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(54) Title: NOVEL TANDEM siRNAS

(57) Abstract: The present invention provides novel molecules, compositions, methods and uses for treating microvascular disorders, eye diseases and respiratory conditions based upon inhibition of two or more target genes.

NOVEL TANDEM siRNAS

PRIORITY

This application claims the benefit of United States Provisional Application No. 60/771238, filed on February 8, 2006, the contents of which are hereby incorporated by reference in their entirety into this application.

FIELD OF THE INVENTION

The present invention relates to siRNA molecules of a novel design and structure, that are capable of inhibiting two or more genes, optionally simultaneously. These novel siRNAs may be used as drugs to treat a variety of diseases and indications.

BACKGROUND OF THE INVENTION

siRNAs and RNA interference

The present invention relates generally to compounds which down-regulate expression of two or more genes, and particularly to novel small interfering RNAs (siRNAs), and to the use of these novel siRNAs in the treatment of various diseases and medical conditions.

The present invention provides methods and compositions for inhibiting expression of the target genes *in vivo*. In general, the method includes administering oligoribonucleotides, such as small interfering RNAs (i.e., siRNAs) that are targeted to two or more particular mRNA and hybridize to, or interact with, it under biological conditions (within the cell), or a nucleic acid material that can produce siRNA in a cell, in an amount sufficient to down-regulate expression of two or more target genes by an RNA interference mechanism. Additionally the siRNAs of the invention can be used *in vitro* as part of a compound screening system to look for small compounds that compete with, or overcome effect of, siRNAs.

RNA interference (RNAi) is a phenomenon involving double-stranded (ds) RNA-dependent gene specific posttranscriptional silencing. Originally, attempts to study this phenomenon and to manipulate mammalian cells experimentally were frustrated by an active, non-specific antiviral defence mechanism which was activated in response to long dsRNA molecules; see Gil et al.

2000, Apoptosis, 5:107-114. Later it was discovered that synthetic duplexes of 21 nucleotide RNAs could mediate gene specific RNAi in mammalian cells, without the stimulation of the generic antiviral defence mechanisms see Elbashir et al. Nature 2001, 411:494-498 and Caplen et al. Proc Natl Acad Sci 2001, 98:9742-9747. As a result, small interfering RNAs (siRNAs), which are short double-stranded RNAs, have become powerful tools in attempting to understand gene function.

Thus RNA interference (RNAi) refers to the process of sequence-specific post-transcriptional gene silencing in mammals mediated by small interfering RNAs (siRNAs) (Fire et al, 1998, Nature 391, 806) or microRNAs (miRNAs) (Ambros V. Nature 431:7006,350-355(2004); and Bartel DP. Cell. 2004 Jan 23;116(2):281-97 *MicroRNAs: genomics, biogenesis, mechanism, and function*). The corresponding process in plants is commonly referred to as specific post transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi.

An siRNA is a double-stranded RNA molecule which down-regulates or silences (prevents) the expression of a gene/ mRNA of its endogenous or cellular counterpart. RNA interference is based on the ability of dsRNA species to enter a specific protein complex, where it is then targeted to the complementary cellular RNA and specifically degrades it. Thus the RNA interference response features an endonuclease complex containing an siRNA, 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 may take place in the middle of the region complementary to the antisense strand of the siRNA duplex (Elbashir et al 2001, Genes Dev., 15, 188). In more detail, longer dsRNAs are digested into short (17-29 bp) dsRNA fragments (also referred to as short inhibitory RNAs – "siRNAs") by type III RNases (DICER, DROSHA, etc ., Bernstein et al., Nature, 2001, v.409, p.363-6; Lee et al., Nature, 2003, .425, p.415-9). These fragments and complementary mRNA are recognized by the RISC protein complex. The whole process is culminated by endonuclease cleavage of target mRNA (McManus&Sharp, Nature Rev Genet , 2002, v.3, p.737-47; Paddison&Hannon, Curr Opin Mol Ther. 2003 Jun;5(3):217-24). For information on these terms and proposed mechanisms, see Bernstein E., Denli AM., Hannon GJ: 2001 *The rest is silence.* RNA. I;7(11):1509-21; Nishikura K.: 2001 *A short primer on RNAi: RNA-directed RNA polymerase acts as a key catalyst.* Cell. I 16;107(4):415-8 and PCT publication WO 01/36646 (Glover et al).

The selection and synthesis of siRNA corresponding to known genes has been widely reported; see for example Chalk AM, Wahlestedt C, Sonnhammer EL. 2004 *Improved and automated prediction of effective siRNA* Biochem. Biophys. Res. Commun. Jun 18;319(1):264-74; Sioud M, Leirdal M., 2004, *Potential design rules and enzymatic synthesis of siRNAs*, Methods Mol Biol.;252:457-69; Levenkova N, Gu Q, Rux JJ.: 2004 ,*Gene specific siRNA selector* Bioinformatics. I 12;20(3):430-2. and Ui-Tei K, Naito Y, Takahashi F, Haraguchi T, Ohki-Hamazaki H, Juni A, Ueda R, Saigo K., *Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference Nucleic Acids* Res. 2004 I 9;32(3):936-48.Se also Liu Y, Braasch DA, Nulf CJ, Corey DR. *Efficient and isoform-selective inhibition of cellular gene expression by peptide nucleic acids*, Biochemistry, 2004 I 24;43(7):1921-7. See also PCT publications WO 2004/015107 (Atugen) and WO 02/44321 (Tuschl et al), and also Chiu YL, Rana TM. *siRNA function in RNAi: a chemical modification analysis*, RNA 2003 Sep;9(9):1034-48 and I Patent Nos.5898031 and 6107094 (Crooke) for production of modified/ more stable siRNAs.

Several groups have described the development of DNA-based vectors capable of generating siRNA within cells. The method generally involves transcription of short hairpin RNAs that are efficiently processed to form siRNAs within cells. Paddison et al. *PNAS* 2002, 99:1443-1448; Paddison et al. *Genes & Dev* 2002, 16:948-958; Sui et al. *PNAS* 2002, 8:5515-5520; and Brummelkamp et al. *Science* 2002, 296:550-553. These reports describe methods to generate siRNAs capable of specifically targeting numerous endogenously and exogenously expressed genes.

siRNA has recently been successfully used for inhibition in primates; for further details see Tolentino et al., Retina 24(1) February 2004 I 132-138. Several studies have revealed that siRNA therapeutics are effective *in vivo* in both mammals and in humans. Bitko et al., have shown that specific siRNA molecules directed against the respiratory syncytial virus (RSV) nucleocapsid N gene are effective in treating mice when administered intranasally (Bitko et al., "Inhibition of respiratory viruses by nasally administered siRNA", Nat. Med. 2005, 11(1):50-55). A review of the use of siRNA in medicine was recently published by Barik S. in J. Mol. Med (2005) 83: 764-773). Furthermore, a phase I clinical study with short siRNA molecule that

targets the VEGFR1 receptor for the treatment of Age-Related Macular Degeneration (AMD) has been conducted in human patients. The siRNA drug administered by an intravitreal inter-ocular injection was found effective and safe in 14 patients tested after a maximum of 157 days of follow up (Boston Globe January 21 2005).

Due to the difficulty in identifying and obtaining regulatory approval for chemical drugs for the treatment of diseases, the molecules of the present invention offer an advantage in that they are non-toxic and may be formulated as pharmaceutical compositions for treatment of any disease. Additionally, the molecules of the present invention have the advantage of being able to efficiently treat diseases and conditions in which two or more genes are involved by targeting said genes with one molecule. Another advantage is their lower effective concentration as compaired to smaller sized siRNAs. Said combined or tandem structures have the advantage that toxicity and/or off-target effects of each siRNA are reduced.

SUMMARY OF THE INVENTION

The invention provides a novel double stranded oligoribonucleotide. This oligoribonucleotide down-regulates the expression of two or more desired genes by the mechanism of RNA interference. The invention also provides a pharmaceutical composition comprising such oligoribonucleotides, and vectors capable of expressing the ribonucleotides.

The present invention also provides a method of treating a patient suffering from a disease or adverse condition, comprising administering to the patient the oligoribonucleotide typically as a pharmaceutical composition, in a therapeutically effective amount so as to thereby treat the patient.

The present invention also relates to functional nucleic acids comprising a double-stranded or triple-stranded or even multistranded structure, their use for the manufacture of a medicament, a pharmaceutical composition comprising such functional nucleic acids and a method for the treatment of a patient.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1-5 present the results of various validation experiments which confirm the efficacy of the molecules of the present invention in down-regulation of various genes. Further information concerning these experiments can be found in Example 4;

Figure 6 presents the structure of different variants of the RNAstar molecule;

Figure 7 presents the general structure of an exemplary RNAstar molecule;

Figure 8 shows the results of Western blot experiments which demonstrate the activity of molecules of the present invention;

Figures 9-10 show further experimental results which demonstrate the efficacy of the molecules of the present invention in down-regulation of various genes – see also Example 6.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to oligonucleotides and oligoribonucleotides which posess therapeutic properties. In particular, the present invention discloses tandem oligoribonucleotides which encode two inhibitory RNA molecules such as siRNAs, wherein each siRNA may be specific for a different gene (or wherein both siRNAs are specific for the same gene). Said combined or tandem structures have the advantage that toxicity and/or off-target effects of each siRNA are minimized, while the efficacy is increased. Further, said tandem structures have the additional advantage in that they can treat two separate therapeutic targets and/or diseases with one single molecule. Additionally, the present invention provides for tandem oligonucleotides which encode three siRNAs, as will be described herein. It is also within the scope of the present invention to provide for oligonucleotides which encode three, four or even five inhibitory RNAs which target the same or as many as five different genes.

