCCACCUCU	1014	
5239	AGUCCUGAUAGCUACCCA	690	5239	AGUCCUGAUAGCUACCCA	690	257	UGGGUAGCUAUCAGGAACU	1015	
5257	ACAGAGCAAGUGAUUUAUA	691	5257	ACAGAGCAAGUGAUUUAUA	691	275	UAUAAUACUUGCUCUGU	1016	

TABLE II-continued

APP, BACE, PSEN1, PSEN2 s1nA AND TARGET SEQUENCES					
5275 AAAUUUGAAAUCCAAACUA	692 5275 AAAUUUGAAAUCCAAACUA	692 5293 UAGUUUGGAUUUCAAUUU	1017		
5293 ACUUUCUAAUAUCACUUU	693 5293 ACUUUCUAAUAUCACUUU	693 5311 AAAGUGAUUUUAAGAAAGU	1018		
5311 UGGUCUCCAUUUUUUCCAG	694 5311 UGGUCUCCAUUUUUUCCAG	694 5329 CUGGGAAAAUUGGAGACCA	1019		
5329 GGACAGGAAAUAUGUCCCC	695 5329 GGACAGGAAAUAUGUCCCC	695 5347 GGGGACAUAUUUCCUGUCC	1020		
5347 CCCUAACUUUCUUGCUUC	696 5347 CCCUAACUUUCUUGCUUC	696 5365 GAAGCAAGAAAGUUAGGGG	1021		
5365 CAAAAAUUAAAAUCCAGCA	697 5365 CAAAAAUUAAAAUCCAGCA	697 5383 UGCUGGAUUUUAAUUUUUG	1022		
5383 AUCCCAAGAUCAUUCUACA	698 5383 AUCCCAAGAUCAUUCUACA	698 5401 UGUAGAAUGAUCUUGGGAU	1023		
5401 AAGUAAUUUUGCACAGACA	699 5401 AAGUAAUUUUGCACAGACA	699 5419 UGUCUGUGCAAAAUUACUU	1024		
5419 AUCUCCUCACCCAGUGCC	700 5419 AUCUCCUCACCCAGUGCC	700 5437 GGCACUGGGUGAGGAGAU	1025		
5437 CUGUCUGGAGCUCACCCAA	701 5437 CUGUCUGGAGCUCACCCAA	701 5455 UUGGGUGAGCUCACAGACAG	1026		
5455 AGGUCACCAAAACAUCUGG	702 5455 AGGUCACCAAAACAUCUGG	702 5473 CCAAGUUGUUUGGUGACCU	1027		
5473 GUUGUGAACCAACUGCCUU	703 5473 GUUGUGAACCAACUGCCUU	703 5491 AAGGCAGUUGGUUACAAC	1028		
5491 UAACCUUCUGGGGAGGGG	704 5491 UAACCUUCUGGGGAGGGG	704 5509 CCCCUCGCCAGAAAGGUUA	1029		
5509 GGAUUAGCUAGACUAGGAG	705 5509 GGAUUAGCUAGACUAGGAG	705 5527 CUCCUAGUCUAGCUAAUCC	1030		
5527 GACCAGAAUGAAUUGGGAA	706 5527 GACCAGAAUGAAUUGGGAA	706 5545 UUCCCAUUCACUUCUGGUC	1031		
5545 AAGGGUGAGGACUUCACAA	707 5545 AAGGGUGAGGACUUCACAA	707 5563 UUGUGAAGUCCUCACCCUU	1032		
5563 AUGUUGGCCUGUCAGAGCU	708 5563 AUGUUGGCCUGUCAGAGCU	708 5581 AGCUCUGACAGGCCAACAU	1033		
5581 UUGAUUAGAAAGCCAAGACA	709 5581 UUGAUUAGAAAGCCAAGACA	709 5599 UGUCUUGGCCUUCUAAUCAA	1034		
5599 AGUGGCAGCAAGGAAGAC	710 5599 AGUGGCAGCAAGGAAGAC	710 5617 GUCUUCUUUUGCUGCCACU	1035		
5617 CUUGGCCAGGAAAAACCU	711 5617 CUUGGCCAGGAAAAACCU	711 5635 AGGUUUUUUUGGGCCAAG	1036		
5635 UGUGGGUUGUCUAAUUUC	712 5635 UGUGGGUUGUCUAAUUUC	712 5653 GAAAUUAGCACAAACCACA	1037		
5653 CUGUCCAGAAAAUAGGGUG	713 5653 CUGUCCAGAAAAUAGGGUG	713 5671 CACCCUAAUUUUGGACAG	1038		
5671 GGACAGAAAGCUUGGGGU	714 5671 GGACAGAAAGCUUGGGGU	714 5689 ACCCCACAAGCUUCUGUCC	1039		
5689 UGCAUGGAGGAAUUGGGAC	715 5689 UGCAUGGAGGAAUUGGGAC	715 5707 GUCCCAAUUCUCCAUUGCA	1040		
5707 CCUGGUUAUGUUGUUAUUC	716 5707 CCUGGUUAUGUUGUUAUUC	716 5725 GAAUAAACAUAUACCAGG	1041		
5725 CUCGGACUGUGAAUUUUGG	717 5725 CUCGGACUGUGAAUUUUGG	717 5743 CCAAAAUUCACAGUCCGAG	1042		
5743 GUGAUGUAAAACAGAAUUA	718 5743 GUGAUGUAAAACAGAAUUA	718 5761 AUUUCUGUUUUACAUCAC	1043		
5761 UUCUGUAAACCUAAUGUCU	719 5761 UUCUGUAAACCUAAUGUCU	719 5779 AGACAUUAGGUUUACAGAA	1044		
5779 UGUUAAAAUAAUGAGCGUU	720 5779 UGUUAAAAUAAUGAGCGUU	720 5797 AACGCUCAUUUUUAUACA	1045		
5797 UAACACAGUAAAAUUAUCA	721 5797 UAACACAGUAAAAUUAUCA	721 5815 UGAAUAAUUUACUGUGUUA	1046		
5815 AAUAAGAAGUCAAAAAAAA	722 5815 AAUAAGAAGUCAAAAAAAA	722 5833 UUUUUUUUGACUUCUUAUU	1047		
5821 AAGUCAAAAAAAA	723 5821 AAGUCAAAAAAAA	723 5839 UUUUUUUUUUUUUGACUU	1048		

PSEN1 NM_007319

Pos Seq	Seq ID	UPos Upper seq	Seq ID	LPos Lower seq	Seq ID
3 GACAGAGUUACCUGCACCG	1049	3 GACAGAGUUACCUGCACCG	1049	21 CGGUGCAGGUAAACUCUGUC	1132
21 GUUGUCCUACUCCAGAAU	1050	21 GUUGUCCUACUCCAGAAU	1050	39 AUUCUGGAAGUAGGACAAC	1133
39 UGCACAGAUGUCUGAGGAC	1051	39 UGCACAGAUGUCUGAGGAC	1051	57 GUCCUCAGACAUCUGUGCA	1134

TABLE II-continued

APP, BACE, PSEN1, PSEN2 siRNA AND TARGET SEQUENCES											
57	CAACCACCUGAGCAAUACU	1052	57	CAACCACCUGAGCAAUACU	1052	75	AGUAUUGCUCAGGUGGUUG	1135			
75	UAAUGACAAUAGAGAACGG	1053	75	UAAUGACAAUAGAGAACGG	1053	93	CCGUUCUCUAUUGUCAUUA	1136			
93	GCAGGAGCACAAACGACAGA	1054	93	GCAGGAGCACAAACGACAGA	1054	111	UCUGUCGUUGUCUCCUGC	1137			
111	ACGGAGCCUUGGCCACCCU	1055	111	ACGGAGCCUUGGCCACCCU	1055	129	AGGGUGGCCAAGGCUCCGU	1138			
129	UGAGCCAUUAUCUAAUGGA	1056	129	UGAGCCAUUAUCUAAUGGA	1056	147	UCCAUUAGAUAAUGGCUCA	1139			
147	ACGACCCCAGGSUAACUCC	1057	147	ACGACCCCAGGSUAACUCC	1057	165	GGAGUUACCCUGGGGUCGU	1140			
165	CCGGCAGGUGGUGGAGCAA	1058	165	CCGGCAGGUGGUGGAGCAA	1058	183	UUGCUCACCACCUGCCGG	1141			
183	AGAUGAGGAAGAAGAUAG	1059	183	AGAUGAGGAAGAAGAUAG	1059	201	CUCAUUCUUCUCCUACU	1142			
201	GGAGCUGACAUGAAUAU	1060	201	GGAGCUGACAUGAAUAU	1060	219	AUAUUUCAAUGCAGCUCC	1143			
219	UGGCGCCAAGCAUGUGAUC	1061	219	UGGCGCCAAGCAUGUGAUC	1061	237	GAUCACAUUCUUGGCGCCA	1144			
237	CAUGCUCUUGUCCUGUG	1062	237	CAUGCUCUUGUCCUGUG	1062	255	CACAGGACAAAGAGCAUG	1145			
255	GACUCUCUGCAUGGUGUG	1063	255	GACUCUCUGCAUGGUGUG	1063	273	CACCACCAUGCAGAGAGUC	1146			
273	GGUCGUGGCUACCAUUAAG	1064	273	GGUCGUGGCUACCAUUAAG	1064	291	CUUAAUGGUAGCCACGACC	1147			
291	GUCAGUCAGCUUUUAUACC	1065	291	GUCAGUCAGCUUUUAUACC	1065	309	GGUAUAAAAGCUGACUGAC	1148			
309	CCGAAGGAUGGGCAGCUA	1066	309	CCGAAGGAUGGGCAGCUA	1066	327	UAGCUGCCAUCCUUCGG	1149			
327	AAUCUAUACCCCAUUCACA	1067	327	AAUCUAUACCCCAUUCACA	1067	345	UGUGAAUGGGGUUAGAUU	1150			
345	AGAAGAUACCGAGACUGUG	1068	345	AGAAGAUACCGAGACUGUG	1068	363	CACAGUCUCGGUAUCUUCU	1151			
363	GGGCCAGAGAGCCUGCAC	1069	363	GGGCCAGAGAGCCUGCAC	1069	381	GUGCAGGGCUCUCUGGCC	1152			
381	CUCAAUUCUGAAUGCUGCC	1070	381	CUCAAUUCUGAAUGCUGCC	1070	399	GGCAGCAUUCAGAAUUGAG	1153			
399	CAUCAUGAUCAGUGUCAU	1071	399	CAUCAUGAUCAGUGUCAU	1071	417	AAUGACACUGAUCUGAUG	1154			
417	UGUUGUCAUGACUACCUC	1072	417	UGUUGUCAUGACUACCUC	1072	435	GAGGAUAGUCAUGACAACA	1155			
435	CCUGGUGGUUCUGUAUAAA	1073	435	CCUGGUGGUUCUGUAUAAA	1073	453	UUUAUACAGAACCACCAGG	1156			
453	AUACAGGUGCUAUAAGGUC	1074	453	AUACAGGUGCUAUAAGGUC	1074	471	GACCUUAUAGCACCUGUAU	1157			
471	CAUCCAUGCCUGGCUUAUU	1075	471	CAUCCAUGCCUGGCUUAUU	1075	489	AAUAAGCCAGGCAUGGAUG	1158			
489	UAUAUCAUCUCUAUUGUUG	1076	489	UAUAUCAUCUCUAUUGUUG	1076	507	CAACAUAUAGAGAUUAUA	1159			
507	GCUGUUCUUUUUUAUUC	1077	507	GCUGUUCUUUUUUAUUC	1077	525	GAAUGAAAAAAGAACAGC	1160			
525	CAUUUACUUGGGGAAGUG	1078	525	CAUUUACUUGGGGAAGUG	1078	543	CACUUCUUCCAAAGUAAAUG	1161			
543	GUUUAAAACCUAUAACGUU	1079	543	GUUUAAAACCUAUAACGUU	1079	561	AACGUUAUAGGUUUUAAAC	1162			
561	UGCUGUGGACUACAUUACU	1080	561	UGCUGUGGACUACAUUACU	1080	579	AGUAUUGUAGUCCACAGCA	1163			
579	UGUUGCACUCCUGAUCUGG	1081	579	UGUUGCACUCCUGAUCUGG	1081	597	CCAGAUCAAGGAGUGCAACA	1164			
597	GAAUUUUGGUGUGGUGGA	1082	597	GAAUUUUGGUGUGGUGGA	1082	615	UCCCAACCAACCAAAUUC	1165			
615	AAUGAUUUCCAUCACUGG	1083	615	AAUGAUUUCCAUCACUGG	1083	633	CCAGUGAAUGGAAAUCAUU	1166			
633	GAAAGGUCCACUUCGACUC	1084	633	GAAAGGUCCACUUCGACUC	1084	651	GAGUCGAAGUGGACCUUUC	1167			
651	CCAGCAGGCAUAUCUCAUU	1085	651	CCAGCAGGCAUAUCUCAUU	1085	669	AAUGAGAUUAGCCUGCUGG	1168			
669	UAUGAUUAGUGCCCUCAUG	1086	669	UAUGAUUAGUGCCCUCAUG	1086	687	CAUGAGGGCACUAUUAUA	1169			
687	GGCCUUGGUGUUUAUAAAG	1087	687	GGCCUUGGUGUUUAUAAAG	1087	705	CUUGAUAAACACAGGGCC	1170			
705	GUACCUCCUGAAUGGACU	1088	705	GUACCUCCUGAAUGGACU	1088	723	AGUCCAUCAGGGAGGUAC	1171			

TABLE II-continued

APP, BACE, PSEN1, PSEN2 s1nA AND TARGET SEQUENCES								
723	UGCUGGGCUCAUCUUGGCU	1089	723	UGCUGGGCUCAUCUUGGCU	1089	741	AGCCAAGAUGAGCCACGCA	1172
741	UGUGAUUUUCGGUAUAUGAU	1090	741	UGUGAUUUUCGGUAUAUGAU	1090	759	AUCAUAUACCGAAAUCACA	1173
759	UUUAGUGGCUGUUUUUGUGU	1091	759	UUUAGUGGCUGUUUUUGUGU	1091	777	ACACAAAACAGCCACUAAA	1174
777	UCCGAAAGGUCCACUUCGU	1092	777	UCCGAAAGGUCCACUUCGU	1092	795	ACGAAGUGGACCUUUCGGA	1175
795	UAUGCUGGUUGAAACAGCU	1093	795	UAUGCUGGUUGAAACAGCU	1093	813	AGCUGUUUCAACCAGCAUA	1176
813	UCAGGAGAGAAAUGAAACG	1094	813	UCAGGAGAGAAAUGAAACG	1094	831	CGUUUCAUUUCUCCUGA	1177
831	GCUUUUCCAGCUCUCAUU	1095	831	GCUUUUCCAGCUCUCAUU	1095	849	AAUGAGAGCUGGAAAAAGC	1178
849	UUACUCCUACAACAAUGGUG	1096	849	UUACUCCUACAACAAUGGUG	1096	867	CACCAUUGUUGAGGAGUAA	1179
867	GUGGUUGUGGAAUAUGGCA	1097	867	GUGGUUGUGGAAUAUGGCA	1097	885	UGCCAUAUUCACCAACCAC	1180
885	AGAAGGAGACCCGGAAGCU	1098	885	AGAAGGAGACCCGGAAGCU	1098	903	AGCUUCCGGGUCUCCUUCU	1181
903	UCAAAGGAGAGUAUCCAAA	1099	903	UCAAAGGAGAGUAUCCAAA	1099	921	UUUGGAUACUCCUUUGA	1182
921	AAAUUCCAAGUAUAUUGCA	1100	921	AAAUUCCAAGUAUAUUGCA	1100	939	UGCAUUAUACUUGGAAUUU	1183
939	AGAAAGAGCCUGUCUGCCU	1101	939	AGAAAGAGCCUGUCUGCCU	1101	957	AGGCAGACAGGCUCUUUCU	1184
957	UCCUGCUGCCAUCAACCUG	1102	957	UCCUGCUGCCAUCAACCUG	1102	975	CAGGUUGAUGGCAGCAGGA	1185
975	GCUGUCUAUAGCUCCCAUG	1103	975	GCUGUCUAUAGCUCCCAUG	1103	993	CAUGGGAGCUAUAGACAGC	1186
993	GGCACCCAGGCUGUUCAUG	1104	993	GGCACCCAGGCUGUUCAUG	1104	1011	CAUGAACAGCCUGGGUGCC	1187
1011	GCCAAAGGGUGCCUGCAGG	1105	1011	GCCAAAGGGUGCCUGCAGG	1105	1029	CCUGCAGGCACCCUUUGGC	1188
1029	GCCCACGGCAGAGAAAGGG	1106	1029	GCCCACGGCAGAGAAAGGG	1106	1047	CCCUUUCUGUGCCGUGGGC	1189
1047	GAGUCACAAGACACUGUUG	1107	1047	GAGUCACAAGACACUGUUG	1107	1065	CAACAGUGUCUUGUGACUC	1190
1065	GCAGAGAAUGAUGAUGGCG	1108	1065	GCAGAGAAUGAUGAUGGCG	1108	1083	CGCCAUAUCAUUCUUGC	1191
1083	GGGUUCAGUGAGGAAUGGG	1109	1083	GGGUUCAGUGAGGAAUGGG	1109	1101	CCCAUUCUCACUGAACCC	1192
1101	GAAGCCCAGAGGGACAGUC	1110	1101	GAAGCCCAGAGGGACAGUC	1110	1119	GACUGUCCCUUGGGCUUC	1193
1119	CAUCUAGGGCCUCAUCGCU	1111	1119	CAUCUAGGGCCUCAUCGCU	1111	1137	AGCGAUGAGGGCCUAGAUG	1194
1137	UCUACACCUGAGUCACGAG	1112	1137	UCUACACCUGAGUCACGAG	1112	1155	CUGGUGACUCAGGUGUAGA	1195
1155	GCUGCUGUCCAGGAACUUU	1113	1155	GCUGCUGUCCAGGAACUUU	1113	1173	AAAGUUCUGGACAGCAGC	1196
1173	UCCAGCAGUAUCCUCGCUG	1114	1173	UCCAGCAGUAUCCUCGCUG	1114	1191	CAGCGAGGAUACUGCUGGA	1197
1191	GGUGAAGACCCAGAGGAAA	1115	1191	GGUGAAGACCCAGAGGAAA	1115	1209	UUUCCUCUGGGUCUUCACC	1198
1209	AGGGGAGUAAAACUUGGAU	1116	1209	AGGGGAGUAAAACUUGGAU	1116	1227	AUCCAAGUUUUAUCCCCU	1199
1227	UUGGGAGAUUUCAUUUUCU	1117	1227	UUGGGAGAUUUCAUUUUCU	1117	1245	AGAAAAUGAAAUCCCCAA	1200
1245	UACAGUGUUCUGGUUGGUA	1118	1245	UACAGUGUUCUGGUUGGUA	1118	1263	UACCAACCAGAAACACUGUA	1201
1263	AAAGCCUCAGCAACAGCCA	1119	1263	AAAGCCUCAGCAACAGCCA	1119	1281	UGGCUGUUGCUGAGGCUUU	1202
1281	AGUGGAGACUGGAACACAA	1120	1281	AGUGGAGACUGGAACACAA	1120	1299	UUGUGUCCAGUCUCCACU	1203
1299	ACCAUAGCCUGUUUUCGUAG	1121	1299	ACCAUAGCCUGUUUUCGUAG	1121	1317	CUACGAAACAGGCUAUGGU	1204
1317	GCCAUUAUUAUUGGUUUGU	1122	1317	GCCAUUAUUAUUGGUUUGU	1122	1335	ACAAACCAUUAUAUUGGC	1205
1335	UGCCUUAUAUUAUACUCC	1123	1335	UGCCUUAUAUUAUACUCC	1123	1353	GGAGUAAUUAUGUAAGGCA	1206
1353	CUUGCCAUUUUAAGAAAG	1124	1353	CUUGCCAUUUUAAGAAAG	1124	1371	CUUUCUUGAAAAUGGCAAG	1207
1371	GCAUUGCCAGCUCUCCAA	1125	1371	GCAUUGCCAGCUCUCCAA	1125	1389	UUGGAAGAGCUGGCAUUGC	1208

TABLE II-continued

APP, BACE, PSEN1, PSEN2 siNA AND TARGET SEQUENCES						
1389 AUCUCCAUCACCUUUGGGC	1126	1389 AUCUCCAUCACCUUUGGGC	1126	1407 GCCCAAAGGUGAUGGAGAU	1209	
1407 CUUGUUUUUCUACUUUGCCA	1127	1407 CUUGUUUUUCUACUUUGCCA	1127	1425 UGGCAAAGUAGAAAACAAG	1210	
1425 ACAGAUUAUCUUGUACAGC	1128	1425 ACAGAUUAUCUUGUACAGC	1128	1443 GCUGUACAAGAUAUUCUGU	1211	
1443 CCUUUUUAUGACCAAUUAG	1129	1443 CCUUUUUAUGACCAAUUAG	1129	1461 CUAAUUGGUCCAUAUAAAG	1212	
1461 GCAUCCAUCAAUUUUUAUA	1130	1461 GCAUCCAUCAAUUUUUAUA	1130	1479 UAUAAAAUUGAUGGAAUGC	1213	
1464 UCCAUCAAUUUUUAUAUCU	1131	1464 UCCAUCAAUUUUUAUAUCU	1131	1482 AGAUAAUAAAUUGAUGGAA	1214	
PSEN2 NM_000447						
Pos Seq	Seq ID	UPos Upper seq	Seq ID	LPos Lower seq	Seq ID	
3 AGCGGCGGCGGAGCAGGCA	1215	3 AGCGGCGGCGGAGCAGGCA	1215	21 UGCCUGCUCGCGCGCCGCU	1339	
21 AUUCCAGCAGUGAGGAGA	1216	21 AUUCCAGCAGUGAGGAGA	1216	39 UCUCUCACUGCUGGAAA	1340	
39 ACAGCCAGAAGCAAGCUAU	1217	39 ACAGCCAGAAGCAAGCUAU	1217	57 AUAGCUUGCUUCUGGCUGU	1341	
57 UUGGAGCUGAAGGAACCUG	1218	57 UUGGAGCUGAAGGAACCUG	1218	75 CAGGUUCCUUCAGCUCCAA	1342	
75 GAGACAGAAGCUAGUCCCC	1219	75 GAGACAGAAGCUAGUCCCC	1219	93 GGGGACUAGCUUCUGUCUC	1343	
93 CCCUCUGAAUUUUACUGAU	1220	93 CCCUCUGAAUUUUACUGAU	1220	111 AUCAGUAAAUAUCAGAGGG	1344	
111 UGAAGAAACUGAGGCCACA	1221	111 UGAAGAAACUGAGGCCACA	1221	129 UGUGGCCUCAGUUUCUUA	1345	
129 AGAGCUAAAGUGACUUUUC	1222	129 AGAGCUAAAGUGACUUUUC	1222	147 GAAAAGUCACUUUAGCUCU	1346	
147 CCCAAGGUCGCCAGCGAG	1223	147 CCCAAGGUCGCCAGCGAG	1223	165 CUCGUGGGGCGACCUUGGG	1347	
165 GGACGUGGGACUUCUCAGA	1224	165 GGACGUGGGACUUCUCAGA	1224	183 UCUGAGAAGUCCACGUCC	1348	
183 ACGUCAGGAGAGUGAUGUG	1225	183 ACGUCAGGAGAGUGAUGUG	1225	201 CACAUACUCUCCUGACGU	1349	
201 GAGGGAGCUGUGUGACCAU	1226	201 GAGGGAGCUGUGUGACCAU	1226	219 AUGGUCACACAGCUCCUC	1350	
219 UAGAAAGUGACGUGUAAA	1227	219 UAGAAAGUGACGUGUAAA	1227	237 UUAACACGUCACUUUCUA	1351	
237 AAACCGAGCGUGCCUCUU	1228	237 AAACCGAGCGUGCCUCUU	1228	255 AAGAGGGCAGCGUGGUUU	1352	
255 UUGAAAGCCAGGAGCAUC	1229	255 UUGAAAGCCAGGAGCAUC	1229	273 GAUGCUCCUGGCUUUCAA	1353	
273 CAUUCAUUUAGCCUGUGA	1230	273 CAUUCAUUUAGCCUGUGA	1230	291 UCAGCAGGCUAAAUGAAUG	1354	
291 AGAAGAAGAAACCAAGUGU	1231	291 AGAAGAAGAAACCAAGUGU	1231	309 ACACUUGGUUUUCUUCUCU	1355	
309 UCCGGGAUUCAGACCUCUC	1232	309 UCCGGGAUUCAGACCUCUC	1232	327 GAGAGGUCUGAAUCCCGGA	1356	
327 CUGCGGCCCAAGUGUUCG	1233	327 CUGCGGCCCAAGUGUUCG	1233	345 CGAACACUUGGGGCCGAG	1357	
345 GUGGUGCUUCCAGAGGCAG	1234	345 GUGGUGCUUCCAGAGGCAG	1234	363 CUGCCUCUGGAAGCACCCAC	1358	
363 GGGCUAUGCUCACAUUCAU	1235	363 GGGCUAUGCUCACAUUCAU	1235	381 AUGAAUGUGAGCAUAGCCC	1359	
381 UGGCCUCUGACAGCGAGGA	1236	381 UGGCCUCUGACAGCGAGGA	1236	399 UCCUCGUGUCAGAGGCCA	1360	
399 AAGAAGUGUGUGAUGAGCG	1237	399 AAGAAGUGUGUGAUGAGCG	1237	417 CGCUCACACACACUUCUU	1361	
417 GGACGUCCUAAUGUCGGC	1238	417 GGACGUCCUAAUGUCGGC	1238	435 GCCGACAUUAGGACGUCC	1362	
435 CCGAGAGCCCCACGCCGCG	1239	435 CCGAGAGCCCCACGCCGCG	1239	453 CGCGGCGUGGGGCUUCGG	1363	
453 GCUCCUGCCAGGAGGGCAG	1240	453 GCUCCUGCCAGGAGGGCAG	1240	471 CUGCCCUCCUGGCAGGAGC	1364	
471 GGCAGGGCCCCAGAGGAUGG	1241	471 GGCAGGGCCCCAGAGGAUGG	1241	489 CCAUCCUCUGGGCCUGCC	1365	
489 GAGAGAACACUGCCAGUG	1242	489 GAGAGAACACUGCCAGUG	1242	507 CACUGGGCAGUGUUCUCUC	1366	

TABLE II-continued

APP, BACE, PSEN1, PSEN2 s1nA AND TARGET SEQUENCES								
507	GGAGAAGCCAGGAGAACGA	1243	507	GGAGAAGCCAGGAGAACGA	1243	525	UCGUUCCUGGCUUCCUCC	1367
525	AGGAGGACGGUGAGGAGGA	1244	525	AGGAGGACGGUGAGGAGGA	1244	543	UCCUCCUACCCGUCCUCCU	1368
543	ACCCUGACCGCUAUGUCUG	1245	543	ACCCUGACCGCUAUGUCUG	1245	561	CAGACAUAGCGGUCAGGGU	1369
561	GUAGUGGGGUUCCCGGGCG	1246	561	GUAGUGGGGUUCCCGGGCG	1246	579	CGCCCGGAACCCACUAC	1370
579	GGCCGCCAGGCCUGGAGGA	1247	579	GGCCGCCAGGCCUGGAGGA	1247	597	UCCUCCAGGCCUGGCGGCC	1371
597	AAGAGCUGACCCUCAAUA	1248	597	AAGAGCUGACCCUCAAUA	1248	615	UAUUUGAGGGUCAGCUCUU	1372
615	ACGGAGCGAAGCAGGUAU	1249	615	ACGGAGCGAAGCAGGUAU	1249	633	AUCACGUGCUCGCUCCGU	1373
633	UCAUGCUGUUUGGCCUGU	1250	633	UCAUGCUGUUUGGCCUGU	1250	651	ACAGGCACAAACAGCAUGA	1374
651	UCACUCUGUGCAUGAUCGU	1251	651	UCACUCUGUGCAUGAUCGU	1251	669	ACGAUCAUGCACAGAGUGA	1375
669	UGGUGGUAGCCACCAUCAA	1252	669	UGGUGGUAGCCACCAUCAA	1252	687	UUGAUGGUGGCUACCACCA	1376
687	AGUCUGUGCGCUUCUACAC	1253	687	AGUCUGUGCGCUUCUACAC	1253	705	GUGUAGAAGCGCACAGACU	1377
705	CAGAGAAGAAUGGACAGCU	1254	705	CAGAGAAGAAUGGACAGCU	1254	723	AGCUGUCCAUUCUUCUCUG	1378
723	UCAUCUACACGACAUUCAC	1255	723	UCAUCUACACGACAUUCAC	1255	741	GUGAAUGUCGUGUAGAUGA	1379
741	CUGAGGACACACCCUCGGU	1256	741	CUGAGGACACACCCUCGGU	1256	759	ACCGAGGGUGUGUCCUCAG	1380
759	UGGGCCAGCGCCUCCUCAA	1257	759	UGGGCCAGCGCCUCCUCAA	1257	777	UUGAGGAGGCGCUGGCCCA	1381
777	ACUCCGUGCUGAACACCCU	1258	777	ACUCCGUGCUGAACACCCU	1258	795	AGGGUGUUCAGCACGGAGU	1382
795	UCAUCAUGAUCAGCGUCAU	1259	795	UCAUCAUGAUCAGCGUCAU	1259	813	AUGACGCUGAUCAUGAUGA	1383
813	UCGUGGUUAUGACCAUCUU	1260	813	UCGUGGUUAUGACCAUCUU	1260	831	AAGAUGGUCAUAAACCAGA	1384
831	UCUUGGUGUGCUCUACAA	1261	831	UCUUGGUGUGCUCUACAA	1261	849	UUGUAGAGCACCACCAAGA	1385
849	AGUACCGCUGCUACAAGUU	1262	849	AGUACCGCUGCUACAAGUU	1262	867	AACUUGUAGCAGCGGUACU	1386
867	UCAUCCAUGGCUGGUUGAU	1263	867	UCAUCCAUGGCUGGUUGAU	1263	885	AUCAACCAGCCAUGGAUGA	1387
885	UCAUGUCUACUGAUGCU	1264	885	UCAUGUCUACUGAUGCU	1264	903	AGCAUCAGUGAAGACAUGA	1388
903	UGCUGUUCCUCUACCUA	1265	903	UGCUGUUCCUCUACCUA	1265	921	UAGGUGAAGAGGAACAGCA	1389
921	AUAUCUACCUUGGGGAAGU	1266	921	AUAUCUACCUUGGGGAAGU	1266	939	ACUUCUCCCAAGGUAGAUU	1390
939	UGCUCAAGACCUACAAGU	1267	939	UGCUCAAGACCUACAAGU	1267	957	ACAUUGUAGGUCUUGAGCA	1391
957	UGGCCAUGGACUACCCAC	1268	957	UGGCCAUGGACUACCCAC	1268	975	GUGGGGUAGUCCAUGGCCA	1392
975	CCUCUUGCUGACUGUCUG	1269	975	CCUCUUGCUGACUGUCUG	1269	993	CAGACAGUCAGCAAGAGGG	1393
993	GGAACUUCGGGGCAGUGGG	1270	993	GGAACUUCGGGGCAGUGGG	1270	1011	CCCACUGCCCCGAAGUUC	1394
1011	GCAUGGUGUGCAUCCACUG	1271	1011	GCAUGGUGUGCAUCCACUG	1271	1029	CAGUGGAUGCACACCAUGC	1395
1029	GGAAGGGCCUUCUGUGCU	1272	1029	GGAAGGGCCUUCUGUGCU	1272	1047	AGCACCAGAGGGCCUUC	1396
1047	UGCAGCAGGCCUACCUCAU	1273	1047	UGCAGCAGGCCUACCUCAU	1273	1065	AUGAGGUAGGCCUUGUGCA	1397
1065	UCAUGAUCAGUGCGCUCAU	1274	1065	UCAUGAUCAGUGCGCUCAU	1274	1083	AUGAGCGCACUGAUCAUGA	1398
1083	UGGCCUAGUGUUCUACAA	1275	1083	UGGCCUAGUGUUCUACAA	1275	1101	UUGAUGAACACUAGGGCCA	1399
1101	AGUACCUCCAGAGUGGUC	1276	1101	AGUACCUCCAGAGUGGUC	1276	1119	GACCACUCUGGGAGGUACU	1400
1119	CCGCGUGGGUACUCCUGGG	1277	1119	CCGCGUGGGUACUCCUGGG	1277	1137	CCCAGGAUGACCCACGCGG	1401
1137	GCGCCAUCUCUGUGUAUGA	1278	1137	GCGCCAUCUCUGUGUAUGA	1278	1155	UCAUACACAGAGAUGGCGC	1402
1155	AUCUCGUGGGCUGUGUGUG	1279	1155	AUCUCGUGGGCUGUGUGUG	1279	1173	CACAGCACAGCCACGAGAU	1403