Thus, in one embodiment, the present invention provides for an oligonucleotide comprising consecutive nucleotides wherein a first segment of such nucleotides encodes a first inhibitory RNA molecule and a second segment of such nucleotides encodes a second inhibitory RNA molecule. Thus, an oligonucleotide which encodes two inhibitory RNA molecules is provided. In a further embodiment, each of the first and the second segment may comprise one strand of a double stranded RNA, and the first and second segments may be joined together by a single stranded RNA linker; a single stranded DNA linker; a linker which comprises a disulfide bond; a linker which comprises a peptide bond; a double stranded RNA linker; a double stranded DNA linker; a linker which comprises a partially single stranded and partially double stranded RNA; or a linker which comprises a partially single stranded and partially double stranded DNA.
Further, the oligonucleotide may comprise modification at the 2' position of one or more sugars, such as 2'Omethyl and/or 2'fluoro substitutions. The 2' modifications may be on alternating nucleotides.

Thus, as detailed above, the oligonucleotide of the present invention may comprise two double stranded RNA sequences linked together by linker, such as a single stranded RNA linker; a single stranded DNA linker; a disulfide linker; a peptide linker; a double stranded RNA linker; a double stranded DNA linker; a partially single stranded and partially double stranded RNA linker; a partially single stranded and partially double stranded DNA linker; or any other kind of

cleavable or non-cleavable chemical linker, *inter alia*. Further, the oligonucleotide may comprise 2'OMethyl or 2'Fluoro or 2'Oallyl or any other 2' modification on preferentially alternate positions. Other stabilizing modifications which do not significantly reduce the enzymatic activity are also possible (e.g.,. terminal modifications). The backbone of the active part of tandem oligonucleotides preferentially comprises phosphate-D-ribose entities but may also contain thiophosphate-D-ribose entities or any other type of modification. Terminal modifications on the 5' and/or 3' part of the tandem oligonucleotides are also possible. Such terminal modifications may be lipids, peptides, sugars or other molecules.

The oligoribonucleotide of the invention may have one of the following general structures:

1)

```
5'  Oligo1 (sense)      LINKER A  Oligo2 (sense)      3'
3'  oligo1 (antisense)  LINKER B  Oligo2 (antisense)  5'
```

wherein either linker A or linker B is present or both linkers A and B are present.

2)

```
5'  oligo1 (antisense)  LINKER A  Oligo2 (antisense)  3'
3'  oligo1 (sense)      LINKER B  Oligo2 (sense)      5'
```

wherein either linker A or linker B is present or both linkers A and B are present.

3)

```
5'  oligo1 (sense)      LINKER A  Oligo1 (antisense)  3'
3'  oligo2 (antisense)  LINKER B  Oligo2 (sense)      5'
```

wherein either linker A or linker B is present or both linkers A and B are present.

4)

```
5'  oligo1 (antisense)  LINKER A    Oligo1 (sense)      3'
3'  oligo2 (sense)      LINKER B    Oligo2 (antisense)  5'
```

wherein either linker A or linker B is present or both linkers A and B are present.

5)

```
5'  oligo1 (sense)       LINKER A  Oligo2 (antisense)     3'
3'  oligo2 (sense)       LINKER B  Oligo1 (antisense)     5'
```

wherein either linker A or linker B is present or both linkers A and B are present.

6)

```
5'  oligo1 (antisense)   LINKER A  Oligo2 (sense)        3'
3'  oligo2 (antisense)   LINKER B  Oligo1 (sense)        5'
```

wherein either linker A or linker B is present or both linkers A and B are present.

It is to be understood that in the context of the present invention, the sense and antisense strands of each RNA represented in the tandem molecule can have varying positions in relation with each other, and any sense / antisense conformation with respect to the position of the linker is possible. Linker A and Linker B may each be present or absent, but at least one of Linker A or Linker B must be present. If both present, they may be identical or different. Whether both or only one of Linker A or Linker B are present, they are collectively referred to herein as a "linker". Thus, the linker may covalently join two or more strands of the resultant tandem molecule. For example, each of the above molecules 1-6 is composed of two sense and two antisense strands, and the linker may covalently join two sense strands, two antisense strands, one sense and one antisense strand, two sense strands and one antisense strand, two antisense strands and one sense strand, or two sense and two antisense strands. Molecules with additional strands are also envisaged, and the linker may join any number of strands as specified for the above molecules. In the case of a nucleic acid linker, the resultant tandem molecule may therefore be comopsed of two continuous strands, or of three strands resulting from one nick or gap in one of the strands, or a multi stranded molecule resulting from two or more nicks or gaps in one or more of the oligos. In the case of a non nucleic acid linker, as will be detailed below, the linker may also join two or more of the strands; in such a case the resultant molecule may have two or more strands, in which the continuous strands contain a non-nucleic acid portion.

Further, the oligoribonucleotides of the present invention may have the following structures:

7)

```
5'  oligo1 (sense)       dTsdTuu       oligo2 (sense)       3'
3'  oligo1 (antisense)   (gap) dTsdT   oligo2 (antisense)   5'
```

wherein the linker dTsdTuu = 5'-2'deoxythymidyl-3'-thiophosphate-5'-2'deoxythymidyl-3'-phosphate-5'-uridyl-3'-phosphate-5'-uridyl-3'-phosphate. Note that the above structure 7 contains a gap.

8)

```
5'  oligo1 (sense)       rUsrU      oligo2 (sense)       3'
3'  oligo1 (antisense)   (gap)      oligo2 (antisense)   5'
```

wherein the linker rUsrU = a thiophosphate linker: 5'-uridyl-3'-thiophosphate-5'-uridyl-3'-phosphate; the linker may also be substituted with an rUrU linker, i.e., a linker having a phosphate backbone. Note that the above structure 8 contains a gap.

9)

```
5'  oligo1 (sense)       dTsdTaa    oligo2 (sense)       3'
3'  oligo1 (antisense)   aadTsdT    oligo2 (antisense)   5'
```

wherein the linker dTsdTaa = aadTsdT = 5'-2'deoxythymidyl-3'-thiophosphate-5'-2'deoxythymidyl-3'-phosphate-5'-adenyl-3'-phosphate-5'-adenyl-3'-phosphate. Note that the above structure 9 does not contain a gap.

10)

```
5'  oligo1 (sense)       dTsdT      oligo2 (sense)       3'
3'  oligo1 (antisense)   dTsdT      oligo2 (antisense)   5'
```

wherein the linker dTsdT = 5'-2'deoxythymidyl-3'-thiophosphate-5'-2'deoxythymidyl-3'-phosphate. Note that the above structure 10 does not contain a gap.

11)

```
5'  Oligo1 (sense)       dTsdTuu    oligo2 (sense)       3'
3'  Oligo1 (antisense)   uudTsdT    oligo2 (antisense)   5'
```

wherein the linker dTsdTuu = uudTsdT = 5'-2'deoxythymidyl-3'-thiophosphate-5'-2'deoxythymidyl-3'-phosphate-5'-uridyl-3'-phosphate-5'-uridyl-3'-phosphate. Note that the above structure 11 does not contain a gap.

12)

5′	oligo1 (sense)	X_n	oligo2 (sense)	3′
3′	oligo1 (antisense)	(gap)	oligo2 (antisense)	5′

wherein the linker X_n = polyRNA (such as, inter alia, poly(5'-adenyl-3'-phosphate - AAAAAAAA) or poly(5'-cytidyl-3'-phosphate-5'-uridyl-3'-phosphate - CUCUCUCU))- a single stranded poly RNA linker wherein n is an integer from 2-50 inclusive, preferable 4-15 inclusive, most preferably 7-8 inclusive. Modified nucleotides or a mixture of nucleotides can also be present in said polyRNA linker. Note that the above structure 12 contains a gap.

13)

5′	oligo1 (sense)	Y_n	oligo2 (sense)	3′
3′	oligo1 (antisense)	(gap)	oligo2 (antisense)	5′

wherein the linker Y_n = polyDNA (such as, inter alia, poly(5'-2'deoxythymidyl-3'-phosphate - TTTTTTTT) a single stranded polyDNA linker wherein n is an integer from 2-50 inclusive, preferable 4-15 inclusive, most preferably 7-8 inclusive. Modified nucleotides or a mixture of nucleotides can also be present in said polyDNA linker. Note that the above structure 13 contains a gap.

14)

5′	oligo1 (sense)	--SS--	oligo2 (sense)	3′
3′	oligo1 (antisense)	(gap)	oligo2 (antisense)	5′

Wherein the linker --SS-- = a linker which comprises a disulfide bond, optionally a bis-hexyl-disulfide linker. Note that the above structure 14 contains a gap.

15)

5′	oligo1 (sense)	1-10 a.a.	oligo2 (sense)	3′
3′	oligo1 (antisense)	(gap)	oligo2 (antisense)	5′

wherein the linker is a linker which comprises a peptide bond, optionally 1-10 amino acid long linker, preferably comprising 4-5 amino acids, optionally X-Gly-Phe-Gly-Y wherein X and Y represent any amino acid. Note that the above structure 15 contains a gap.

Further, with respect to molecules containing any of the linkers as presented in structures 7-15 above, it is to be noted that the order of the sense and antisense strands may be altered, such that any conformation is possible, including but not limited to the general structures 1-7 above comprising any of the linkers of the structures 7-15 above.

In an additional embodiment, the present invention provides for an oligonucleotide as above wherein the oligonucleotide is an oligoribonucleotide which encodes two siRNAs.

An additional novel molecule provided by the present invention is an oligonucleotide comprising consecutive nucleotides wherein a first segment of such nucleotides encode a first inhibitory RNA molecule, a second segment of such nucleotides encode a second inhibitory RNA molecule, and a third segment of such nucleotides encode a third inhibitory RNA molecule. Each of the first, the second and the third segment may comprise one strand of a double stranded RNA and the first, second and third segments may be joined together by a linker. Further, the oligonucleotide may comprise three double stranded segments joined together by one or more linker.

Thus, one molecule provided by the present invention is an oligonucleotide comprising consecutive nucleotides which encode three inhibitory RNA molecules; said oligonucleotide may possess a triple stranded structure, such that three double stranded arms are linked together by one or more linker, such as any of the linkers presented hereinabove.

This molecule forms a "star"-like structure, and may also be referred to herein as RNAstar.

Said triple-stranded oligonucleotide may be an oligoribonucleotide having the general structure:

```
5'  oligo1 (sense)       LINKER A   Oligo2 (sense)       3'
3'  oligo1 (antisense)   LINKER B   Oligo3 (sense)       5'
3'  oligo3 (antisense)   LINKER C   oligo2 (antisense)   5'
```

or

```
5'  oligo1 (sense)       LINKER A   Oligo2 (antisense)   3'
```

```
3'  oligo1 (antisense)   LINKER B   Oligo3 (sense)       5'
3'  oligo3 (antisense)   LINKER C   oligo2 (sense)       5'
```

or

```
5'  oligo1 (sense)       LINKER A   oligo3 (antisense)   3'
3'  oligo1 (antisense)   LINKER B   oligo2 (sense)       5'
5'  oligo3 (sense)       LINKER C   oligo2 (antisense)   3'
```

wherein one or more of linker A, linker B or linker C is present; any combination of two or more oligonucleotides and one or more of linkers A-C is possible, so long as the polarity of the strands and the general structure of the molecule remains. Further, if two or more of linkers A-C are present, they may be identical or different.

Thus, a triple-armed structure is formed, wherein each arm comprises a sense strand and complementary antisense strand. The triple armed structure may be triple stranded, whereby each arm possesses base pairing. An exemplary structure is presented in Figure 7.

Further, the above triple stranded structure may have a gap instead of a linker in one or more of the strands. Such a molecule with one gap is technically quadruple stranded and not triple stranded; inserting additional gaps or nicks will lead to the molecule having additional strands. Preliminary results obtained by the inventors of the present invention indicate that said gapped molecules are more active in inhibiting certain target genes than the similar but non-gapped molecules. This may also be the case for nicked molecules.

In the context of the present invention, a gap in a nucleic acid means that the molecule is missing one or more nucleotide at the site of the gap, while a nick in a nucleic acid means that there are no missing nucleotides, but rather, there is no phospho-diester bond between 2 adjacent nucleotides at the site of the nick. Any of the molecules of the present invention may contain one or more gap and/or one or more nick.

Examples of the structure of the triple-stranded molecule include the following:

16)

```
5'  oligo1 (sense)       HEG        Oligo2 (sense)       3'
```

3′	oligo1 (antisense)	HEG	Oligo3 (sense)	5′
3′	oligo3 (antisense)	HEG	oligo2 (antisense)	5′
5′	oligo1 (sense)	HEG	oligo3 (antisense)	3′
3′	oligo1 (antisense)	HEG	oligo2 (sense)	5′
5′	oligo3 (sense)	HEG	oligo2 (antisense)	3′

wherein the linker designated HEG is a hexaethylenglycol linker.

17)

5′	Oligo1 (sense)	Nn	oligo3 (antisense)	3′
3′	Oligo1 (antisense)	Nn	oligo2 (sense)	5′
5′	Oligo3 (sense)	Nn	oligo2 (antisense)	3′

wherein the linker designated Nn is a nucleotide linker, optionally a single stranded nucleotide linker which can be composed of any DNA nucleotides, RNA nucleotides, synthetic nucleotides or any combination thereof (such as, for example, poly(5'-2'deoxythymidyl-3'-phosphate) - a single stranded poly T DNA linker having 4 nucleotides). Further, said linker may be composed of 1-50, typically 1-20 or 2-10 nucleotides, wherein the linker on each of the 3 strands may differ in length.

Additionally, said triple-stranded molecules of the invention may be joined together by any of the linkers disclosed herein. Further, as above in the case of the double stranded molecules, the triple-stranded molecules of the present invention may have a conformation as above except that the order of the sense and antisense strands is altered. Any conformation is possible, as long as at least one sense and one antisense strand are included for each gene target desired for inhibition.

It is to be noted that all of the linkers disclosed herein may have additional recognition sites for cleavage or processing by enzymes or by the chemical environment inside the cell/cell compartments which inrease the efficiency of the conversion of said molecules into several separate inhibitory modules.

Further provided by the present invention is a vector comprising any of the oligonucleotide molecules disclosed herein, a vector encoding any of the oligonucleotide molecules disclosed

herein, a vector which upopn transcription gives rise to any of the oligonucleotide molecules disclosed herein, and a pharmaceutical composition comprising any of the oligonucleotide molecules disclosed herein or any of said vectors comprising or encoding or giving rise to them and a pharmaceutically acceptable carrier.

Said pharmaceutical compositions may be used in the treatment of a variety of diseases and indications and, as discussed herein, they have a particular advantage in that they increase efficacy and minimize side effects, especially when used to treat two separate indications or targets (as opposed to two different drugs). In particular, the pharmaceutical compositions of the present invention can be used to treat a respiratory disorder such as COPD, a microvascular disorder such as acute renal failure (ARF) or diabetic retinopathy and in particular an eye disease such as ocular scarring or macular degeneration.

"Respiratory disorder" refers to conditions, diseases or syndromes of the respiratory system including but not limited to pulmonary disorders of all types including chronic obstructive pulmonary disease (COPD), emphysema, chronic bronchitis, asthma and lung cancer, *inter alia*. Emphysema and chronic bronchitis may occur as part of COPD or independently.

"Microvascular disorder" refers to any condition that affects microscopic capillaries and lymphatics, in particular vasospastic diseases, vasculitic diseases and lymphatic occlusive diseases. Examples of microvascular disorders include, inter alia: eye disorders such as Amaurosis Fugax (embolic or secondary to SLE), apla syndrome, Prot CS and ATIII deficiency, microvascular pathologies caused by IV drug use, dysproteinemia, temporal arteritis, anterior ischemic optic neuropathy, optic neuritis (primary or secondary to autoimmune diseases), glaucoma, von Hippel Lindau syndrome, corneal disease, corneal transplant rejection cataracts, Eales' disease, frosted branch angiitis, encircling buckling operation, uveitis including pars planitis, choroidal melanoma, choroidal hemangioma, optic nerve aplasia; retinal conditions such as retinal artery occlusion, retinal vein occlusion, retinopathy of prematurity, HIV retinopathy, Purtscher retinopathy, retinopathy of systemic vasculitis and autoimmune diseases, diabetic retinopathy, hypertensive retinopathy, radiation retinopathy, branch retinal artery or vein occlusion, idiopathic retinal vasculitis, aneurysms, neuroretinitis, retinal embolization, acute retinal necrosis, Birdshot retinochoroidopathy, long-standing retinal detachment; systemic

conditions such as Diabetes mellitus, diabetic retinopathy (DR), diabetes-related microvascular pathologies (as detailed herein), hyperviscosity syndromes, aortic arch syndromes and ocular ischemic syndromes, carotid–cavernous fistula, multiple sclerosis, systemic lupus erythematosus, arteriolitis with SS-A autoantibody, acute multifocal hemorrhagic vasculitis, vasculitis resulting from infection, vasculitis resulting from Behçet's disease, sarcoidosis, coagulopathies, neuropathies, nephropathies, microvascular diseases of the kidney, and ischemic microvascular conditions, inter alia. Microvascular disorders may comprise a neovascular element. The term "neovascular disorder" refers to those conditions where the formation of blood vessels (neovascularization) is harmful to the patient. Examples of ocular neovascularization include: retinal diseases (diabetic retinopathy, diabetic Macular Edema, chronic glaucoma, retinal detachment, and sickle cell retinopathy); rubeosis iritis; proliferative vitreo-retinopathy; inflammatory diseases; chronic uveitis; neoplasms (retinoblastoma, pseudoglioma and melanoma); Fuchs' heterochromic iridocyclitis; neovascular glaucoma; corneal neovascularization (inflammatory, transplantation and developmental hypoplasia of the iris); neovascularization following a combined vitrectomy and lensectomy; vascular diseases (retinal ischemia, choroidal vascular insufficiency, choroidal thrombosis and carotid artery ischemia); neovascularization of the optic nerve; and neovascularization due to penetration of the eye or contusive ocular injury. All these neovascular conditions may be treated using the compounds and pharmaceutical compositions of the present invention.

"Eye disease" refers to refers to conditions, diseases or syndromes of the eye including but not limited to any conditions involving choroidal neovascularization (CNV), wet and dry AMD, ocular histoplasmosis syndrome, angiod streaks, ruptures in Bruch's membrane, myopic degeneration, ocular tumors, ocular scarring, retinal degenerative diseases and retinal vein occlusion (RVO).