TABLE II-continued

APP, BACE, PSEN1, PSEN2 siRNA AND TARGET SEQUENCES											
1173 GUCCCAAAGGGCCUCUGAG	1280	1173 GUCCCAAAGGGCCUCUGAG	1280	1191 CUCAGAGGCCCUUUGGGAC	1404						
1191 GAAUGCUGGUAGAAACUGC	1281	1191 GAAUGCUGGUAGAAACUGC	1281	1209 GCAGUUUCUACCAGCAUUC	1405						
1209 CCCAGGAGAGAAAUGAGCC	1282	1209 CCCAGGAGAGAAAUGAGCC	1282	1227 GGCUCAUUUCUCCUGGG	1406						
1227 CCAUAUUCUCCUGCCUGAU	1283	1227 CCAUAUUCUCCUGCCUGAU	1283	1245 AUCAGGGCAGGGAUAUUG	1407						
1245 UAUACUCAUCUGCCAUGGU	1284	1245 UAUACUCAUCUGCCAUGGU	1284	1263 ACCAUGGCAGAUAGAUUA	1408						
1263 UGUGGACGGUUGGCAUGGC	1285	1263 UGUGGACGGUUGGCAUGGC	1285	1281 GCCAUGCCAACCGUCCACA	1409						
1281 CGAAGCUGGACCCUCCUC	1286	1281 CGAAGCUGGACCCUCCUC	1286	1299 GAGGAGGGGUCCAGCUUCG	1410						
1299 CUCAGGGUGCCUCCAGCU	1287	1299 CUCAGGGUGCCUCCAGCU	1287	1317 AGCUGGAGGSCACCCUGAG	1411						
1317 UCCCCUACGACCCGGAGAU	1288	1317 UCCCCUACGACCCGGAGAU	1288	1335 AUCUCCGGGUCGUAGGGGA	1412						
1335 UGGAAGAAGACUCCUAUGA	1289	1335 UGGAAGAAGACUCCUAUGA	1289	1353 UCAUAGGAGUCUUCUCCA	1413						
1353 ACAGUUUUGGGAGCCUUC	1290	1353 ACAGUUUUGGGAGCCUUC	1290	1371 GAAGGCUCCCCAAAACUGU	1414						
1371 CAUACCCCGAAGUCUUUGA	1291	1371 CAUACCCCGAAGUCUUUGA	1291	1389 UCAAAGACUUCGGGGUAUG	1415						
1389 AGCCUCCCUUGACUGGCUA	1292	1389 AGCCUCCCUUGACUGGCUA	1292	1407 UAGCCAGUCAAGGGAGGCU	1416						
1407 ACCCAGGGGAGGAGCUGGA	1293	1407 ACCCAGGGGAGGAGCUGGA	1293	1425 UCCAGCUCCUCCUUGGGU	1417						
1425 AGGAAGAGGAGGAAAGGGG	1294	1425 AGGAAGAGGAGGAAAGGGG	1294	1443 CCCC UUCCUCCUUCUCCU	1418						
1443 GCGUGAAGCUUGGCCUCGG	1295	1443 GCGUGAAGCUUGGCCUCGG	1295	1461 CCGAGGCCAAGCUUCACGC	1419						
1461 GGGACUUCUUCUUCACAG	1296	1461 GGGACUUCUUCUUCACAG	1296	1479 CUGUAGAAGUAGAGUCCC	1420						
1479 GUGUGCUGGUGGCAAGGC	1297	1479 GUGUGCUGGUGGCAAGGC	1297	1497 GCCUUGCCACCAGCACAC	1421						
1497 CGGCUGCCACGGGCAGCGG	1298	1497 CGGCUGCCACGGGCAGCGG	1298	1515 CCGCUGCCCGUGGCAGCGG	1422						
1515 GGGACUGGAUACACGCU	1299	1515 GGGACUGGAUACACGCU	1299	1533 AGCGUGUAUUCAGUCCC	1423						
1533 UGGCCUGCUUCUGGCGCAU	1300	1533 UGGCCUGCUUCUGGCGCAU	1300	1551 AUGGCCACGAAGCAGGCCA	1424						
1551 UCCUCAUUGGCUUGUGUCU	1301	1551 UCCUCAUUGGCUUGUGUCU	1301	1569 AGACACAAGCCAUGAGGA	1425						
1569 UGACCCUCCUGCUGCUUGC	1302	1569 UGACCCUCCUGCUGCUUGC	1302	1587 GCAAGCAGCAGGAGGGUCA	1426						
1587 CUGUGUUAAGAAGCGCGU	1303	1587 CUGUGUUAAGAAGCGCGU	1303	1605 AGCGCCUUCUUGAACACAG	1427						
1605 UGCCC GCCUCCCAUCUC	1304	1605 UGCCC GCCUCCCAUCUC	1304	1623 GAGAUGGGGAGGCGGGCA	1428						
1623 CCAUCACGUUCGGGCUAU	1305	1623 CCAUCACGUUCGGGCUAU	1305	1641 AUGAGCCGAACGUGAUGG	1429						
1641 UCUUUUAUUCUCCACGGA	1306	1641 UCUUUUAUUCUCCACGGA	1306	1659 UCCGUGGAGAAGUAAAAGA	1430						
1659 ACAACUGGUGCGGCCGUU	1307	1659 ACAACUGGUGCGGCCGUU	1307	1677 AACGGCCGACACAGGUUGU	1431						
1677 UCAUGGACACCCUGGCCUC	1308	1677 UCAUGGACACCCUGGCCUC	1308	1695 GAGGCCAGGGUGUCCAUGA	1432						
1695 CCCAUCAGCUCUACAUCUG	1309	1695 CCCAUCAGCUCUACAUCUG	1309	1713 CAGAUGUAGAGCUGAUGGG	1433						
1713 GAGGGACAUGGUGGCCAC	1310	1713 GAGGGACAUGGUGGCCAC	1310	1731 GUGGCACACCAUGUCCUCC	1434						
1731 CAGGCUGCAAGCUGCAGGG	1311	1731 CAGGCUGCAAGCUGCAGGG	1311	1749 CCCUGCAGCUUGCAGCCUG	1435						
1749 GAAUUUUAUUGGAUGCAG	1312	1749 GAAUUUUAUUGGAUGCAG	1312	1767 CUGCAUCCAAUGAAAUUC	1436						
1767 GUUGUAUAGUUUACACUC	1313	1767 GUUGUAUAGUUUACACUC	1313	1785 GAGUGUAAAACUAUACAAC	1437						
1785 CUAGUGCCAUAUAUUUUUA	1314	1785 CUAGUGCCAUAUAUUUUUA	1314	1803 UAAAAUAUAUUGGCACUAG	1438						
1803 AAGACUUUUUUUCCUUAA	1315	1803 AAGACUUUUUUUCCUUAA	1315	1821 UUAAGGAAGAAAAGUCUU	1439						
1821 AAAAAUAAAGUACGUGUUU	1316	1821 AAAAAUAAAGUACGUGUUU	1316	1839 AAACACGUACUUUAUUUUU	1440						

TABLE II-continued

APP, BACE, PSEN1, PSEN2 siNA AND TARGET SEQUENCES									
1839	UACUUGGUGAGGAGGAGGC	1317	1839	UACUUGGUGAGGAGGAGGC	1317	1857	GCCUCCUCCUACCAAGUA	1441	
1857	CAGAACCAGCUCUUUGGUG	1318	1857	CAGAACCAGCUCUUUGGUG	1318	1875	CACCAAAGAGCUGGUUCUG	1442	
1875	GCCAGCUGUUUCAUACCA	1319	1875	GCCAGCUGUUUCAUACCA	1319	1893	UGGUGAUGAAACAGCUGGC	1443	
1893	AGACUUUGGCUCGCCUUCU	1320	1893	AGACUUUGGCUCGCCUUCU	1320	1911	AAAGCGGAGCCAAAGUCU	1444	
1911	UGGGGAGCGCCUCGCUUCA	1321	1911	UGGGGAGCGCCUCGCUUCA	1321	1929	UGAAGCGAGCGCUCCCA	1445	
1929	ACGGACAGGAAGCACAGCA	1322	1929	ACGGACAGGAAGCACAGCA	1322	1947	UGCUGUCUUCUGUCCGU	1446	
1947	AGGUUUUCCAGAUAAACU	1323	1947	AGGUUUUCCAGAUAAACU	1323	1965	AGUUCUUCGUAUAAACU	1447	
1965	UGAGAAGGUCAGAUUAGGG	1324	1965	UGAGAAGGUCAGAUUAGGG	1324	1983	CCCUAAUCUGACCUUCUA	1448	
1983	GCGGGGAGAAGAGCAUCCG	1325	1983	GCGGGGAGAAGAGCAUCCG	1325	2001	CGGAUGCUCUUCUCCCGC	1449	
2001	GGCAUGAGGGCUGAGAUGC	1326	2001	GGCAUGAGGGCUGAGAUGC	1326	2019	GCAUCUCAGCCCUCAUGCC	1450	
2019	CGCAAAGAGUGUCUGGG	1327	2019	CGCAAAGAGUGUCUGGG	1327	2037	CCCAGCACACUCUUUGCG	1451	
2037	GAGUGGCCCCUGGCACCUG	1328	2037	GAGUGGCCCCUGGCACCUG	1328	2055	CAGGUGCCAGGGGCCACUC	1452	
2055	GGGUGCUCUGGCUGGAGAG	1329	2055	GGGUGCUCUGGCUGGAGAG	1329	2073	CUCUCCAGCCAGAGCACCC	1453	
2073	GGAAAAGCCAGUCCCUAC	1330	2073	GGAAAAGCCAGUCCCUAC	1330	2091	GUAGGGAACUGGCUUUUCC	1454	
2091	CGAGGAGUGUCCCAAUGC	1331	2091	CGAGGAGUGUCCCAAUGC	1331	2109	GCAUUGGGAACACUCCUG	1455	
2109	CUUUGUCCAUGAUGUCCUU	1332	2109	CUUUGUCCAUGAUGUCCUU	1332	2127	AAGGACAUCAUGGACAAAG	1456	
2127	UGUUAUUUUUUGCCUUUA	1333	2127	UGUUAUUUUUUGCCUUUA	1333	2145	UAAAGGCAUAAAUACA	1457	
2145	AGAAACUGAGUCCUGUUCU	1334	2145	AGAAACUGAGUCCUGUUCU	1334	2163	AGAACAGGACUCAGUUUCU	1458	
2163	UUGUUACGGCAGUCACACU	1335	2163	UUGUUACGGCAGUCACACU	1335	2181	AGUGUGACUGCCGUAACAA	1459	
2181	UGCUGGGAAGUGGCUUAU	1336	2181	UGCUGGGAAGUGGCUUAU	1336	2199	AUUAAGCCACUCCAGCA	1460	
2199	UAGUAAUUAUAAAUAG	1337	2199	UAGUAAUUAUAAAUAG	1337	2217	CUAUUUUUGAUUUUACUA	1461	
2216	AGAUGAGUCCUGUAGAAA	1338	2216	AGAUGAGUCCUGUAGAAA	1338	2234	UUUCUACAGGACUCAUCU	1462	

[0437] The 3'-ends of the Upper sequence and the Lower sequence of the siNA construct can include an overhang sequence, for example about 1, 2, 3, or 4 nucleotides in length, preferably 2 nucleotides in length, wherein the overhanging sequence of the lower sequence is optionally

complementary to a portion of the target sequence. The upper sequence is also referred to as the sense strand, whereas the lower sequence is also referred to as the antisense strand. The upper and lower sequences in the Table can further comprise a chemical modification having Formulae I-VII or any combination thereof.

TABLE III

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS						
Tar- get Pos	Target	Seq ID	APP			Seq ID
			Cmpd #	Aliases	Sequence	
791	CAGACUAUGCAGAUUGGAGUGAA	1463		APP:793U21 sense siNA	GACUAUGCAGAUUGGAGUGTT	1495
829	GUAGCAGAGGAGGAAGAAGUGGC	1464		APP:831U21 sense siNA	AGCAGAGGAGGAAGAAGUGTT	1496
851	CUGAGGUGGAAGAAGAAGAGCC	1465		APP:853U21 sense siNA	GAGGUGGAAGAAGAAGAGTT	1497
1356	AGAGAGAAUUGUCCAGGUCAUGA	1466		APP:1358U21 sense siNA	AGAGAAUUGUCCAGGUCAUTT	1498

TABLE III-continued

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS					
1568 AGAACUACAUACCCGCUCUGCAG	1467	APP:1570U21 sense siNA	AACUACAUACCCGCUCUGCTT	1499	
2012 AUUCUUUUGGGGUGACUCUGUG	1468	APP:2014U21 sense siNA	UCUUUUGGGGUGACUCUGTT	1500	
2481 UGAAGUUGGACAGCAAAACCAUU	1469	APP:2483U21 sense siNA	AAGUUGGACAGCAAAACCATT	1501	
2482 GAAGUUGGACAGCAAAACCAUUG	1470	APP:2484U21 sense siNA	AGUUGGACAGCAAAACCAUTT	1502	
791 CAGACUAUGCAGAUGGGAGUGAA	1463	APP:811L21 antisense siNA (793C)	CACUCCCAUCUGCAUAGUCTT	1503	
829 GUAGCAGAGGAGGAAGAAGUGGC	1464	APP:849L21 antisense siNA (831C)	CACUUCUUCUCCUCUGCUTT	1504	
851 CUGAGGUGGAAGAAGAAGAAGCC	1465	APP:871L21 antisense siNA (853C)	CUUCUUCUUCUCCACCUCTT	1505	
1356 AGAGAGAAUGUCCAGGUCAUGA	1466	APP:1376L21 antisense siNA (1358C)	AUGACCUGGACAUCUCUTT	1506	
1568 AGAACUACAUACCCGCUCUGCAG	1467	APP:1588L21 antisense siNA (157CC)	GCAGAGCGGUGAUGUAGUUTT	1507	
2012 AUUCUUUUGGGGUGACUCUGUG	1468	APP:2032L21 antisense siNA (2014C)	CAGAGUCAGCCCCAAAAGATT	1508	
2481 UGAAGUUGGACAGCAAAACCAUU	1469	APP:2501L21 antisense siNA (2483C)	UGGUUUUGCUGUCCAACUUTT	1509	
2482 GAAGUUGGACAGCAAAACCAUUG	1470	APP:2502L21 antisense siNA (2484C)	AUGGUUUUGCUGUCCAACUTT	1510	
791 CAGACUAUGCAGAUGGGAGUGAA	1463	APP:793U21 sense siNA stab04	B GAcuAuGcAGAuGGGAGuGTT B	1511	
829 GUAGCAGAGGAGGAAGAAGUGGC	1464	APP:831U21 sense siNA stab04	B AGcAGAGGAGGAAGAAGuGTT B	1512	
851 CUGAGGUGGAAGAAGAAGAAGCC	1465	APP:853U21 sense siNA stab04	B GAGGuGGAAGAAGAAGAGTT B	1513	
1356 AGAGAGAAUGUCCAGGUCAUGA	1466	APP:1358U21 sense siNA stab04	B AGAGAAuGucccAGGucAuTT B	1514	
1568 AGAACUACAUACCCGCUCUGCAG	1467	APP:1570U21 sense siNA stab04	B AAcuAcAucAccGcucuGcTT B	1515	
2012 AUUCUUUUGGGGUGACUCUGUG	1468	APP:2014U21 sense siNA stab04	B ucuuuuGGGGcuGAcucuGTT B	1516	
2481 UGAAGUUGGACAGCAAAACCAUU	1469	APP:2483U21 sense siNA stab04	B AAGuuGGAcAGcAAAaccATT B	1517	
2482 GAAGUUGGACAGCAAAACCAUUG	1470	APP:2484U21 sense siNA stab04	B AGuuGGAcAGcAAAaccAuTT B	1518	
791 CAGACUAUGCAGAUGGGAGUGAA	1463	APP:811L21 antisense siNA (793C) stab05	cAcucccAucGcAuAGucTsT	1519	
829 GUAGCAGAGGAGGAAGAAGUGGC	1464	APP:849L21 antisense siNA (831C) stab05	cAcuucuccuccucuGcuTsT	1520	
851 CUGAGGUGGAAGAAGAAGAAGCC	1465	APP:871L21 antisense siNA (853C) stab05	cuucuucuuccuccAccucTsT	1521	
1356 AGAGAGAAUGUCCAGGUCAUGA	1466	APP:1376L21 antisense siNA (1358C) stab05	AuGAccuGGGAcAuucucuTsT	1522	
1568 AGAACUACAUACCCGCUCUGCAG	1467	APP:1588L21 antisense siNA (1570C) stab05	GcAGAGcGGUGAuGuAGuuTsT	1523	
2012 AUUCUUUUGGGGUGACUCUGUG	1468	APP:2032L21 antisense siNA (2014C) stab05	cAGAGucAGccccAAAAGATsT	1524	
2481 UGAAGUUGGACAGCAAAACCAUU	1469	APP:2501L21 antisense siNA (2483C) stab05	uGGuuuuGcuGuccAAcuuTsT	1525	
2482 GAAGUUGGACAGCAAAACCAUUG	1470	APP:2502L21 antisense siNA (2484C) stab05	AuGGuuuuGcuGuccAAcuTsT	1526	