The pharmaceutical composition is in its various embodiments is adapted for administration in various ways. Such administration comprises systemic and local administration as well as oral, subcutaneous, parenteral, intravenous, intraarterial, intramuscular, intraperitonial, intranasal, aerosol and intrategral administration, and administration by inhalation.

It will be acknowledged by those skilled in the art that the amount of the pharmaceutical composition and the respective nucleic acid and vector, respectively, depends on the clinical

condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, bodyweight and other factors known to medical practitioners. The pharmaceutically effective amount for purposes of prevention and/or treatment is thus determined by such considerations as are known in the medical arts. Preferably, the amount is effective to achieve improvement including but limited to improve the diseased condition or to provide for a more rapid recovery, improvement or elimination of symptoms and other indicators as are selected as appropriate measures by those skilled in the medical arts.

In a preferred embodiment, the pharmaceutical composition according to the present invention may comprise other pharmaceutically active compounds. Preferably, such other pharmaceutically active compounds are selected from the group comprising compounds which allow for uptake intracellular cell delivery, compounds which allow for endosomal release, compounds which allow for, longer circulation time and compounds which allow for targeting of endothelial cells or pathogenic cells. Preferred compounds for endosomal release are chloroquine, and inhibitors of ATP dependent H^+ pumps. The pharmaceutical composition is preferably formulated so as to provide for a single dosage administration or a multi-dosage administration. For further information on dosage, formulation and delivery of the compounds of the present invention see Example 7.

"Treating a disease" refers to administering a therapeutic substance effective to ameliorate symptoms associated with a disease, to lessen the severity or cure the disease, or to prevent the disease from occurring.
The term "disease" comprises any illness or adverse condition.

A "therapeutically effective dose" refers to an amount of a pharmaceutical compound or composition which is effective to achieve an improvement in a patient or his physiological systems including, but not limited to, improved survival rate, more rapid recovery, or improvement or elimination of symptoms, and other indicators as are selected as appropriate determining measures by those skilled in the art.

An "inhibitor" is a compound which is capable of inhibiting the activity of a gene or the product of such gene to an extent sufficient to achieve a desired biological or physiological effect. Such

inhibitors include substances that affect the transcription or translation of the gene as well as substances that affect the activity of the gene product. Examples of such inhibitors may include, *inter alia*: polynucleotides such as antisense (AS) fragments, siRNA, or vectors comprising them; polypeptides such as dominant negatives, antibodies, and enzymes; catalytic RNAs such as ribozymes; and chemical molecules with a low molecular weight e.g. a molecular weight below 2000 daltons.

"Expression vector" refers to a vector that has the ability to incorporate and express heterologous DNA fragments in a foreign cell. Many prokaryotic and eukaryotic expression vectors are known and/or commercially available. Selection of appropriate expression vectors is within the knowledge of those having skill in the art.

By "small interfering RNA" (siRNA) is meant an RNA molecule which decreases or silences (prevents) the expression of a gene/ mRNA of its endogenous cellular counterpart. The term is understood to encompass "RNA interference" (RNAi). RNA interference (RNAi) refers to the process of sequence-specific post transcriptional gene silencing in mammals mediated by small interfering RNAs (siRNAs) (Fire et al, 1998, Nature 391, 806). The corresponding process in plants is commonly referred to as specific post transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The RNA interference response may feature an endonuclease complex containing an siRNA, 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 may take place in the middle of the region complementary to the antisense strand of the siRNA duplex (Elbashir et al 2001, Genes Dev., 15, 188). For recent information on these terms and proposed mechanisms, see Bernstein E., Denli AM., Hannon GJ: *The rest is silence. RNA.* 2001 Nov;7(11):1509-21; and Nishikura K.: *A short primer on RNAi: RNA-directed RNA polymerase acts as a key catalyst. Cell.* 2001 Nov 16;107(4):415-8.

During recent years, RNAi has emerged as one of the most efficient methods for inactivation of genes (Nature Reviews, 2002, v.3, p.737-47; Nature, 2002, v.418,p.244-51). As a method, it is based on the ability of dsRNA species to enter a specific protein complex, where it is then targeted to the complementary cellular RNA and specifically degrades it. In more detail,

dsRNAs are digested into short (17-29 bp) inhibitory RNAs (siRNAs) by type III RNAses (DICER, Drosha, etc) (Nature, 2001, v.409, p.363-6; Nature, 2003, .425, p.415-9). These fragments and complementary mRNA are recognized by the specific RISC protein complex. The whole process is culminated by endonuclease cleavage of target mRNA (Nature Reviews, 2002, v.3, p.737-47; Curr Opin Mol Ther. 2003 Jun;5(3):217-24).

For disclosure on how to design and prepare siRNA to known genes see for example Chalk AM, Wahlestedt C, Sonnhammer EL. *Improved and automated prediction of effective siRNA* Biochem. Biophys. Res. Commun. 2004 Jun 18;319(1):264-74; Sioud M, Leirdal M., *Potential design rules and enzymatic synthesis of siRNAs,* Methods Mol Biol.2004;252:457-69; Levenkova N, Gu Q, Rux JJ.: Gene specific siRNA selector Bioinformatics. 2004 Feb 12;20(3):430-2. and Ui-Tei K, Naito Y, Takahashi F, Haraguchi T, Ohki-Hamazaki H, Juni A, Ueda R, Saigo K., *Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference* Nucleic Acids Res. 2004 Feb 9;32(3):936-48. See also Liu Y, Braasch DA, Nulf CJ, Corey DR. *Efficient and isoform-selective inhibition of cellular gene expression by peptide nucleic acids* Biochemistry, 2004 Feb 24;43(7):1921-7. See also PCT publications WO 2004/015107 (Atugen) and WO 02/44321 (Tuschl et al), and also Chiu YL, Rana TM. siRNA function in RNAi: a chemical modification analysis, RNA 2003 Sep;9(9):1034-48 and US Patent Nos.5898031 and 6107094 (Crooke) for production of modified/ more stable siRNAs.

DNA-based vectors capable of generating siRNA within cells have been developed. The method generally involves transcription of short hairpin RNAs that are efficiently processed to form siRNAs within cells. Paddison et al. *PNAS* 2002, 99:1443-1448; Paddison et al. *Genes & Dev* 2002, 16:948-958; Sui et al. *PNAS* 2002, 8:5515-5520; and Brummelkamp et al. *Science* 2002, 296:550-553. These reports describe methods to generate siRNAs capable of specifically targeting numerous endogenously and exogenously expressed genes.

For delivery of siRNAs, see, for example, Shen et al (FEBS letters 539: 111-114 (2003)), Xia et al., Nature Biotechnology 20: 1006-1010 (2002), Reich et al., Molecular Vision 9: 210-216 (2003), Sorensen et al. (J.Mol.Biol. 327: 761-766 (2003), Lewis et al., Nature Genetics 32: 107-108 (2002) and Simeoni et al., Nucleic Acids Research 31, 11: 2717-2724 (2003). siRNA has recently been successfully used for inhibition in primates; for further details see Tolentino et al., Retina 24(1) February 2004 pp 132-138.

siRNAs of the present invention

General specifications of siRNAs of the present invention

Generally, the siRNAs used in the present invention comprise a ribonucleic acid comprising a double stranded structure, whereby the double- stranded structure comprises a first strand and a second strand, whereby the first strand comprises a first stretch of contiguous nucleotides and whereby said first stretch is at least partially complementary to a target nucleic acid, and the second strand comprises a second stretch of contiguous nucleotides and whereby said second stretch is at least partially identical to a target nucleic acid, whereby said first strand and/or said second strand comprises a plurality of groups of modified nucleotides having a modification at the 2'-position whereby within the strand each group of modified nucleotides is flanked on one or both sides by a flanking group of nucleotides whereby the flanking nucleotides forming the flanking group of nucleotides is either an unmodified nucleotide or a nucleotide having a modification different from the modification of the modified nucleotides. Further, said first strand and/or said second strand may comprise said plurality of modified nucleotides and may comprises said plurality of groups of modified nucleotides.

The group of modified nucleotides and/or the group of flanking nucleotides may comprise a number of nucleotides whereby the number is selected from the group comprising one nucleotide to 10 nucleotides. In connection with any ranges specified herein it is to be understood that each range discloses any individual integer between the respective figures used to define the range including said two figures defining said range. In the present case the group thus comprises one nucleotide, two nucleotides, three nucleotides, four nucleotides, five nucleotides, six nucleotides, seven nucleotides, eight nucleotides, nine nucleotides and ten nucleotides.

The pattern of modified nucleotides of said first strand may be the same as the pattern of modified nucleotides of said second strand, and may align with the pattern of said second strand. Additionally, the pattern of said first strand may be shifted by one or more nucleotides relative to the pattern of the second strand.

The modifications discussed above may be selected from the group comprising sugar modifications such as amino, fluoro, alkoxy (including LNAs [linked nucleic acids] – which are circularized alkoxy modifications) or alkyl and base modifications such as 5-Alkyl-pyrimidines, 7-Deaza-purines, 8-Alkyl-purines or many other base modifications.

The double stranded structure of the siRNA may be blunt ended, on one or both sides. More specifically, the double stranded structure may be blunt ended on the double stranded structure's side which is defined by the S'- end of the first strand and the 3'-end of the second strand, or the double stranded structure may be blunt ended on the double stranded structure's side which is defined by at the 3'-end of the first strand and the 5'-end of the second strand.