TABLE III-continued

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS					
791 CAGACUAUGCAGAU ~~GGG~~ GUGAA	1463	APP:793U21 sense siNA stab07	B ~~G~~ AcuAuGcAGAU ~~GGG~~ GuGTT B	1527	
829 GUAGCAGAGGAGGAAGAAGUGGC	1464	APP:831U21 sense siNA stab07	B AGcAGAGGAGGAAGAAGuGTT B	1528	
851 CUGAGGUGGAAGAAGAAGGCC	1465	APP:853U21 sense siNA stab07	B GAGGuGGAAGAAGAAGATT B	1529	
1356 AGAGAGAAUGUCCAGGUCAUGA	1466	APP:1358U21 sense siNA stab07	B AGAGAAuGu ~~ccc~~ AGGuCAuTT B	1530	
1568 AGAACUACAUCACCGCUCUGCAG	1467	APP:1570U21 sense siNA stab07	B AAcuAcAucAccGcucuGcTT B	1531	
2012 AUUCUUUUGGGGUGACUCUGUG	1468	APP:2014U21 sense siNA stab07	B uuuuuuGGGGuGAcucuGTT B	1532	
2481 UGAAGUUGGACAGCAAAACCAUU	1469	APP:2483U21 sense siNA stab07	B AAGuuGGAcAGcAAAAccATT B	1533	
2482 GAAGUUGGACAGCAAAACCAUUG	1470	APP:2484U21 sense siNA stab07	B AGuuGGAcAGcAAAAccAuTT B	1534	
791 CAGACUAUGCAGAU ~~GGG~~ GUGAA	1463	APP:811L21 antisense siNA (793C) stab11	cAcucccAucUGcAuAGucTsT	1535	
829 GUAGCAGAGGAGGAAGAAGUGGC	1464	APP:849L21 antisense siNA (831C) stab11	cAcuuuuccuccucuGcuTsT	1536	
851 CUGAGGUGGAAGAAGAAGGCC	1465	APP:871L21 antisense siNA (853C) stab11	cuucuucuuccuccAccucTsT	1537	
1356 AGAGAGAAUGUCCAGGUCAUGA	1466	APP:1376L21 antisense siNA (1358C) stab11	AuGAccuGGGAcAuucucuTsT	1538	
1568 AGAACUACAUCACCGCUCUGCAG	1467	APP:1588L21 antisense siNA (1570C) stab11	GcAGAGcGGuGAuGuAGuuTsT	1539	
2012 AUUCUUUUGGGGUGACUCUGUG	1468	APP:2032L21 antisense siNA (2014C) stab11	cAGAGucAGccccAAAAGATsT	1540	
2481 UGAAGUUGGACAGCAAAACCAUU	1469	APP:2501L21 antisense siNA (2483C) stab11	uGGuuuuGcuGuccAAcuuTsT	1541	
2482 GAAGUUGGACAGCAAAACCAUUG	1470	APP:2502L21 antisense siNA (2484C) stab11	AuGGuuuuGcuGuccAAcuTsT	1542	
791 CAGACUAUGCAGAU ~~GGG~~ GUGAA	1463	APP:793U21 sense siNA stab18	B ~~G~~ AcuAuGcAGAU ~~GGG~~ GuGTT B	1543	
829 GUAGCAGAGGAGGAAGAAGUGGC	1464	APP:831U21 sense siNA stab18	B AGcAGAGGAGGAAGAAGuGTT B	1544	
851 CUGAGGUGGAAGAAGAAGGCC	1465	APP:853U21 sense siNA stab18	B GAGGuGGAAGAAGAAGATT B	1545	
1356 AGAGAGAAUGUCCAGGUCAUGA	1466	APP:1358U21 sense siNA stab18	B AGAGAAuGu ~~ccc~~ AGGuCAuTT B	1546	
1568 AGAACUACAUCACCGCUCUGCAG	1467	APP:1570U21 sense siNA stab18	B AAcuAcAucAccGcucuGcTT B	1547	
2012 AUUCUUUUGGGGUGACUCUGUG	1468	APP:2014U21 sense siNA stab18	B uuuuuuGGGGuGAcucuGTT B	1548	
2481 UGAAGUUGGACAGCAAAACCAUU	1469	APP:2483U21 sense siNA stab18	B AAGuuGGAcAGcAAAAccATT B	1549	
2482 GAAGUUGGACAGCAAAACCAUUG	1470	APP:2484U21 sense siNA stab18	B AGuuGGAcAGcAAAAccAuTT B	1550	
791 CAGACUAUGCAGAU ~~GGG~~ GUGAA	1463	33885 APP:811L21 antisense siNA (793C) stab08	cAcucccAucUGcAuAGucTsT	1551	
829 GUAGCAGAGGAGGAAGAAGUGGC	1464	33886 APP:849L21 antisense siNA (831C) stab08	cAcuuuuccuccucuGcuTsT	1552	
851 CUGAGGUGGAAGAAGAAGGCC	1465	33887 APP:871L21 antisense siNA (853C) stab08	cuucuucuuccuccAccucTsT	1553	
1356 AGAGAGAAUGUCCAGGUCAUGA	1466	33888 APP:1376L21 antisense siNA (1358C) stab08	AuGAccuGGGAcAuucucuTsT	1554	
1568 AGAACUACAUCACCGCUCUGCAG	1467	33889 APP:1588L21 antisense siNA (1570C) stab08	GcAGAGcGGuGAuGuAGuuTsT	1555	
2012 AUUCUUUUGGGGUGACUCUGUG	1468	33890 APP:2032L21 antisense siNA (2014C) stab08	cAGAGucAGccccAAAAGATsT	1556	

TABLE III-continued

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS					
2481 UGAAGUUGGACAGCAAAACCAUU	1469	33891	APP:2501L21 antisense siNA (2483C) stab08	uGGuuuuGcuGuccAAcuuTsT	1557
2482 GAAGUUGGACAGCAAAACCAUUG	1470	33892	APP:2502L21 antisense siNA (2484C) stab08	AuGGuuuuGcuGuccAAcuTsT	1558
791 CAGACUAUGCAGAUUGGGAGUGAA	1463	33869	APP:793U21 sense siNA stab09	B GACUAUGCAGAUUGGGAGUGTT B	1559
829 GUAGCAGAGGAGGAAGAAGUGGC	1464	33870	APP:831U21 sense siNA stab09	B AGCAGAGGAGGAAGAAGUGTT B	1560
851 CUGAGGUGGAAGAAGAAGAGCC	1465	33871	APP:853U21 sense siNA stab09	B GAGGUGGAAGAAGAAGAAGTT B	1561
1356 AGAGAGAAUGUCCAGGUCAUGA	1466	33872	APP:1358U21 sense siNA stab09	B AGAGAAUGUCCAGGUCAUTT B	1562
1568 AGAACUACAUCACCGCUCUGCAG	1467	33873	APP:1570U21 sense siNA stab09	B AACUACAUCACCGCUCUGCTT B	1563
2012 AUUCUUUUGGGGUGACUCUGUG	1468	33874	APP:2014U21 sense siNA stab09	B UCUCUUUGGGGUGACUCUGTT B	1564
2481 UGAAGUUGGACAGCAAAACCAUU	1469	33875	APP:2483U21 sense siNA stab09	B AAGUUGGACAGCAAAACCATT B	1565
2482 GAAGUUGGACAGCAAAACCAUUG	1470	33876	APP:2484U21 sense siNA stab09	B AGUUGGACAGCAAAACCAUTT B	1566
791 CAGACUAUGCAGAUUGGGAGUGAA	1463	33877	APP:811L21 antisense siNA (793C) stab10	CACUCCCAUCUGCAUAGUCTsT	1567
829 GUAGCAGAGGAGGAAGAAGUGGC	1464	33878	APP:849L21 antisense siNA (831C) stab10	CACUUCUUCUCCUCUCUGUTsT	1568
851 CUGAGGUGGAAGAAGAAGAGCC	1465	33879	APP:871L21 antisense siNA (853C) stab10	CUUCUUCUUCUCCACCUCTsT	1569
1356 AGAGAGAAUGUCCAGGUCAUGA	1466	33880	APP:1376L21 antisense siNA (1358C) stab10	AUGACCUGGGACAUCUCUTsT	1570
1568 AGAACUACAUCACCGCUCUGCAG	1467	33881	APP:1588L21 antisense siNA (1570C) stab10	GCAGAGCGGUGAUGUAGUUTsT	1571
2012 AUUCUUUUGGGGUGACUCUGUG	1468	33882	APP:2032L21 antisense siNA (2014C) stab10	CAGAGUCAGCCCCAAAAGATsT	1572
2481 UGAAGUUGGACAGCAAAACCAUU	1469	33883	APP:2501L21 antisense siNA (2483C) stab10	UGGUUUUGCUGUCCAACUUTsT	1573
2482 GAAGUUGGACAGCAAAACCAUUG	1470	33884	APP:2502L21 antisense siNA (2484C) stab10	AUGGUUUUGCUGUCCAACUTsT	1574
791 CAGACUAUGCAGAUUGGGAGUGAA	1463		APP:811L21 antisense siNA (793C) stab19	cAcucccAucUgcAuAGucTT B	1575
829 GUAGCAGAGGAGGAAGAAGUGGC	1464		APP:849L21 antisense siNA (831C) stab19	cAcuuucuccuccucucUcUcTT B	1576
851 CUGAGGUGGAAGAAGAAGAGCC	1465		APP:871L21 antisense siNA (853C) stab19	cuuucuuucuccAccucTT B	1577
1356 AGAGAGAAUGUCCAGGUCAUGA	1466		APP:1376L21 antisense siNA (1358C) stab19	AuGAccuGGGAcAuucucUcTT B	1578
1568 AGAACUACAUCACCGCUCUGCAG	1467		APP:1588L21 antisense siNA (1570C) stab19	GcAGAGcGGUGAuGuAGuuTT B	1579
2012 AUUCUUUUGGGGUGACUCUGUG	1468		APP:2032L21 antisense siNA (2014C) stab19	cAGAGucAGccccAAAAGATT B	1580
2481 UGAAGUUGGACAGCAAAACCAUU	1469		APP:2501L21 antisense siNA (2483C) stab19	uGGuuuuGcuGuccAAcuuTT B	1581
2482 GAAGUUGGACAGCAAAACCAUUG	1470		APP:2502L21 antisense siNA (2484C) stab19	AuGGuuuuGcuGuccAAcuTT B	1582
791 CAGACUAUGCAGAUUGGGAGUGAA	1463		APP:811L21 antisense siNA (793C) stab22	CACUCCCAUCUGCAUAGUCTT B	1583

TABLE III-continued

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS						
829 GUAGCAGAGGAGGAAGAAGUGGC	1464	APP:849L21 antisense siNA (831C) stab22	CACUUCUCCUCCUCUCUUTT	B	1584	
851 CUGAGGUGGAAGAAGAAGGCC	1465	APP:871L21 antisense siNA (853C) stab22	CUUCUUCUCCUCCUCCUUTT	B	1585	
1356 AGAGAGAAUGUCCAGGUCAUGA	1466	APP:1376L21 antisense siNA (1358C) stab22	AUGACCUGGGACAUCUCUUTT	B	1586	
1568 AGAACUACAUCACCCGUCUGCAG	1467	APP:1588L21 antisense siNA (1570C) stab22	GCAGAGCGGUGAUGUAGUUTT	B	1587	
2012 AUUCUUUUGGGGUGACUCUGUG	1468	APP:2032L21 antisense siNA (2014C) stab22	CAGAGUCAGCCCCAAAGATT	B	1588	
2481 UGAAGUUGGACAGCAAAACCAU	1469	APP:2501L21 antisense siNA (2483C) stab22	UGGUUUUGCUGUCCAACUUTT	B	1589	
2482 GAAGUUGGACAGCAAAACCAUUG	1470	APP:2502L21 antisense siNA (2484C) stab22	AUGGUUUUGCUGUCCAACUUTT	B	1590	
BACE						
Tar- get Pos Target	Seq ID	Cmpd # Aliases	Sequence		Seq ID	
1025 CCUGGAGCCUUUCUUGACUCUC	1471	BACE:1027U21 sense siNA	UGGAGCCUUUCUUGACUUCTT		1591	
1028 GGAGCCUUUCUUGACUCUCUGG	1472	BACE:1030U21 sense siNA	AGCCUUUCUUGACUCUCUUTT		1592	
1393 AGAAGUCCUGAUGGUUUCUGG	1473	BACE:1395U21 sense siNA	AAGUCCUGAUGGUUUCUUTT		1593	
1490 AAUGGGUGAGGUUACCAACCAGU	1474	31005 BACE:1492U21 sense siNA	UGGGUGAGGUUACCAACCATT		1594	
1753 UCACCUUGGACAUGGAAGACUGU	1475	31006 BACE:1755U21 sense siNA	ACCUUGGACAUGGAAGACUUTT		1595	
1803 UCAACCCUCAUGACCAUAGCCUA	1476	BACE:1805U21 sense siNA	AACCCUCAUGACCAUAGCCTT		1596	
2457 CCUAAACUUGGUGCAAAGAUUGC	1477	31007 BACE:2459U21 sense siNA	UAACAUUGGUGCAAAGAUUTT		1597	
3583 UAUGGACCUGCUAAGUGUGGAA	1478	31008 BACE:3585U21 sense siNA	UGGGACCUGCUAAGUGUGGTT		1598	
1025 CCUGGAGCCUUUCUUGACUCUC	1471	BACE:1045L21 antisense siNA (1027C)	GAGUCAAGAAAGGCUCUCCATT		1599	
1028 GGAGCCUUUCUUGACUCUCUGG	1472	BACE:1048L21 antisense siNA (1030C)	AGAGAGUCAAGAAAGGCUUTT		1600	
1393 AGAAGUCCUGAUGGUUUCUGG	1473	BACE:1413L21 antisense siNA (1395C)	AGAAACCAUCAGGGAACUUTT		1601	
1490 AAUGGGUGAGGUUACCAACCAGU	1474	31081 BACE:1510L21 antisense siNA (1492C)	UGGUUGGUAAACCUCACCCATT		1602	
1753 UCACCUUGGACAUGGAAGACUGU	1475	31082 BACE:1773L21 antisense siNA (1755C)	AGUCUCCAUGUCCAAGGUTT		1603	
1803 UCAACCCUCAUGACCAUAGCCUA	1476	BACE:1823L21 antisense siNA (1805C)	GGCUAUGGUCAUGAGGGUUTT		1604	
2457 CCUAAACUUGGUGCAAAGAUUGC	1477	31083 BACE:2477L21 antisense siNA (2459C)	AAUCUUUGCACCAAGUUAATT		1605	
3583 UAUGGACCUGCUAAGUGUGGAA	1478	31084 BACE:3603L21 antisense siNA (3585C)	CCACACUAGCAGGUCCUCCATT		1606	
1025 CCUGGAGCCUUUCUUGACUCUC	1471	BACE:1027U21 sense siNA stab04	B uGGAGccuuuuuuGAcuTT	B	1607	
1028 GGAGCCUUUCUUGACUCUCUGG	1472	BACE:1030U21 sense siNA stab04	B AGccuuuuuuGAcucucTT	B	1608	