Additionally, at least one of the two strands may have an overhang of at least one nucleotide at the 5'-end; the overhang may consist of at least one deoxyribonucleotide. At least one of the strands may also optionally have an overhang of at least one nucleotide at the 3'-end.

The length of the double-stranded structure of the siRNA is typically from about 17 to 21 and more preferably 18 or 19 bases. Further, the length of said first strand and/or the length of said second strand may independently from each other be selected from the group comprising the ranges of from about 15 to about 23 bases, 17 to 21 bases and 18 or 19 bases.

Additionally, the complementarily between said first strand and the target nucleic acid may be perfect, or the duplex formed between the first strand and the target nucleic acid may comprise at least 15 nucleotides wherein there is one mismatch or two mismatches between said first strand and the target nucleic acid forming said double-stranded structure.

In some cases both the first strand and the second strand each comprise at least one group of modified nucleotides and at least one flanking group of nucleotides, whereby each group of modified nucleotides comprises at least one nucleotide and whereby each flanking group of nucleotides comprising at least one nucleotide with each group of modified nucleotides of the first strand being aligned with a flanking group of nucleotides on the second strand, whereby the most terminal S' nucleotide of the first strand is a nucleotide of the group of modified nucleotides, and the most terminal 3' nucleotide of the second strand is a nucleotide of the flanking group of nucleotides. Each group of modified nucleotides may consist of a single nucleotide and/or each flanking group of nucleotides may consist of a single nucleotide.

Additionally, it is possible that on the first strand the nucleotide forming the flanking group of nucleotides is an unmodified nucleotide which is arranged in a 3' direction relative to the nucleotide forming the group of modified nucleotides, and on the second strand the nucleotide forming the group of modified nucleotides is a modified nucleotide which is arranged in 5' direction relative to the nucleotide forming the flanking group of nucleotides.

Further the first strand of the siRNA may comprise eight to twelve, preferably nine to eleven, groups of modified nucleotides, and the second strand may comprise seven to eleven, preferably

eight to ten, groups of modified nucleotides.

The first strand and the second strand may be linked by a loop structure, which may be comprised of a non-nucleic acid polymer such as, *inter alia*, polyethylene glycol. Alternatively, the loop structure may be comprised of a nucleic acid. The loop structure may additionally be comprised of amino acids or PNAs.

Further, the 5'-terminus of the first strand of the siRNA may be linked to the 3'-terminus of the second strand, or the 3'-end of the first strand may be linked to the 5'-terminus of the second strand, said linkage being via a nucleic acid linker typically having a length between 10-2000 nucleobases.

The siRNAs of the present invention, the various possible properties of which are desribed herein, are linked together by a variety of linkers as described above, such that a molecule which ciomprises two siRNA moieties is created. Such molecules are novel and may be used to treat a veriety of indications, as described herein.

Particular specifications of siRNAs of the present invention

The invention provides a molecule comprising a compound having the structure:

5' $(N)_x$ – Z 3' (antisense strand)

3' Z'-$(N')_y$ 5' (sense strand)

wherein each N and N' is a ribonucleotide which may be modified or unmodified in its sugar and/or base and/or backbone and $(N)_x$ and $(N')_y$ is oligomer in which each consecutive N or N' is joined to the next N or N' by a covalent bond ;

wherein each of x and y is an integer between 19 and 40;

and wherein each of Z and Z' may be present or absent, but if present is dTdT, rUrU, dUdU or rTrT and is covalently attached at the 3' terminus of the strand in which it is present.

In particular, the invention provides the above compound wherein the covalent bond is a phosphodiester bond, wherein x = y or y-1, preferably wherein x = y = 19 or 20; or x=20 and

y=19; or x=19 and y=20, wherein Z and Z' are both absent, wherein at least one ribonucleotide is modified in its sugar residue at the 2' position, wherein the moiety at the 2' position is methoxy (2'-O-Methyl) wherein alternating ribonucleotides are modified in both the antisense and the sense strands and wherein the ribonucleotides at the 5' and 3' termini of the antisense strand are modified in their sugar residues, and the ribonucleotides at the 5' and 3' termini of the sense strand are unmodified in their sugar residues.

Additionally, stabilizing terminal modifications are also possible, according to the following examples, *inter alia*:

Example (1) agagcgagaugaucuggaa-rUsrU-agagaagaucuacguguua

Example (2) agagcgagaugaucuggaa-rUsrU-agagaagaucuacguguua

Note that s indicates thiophosphate; underlining indicates modification, such as 2'O-methyl.

Further, this aspect provides for a pharmaceutical composition comprising two or more compounds of the above structure covalently or non-covalently linked, preferably by a linker, for the treatment of any disease or condition. Said two compounds may be covalently or non-covalently bound, or joined together by a nucleic acid linker of a length ranging from 2-100, preferably 2-50 or 2-30 nucleotides; or by a non nucleic acid linker such as HEG, diasulfide or a peptide linker. Such siRNA molecules are therefore comprised of a double-stranded nucleic acid structure as described herein. Such tandem siRNA molecules comprising two siRNA sequences would typically be of 38-150 nucleotides in length, more preferably 38 or 40- 60 nucleotides in length, and longer accordingly if more than two siRNA sequences are included in the tandem molecule. A longer tandem molecule comprised of two or more longer sequences which encode a molecule comprising siRNA which is produced via internal cellular processing, e.g., long dsRNAs, is also envisaged, as is a tandem molecule encoding two or more shRNAs. Such tandem molecules are also considered to be a part of the present invention.

Said combined or tandem structures have the advantage that toxicity and/or off-target effects of each siRNA are minimized, while the efficacy is increased – all as described herein.

Additionally the siRNA molecule used in the present invention may be an oligoribonucleotide an oligoribonucleotide wherein the dinucleotide dTdT is covalently attached to the 3' terminus, and/or in at least one nucleotide a sugar residue is modified, possibly with a modification comprising a 2'-O-Methyl modification. Further, the 2' OH group may be replaced by a group or moiety selected from the group comprising $-OCH_3$, $-OCH_2CH_3$, $-OCH_2CH_2CH_3$, $-O-CH_2CHCH_2$, $-NH_2$, -O-alkoxy, -O-LNA (linked to the 4' carbon of the sugar) and F. Further, the preferable compounds of the present invention as disclosed above may be phosphorylated or non-phosphorylated.

Additionally, the siRNA used in the present invention may be an oligoribonucleotide wherein in alternating nucleotides modified sugars are located in both strands. Particularly, the oligoribonucleotide may comprise one of the sense strands wherein the sugar is unmodified in the terminal 5'and 3' nucleotides, or one of the antisense strands wherein the sugar is modified in the terminal 5'and 3' nucleotides.

As detailed above, possible modification of the molecules of the present invention include modification of a sugar moiety, optionally at the 2' position, whereby the 2' OH group is replaced by a group or moiety selected from the group comprising $-H-OCH_3$, $-OCH_2CH_3$, $-OCH_2CH_2CH_3$, $-O-CH_2CHCH_2$, $-NH_2$, and –F.

Further possible modifications include modification of the nucleobase moiety and the modification or modified nucleobase may be selected from the group comprising inosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyladenines, 5-halo-uracil, 5-halo-cytosine, 5-halo-cytosine, 6-aza-cytosine, 6-aza-thymine, pseudouracil, 4-thio-uracil, 8-halo-adenine, 8-amino-adenine, 8-thiol-adenine, 8-thioalkyl-adenines, 8-hydroxyl-adenine and other 8-substituted adenines, 8-halo-guanines, 8-amino-guanine, 8-thiol-guanine, 8-thioalkyl-guanine, 8-hydroxyl-guanine and other substituted guanines, other aza- and deaza adenines, other aza- and deaza guanines, 5-trifluoromethyl-uracil and 5-trifluoro-cytosine.

In an additional embodiment the modification is a modification of the phosphate moiety, whereby the modified phosphate moiety is selected from the group comprising phosphothioate or lack of a phosphate group.

The tandem molecules of the present invention may comprise siRNAs, synthetic siRNAs, shRNAs and synthetic shRNAs, in addition to other nucleic acid sequences or molecules which encode such molecules or other inhibitory nucleotide moelcules. As used herein siRNAs may additionally comprise expression vector derived siRNAs, whereby the expression vector is in a preferred embodiment a virus such as Adenoviruses, Adenoassociated viruses, Herpes viruses and Lentiviruses. As used herein shRNA preferably means short hairpin RNAs. Such shRNA can be made synthetically or can be generated using vector encoded expression systems, preferably using RNA polymerase III promoters.

As used herein with any strategy for the design of tandem molecules, RNAi or any embodiment of RNAi disclosed herein, the term end modification means a chemical entity added to the most 5' or 3' nucleotide of the first and/or second strand. Examples for such end modifications include, but are not limited to, 3' or 5' phosphate, inverted abasic, abasic, amino, fluoro, chloro, bromo, CN, CF_3, methoxy, imidazolyl, caboxylate, phosphothioate, C_1 to C_{22} and lower alkyl, lipids, sugars and polyaminoacids (i.e. peptides), substituted lower alkyl, alkaryl or aralkyl, OCF_3, OCN, O-, S-, or N-alkyl; O-, S-, or N-alkenyl; $SOCH_3$; SO_2CH_3; ONO_2; NO_2, N_3; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino or substituted silyl, as, among others, described in European patents EP 0 586 520 B1 or EP 0 618 925 B1.

A further end modification is a biotin group. Such biotin group may preferably be attached to either the most 5' or the most 3' nucleotide of the first and/or second strand or to both ends. In a more preferred embodiment the biotin group is coupled to a polypeptide or a protein. It is also within the scope of the present invention that the polypeptide or protein is attached through any of the other aforementioned end modifications.

The various end modifications as disclosed herein are preferably located at the ribose moiety of a nucleotide of the nucleic acid according to the present invention. More particularly, the end modification may be attached to or replace any of the OH-groups of the ribose moiety, including but not limited to the 2'OH, 3'OH and 5'OH position, provided that the nucleotide thus modified is a terminal nucleotide. Inverted abasic or abasic are nucleotides, either desoxyribonucleotides or ribonucleotides which do not have a nucleobase moiety. This kind of

compound is, among others, described in Sternberger, M., Schmiedeknecht, A., Kretschmer, A., Gebhardt, F., Leenders, F., Czauderna, F., Von Carlowitz, I., Engle, M., Giese, K., Beigelman, L. & Klippel, A. (2002). Antisense Nucleic Acid Drug Dev, 12, 131-43

Further modifications can be related to the nucleobase moiety, the sugar moiety or the phosphate moiety of the individual nucleotide.

Such modification of the nucleobase moiety can be such that the derivatives of adenine, guanine, cytosine and thymidine and uracil, respectively, are modified. Particularly preferred modified nucleobases are selected from the group comprising inosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyladenines, 5-halo-uracil, 5-halo-cytosine, 5-halo-cytosine, 6-aza-cytosine, 6-aza-thymine, pseudouracil, 4-thio-uracil, 8-halo-adenine, 8-amino-adenine, 8-thiol-adenine, 8-thioalkyl-adenines, 8-hydroxyl-adenine and other 8-substituted adenines, 8-halo-guanines, 8-amino-guanine, 8-thiol-guanine, 8-thioalkyl-guanine, 8-hydroxyl-guanine and other substituted guanines, other aza- and deaza adenines, other aza- and deaza guanines, 5-trifluoromethyl-uracil and 5-trifluoro-cytosine. In another preferred embodiment, the sugar moiety of the nucleotide is modified, whereby such modification preferably is at the 2' position of the ribose and desoxyribose moiety, respectively, of the nucleotide. More preferably, the 2' OH group is replaced by a group or moiety selected from the group comprising amino, fluoro, alkoxy and alkyl. Preferably, alkoxy is either methoxy or ethoxy. Also preferably alkyl means methyl, ethyl, propyl, isobutyl, butyl and isobutyl. It is even more preferred that, regardless of the type of modification, the nucleotide is preferably a ribonucleotide.

A further form of nucleotides used may be siNA which is described in international patent application WO 03/070918, *inter alia*.

It is to be understood that, in the context of the present invention, any of the siRNA molecules disclosed herein, or any long double-stranded RNA molecules (typically 25-500 nucleotides in length) which are processed by endogenous cellular complexes (such as DICER – see above) to form the siRNA molecules disclosed herein, or molecules which comprise the siRNA molecules

disclosed herein, can be incorporated into the tandem molecules of the present invention to form additional novel molecules, and can employed in the treatment of the diseases or disorders described herein.

In particular, it is envisaged that a long oligonucleotide (typically about 80-500 nucleotides in length) comprising one or more stem and loop structures, where stem regions comprise the sequences of the oligonucleotides of the invention, may be delivered in a carrier, preferably a pharmaceutically acceptable carrier, and may be processed intracellularly by endogenous cellular complexes (e.g. by DROSHA and DICER as described above) to produce one or more smaller double stranded oligonucleotides (siRNAs) which are oligonucleotides of the invention. This oligonucleotide can be termed a tandem shRNA construct. It is envisaged that this long oligonucleotide is a single stranded oligonucleotide comprising one or more stem and loop structures, wherein each stem region comprises a sense and corresponding antisense siRNA sequence. Such a molecule and other similar molecules may encode two, three, four or even five siRNAs which may target one or more genes and function in a manner similar to the other tandem siRNA molecules disclosed herein. Such a molecule will include the linkers disclosed herein.

Any molecules, such as, for example, antisense DNA molecules which comprise the inhibitory sequences disclosed herein (with the appropriate nucleic acid modifications) are particularly desirable and may be used in the same capacity as their corresponding RNAs / siRNAs for all uses and methods disclosed herein.

By the term "antisense" (AS) or "antisense fragment" is meant a polynucleotide fragment (comprising either deoxyribonucleotides, ribonucleotides, synthetic nucleotides or a mixture thereof) having inhibitory antisense activity, said activity causing a decrease in the expression of the endogenous genomic copy of the corresponding gene. The sequence of the AS is designed to complement a target mRNA of interest and form an RNA:AS duplex. This duplex formation can prevent processing, splicing, transport or translation of the relevant mRNA. Moreover, certain AS nucleotide sequences can elicit cellular RNase H activity when hybridized with the target mRNA, resulting in mRNA degradation (Calabretta et al, 1996: *Antisense strategies in the treatment of leukemias. Semin Oncol. 23(1):78-87*). In that case, RNase H will cleave the RNA component of the duplex and can potentially release the AS to further hybridize with additional

molecules of the target RNA. An additional mode of action results from the interaction of AS with genomic DNA to form a triple helix which can be transcriptionally inactive.

All analogues of, or modifications to, a nucleotide / oligonucleotide may be employed with the present invention, provided that said analogue or modification does not substantially affect the function of the nucleotide / oligonucleotide. The nucleotides can be selected from naturally occurring or synthetic modified bases. Naturally occurring bases include adenine, guanine, cytosine, thymine and uracil. Modified bases of nucleotides include inosine, xanthine, hypoxanthine, 2- aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza cytosine and 6-aza thymine, psuedo uracil, 4- thiuracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thioalkyl guanines, 8- hydroxyl guanine and other substituted guanines, other aza and deaza adenines, other aza and deaza guanines, 5-trifluoromethyl uracil and 5- trifluoro cytosine.

In addition, analogues of polynucleotides can be prepared wherein the structure of the nucleotide is fundamentally altered and that are better suited as therapeutic or experimental reagents. An example of a nucleotide analogue is a peptide nucleic acid (PNA) wherein the deoxyribose (or ribose) phosphate backbone in DNA (or RNA is replaced with a polyamide backbone which is similar to that found in peptides. PNA analogues have been shown to be resistant to degradation by enzymes and to have extended lives in vivo and in vitro. Further, PNAs have been shown to bind stronger to a complementary nucleic acid - such as a DNA sequence - than a DNA molecule. This observation is attributed to the lack of charge repulsion between the PNA strand and the DNA strand. Other modifications that can be made to oligonucleotides include polymer backbones, cyclic backbones, or acyclic backbones.

By "homolog/homology", as utilized in the present invention, is meant at least about 70%, preferably at least about 75% homology, advantageously at least about 80% homology, more advantageously at least about 90% homology, even more advantageously at least about 95%, e.g., at least about 97%, about 98%, about 99% or even about 100% homology. The invention also comprehends that these nucleotides / oligonucleotides / polynucleotides can be used in the same fashion as the herein or aforementioned polynucleotides and polypeptides.

Alternatively or additionally, "homology", with respect to sequences, can refer to the number of

positions with identical nucleotides, divided by the number of nucleotides in the shorter of the two sequences, wherein alignment of the two sequences can be determined in accordance with the Wilbur and Lipman algorithm ((1983) Proc. Natl. Acad. Sci. USA 80:726); for instance, using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4, computer-assisted analysis and interpretation of the sequence data, including alignment, can be conveniently performed using commercially available programs (e.g., Intelligenetics™ Suite, Intelligenetics Inc., CA). When RNA sequences are said to be similar, or to have a degree of sequence identity or homology with DNA sequences, thymidine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence. RNA sequences within the scope of the invention can be derived from DNA sequences or their complements, by substituting thymidine (T) in the DNA sequence with uracil (U).

Additionally or alternatively, amino acid sequence similarity or homology can be determined, for instance, using the BlastP program (Altschul *et al.*, Nucl. Acids Res. 25:3389-3402) and available at NCBI. The following references provide algorithms for comparing the relative identity or homology of amino acid residues of two polypeptides, and additionally, or alternatively, with respect to the foregoing, the teachings in these references can be used for determining percent homology: Smith *et al.*, (1981) Adv. Appl. Math. 2:482-489; Smith *et al.*, (1983) Nucl. Acids Res. 11:2205-2220; Devereux *et al.*, (1984) Nucl. Acids Res. 12:387-395; Feng *et al.*, (1987) J. Molec. Evol. 25:351-360; Higgins *et al.*, (1989) CABIOS 5:151-153; and Thompson *et al.*, (1994) Nucl. Acids Res. 22:4673-4680.

"Having at least X% homolgy" - with respect to two amino acid or nucleotide sequences, refers to the percentage of residues that are identical in the two sequences when the sequences are optimally aligned. Thus, 90% amino acid sequence identity means that 90% of the amino acids in two or more optimally aligned polypeptide sequences are identical.

The invention has been described in an illustrative manner, and it is to be understood that the terminology used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention can be practiced otherwise than as specifically described.

Throughout this application, various publications, including United States patents, are referenced by author and year and patents by number. The disclosures of these publications and patents and patent applications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

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EXAMPLES

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the claimed invention in any way.