TABLE III-continued

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS					
1393 AGAAGUCCCCUGAUGGUUUCUGG	1473	BACE:1395U21 sense siNA stab04	B AAGUucccuGAUGGuuucUTT B	1609	
1490 AAUGGGUGAGGUUACCAACCAGU	1474 30729	BACE:1492U21 sense siNA stab04	B uGGGuGAGGuuAccAAccATT B	1610	
1753 UCACCUUGGACAUGGAAGACUGU	1475 30730	BACE:1755U21 sense siNA stab04	B AccuuGGACAuGGAAGAcuTT B	1611	
1803 UCAACCCUCAUGACCAUAGCCUA	1476	BACE:1805U21 sense siNA stab04	B AAcccucAuGAccAuAGccTT B	1612	
2457 CCUAAACAUUGGUGCAAAGAUUGC	1477 31378	BACE:2459U21 sense siNA stab04	B uAAcAuUGGuGcAAAGAuTT B	1613	
3583 UAUGGGACCUGCUAAGUGUGGAA	1478 30732	BACE:3585U21 sense siNA stab04	B uGGGAccuGcuAAAGuGuGTT B	1614	
1025 CCUGGAGCCUUUCUUGACUCUC	1471	BACE:1045L21 antisense siNA (1027C) stab05	GAGucAAAGAAAGGuccATsT	1615	
1028 GGAGCCUUUCUUGACUCUCUGG	1472	BACE:1048L21 antisense siNA (1030C) stab05	AGAGAGucAAAGAAAGGcuTsT	1616	
1393 AGAAGUCCCCUGAUGGUUUCUGG	1473	BACE:1413L21 antisense siNA (1395C) stab05	AGAAAccAucAGGGAACuuTsT	1617	
1490 AAUGGGUGAGGUUACCAACCAGU	1474 30733	BACE:1510L21 antisense siNA (1492C) stab05	uGGuuGGuAAccucAcccATsT	1618	
1753 UCACCUUGGACAUGGAAGACUGU	1475 30734	BACE:1773L21 antisense siNA (1755C) stab05	AGucuuccAuGuccAAGGuTsT	1619	
1803 UCAACCCUCAUGACCAUAGCCUA	1476	BACE:1823L21 antisense siNA (1805C) stab05	GGcuAuGGucAuGAGGGuTsT	1620	
2457 CCUAAACAUUGGUGCAAAGAUUGC	1477 31381	BACE:2477L21 antisense siNA (2459C) stab05	AAucuuuGcAccAAuGuuATsT	1621	
3583 UAUGGGACCUGCUAAGUGUGGAA	1478 30736	BACE:3603L21 antisense siNA (3585C) stab05	ccAcAcuuAGcAGGucccATsT	1622	
1025 CCUGGAGCCUUUCUUGACUCUC	1471	BACE:1027U21 sense siNA stab07	B uGGAGccuuuuuuGAcucTT B	1623	
1028 GGAGCCUUUCUUGACUCUCUGG	1472	BACE:1030U21 sense siNA stab07	B AGccuuuuuuGAcucucUTT B	1624	
1393 AGAAGUCCCCUGAUGGUUUCUGG	1473	BACE:1395U21 sense siNA stab07	B AAGUucccuGAUGGuuucUTT B	1625	
1490 AAUGGGUGAGGUUACCAACCAGU	1474	BACE:1492U21 sense siNA stab07	B uGGGuGAGGuuAccAAccATT B	1626	
1753 UCACCUUGGACAUGGAAGACUGU	1475	BACE:1755U21 sense siNA stab07	B AccuuGGACAuGGAAGAcuTT B	1627	
1803 UCAACCCUCAUGACCAUAGCCUA	1476	BACE:1805U21 sense siNA stab07	B AAcccucAuGAccAuAGccTT B	1628	
2457 CCUAAACAUUGGUGCAAAGAUUGC	1477 31384	BACE:2459U21 sense siNA stab07	B uAAcAuUGGuGcAAAGAuTT B	1629	
3583 UAUGGGACCUGCUAAGUGUGGAA	1478	BACE:3585U21 sense siNA stab07	B uGGGAccuGcuAAAGuGuGTT B	1630	
1025 CCUGGAGCCUUUCUUGACUCUC	1471	BACE:1045L21 antisense siNA (1027C) stab11	GAGucAAAGAAAGGuccATsT	1631	
1028 GGAGCCUUUCUUGACUCUCUGG	1472	BACE:1048L21 antisense siNA (103CC) stab11	AGAGAGucAAAGAAAGGcuTsT	1632	

TABLE III-continued

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS					
1393	AGAAGUCCCGAUGGUUUCUGG	1473	BACE:1413L21 antisense siNA (1395C) stab11	AGAAAccAucAGGGAacuuTsT	1633
1490	AAUGGGUGAGGUUACCAACCAGU	1474	BACE:1510L21 antisense siNA (1492C) stab11	GGuuGGuAAccucAcccATsT	1634
1753	UCACCUUGGACAUGGAAGACUGU	1475 (1755C) stab11	BACE:1773L21 antisense siNA	GucuuccAuGuccAAGGuTsT	1635
1803	UCAACCCUCAUGACCAUAGCCUA	1476	BACE:1823L21 antisense siNA (1805C) stab11	GcuAuGGucAuGAGGGuuTsT	1636
2457	CCUAACAUUGGUGCAAAGAUUGC	1477	31387BACE:2477L21 antisense siNA (2459C) stab11	AucuuuGcAccAAuGuuATsT	1637
3583	UAUGGGACCGCUAAGUGUGGAA	1478	BACE:3603L21 antisense siNA (3585C) stab11	ccAcAcuuAGcAGGucccATsT	1638
1025	CCUGGAGCCUUUCUUUGACUCUC	1471	BACE:1027U21 sense siNA stab18	B uGGAGccuuucuuuGAcucTT B	1639
1028	GGAGCCUUUCUUUGACUCUCUGG	1472	BACE:1030U21 sense siNA stab18	B AGccuuucuuuGAcucucTT B	1640
1393	AGAAGUCCCGAUGGUUUCUGG	1473	BACE:1395U21 sense siNA stab18	B AAGuucccuGAuGGuuucTT B	1641
1490	AAUGGGUGAGGUUACCAACCAGU	1474	BACE:1492U21 sense siNA stab18	B uGGGuGAGGuuAccAACcATT B	1642
1753	UCACCUUGGACAUGGAAGACUGU	1475	BACE:1755U21 sense siNA stab18	B AccuuGGAcAuGGAAGAcTT B	1643
1803	UCAACCCUCAUGACCAUAGCCUA	1476	BACE:1805U21 sense siNA stab18	B AAccucAuGAccAuAGcTT B	1644
2457	CCUAACAUUGGUGCAAAGAUUGC	1477	BACE:2459U21 sense siNA stab18	B uAAcAuuGGuGcAAAGAuTT B	1645
3583	UAUGGGACCGCUAAGUGUGGAA	1478	BACE:3585U21 sense siNA stab18	B uGGGAccuGcuAAGuGuGTT B	1646
1025	CCUGGAGCCUUUCUUUGACUCUC	1471	BACE:1045L21 antisense siNA (1027C) stab08	GAGucAAAGAAAGGucccATsT	1647
1028	GGAGCCUUUCUUUGACUCUCUGG	1472	BACE:1048L21 antisense siNA (1030C) stab08	AGAGAGucAAAGAAAGGcuTsT	1648
1393	AGAAGUCCCGAUGGUUUCUGG	1473	BACE:1413L21 antisense siNA (1395C) stab08	AGAAAccAucAGGGAacuuTsT	1649
1490	AAUGGGUGAGGUUACCAACCAGU	1474	BACE:1510L21 antisense siNA (1492C) stab08	uGGuuGGuAAccucAcccATsT	1650
1753	UCACCUUGGACAUGGAAGACUGU	1475	BACE:1773L21 antisense siNA (1755C) stab08	AGucuuccAuGuccAAGGuTsT	1651
1803	UCAACCCUCAUGACCAUAGCCUA	1476	BACE:1823L21 antisense siNA (1805C) stab08	GGcuAuGGucAuGAGGGuuTsT	1652
2457	CCUAACAUUGGUGCAAAGAUUGC	1477	BACE:2477L21 antisense siNA (2459C) stab08	AAucuuuGcAccAAuGuuATsT	1653
3583	UAUGGGACCGCUAAGUGUGGAA	1478	BACE:3603L21 antisense siNA (3585C) stab08	ccAcAcuuAGcAGGucccATsT	1654
1025	CCUGGAGCCUUUCUUUGACUCUC	1471	BACE:1027U21 sense siNA stab09	B UGGAGCCUUUCUUUGACUCUTT B	1655
1028	GGAGCCUUUCUUUGACUCUCUGG	1472	BACE:1030U21 sense siNA stab09	B AGCCUUUCUUUGACUCUCUTT B	1656

TABLE III-continued

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS					
1393 AGAAGUCCUGAUGGUUUCUGG	1473	BACE:1395U21 sense siNA stab09	B AAGUCCUGAUGGUUUCUTT	B	1657
1490 AAUGGGUGAGGUUACCAACCAGU	1474	BACE:1492U21 sense siNA stab09	B UGGGUGAGGUUACCAACCATT	B	1658
1753 UCACCUUGGACAUGGAAGACUGU	1475	BACE:1755U21 sense siNA stab09	B ACCUUGGACAUGGAAGACUTT	B	1659
1803 UCAACCCUCAUGACCAUAGCCUA	1476	BACE:1805U21 sense siNA stab09	B AACCCUCAUGACCAUAGCCTT	B	1660
2457 CCUAACAUGGUGCAAAGAUGC	1477	BACE:2459U21 sense siNA stab09	B UAACAUGGUGCAAAGAUAUTT	B	1661
3583 UAUGGGACUGCUAAGUGUGGAA	1478	BACE:3585U21 sense siNA stab09	B UGGGACUGCUAAGUGUGGTT	B	1662
1025 CCUGGAGCCUUUCUUGACUCUC	1471	BACE:1045L21 antisense siNA (1027C) stab10	GAGUCAAGAAAGGCCUCCATsT		1663
1028 GGAGCCUUUCUUGACUCUCUGG	1472	BACE:1048L21 antisense siNA (1030C) stab10	AGAGAGUCAAGAAAGGCUTsT		1664
1393 AGAAGUCCUGAUGGUUUCUGG	1473	BACE:1413L21 antisense siNA (1395C) stab10	AGAAACCAUCAGGGAACUUTsT		1665
1490 AAUGGGUGAGGUUACCAACCAGU	1474	BACE:1510L21 antisense siNA (1492C) stab10	UGGUUGGUAACCUCACCCATsT		1666
1753 UCACCUUGGACAUGGAAGACUGU	1475	BACE:1773L21 antisense siNA (1755C) stab10	AGUCUCCAUGUCCAAGGUTsT		1667
1803 UCAACCCUCAUGACCAUAGCCUA	1476	BACE:1823L21 antisense siNA (1805C) stab10	GGCUAUGGUCAUGAGGGUUTsT		1668
2457 CCUAACAUGGUGCAAAGAUGC	1477	BACE:2477L21 antisense siNA (2459C) stab10	AAUCUUUGCACCAUGUUAATsT		1669
3583 UAUGGGACUGCUAAGUGUGGAA	1478	BACE:3603L21 antisense siNA (3585C) stab10	CCACACUAGCAGGUCCCATsT		1670
1025 CCUGGAGCCUUUCUUGACUCUC	1471	BACE:1045L21 antisense siNA (1027C) stab19	GAGucAAAGAAAGGcuccATT	B	1671
1028 GGAGCCUUUCUUGACUCUCUGG	1472	BACE:1048L21 antisense siNA (1030C) stab19	AGAGAGucAAAGAAAGGcuTT	B	1672
1393 AGAAGUCCUGAUGGUUUCUGG	1473	BACE:1413L21 antisense siNA (1395C) stab19	AGAAAccAucAGGGAAcuuTT	B	1673
1490 AAUGGGUGAGGUUACCAACCAGU	1474	BACE:1510L21 antisense siNA (1492C) stab19	uGGuuGGuAAccucAcccATT	B	1674
1753 UCACCUUGGACAUGGAAGACUGU	1475	BACE:1773L21 antisense siNA (1755C) stab19	AGucuuuccAuGuccAAGGuTT	B	1675
1803 UCAACCCUCAUGACCAUAGCCUA	1476	BACE:1823L21 antisense siNA (1805C) stab19	GGcuAuGGucAuGAGGGuuTT	B	1676
2457 CCUAACAUGGUGCAAAGAUGC	1477	BACE:2477L21 antisense siNA (2459C) stab19	AAucuuuGcAccAAuGuuATT	B	1677
3583 UAUGGGACUGCUAAGUGUGGAA	1478	BACE:3603L21 antisense siNA (3585C) stab19	ccAcAcuuAGcAGGucccATT	B	1678
1025 CCUGGAGCCUUUCUUGACUCUC	1471	BACE:1045L21 antisense siNA (1027C) stab22	GAGUCAAGAAAGGCCUCCATT	B	1679
1028 GGAGCCUUUCUUGACUCUCUGG	1472	BACE:1048L21 antisense siNA (1030C) stab22	AGAGAGUCAAGAAAGGCUTT	B	1680
1393 AGAAGUCCUGAUGGUUUCUGG	1473	BACE:1413L21 antisense siNA (1395C) stab22	AGAAACCAUCAGGGAACUUTT	B	1681

TABLE III-continued

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS						
1490	AAUGGGUGAGGUUACCAACCAGU	1474	BACE:1510L21 antisense siNA (1492C) stab22	UGGUUGGUAACCUCACCCATT B	1682	
1753	UCACCUUGGACAUGGAAGACUGU	1475	BACE:1773L21 antisense siNA (1755C) stab22	AGUCUCCAUGUCCAAGGUTT B	1683	
1803	UCAACCCUCAUGACCAUAGCCUA	1476	BACE:1823L21 antisense siNA (1805C) stab22	GGCUAUGGUCAUGAGGGUUTT B	1684	
2457	CCUAAACAUUGGUGCAAAGAUUGC	1477	BACE:2477L21 antisense siNA (2459C) stab22	AAUCUUUGCACCAAUGUUATT B	1685	
3583	UAUGGGACCUUGCUAAGUGUGGAA	1478	BACE:3603L21 antisense siNA (3585C) stab22	CCACACUUAGCAGGUCCCATT B	1686	
2457	CCUAAACAUUGGUGCAAAGAUUGC	657 31390	BACE:2459U21 sense siNA inv stab04	B uuAGAAAcGuGGuuAcAAuTT B	1687	
2457	CCUAAACAUUGGUGCAAAGAUUGC	657 31393	BACE:2477L21 antisense siNA (2459C) inv stab05	AuuGuAAccAcGuuucuAATsT	1688	
2457	CCUAAACAUUGGUGCAAAGAUUGC	657 31396	BACE:2459U21 sense siNA inv stab07	B uuAGAAAcGuGGuuAcAAuTT B	1689	
2457	CCUAAACAUUGGUGCAAAGAUUGC	657 31399	BACE:2477L21 antisense siNA (2459C) inv stab11	AuuGuAAccAcGuuucuAATsT	1690	
PSEN1						
Tar- get Pos	Target	Seq ID	Cmpd # Aliases	Sequence	Seq ID	
693	CUAAUGGACGACCCAGGGUAAC	1479	PSEN1:695U21 sense siNA	AAUGGACGACCCAGGGUATT	1691	
1131	CUGUUGCACUCCUGAUCUGGAU	1480	PSEN1:1133U21 sense siNA	GUUGCACUCCUGAUCUGGATT	1692	
1493	GAAAGCACAGAAAGGAGUCACA	1481	PSEN1:1495U21 sense siNA	AAGCACAGAAAGGAGUCATT	1693	
1505	AGGGAGUCACAAGACACUGUUGC	1482	PSEN1:1507U21 sense siNA	GGAGUCACAAGACACUGUUTT	1694	
1748	GACUGGAACACAACCAUAGCCUG	1483	PSEN1:1750U21 sense siNA	CUGGAACACAACCAUAGCCTT	1695	
1751	UGGAACACAACCAUAGCCUGUU	1484	PSEN1:1753U21 sense siNA	GAACACAACCAUAGCCUGUTT	1696	
2184	CUACCAGAUUUGAGGGACGAGGU	1485	PSEN1:2186U21 sense siNA	ACCAGAUUUGAGGGACGAGTT	1697	
3007	UGUUGCCCAAAGCGGUAGAAU	1486	PSEN1:3009U21 sense siNA	UAUGCCCAAAGCGGUAGAATT	1698	
693	CUAAUGGACGACCCAGGGUAAC	1479	PSEN1:713L21 antisense siNA (695C)	UACCCUGGGGUCGUCCAUTT	1699	
1131	CUGUUGCACUCCUGAUCUGGAU	1480	PSEN1:1151L21 antisense siNA (1133C)	UCCAGAUCCAGGAGUGCAACTT	1700	
1493	GAAAGCACAGAAAGGAGUCACA	1481	PSEN1:1513L21 antisense siNA (1495C)	UGACUCCUUUCUGUGCUUTT	1701	
1505	AGGGAGUCACAAGACACUGUUGC	1482	PSEN1:1525L21 antisense siNA (1507C)	AACAGUGUCUUGUGACUCCTT	1702	
1748	GACUGGAACACAACCAUAGCCUG	1483	PSEN1:1768L21 antisense siNA (1750C)	GGCUAUGGUUGUGUUCAGTT	1703	
1751	UGGAACACAACCAUAGCCUGUU	1484	PSEN1:1771L21 antisense siNA (1753C)	ACAGGCUAUGGUUGUGUUCTT	1704	
2184	CUACCAGAUUUGAGGGACGAGGU	1485	PSEN1:2204L21 antisense siNA (2186C)	CUCGUCCCUCAAUUCUGGUTT	1705	
3007	UGUUGCCCAAAGCGGUAGAAU	1486	PSEN1:3027L21 antisense siNA (3009C)	UUCUACCGCUUUGGGCAUATT	1706	

TABLE III-continued

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS					
693 CUAUUGGACGACCCAGGGUAAC	1479	PSEN1:695U21 sense siNA stab04	B AAUGGACGACCCAGGGuATT	B	1707
1131 CUGUUGCACUCCUGAUCUGGAU	1480	PSEN1:1133U21 sense siNA stab04	B GuuGcAcuccuGAucUGATT	B	1708
1493 GAAAGCACAGAAAGGGAGUCACA	1481	PSEN1:1495U21 sense siNA stab04	B AAGcAcAGAAAGGGAGucATT	B	1709
1505 AGGGAGUCACAAGACACUGUUGC	1482	PSEN1:1507U21 sense siNA stab04	B GGAGucAcAAGAcAcuGuuTT	B	1710
1748 GACUGGAACACAACCAUAGCCUG	1483	PSEN1:1750U21 sense siNA stab04	B cuGGAACAcAAccAuAGccTT	B	1711
1751 UGGAACACAACCAUAGCCUGUUU	1484	PSEN1:1753U21 sense siNA stab04	B GAAcAcAAccAuAGccuGuTT	B	1712
2184 CUACCAGAUUUGAGGGACGAGGU	1485	PSEN1:2186U21 sense siNA stab04	B AccAGAuuuGAGGGAcGAGTT	B	1713
3007 UGU AUGCCCAAAGCGGUAGAAU	1486	PSEN1:3009U21 sense siNA stab04	B uAuGcccAAAGcGGuAGAATT	B	1714
693 CUAUUGGACGACCCAGGGUAAC	1479	PSEN1:713L21 antisense siNA (695C) stab05	uAcccuGGGGucGuccAuTsT		1715
1131 CUGUUGCACUCCUGAUCUGGAU	1480	PSEN1:1151L21 antisense siNA (1133C) stab05	uccAGAUcAGGAGuGcAAcTsT		1716
1493 GAAAGCACAGAAAGGGAGUCACA	1481	PSEN1:1513L21 antisense siNA (1495C) stab05	uGAcuccuuucGuGcuTsT		1717
1505 AGGGAGUCACAAGACACUGUUGC	1482	PSEN1:1525L21 antisense siNA (1507C) stab05	AAcAGUGucuuGuGAcuccTsT		1718
1748 GACUGGAACACAACCAUAGCCUG	1483	PSEN1:1768L21 antisense siNA (1750C) stab05	GGcuAuGGuuGuGuuccAGTsT		1719
1751 UGGAACACAACCAUAGCCUGUUU	1484	PSEN1:1771L21 antisense siNA (1753C) stab05	AcAGGcuAuGGuuGuGuucTsT		1720
2184 CUACCAGAUUUGAGGGACGAGGU	1485	PSEN1:2204L21 antisense siNA (2186C) stab05	cucGucccucAAAucUGuTsT		1721
3007 UGU AUGCCCAAAGCGGUAGAAU	1486	PSEN1:3027L21 antisense siNA (3009C) stab05	uuuAccGcuuuGGGcAuATsT		1722
693 CUAUUGGACGACCCAGGGUAAC	1479	PSEN1:695U21 sense siNA stab07	B AAUGGACGACCCAGGGuATT	B	1723
1131 CUGUUGCACUCCUGAUCUGGAU	1480	PSEN1:1133U21 sense siNA stab07	B GuuGcAcuccuGAucUGATT	B	1724
1493 GAAAGCACAGAAAGGGAGUCACA	1481	PSEN1:1495U21 sense siNA stab07	B AAGcAcAGAAAGGGAGucATT	B	1725
1505 AGGGAGUCACAAGACACUGUUGC	1482	PSEN1:1507U21 sense siNA stab07	B GGAGucAcAAGAcAcuGuuTT	B	1726
1748 GACUGGAACACAACCAUAGCCUG	1483	PSEN1:1750U21 sense siNA stab07	B cuGGAACAcAAccAuAGccTT	B	1727
1751 UGGAACACAACCAUAGCCUGUUU	1484	PSEN1:1753U21 sense siNA stab07	B GAAcAcAAccAuAGccuGuTT	B	1728
2184 CUACCAGAUUUGAGGGACGAGGU	1485	PSEN1:2186U21 sense siNA stab07	B AccAGAuuuGAGGGAcGAGTT	B	1729
3007 UGU AUGCCCAAAGCGGUAGAAU	1486	PSEN1:3009U21 sense siNA stab07	B uAuGcccAAAGcGGuAGAATT	B	1730
693 CUAUUGGACGACCCAGGGUAAC	1479	PSEN1:713L21 antisense siNA (695C) stab11	uAcccuGGGGucGuccAuTsT		1731

TABLE III-continued

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS					
1131	CUGUUGCACUCCUGAUCUGGAAU	1480	PSEN1:1151L21 antisense siNA (1133C) stab11	uccAGAuCAGGAGuGcAAcTsT	1732
1493	GAAAGCACAGAAAGGAGUCACA	1481	PSEN1:1513L21 antisense siNA (1495C) stab11	uGAcuccuuuuGuGcuuTsT	1733
1505	AGGGAGUCACAAGACACUGUUGC	1482	PSEN1:1525L21 antisense siNA (1507C) stab11	AAcAGuGucuuGuGAcuccTsT	1734
1748	GACUGGAACACAACCAUAGCCUG	1483	PSEN1:1768L21 antisense siNA (1750C) stab11	GGcuAuGGuuGuGuuccAGTsT	1735
1751	UGGAACACAACCAUAGCCUGUUU	1484	PSEN1:1771L21 antisense siNA (1753C) stab11	AcAGGcuAuGGuuGuGuucTsT	1736
2184	CUACCAGAUUUGAGGGACGAGGU	1485	PSEN1:2204L21 antisense siNA (2186C) stab11	cucGucccucAAAucuuGGuTsT	1737
3007	UGUAUGCCCAAAGCGUAGAAUU	1486	PSEN1:3027L21 antisense siNA (3009C) stab11	uuuuAccGcuuuGGGcAuATsT	1738
693	CUAAUGGACGACCCAGGGUAAAC	1479	PSEN1:695U21 sense siNA stab18	B AAUGGAcGAcccccAGGSuATT B	1739
1131	CUGUUGCACUCCUGAUCUGGAAU	1480	PSEN1:1133U21 sense siNA stab18	B GuuGcAcuccuGAucuGGAATT B	1740
1493	GAAAGCACAGAAAGGAGUCACA	1481	PSEN1:1495U21 sense siNA stab18	B AAGcAcAGAAAGGGAGucATT B	1741
1505	AGGGAGUCACAAGACACUGUUGC	1482	PSEN1:1507U21 sense siNA stab18	B GGAGucAcAAGAcAcuGuuTT B	1742
1748	GACUGGAACACAACCAUAGCCUG	1483	PSEN1:1750U21 sense siNA stab18	B cuGGAAcAcAAccAuAGccTT B	1743
1751	UGGAACACAACCAUAGCCUGUUU	1484	PSEN1:1753U21 sense siNA stab18	B GAAcAcAAccAuAGccuGuTT B	1744
2184	CUACCAGAUUUGAGGGACGAGGU	1485	PSEN1:2186U21 sense siNA stab18	B AccAGAuuuGAGGGAcGAGTT B	1745
3007	UGUAUGCCCAAAGCGUAGAAUU	1486	PSEN1:3009U21 sense siNA stab18	B uAuGccccAAAGcGGuAGAATT B	1746
693	CUAAUGGACGACCCAGGGUAAAC	1479	33933 PSEN1:713L21 antisense siNA (695C) stab08	uAcccuGGGGuGuccAuTsT	1747
1131	CUGUUGGACUCCUGAUCUGGAAU	1480	33934 PSEN1:1151L21 antisense siNA (1133C) stab08	uccAGAuCAGGAGuGcAAcTsT	1748
1493	GAAAGCACAGAAAGGAGUCACA	1481	33935 PSEN1:1513L21 antisense siNA (1495C) stab08	uGAcuccuuuuGuGcuuTsT	1749
1505	AGGGAGUCACAAGACACUGUUGC	1482	33936 PSEN1:1525L21 antisense siNA (1507C) stab08	AAcAGuGucuuGuGAcuccTsT	1750
1748	GACUGGAACACAACCAUAGCCUG	1483	33937 PSEN1:1768L21 antisense siNA (1750C) stab08	GGcuAuGGuuGuGuuccAGTsT	1751
1751	UGGAACACAACCAUAGCCUGUUU	1484	33938 PSEN1:1771L21 antisense siNA (1753C) stab08	AcAGGcuAuGGuuGuGuucTsT	1752
2184	CUACCAGAUUUGAGGGACGAGGU	1485	33939 PSEN1:2204L21 antisense siNA (2186C) stab08	cucGucccucAAAucuuGGuTsT	1753
3007	UGUAUGCCCAAAGCGUAGAAUU	1486	33940 PSEN1:3027L21 antisense siNA (3009C) stab08	uuuuAccGcuuuGGGcAuATsT	1754
693	CUAAUGGACGACCCAGGGUAAAC	1479	33917 PSEN1:695U21 sense siNA stab09	B AAUGGACGACCCAGGGUATT B	1755

TABLE III-continued

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS									
1131	CUGUUGCACUCCUGAUCUGGAAU	1480	33918	PSEN1:1133U21 sense siNA stab09	B	GUUGCACUCCUGAUCUGGATT	B	1756	
1493	GAAAGCACAGAAAGGGAGUCACA	1481	33919	PSEN1:1495U21 sense siNA stab09	B	AAGCACAGAAAGGGAGUCATT	B	1757	
1505	AGGGAGUCACAAGACACUGUUGC	1482	33920	PSEN1:1507U21 sense siNA stab09	B	GGAGUCACAAGACACUGUUTT	B	1758	
1748	GACUGGAACACAACCAUAGCCUG	1483	33921	PSEN1:1750U21 sense siNA stab09	B	CUGGAACACAACCAUAGCCTT	B	1759	
1751	UGGAACACAACCAUAGCCUGUUU	1484	33922	PSEN1:1753U21 sense siNA stab09	B	GAACACAACCAUAGCCUGUTT	B	1760	
2184	CUACCAGAUUUGAGGGACGAGGU	1485	33923	PSEN1:2186U21 sense siNA stab09	B	ACCAGAUUUGAGGGACGAGTT	B	1761	
3007	UGUAUGCCCAAAGCGGUAGAAUU	1486	33924	PSEN1:3009U21 sense siNA stab09	B	UAUGCCCAAAGCGGUAGAATT	B	1762	
693	CUAUUGGACGACCCAGGUUAC	1479	33925	PSEN1:713L21 antisense siNA (695C) stab10	U	ACCUGGGGUCGUCCAUTtT		1763	
1131	CUGUUGCACUCCUGAUCUGGAAU	1480	33926	PSEN1:1151L21 antisense siNA (1133C) stab10	U	CCAGAUCCAGGAGUGCAACTtT		1764	
1493	GAAAGCACAGAAAGGGAGUCACA	1481	33927	PSEN1:1513L21 antisense siNA (1495C) stab10	U	GACUCCCUUUCUGUGCUUttT		1765	
1505	AGGGAGUCACAAGACACUGUUGC	1482	33928	PSEN1:1525L21 antisense siNA (1507C) stab10	A	ACAGUGUCUUGUGACUCCttT		1766	
1748	GACUGGAACACAACCAUAGCCUG	1483	33929	PSEN1:1768L21 antisense siNA (1750C) stab10	G	CUAUGGUUGUGUCCAGTtT		1767	
1751	UGGAACACAACCAUAGCCUGUUU	1484	33930	PSEN1:1771L21 antisense siNA (1753C) stab10	A	CAGGCUAUGGUUGUGUUCttT		1768	
2184	CUACCAGAUUUGAGGGACGAGGU	1485	33931	PSEN1:2204L21 antisense siNA (2186C) stab10	C	UGUCCCUCAAUUCUGGUTtT		1769	
3007	UGUAUGCCCAAAGCGGUAGAAUU	1486	33932	PSEN1:3027L21 antisense siNA (3009C) stab10	U	UCUACCGCUUUGGGCAUAtT		1770	
693	CUAUUGGACGACCCAGGUUAC	1479		PSEN1:713L21 antisense siNA (695C) stab19	u	AcccuGGGGuGuccAuuTT	B	1771	
1131	CUGUUGCACUCCUGAUCUGGAAU	1480		PSEN1:1151L21 antisense siNA (1133C) stab19	ucc	AGAuCAGGAGuGcAAcTT	B	1772	
1493	GAAAGCACAGAAAGGGAGUCACA	1481		PSEN1:1513L21 antisense siNA (1495C) stab19	u	GAuccccuuucGuGcuTT	B	1773	
1505	AGGGAGUCACAAGACACUGUUGC	1482		PSEN1:1525L21 antisense siNA (1507C) stab19	A	AcAGuGuuuGuGAcuccTT	B	1774	
1748	GACUGGAACACAACCAUAGCCUG	1483		PSEN1:1768L21 antisense siNA (1750C) stab19	GGcuAu	GGuuGuGuuccAGTT	B	1775	
1751	UGGAACACAACCAUAGCCUGUUU	1484		PSEN1:1771L21 antisense siNA (1753C) stab19	Ac	AGGcuAuGGuuGuGuucTT	B	1776	
2184	CUACCAGAUUUGAGGGACGAGGU	1485		PSEN1:2204L21 antisense siNA (2186C) stab19	cuc	GuccuccAAAuCuGGTT	B	1777	
3007	UGUAUGCCCAAAGCGGUAGAAUU	1486		PSEN1:3027L21 antisense siNA (3009C) stab19	uucu	AccGcuuuGGGcAuATT	B	1778	
693	CUAUUGGACGACCCAGGUUAC	1479		PSEN1:713L21 antisense siNA (695C) stab22	U	ACCUGGGGUCGUCCAUTT	B	1779	