Standard molecular biology protocols known in the art not specifically described herein are generally followed essentially as in Sambrook et al., *Molecular cloning: A laboratory manual*, Cold Springs Harbor Laboratory, New-York (1989, 1992), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1988).

Standard organic synthesis protocols known in the art not specifically described herein are generally followed essentially as in *Organic syntheses: Vol.1- 79,* editors vary, J. Wiley, New York, (1941 - 2003); Gewert et al., *Organic synthesis workbook*, Wiley-VCH, Weinheim (2000); Smith & March, *Advanced Organic Chemistry*, Wiley-Interscience; 5th edition (2001).

Standard medicinal chemistry methods known in the art not specifically described herein are generally followed essentially as in the series "Comprehensive Medicinal Chemistry", by various authors and editors, published by Pergamon Press.

The features of the present invention disclosed in the specification, the claims and/or the drawings may both separately and in any combination thereof be material for realizing the invention in various forms thereof.

Example 1

General Materials and methods

If not indicated to the contrary, the following materials and methods were used in Examples 1-5:

Cell culture

The first human cell line, namely HeLa cells (American Type Culture Collection) were cultured as follows: Hela cells (American Type Culture Collection) were cultured as described in Czauderna F et al. (Czauderna, F., Fechtner, M., Aygun, H., Arnold, W., Klippel, A., Giese, K. & Kaufmann, J. (2003). Nucleic Acids Res, 31, 670-82).

The second human cell line was a human keratinozyte cell line which was cultivated as follows: Human keratinocytes were cultured at 37 °C in Dulbecco's modified Eagle medium (DMEM) containing 10% FCS.

The mouse cell line was B16V (American Type Culture Collection) cultured at 37 °C in Dulbecco's modified Eagle medium (DMEM) containing 10% FCS. Culture conditions were as described in Methods Find Exp Clin Pharmacol. 1997 May; 19(4):231-9:

In each case, the cells were subject to the experiments as described herein at a density of about 50,000 cells per well and the double-stranded nucleic acid according to the present invention was added at 20 nM, whereby the double-stranded nucleic acid was complexed using 1 µg/ml of a proprietary lipid.as described below.

Induction of hypoxia-like conditions

The cells were treated with $CoCl_2$ for inducing a hypoxia-like condition as follows: siRNA transfections were carried out in 10-cm plates (30-50% confluency) as described by (Czauderna et al., 2003; Kretschmer et al., 2003). Briefly, siRNA were transfected by adding a preformed 10x concentrated complex of GB and lipid in serum-free medium to cells in complete medium. The total transfection volume was 10 ml. The final lipid concentration was 1.0 µg/ml; the final siRNA concentration was 20 nM unless otherwise stated. Induction of the hypoxic responses was carried out by adding $CoCl_2$ (100µM) directly to the tissue culture medium 24 h before lysis.

Preparation of cell extracts and immuno blotting

The preparation of cell extracts and immuno blot analysis were carried out essentially as described by Klippel et al. (Klippel, A., Escobedo, M.A., Wachowicz, M.S., Apell, G., Brown, T.W., Giedlin, M.A., Kavanaugh, W.M. & Williams, L.T. (1998). Mol Cell Biol, 18, 5699-711; Klippel, A., Reinhard, C., Kavanaugh, W.M., Apell, G., Escobedo, M.A. & Williams, L.T. (1996). Mol Cell Biol, 16, 4117-27). Polyclonal antibodies against full length RTP801 were generated by immunising rabbits with recombinant RTP801 protein producing bacteria from pET19-b expression vector (Merck Biosciences GmbH, Schwalbach, Germany). The murine monoclonal anti-p110a and anti-p85 antibodies have been described by Klippel et al. (supra).

Example 2

Preparation of nucleic acid molecules / siRNAs

The molecules and compounds of the present invention can be synthesized by any of the methods which are well-known in the art for synthesis of ribonucleic (or deoxyribonucleic) oligonucleotides. For example, a commercially available machine (available, *inter alia*, from Applied Biosystems) can be used; the oligonucleotides are prepared for example according to the sequences disclosed herein and also according to known genes.

The strands are synthesized separately and then are annealed to each other in the tube.

The molecules of the invention may be synthesized by procedures known in the art e.g. the procedures as described in Usman et al., 1987, *J. Am. Chem. Soc.*, 109, 7845; Scaringe et al., 1990, *Nucleic Acids Res.*, 18, 5433; Wincott et al., 1995, *Nucleic Acids Res.* 23, 2677-2684; and Wincott et al., 1997, *Methods Mol. Bio.*, 74, 59, and may make use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. The modified (e.g. 2'-O-methylated) nucleotides and unmodified nucleotides are incorporated as desired.

The linker can be a polynucleotide linker or a non-nucleotide linker.

For further information, see for example PCT publication No. WO 2004/015107 (atugen AG).

In the context of the RNAster molecule disclosed herein, having one of the following structures:

5′	oligo1 (sense)	LINKER A	Oligo2 (sense)	3′	(Strand 1)
3′	oligo1 (antisense)	LINKER B	Oligo3 (sense)	5′	(Strand 2)
3′	oligo3 (antisense)	LINKER C	oligo2 (antisense)	5′	(Strand 3)

or

5′	oligo1 (sense)	LINKER A	Oligo2 (antisense)	3′	(Strand 1)
3′	oligo1 (antisense)	LINKER B	Oligo3 (sense)	5′	(Strand 2)
3′	oligo3 (antisense)	LINKER C	oligo2 (sense)	5′	(Strand 3)

or

5′	oligo1 (sense)	LINKER A	oligo3 (antisense)	3′	(Strand 1)
3′	oligo1 (antisense)	LINKER B	oligo2 (sense)	5′	(Strand 2)
5′	oligo3 (sense)	LINKER C	oligo2 (antisense)	3′	(Strand 3)

each of strand 1, strand 2 and strand 3 is synthesized separately and the three strands are then mixed together to form the RNAstar molecule. Therefore, although the three structures represented above give rise to a similar RNAstar molecule, they are not identical. Note that the numbering of the strands as above is for the purpose of simplification only and is not intended to be limiting in any way.

Example 3

Structures of the molecules of the present invention

As will be described below, the structures of the molecules of the present invention are exemplified using two model siRNA sequences, one which targets the TGaseII gene (see co-assigned patent application publication no. WO 2005/072057) and another which targets the HNOEL gene (see co-assigned patent application publication no. WO 2004/112565). However, this is not intended to be limiting in any way, and the siRNA sequences can be replaced with an appropriate sequence which will target any gene, siRNA inhibition of which is desirable. The sequences used herein are intended as teaching examples which demontrate the molecular structure only.

1) Structure 1: Interrupted antisense strand with a minimal linker

Variant Ia

```
Seq02-s     5'      aagagcgagaugaucuggaadTsdTuuagagaagaucuacguguuagdTsdT 3'
Seq04a-as   3' dTsdTuucucgcucuacuagaccuu  dTsdTucucuucuagaugcacaauc      5'
```

Variant Ib

```
Seq02b-s    5'      agagcgagaugaucuggaadTsdTuuagagaagaucuacguguuadTsdT 3'
TGas+HNas   3' dTsdTucucgcucuacuagaccuu  dTsdTucucuucuagaugcacaau      5'
```

Variant Ic

```
Seq02c-s    5'      aagagcgagaugaucuggaadTsdTuuagagaagaucuacguguuagdTsdT 3'
Seq04c-as   3' dTsdTuucucgcucuacuagaccuu  dTsdTucucuucuagaugcacaauc      5'
```

Variant Ib/2

```
Seq02b/2-s     5'      agagcgagaugaucuggaarUsrUagagaagaucuacguguua      3'
TGas/2+HNas/2  3'      ucucgcucuacuagaccuu      ucucuucuagaugcacaau      5'
```

2) Structure 2: Complete base-pairing with a minimal linker

Variant Id

```
Seq01-s     5'      agagcgagaugaucuggaadTsdTaaagagaagaucuacguguuadTsdT 3'
Seq01-as    3' dTsdTucucgcucuacuagaccuuaadTsdTucucuucuagaugcacaau      5'
```

3) Structure 3: Minimal loop linker

Variant Ie

```
Seq03-s     5'      agagcgagaugaucuggaadTsdTagagaagaucuacguguuadTsdT 3'
Seq03-as    3' dTsdTucucgcucuacuagaccuudTsdTucucuucuagaugcacaau      5'
```

Variant If

```
Seq02-s     5'      aagagcgagaugaucuggaadTsdTuuagagaagaucuacguguuagdTsdT 3'
Seq02-as    3' dTsdTuucucgcucuacuagaccuuuudTsdTucucuucuagaugcacaauc      5'
```

4) Structure 4: triple stranded "star"-like structure

HEG-Linker: Hexaethylenglycol

SeqX1 5' agagcgagaugaucuggaa**dTsdTaaHEG**uuaaagagaagaucuacguguua**dTsdT** 3'
SeqX2 3' **dTsdT**ucucgcucuacuagaccuuaaauu**HEG**aa**dTsdTuuuaguagguaacgaaccc** 5'
SeqX3 3' **dTsdT**ggguucguuaccuacuaaaaauu**HEG**aa**dTsdT**ucucuucuagaugcacaau 5'

TTTT-Linker: poly(5'-2'deoxythymidyl-3'-phosphate)

Variant Ig

SeqX1 5' agagcgagaugaucuggaa**dTsdTaaTTTT**uuaaagagaagaucuacguguua**dTsdT** 3'
SeqX2 3' **dTsdT**ucucgcucuacuagaccuuaaauu**TTTTaadTsdT**uuuaguagguaacgaaccc 5'
SeqX3 3' **dTsdT**ggguucguuaccuacuaaaaauu**TTTTaadTsdT**ucucuucuagaugcacaau 5'

Note that it may be easier to synthesize the TTTT linker molecule than the HEG-linker molecule.