TABLE III-continued

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS						
1131	CUGUUGCACUCCUGAUCUGGAU	1480	PSEN1:1151L21 antisense siNA (1133C) stab22	UCCAGAUCAAGAGUGCAACTT B	1780	
1493	GAAAGCACAGAAAGGGAGUCACA	1481	PSEN1:1513L21 antisense siNA (1495C) stab22	UGACUCCCUUUCUGUCUUTT B	1781	
1505	AGGGAGUCACAAGACACUGUUGC	1482	PSEN1:1525L21 antisense siNA (1507C) stab22	AACAGUGUCUUGGACUCCTT B	1782	
1748	GACUGGAACACAACCAUAGCCUG	1483	PSEN1:1768L21 antisense siNA (1750C) stab22	GGCUAUGGUUGUGUCCAGTT B	1783	
1751	UGGAACACAACCAUAGCCUGUUU	1484	PSEN1:1771L21 antisense siNA (1753C) stab22	ACAGGCUAUGGUUGUGUUCTT B	1784	
2184	CUACCAGAUUUGAGGGACGAGGU	1485	PSEN1:2204L21 antisense siNA (2186C) stab22	CUCGUCCCUCAAUUGGUTT B	1785	
3007	UGUAUGCCCAAAGCGGUAGAAUU	1486	PSEN1:3027L21 antisense siNA (3009C) stab22	UUCUACCGCUUUGGGCAUATT B	1786	
PSEN2						
Tar- get Pos	Target	Seq ID	Cmpd # Aliases	Sequence	Seq ID	
104	UUACUGAUGAAGAAACUGAGGCC	1487	PSEN2:106U21 sense siNA	ACUGAUGAAGAAACUGAGGTT	1787	
260	AGCCAGGGAGCAUCAUUAUUUA	1488	PSEN2:262U21 sense siNA	CCAGGGAGCAUCAUUAUUTT	1788	
549	ACCGCUAUGUCUGUAGUGGGGUU	1489	PSEN2:551U21 sense siNA	CGCUAUGUCUGUAGUGGGGTT	1789	
597	AAGAGCUGACCCUCAAAUACGGA	1490	PSEN2:599U21 sense siNA	GAGCUGACCCUCAAAUACGTT	1790	
730	CACGACAUUCACUGAGGACACAC	1491	PSEN2:732U21 sense siNA	CGACAUUCACUGAGGACACTT	1791	
938	GUGCUCAGACCUACAAGUGGGC	1492	PSEN2:940U21 sense siNA	GCUCAAGACCUACAAGUGGTT	1792	
947	ACCUACAAGUGGCCAUGGACUA	1493	PSEN2:949U21 sense siNA	CUACAAGUGGCCAUGGACTT	1793	
2095	GAGUGUCCCCAUGCUUUGUCCA	1494	PSEN2:2097U21 sense siNA	GUGUCCCCAUGCUUUGUCTT	1794	
104	UUACUGAUGAAGAAACUGAGGCC	1487	PSEN2:124L21 antisense siNA (106C)	CCUCAGUUUCUUAUCAGUTT	1795	
260	AGCCAGGGAGCAUCAUUAUUUA	1488	PSEN2:280L21 antisense siNA (262C)	AAUGAAUGAUGCUCUGGTT	1796	
549	ACCGCUAUGUCUGUAGUGGGGUU	1489	PSEN2:569L21 antisense siNA (551C)	CCCCACUACAGACAAGCGTT	1797	
597	AAGAGCUGACCCUCAAAUACGGA	1490	PSEN2:617L21 antisense siNA (599C)	CGUAUUGAGGGUCAGCUCTT	1798	
730	CACGACAUUCACUGAGGACACAC	1491	PSEN2:750L21 antisense siNA (732C)	GUGUCCUCAGUGAAUGCGTT	1799	
938	GUGCUCAGACCUACAAGUGGGC	1492	PSEN2:958L21 antisense siNA (940C)	CACAUUGUAGGUCUUGAGCTT	1800	
947	ACCUACAAGUGGCCAUGGACUA	1493	PSEN2:967L21 antisense siNA (949C)	GUCCAUGGCCACAUGUAGTT	1801	
2095	GAGUGUCCCCAUGCUUUGUCCA	1494	PSEN2:2115L21 antisense siNA (2097C)	GACAAAGCAUUGGGAACACTT	1802	
104	UUACUGAUGAAGAAACUGAGGCC	1487	PSEN2:106U21 sense siNA stab04	B AcuGAUGAAGAAAcuGAGGTT B	1803	
260	AGCCAGGGAGCAUCAUUAUUUA	1488	PSEN2:262U21 sense siNA stab04	B ccAGGGAGcAucAucAuuTT B	1804	

TABLE III-continued

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS					
549	ACCGCUAUGUCUGUAGUGGGGUU	1489	PSEN2:551U21 sense siNA stab04	B cGcuAuGucuGuAGUGGGGTT B	1805
597	AAGAGCUGACCCUCAAUACGGA	1490	PSEN2:599U21 sense siNA stab04	B GAGcuGAcccucAAAUAcGTT B	1806
730	CACGACAUCACUGAGGACACAC	1491	PSEN2:732U21 sense siNA stab04	B cGAcAuucAcuGAGGAcAcTT B	1807
938	GUGCUCAGACCUACAUGUGGC	1492	PSEN2:940U21 sense siNA stab04	B GcucAAGAccuAcAAUGuGTT B	1808
947	ACCUACAAGUGGCCAUGGACUA	1493	PSEN2:949U21 sense siNA stab04	B cuAcAAuGuGGccAuGGAcTT B	1809
2095	GAGUGUCCCCAUGCUUUGUCCA	1494	PSEN2:2097U21 sense siNA stab04	B GuGuucccAAuGcuuuGucTT B	1810
104	UUACUGAUGAAGAAACUGAGGCC	1487	PSEN2:124L21 antisense siNA (106C) stab05	ccucAGuuucuuAcuAGuTsT	1811
260	AGCCAGGGAGCAUCAUUAUUUA	1488	PSEN2:280L21 antisense siNA (262C) stab05	AAuGAAuGAuGcuuccuGGTsT	1812
549	ACCGCUAUGUCUGUAGUGGGGUU	1489	PSEN2:569L21 antisense siNA (551C) stab05	ccccAcuAcAGAcAuAGcGTsT	1813
597	AAGAGCUGACCCUCAAUACGGA	1490	PSEN2:617L21 antisense siNA (5990) stab05	cGuAuuuGAGGGucAGcucTsT	1814
730	CACGACAUCACUGAGGACACAC	1491	PSEN2:750L21 antisense siNA (7320) stab05	GuGuccucAGuGAAuGucGTsT	1815
938	GUGCUCAGACCUACAUGUGGC	1492	PSEN2:958L21 antisense siNA (940C) stab05	cAcAuGuAGGucuuGAGcTsT	1816
947	ACCUACAAGUGGCCAUGGACUA	1493	PSEN2:967L21 antisense siNA (949C) stab05	GuccAuGGccAcAuGuAGTsT	1817
2095	GAGUGUCCCCAUGCUUUGUCCA	1494	PSEN2:2115L21 antisense siNA (20970) stab05	GAcAAAGcAuGGGAACAcTsT	1818
104	UUACUGAUGAAGAAACUGAGGCC	1487	PSEN2:106U21 sense siNA stab07	B AcuGAuGAAGAAAcuGAGGTT B	1819
260	AGCCAGGGAGCAUCAUUAUUUA	1488	PSEN2:262U21 sense siNA stab07	B ccAGGGAGcAuAcuucAuTT B	1820
549	ACCGCUAUGUCUGUAGUGGGGUU	1489	PSEN2:551U21 sense siNA stab07	B cGcuAuGucuGuAGUGGGGTT B	1821
597	AAGAGCUGACCCUCAAUACGGA	1490	PSEN2:599U21 sense siNA stab07	B GAGcuGAcccucAAAUAcGTT B	1822
730	CACGACAUCACUGAGGACACAC	1491	PSEN2:732U21 sense siNA stab07	B cGAcAuucAcuGAGGAcAcTT B	1823
938	GUGCUCAGACCUACAUGUGGC	1492	PSEN2:940U21 sense siNA stab07	B GcucAAGAccuAcAAUGuGTT B	1824
947	ACCUACAAGUGGCCAUGGACUA	1493	PSEN2:949U21 sense siNA stab07	B cuAcAAuGuGGccAuGGAcTT B	1825
2095	GAGUGUCCCCAUGCUUUGUCCA	1494	PSEN2:2097U21 sense siNA stab07	B GuGuucccAAuGcuuuGucTT B	1826
104	UUACUGAUGAAGAAACUGAGGCC	1487	PSEN2:124L21 antisense siNA (1060) stab11	cucAGuuucuuAcuAGuTsT	1827
260	AGCCAGGGAGCAUCAUUAUUUA	1488	PSEN2:280L21 antisense siNA (2620) stab11	AuGAAuGAuGcuuccuGGTsT	1828
549	ACCGCUAUGUCUGUAGUGGGGUU	1489	PSEN2:569L21 antisense siNA (5510) stab11	cccAcuAcAGAcAuAGcGTsT	1829

TABLE III-continued

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS					
597 AAGAGCUGACCCUCAAUACGGA	1490	PSEN2:617L21 antisense siNA (599C) stab11	cGuAuuuGAGGGucAGcucTsT	1830	
730 CACGACAUUCACUGAGGACACAC	1491	PSEN2:750L21 antisense siNA (732C) stab11	GuGuccucAGuGAAuGucGTsT	1831	
938 GUGCUCAAGACCUACAAGUGGCG	1492	PSEN2:958L21 antisense siNA (940C) stab11	cAcAuuGuAGGucuuGAGcTsT	1832	
947 ACCUACAAGUGGCGCAUGGACUA	1493	PSEN2:967L21 antisense siNA (949C) stab11	GuccAuGGccAcAuuGuAGTsT	1833	
2095 GAGUGUUCCCAAUGCUUUGUCCA	1494	PSEN2:2115L21 antisense siNA (2097C) stab11	GACAAAGcAuuGGGAACAcTsT	1834	
104 UUACUGAUGAAGAAACUGAGGCC	1487	PSEN2:106U21 sense siNA stab18	B <u>A</u> c <u>u</u> G <u>A</u> uG <u>A</u> G <u>A</u> A <u>A</u> c <u>u</u> GAGGTT B	1835	
260 AGCCAGGGAGCAUCAUUAUUUA	1488	PSEN2:262U21 sense siNA stab18	B <u>cc</u> AGGGAGc <u>A</u> uc <u>A</u> uuc <u>A</u> uuTT B	1836	
549 ACCGCUAUGUCUGUAGUGGGGUU	1489	PSEN2:551U21 sense siNA stab18	B <u>c</u> G <u>c</u> u <u>A</u> uG <u>u</u> c <u>u</u> G <u>u</u> A <u>G</u> uGGGTT B	1837	
597 AAGAGCUGACCCUCAAUACGGA	1490	PSEN2:599U21 sense siNA stab18	B <u>G</u> AGc <u>u</u> G <u>A</u> cc <u>c</u> u <u>c</u> AA <u>A</u> uAcGTT B	1838	
730 CACGACAUUCACUGAGGACACAC	1491	PSEN2:732U21 sense siNA stab18	B <u>c</u> G <u>A</u> c <u>A</u> uuc <u>A</u> c <u>u</u> GAGGAcAcTT B	1839	
938 GUGCUCAAGACCUACAAGUGGCG	1492	PSEN2:940U21 sense siNA stab18	B <u>G</u> cuc <u>A</u> AGAc <u>c</u> uAc <u>A</u> AuGuGTT B	1840	
947 ACCUACAAGUGGCGCAUGGACUA	1493	PSEN2:949U21 sense siNA stab18	B <u>c</u> uAc <u>A</u> AuGuGGcc <u>A</u> uGGAcTT B	1841	
2095 GAGUGUUCCCAAUGCUUUGUCCA	1494	PSEN2:2097U21 sense siNA stab18	B <u>G</u> uGuuccc <u>A</u> AuGcuuGuGTT B	1842	
104 UUACUGAUGAAGAAACUGAGGCC	1487	33957 PSEN2:124L21 antisense siNA (106C) stab08	ccucAGuuuc <u>u</u> uc <u>A</u> uc <u>A</u> GuTsT	1843	
260 AGCCAGGGAGCAUCAUUAUUUA	1488	33958 PSEN2:280L21 antisense siNA (262C) stab08	AAuGAAuG <u>A</u> uGcuccc <u>u</u> GGTsT	1844	
549 ACCGCUAUGUCUGUAGUGGGGUU	1489	33959 PSEN2:569L21 antisense siNA (551C) stab08	ccccAcuAcAGAc <u>A</u> uAGcGTsT	1845	
597 AAGAGCUGACCCUCAAUACGGA	1490	33960 PSEN2:617L21 antisense siNA (599C) stab08	cGuAuuuGAGGGucAGcucTsT	1846	
730 CACGACAUUCACUGAGGACACAC	1491	33961 PSEN2:750L21 antisense siNA (732C) stab08	GuGuccucAGuGAAuGucGTsT	1847	
938 GUGCUCAAGACCUACAAGUGGCG	1492	33962 PSEN2:958L21 antisense siNA (940C) stab08	cAcAuuGuAGGucuuGAGcTsT	1848	
947 ACCUACAAGUGGCGCAUGGACUA	1493	33963 PSEN2:967L21 antisense siNA (949C) stab08	GuccAuGGccAcAuuGuAGTsT	1849	
2095 GAGUGUUCCCAAUGCUUUGUCCA	1494	33964 PSEN2:2115L21 antisense siNA (2097C) stab08	GAcAAAGcAuuGGGAACAcTsT	1850	
104 UUACUGAUGAAGAXACUGAGGCC	1487	33941 PSEN2:106U21 sense siNA stab09	B ACUGAUGAAGAAACUGAGGTT B	1851	
260 AGCCAGGGAGCAUCAUUAUUUA	1488	33942 PSEN2:262U21 sense siNA stab09	B CCAGGGAGCAUCAUUAUUTT B	1852	
549 ACCGCUAUGUCUGUAGUGGGGUU	1489	33943 PSEN2:551U21 sense siNA stab09	B CGCUAUGUCUGUAGUGGGTT B	1853	

TABLE III-continued

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS						
597 AAGAGCUGACCCUCAAUACGGA	1490	33944	PSEN2:599U21 sense siNA stab09	B GAGCUGACCCUCAAUACGTT	B	1854
730 CACGACAUUCACUGAGGACACAC	1491	33945	PSEN2:732U21 sense siNA stab09	B CGACAUUCACUGAGGACACTT	B	1855
938 GUGCUCAAGACCUCAAUGUGGC	1492	33946	PSEN2:940U21 sense siNA stab09	B GCUCAAGACCUCAAUGUGTT	B	1856
947 ACCUACAAUGUGGCCAUGGACUA	1493	33947	PSEN2:949U21 sense siNA stab09	B CUACAAUGUGGCCAUGGACTT	B	1857
2095 GAGUGUCCCAAUGCUUUGUCCA	1494	33948	PSEN2:2097U21 sense siNA stab09	B GUGUCCCAAUGCUUUGUCTT	B	1858
104 UUACUGAUGAAGAAACUGAGGCC	1487	33949	PSEN2:124L21 antisense siNA (106C) stab10	CCUCAGUUUCUUCAGUTsT		1859
260 AGCCAGGGAGCAUCAUUAUUUA	1488	33950	PSEN2:280L21 antisense siNA (262C) stab10	AAUGAAUGAUGCUCCUGGTsT		1860
549 ACCGCUAUGUCUGUAGUGGGUU	1489	33951	PSEN2:569L21 antisense siNA (551C) stab10	CCCCACUACAGACAUAGCGTsT		1861
597 AAGAGCUGACCCUCAAUACGGA	1490	33952	PSEN2:617L21 antisense siNA (599C) stab10	CGUAUUUGAGGGUCAGUCTsT		1862
730 CACGACAUUCACUGAGGACACAC	1491	33953	PSEN2:750L21 antisense siNA (732C) stab10	GUGUCCUCAGUGAAUGCGTsT		1863
938 GUGCUCAAGACCUCAAUGUGGC	1492	33954	PSEN2:958L21 antisense siNA (940C) stab10	CACAUUGUAGGUCUUGAGCTsT		1864
947 ACCUACAAUGUGGCCAUGGACUA	1493	33955	PSEN2:967L21 antisense siNA (949C) stab10	GUCCAUGGCCACAUUGUAGTsT		1865
2095 GAGUGUCCCAAUGCUUUGUCCA	1494	33956	PSEN2:2115L21 antisense siNA (2097C) stab10	GACAAAGCAUUGGGAACACTsT		1866
104 UUACUGAUGAAGAAACUGAGGCC	1487		PSEN2:124L21 antisense siNA (106C) stab19	ccucAGuuucucBucAGuTT	B	1867
260 AGCCAGGGAGCAUCAUUAUUUA	1488		PSEN2:280L21 antisense siNA (262C) stab19	AAUGAAUGAUGcucccuGTT	B	1868
549 ACCGCUAUGUCUGUAGUGGGUU	1489		PSEN2:569L21 antisense siNA (551C) stab19	ccccAcuAcAGAcAuAGcGTT	B	1869
597 AAGAGCUGACCCUCAAUACGGA	1490		PSEN2:617L21 antisense siNA (599C) stab19	cGuAuuuGAGGGuAcGcucTT	B	1870
730 CACGACAUUCACUGAGGACACAC	1491		PSEN2:750L21 antisense siNA (732C) stab19	GuGuuccucAGuGAAuGucGTT	B	1871
938 GUGCUCAAGACCUCAAUGUGGC	1492		PSEN2:958L21 antisense siNA (940C) stab19	cAcAuGuAGGucuuGAGcTT	B	1872
947 ACCUACAAUGUGGCCAUGGACUA	1493		PSEN2:967L21 antisense siNA (949C) stab19	GuccAuGGccAcAuGuAGTT	B	1873
2095 GAGUGUCCCAAUGCUUUGUCCA	1494		PSEN2:2115L21 antisense siNA (2097C) stab19	GAcAAAGcAuGGGAAcAcTT	B	1874
104 UUACUGAUGAAGAAACUGAGGCC	1487		PSEN2:124L21 antisense siNA (106C) stab22	CCUCAGUUUCUUCAGUTT	B	1875
260 AGCCAGGGAGCAUCAUUAUUUA	1488		PSEN2:280L21 antisense siNA (262C) stab22	AAUGAAUGAUGCUCCUGGTT	B	1876
549 ACCGCUAUGUCUGUAGUGGGUU	1489		PSEN2:569L21 antisense siNA (551C) stab22	CCCCACUACAGACAUAGCGTT	B	1877

TABLE III-continued

APP, BACE, PSEN1, PSEN2, SYNTHETIC MODIFIED siNA CONSTRUCTS					
597 AAGAGCUGACCCUCAAUACGGA	1490	PSEN2:617L21 antisense siNA (599C) stab22	CGUAUUUGAGGGUCAGCUCTT B	1878	
730 CACGACAUUCACUGAGGACACAC	1491	PSEN2:750L21 antisense siNA (732C) stab22	GUGUCCUCAGUGAUGUCGTT B	1879	
938 GUGUCUACAGACCUCAAUGUGGC	1492	PSEN2:958L21 antisense siNA (940C) stab22	CACAUUGUAGGUCUUGAGCTT B	1880	
947 ACCUACAAGUGGCCAUGGACUA	1493	PSEN2:967L21 antisense siNA (949C) stab22	GUCCAUGGCCACAUGUAGTT B	1881	
2095 GAGUGUCCCAUUGCUUUGUCCA	1494	PSEN2:2115L21 antisense siNA (2097C) stab22	GACAAAGCAUUGGGAACACTT B	1882	

Uppercase = ribonucleotide

u,c = 2'-deoxy-2'-fluoro U,C
 T = thymidine
 B = inverted deoxy abasic
 s = phosphorothioate linkage
 A = deoxy Adenosine
 G = deoxy Guanosine
G = 2'-O-methyl Guanosine
A = 2'-O-methyl Adenosine

[0438]

TABLE IV

Non-limiting examples of Stabilization Chemistries for chemically modified siNA constructs					
Chemistry	pyrimidine	Purine	cap	p = S	Strand
"Stab 00"	Ribo	Ribo	TT at 3'-ends		S/AS
"Stab 1"	Ribo	Ribo	—	5 at 5'-end 1 at 3'-end	S/AS
"Stab 2"	Ribo	Ribo	—	All linkages	Usually AS
"Stab 3"	2'-fluoro	Ribo	—	4 at 5'-end 4 at 3'-end	Usually S
"Stab 4"	2'-fluoro	Ribo	5' and 3'-ends	—	Usually S
"Stab 5"	2'-fluoro	Ribo	—	1 at 3'-end	Usually AS
"Stab 6"	2'-O-Methyl	Ribo	5' and 3'-ends	—	Usually S
"Stab 7"	2'-fluoro	2'-deoxy	5' and 3'-ends	—	Usually S
"Stab 8"	2'-fluoro	2'-O-Methyl	—	1 at 3'-end	Usually AS
"Stab 9"	Ribo	Ribo	5' and 3'-ends	—	Usually S
"Stab 10"	Ribo	Ribo	—	1 at 3'-end	Usually AS
"Stab 11"	2'-fluoro	2'-deoxy	—	1 at 3'-end	Usually AS
"Stab 12"	2'-fluoro	LNA	5' and 3'-ends		Usually S
"Stab 13"	2'-fluoro	LNA		1 at 3'-end	Usually AS
"Stab 14"	2'-fluoro	2'-deoxy		2 at 5'-end 1 at 3'-end	Usually AS
"Stab 15"	2'-deoxy	2'-deoxy		2 at 5'-end 1 at 3'-end	Usually AS
"Stab 16"	Ribo	2'-O-Methyl	5' and 3'-ends		Usually S
"Stab 17"	2'-O-Methyl	2'-O-Methyl	5' and 3'-ends		Usually S
"Stab 18"	2'-fluoro	2'-O-Methyl	5' and 3'-ends		Usually S
"Stab 19"	2'-fluoro	2'-O-Methyl	3'-end		Usually AS

TABLE IV-continued

Non-limiting examples of Stabilization Chemistries for chemically modified siNA constructs					
Chemistry	pyrimidine	Purine	cnp	p = S	Strand
"Stab 20"	2'-fluoro	2'-deoxy	3'-end		Usually AS
"Stab 21"	2'-fluoro	Ribo	3'-end		Usually AS
"Stab 22"	Ribo	Ribo	3'-end		Usually AS
"Stab 23"	2'-fluoro*	2'-deoxy*	5' and 3'-ends		Usually S
"Stab 24"	2'-fluoro*	2'-O-Methyl*	—	1 at 3'-end	Usually AS
"Stab 25"	2'-fluoro*	2'-O-Methyl*	—	1 at 3'-end	Usually AS

CAP = any terminal cap, see for example FIG. 10.

All Stab 00-25 chemistries can comprise 3'-terminal thymidine (TT) residues

All Stab 00-25 chemistries typically comprise about 21 nucleotides, but can vary as described herein.

S = sense strand

AS = antisense strand

*Stab 23 has single ribonucleotide adjacent to 3'-CAP

*Stab 24 has single ribonucleotide at 5'-terminus

*Stab 25 has three ribonucleotides at 5'-terminus

[0439]

TABLE V

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
A. 2.5 μ mol Synthesis Cycle ABI 394 Instrument					
Phosphoramidites	6.5	163 μ L	45 sec	2.5 min	7.5 min
S-Ethyl Tetrazole	23.8	238 μ L	45 sec	2.5 min	7.5 min
Acetic Anhydride	100	233 μ L	5 sec	5 sec	5 sec
N-Methyl	186	233 μ L	5 sec	5 sec	5 sec
Imidazole					
TCA	176	2.3 mL	21 sec	21 sec	21 sec
Iodine	11.2	1.7 mL	45 sec	45 sec	45 sec
Beaucage	12.9	645 μ L	100 sec	300 sec	300 sec
Acetonitrile	NA	6.67 mL	NA	NA	NA
B. 0.2 μ mol Synthesis Cycle ABI 394 Instrument					
Phosphoramidites	15	31 μ L	45 sec	233 sec	465 sec
S-Ethyl Tetrazole	38.7	31 μ L	45 sec	233 min	465 sec
Acetic Anhydride	655	124 μ L	5 sec	5 sec	5 sec
N-Methyl	1245	124 μ L	5 sec	5 sec	5 sec
Imidazole					
TCA	700	732 μ L	10 sec	10 sec	10 sec
Iodine	20.6	244 μ L	15 sec	15 sec	15 sec
Beaucage	7.7	232 μ L	100 sec	300 sec	300 sec
Acetonitrile	NA	2.64 mL	NA	NA	NA
Reagent	Equivalents: DNA/2'-O- methyl/Ribo	Amount: DNA/2'-O- methyl/Ribo	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time* Ribo
C. 0.2 μ mol Synthesis Cycle 96 well Instrument					
Phosphoramidites	22/33/66	40/60/120 μ L	60 sec	180 sec	360 sec
S-Ethyl Tetrazole	70/105/210	40/60/120 μ L	60 sec	180 min	360 sec
Acetic Anhydride	265/265/265	50/50/50 μ L	10 sec	10 sec	10 sec
N-Methyl	502/502/502	50/50/50 μ L	10 sec	10 sec	10 sec
Imidazole					
TCA	238/475/475	250/500/500 μ L	15 sec	15 sec	15 sec
Iodine	6.8/6.8/6.8	80/80/80 μ L	30 sec	30 sec	30 sec
Beaucage	34/51/51	80/120/120	100 sec	200 sec	200 sec
Acetonitrile	NA	1150/1150/1150 μ L	NA	NA	NA

*Wait time does not include contact time during delivery.

*Tandem synthesis utilizes double coupling of linker molecule

[0440]

SEQUENCE LISTING

The patent application contains a lengthy "Sequence Listing" section. A copy of the "Sequence Listing" is available in electronic form from the USPTO web site (<http://seqdata.uspto.gov/sequence.html?DocID=20050209179>). An electronic copy of the "Sequence Listing" will also be available from the USPTO upon request and payment of the fee set forth in 37 CFR 1.19(b)(3).

What we claim is:

1. A chemically synthesized double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of an amyloid precursor protein (APP)RNA via RNA interference (RNAi), wherein:

a. each strand of said siNA molecule is about 18 to about 23 nucleotides in length; and

b. one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to said APP RNA for the siNA molecule to direct cleavage of the APP RNA via RNA interference.

2. The siNA molecule of claim 1, wherein said siNA molecule comprises no ribonucleotides.

3. The siNA molecule of claim 1, wherein said siNA molecule comprises one or more ribonucleotides.

4. The siNA molecule of claim 1, wherein one strand of said double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence of a APP gene or a portion thereof, and wherein a second strand of said double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of said APP RNA.

5. The siNA molecule of claim 4, wherein each strand of the siNA molecule comprises about 18 to about 23 nucleotides, and wherein each strand comprises at least about 19 nucleotides that are complementary to the nucleotides of the other strand.

6. The siNA molecule of claim 1, wherein said siNA molecule comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence of a APP gene or a portion thereof, and wherein said siNA further comprises a sense region, wherein said sense region comprises a nucleotide sequence substantially similar to the nucleotide sequence of said APP gene or a portion thereof.

7. The siNA molecule of claim 6, wherein said antisense region and said sense region comprise about 18 to about 23 nucleotides, and wherein said antisense region comprises at least about 18 nucleotides that are complementary to nucleotides of the sense region.

8. The siNA molecule of claim 1, wherein said siNA molecule comprises a sense region and an antisense region, and wherein said antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by a APP gene, or a portion thereof, and said sense region comprises a nucleotide sequence that is complementary to said antisense region.

9. The siNA molecule of claim 6, wherein said siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and a second fragment comprises the antisense region of said siNA molecule.

10. The siNA molecule of claim 6, wherein said sense region is connected to the antisense region via a linker molecule.

11. The siNA molecule of claim 10, wherein said linker molecule is a polynucleotide linker.

12. The siNA molecule of claim 10, wherein said linker molecule is a non-nucleotide linker.

13. The siNA molecule of claim 6, wherein pyrimidine nucleotides in the sense region are 2'-O-methylpyrimidine nucleotides.

14. The siNA molecule of claim 6, wherein purine nucleotides in the sense region are 2'-deoxy purine nucleotides.

15. The siNA molecule of claim 6, wherein pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides.

16. The siNA molecule of claim 9, wherein the fragment comprising said sense region includes a terminal cap moiety at a 5'-end, a 3'-end, or both of the 5' and 3' ends of the fragment comprising said sense region.

17. The siNA molecule of claim 16, wherein said terminal cap moiety is an inverted deoxy abasic moiety.

18. The siNA molecule of claim 6, wherein pyrimidine nucleotides of said antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides.

19. The siNA molecule of claim 6, wherein purine nucleotides of said antisense region are 2'-O-methyl purine nucleotides.

20. The siNA molecule of claim 6, wherein purine nucleotides present in said antisense region comprise 2'-deoxy-purine nucleotides.

21. The siNA molecule of claim 18, wherein said antisense region comprises a phosphorothioate internucleotide linkage at the 3' end of said antisense region.

22. The siNA molecule of claim 6, wherein said antisense region comprises a glyceryl modification at a 3' end of said antisense region.

23. The siNA molecule of claim 9, wherein each of the two fragments of said siNA molecule comprise about 21 nucleotides.

24. The siNA molecule of claim 23, 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.

25. The siNA molecule of claim 24, wherein each of the two 3' terminal nucleotides of each fragment of the siNA molecule are 2'-deoxy-pyrimidines.

26. The siNA molecule of claim 25, wherein said 2'-deoxy-pyrimidine is 2'-deoxy-thymidine.

27. The siNA molecule of claim 23, wherein all of the about 21 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule.

28. The siNA molecule of claim 23, wherein about 19 nucleotides of the antisense region are base-paired to the nucleotide sequence of the RNA encoded by a APP gene or a portion thereof.

29. The siNA molecule of claim 23, wherein about 21 nucleotides of the antisense region are base-paired to the nucleotide sequence of the RNA encoded by a APP gene or a portion thereof.

30. The siNA molecule of claim 9, wherein a 5'-end of the fragment comprising said antisense region optionally includes a phosphate group.

31. A composition comprising the siNA molecule of claim 1 in an pharmaceutically acceptable carrier or diluent.

32. A siNA according to claim 1 wherein the APP RNA comprises Genbank Accession No. NM_000484.

33. A siNA according to claim 1 wherein said siNA comprises any of SEQ ID NOs. 1-199, 200-398, 1463-1470, and 1495-1590.

34. A composition comprising the siNA of claim 32 together with a pharmaceutically acceptable carrier or diluent.

35. A composition comprising the siNA of claim 33 together with a pharmaceutically acceptable carrier or diluent.

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