5) Structure 5: Combination of dsRNA(1) and dsRNA(2) by RNA-Linker

Example: Variant IIa/2

Seq02b/3-s 5' agagcgagaugaucuggaa**AAAAAAAAA**agagaagaucuacguguua 3'
TGas/2+HNas/2 3' ucucgcucuacuagaccuu ucucuucuagaugcacaau 5'

Note that the above structure may be cleaved endogenously by RNase.

6) Structure 6: Combination of dsRNA(1) and dsRNA(2) by DNA-Linker

Example

5' agagcgagaugaucuggaa**TTTTTTTTT**agagaagaucuacguguua 3'
TGas/2+HNas/2 3' ucucgcucuacuagaccuu ucucuucuagaugcacaau 5'

Note that the above structure may be cleaved endogenously by DNase.

7) Structure 7: Combination of dsRNA(1) and dsRNA(2) by a Disulfid linker

Example: Variant IIIa/1

```
Seq02b/5-s      5' agagcgagaugaucuggaa--SS--agagaagaucuacguguua 3'
TGas/2+HNas/2   3' ucucgcucuacuagaccuu       ucucuucuagaugcacaau 5'
```

Note that the above structure may be cleaved endogenously under a reducing enviroment or by disulfide reductases. Additional references for the preparation of molecules possessing this structure include: BA Connolly and P Rider "Chemical synthesis of oligonucleotides containing a free sulphydryl group and subsequent attachment of thiol specific probes" Nucleic Acids Res., Jun **1985**; 13: 4485 - 4502.; ND Sinha and RM Cook "The preparation and application of functionalised synthetic oligonucleotides: III. Use of H-phosphonate derivatives of protected amino-hexanol and mercapto-propanol or –hexanol" Nucleic Acids Res., Mar **1988**; 16: 2659 - 2669.; RK Gaur, P Sharma, and KC Gupta "A simple method for the introduction of thiol group at 5'-termini of oligodeoxynucleotides" Nucleic Acids Res., Jun **1989**; 17: 4404.; A Kumar, S Advani, H Dawar, and GP Talwar "A simple method for introducing a thiol group at the 5'-end of synthetic oligonucleotides" Nucleic Acids Res., Aug **1991**; 19: 4561.

8) Structure 8: Combination of dsRNA(1) and dsRNA(2) by a peptide linker

Example: Variant Ib/2

```
Seq02b/2-s      5' agagcgagaugaucuggaaX-Gly-Phe-Gly-Yagagaagaucuacguguua 3'
TGas/2+HNas/2   3' ucucgcucuacuagaccuu              ucucuucuagaugcacaau 5'
```

Note that the above structure may be cleaved endogenously by several specific or non-specific peptidases. Additional references for the preparation of molecules possessing this structure include: M Antopolsky and A Azhayev "Stepwise Solid-Phase Synthesis of Peptide-Oligonucleotide Conjugates on New Solid Supports" in Perspectives in Nucleoside and Nucleic Acid Chemistry, p.275-285, Edited by V. Kisakürek and H. Rosemeyer, Wiley, Verlag Helvetica Chimica Acta 2000.; DA Stetsenko and MJ Gait „Chemical Methods for Peptide-

Oligonucleotide Conjugate Synthesis" in Oligonucleotide Synthesis Methods and Applications, Edited by Piet Herdewijn, Methods in Molecular Biology Volume 288, Humana Press 2005.

All the above structures can be constructed with or without 5'-6FAM (6-Carboxy-Fluoresceine) on the sense strandsense; additionally, alternative modifications for the RNA nucleotides include 2'-O-Methyl, 2'-Fluoro, 2'-OAllyl and also some base modifications (Beaucage, S.L.; Iyer, R.P.; *Tetrahedron*, **1992**, *48*, 2223-2311 and Beaucage, S.L.; Iyer, R.P.; *Tetrahedron*, **1993**, *49*, 6123-6194). Alternative modifications for the DNA nucleotides include base modified DNA; see also Beaucage, S.L.; Iyer, R.P.; *Tetrahedron*, **1992**, *48*, 2223-2311 and Beaucage, S.L.; Iyer, R.P.; *Tetrahedron*, **1993**, *49*, 6123-6194.

Note that in the above structures, dT or T indicate desoxyribothymidine (DNA); s indicates phosphorothioate (PO_3S instead of PO_4); U, A, G, and C indicate 2'-O-Methyl-Ribouridine (RNA), 2'-O-Methyl-Riboadenosine (RNA), 2'-O-Methyl-Riboguanosine (RNA) and 2'-O-Methyl-Ribocytidine (RNA) respectively; U, A, G and C indicate ribouridine (RNA), riboadenosine (RNA), riboguanosine (RNA) and ribocytidine (RNA) respectively; HEG indicates hexaethylenglycol and 6FAM indicates 6-Carboxy-Fluoresceine.

Example 4

Experimental Results

Controls used for the testing of the molecules of the present invention were two siRNAs against two separate genes, TGaseII and HNOEL, each which the assignee of the present invention has already showed to inhibit effectively the corresponding gene (see PCT publications WO 2005/072057 and WO 2004/112565 respectively). The two molecules were combined in the test tube and compared to the efficacy of molecules possessing the above described structures, wherein both these siRNAs are encoded in the one molecule. p53 siRNA was also used as a control.

TGaseII

TGaseII-s	5'	agagcgagaugaucuggaa**dTsdT** 3'
TGaseII-as	3'	**dTsdT**ucucgcucuacuagaccuu 5'

HNOEL

HNOEL-s	5′	agagaagaucuacguguuadTsdT 3′
HNOEL-as	3′	dTsdTucucuucuagaugcacaau 5′

p53

p53-s	5′	cccaagcaauggaugauuudTsdT 3′
p53-as	3′	dTsdTggguucguuaccuacuaaa 5′

Additional molecules used as controls were the above molecules with and without sense 5'FAM, designated TGaseII-s and HNOEL-s.

siRNA transfection with monomers, tandem and RNAstar siRNA molecules

$2x10^5$ tested cells were seeded per well in 6-well plates (70-80% confluent). After 24 hours, cells were transfected with siRNA oligos using Lipofectamine 2000 reagent (Invitrogen) at a final concentration of 500pM, 5nM and 20nM. PTEN-Cy3 oligos or FAM labeled oligos were used as a positive control for transfection; PTEN-Cy3, MR3 and GFPsi molecules were used as negative control for siRNA activity. 48h-72h after transfection cells were harvested and RNA or proteins were extracted from cells. Transfection efficiency was tested by fluorescent microscopy or by FACS (using FL-2 filter (for Cy3) or FL-1 filter (for FAM)).

siRNA sample preparation:

For each transfected well:

Dilute 3ul lipofectamine 2000 reagent in 250ul serum free medium, and incubate for 5min at RT.

Dilute siRNA molecules as mention below:

Oligos:

- PTEN Cy3 stock $1.5x10^6$nM (dilute 1:150 to final concentration of 10uM in PBS)
- Monomers/tandem/RNAstar stock 100uM (dilute 1:10 to final concentration of 10uM in PBS)

Table 1

Oligos	No of wells	Final volume	SiRNA stock 10uM
20nM (1:500)	3	6ml	750ul medium+12ul SiRNA
5nM (1:4 of 20nM)			250 ul from 20nM + 750ul Medium (drop 450ul before adding lipofectamine)
500pM (1:10 of 5nM)			50ul from 5nM +450ul medium

Combine Lipofectamine 2000 Reagent with siRNA (1:1 volume), mix gently and incubate at RT for 20min

Transfection

Replace medium with 1.5ml fresh growth medium (containing serum).

Add Lipofectamine/siRNA complex onto cells (500ul per well), and rock the plate back and forth (2ml final volume in each well)

Incubate cells at 37^0C in a CO2 incubator.

Cells used for siRNA activity examination

Table 2

Cell line	Tested gene	Species tested gene	Expression type
HFL-1	TGaseII, p53, HNOEL	Human	Endogenous
293	TGASEII	Rat	Exogenous
293	HNOEL	Human	Exogenous
NRK49	TGASEII	Rat	Exogenous
HCT116	P53	Human	Endogenous/5Fu

p53 was induced in HCT116 cells following treatment with 25ug/ml 5Fu (5Fu was added in the last 8h of experiment, before harvesting cells).

Results:

HNOEL monomer molecule activity:

Western blot analysis of HNOEL expression in 293 cells expressing exogenous human HNOEL cDNA, following HNOEL+2nt (additional overhangs) and blunt siRNA transfection is presented in figure 1a.

TGASEII monomer activity:

qPCR analysis of TGASEII expression in 293 cells expressing exogenous rat TGASEII cDNA, following TGASE+2nt and blunt siRNA transfection is presented in Table 3 as % of the control expression

Table 3

SiRNA conc.	20nM	5nM	0.5nM
293 cellls	100%		
PTEN-Cy3	143%		
MR3	174%		
TGASEII	22%	7.8%	67%
TGASEII+2nt	34%	27%	45%

p53 monomer activity:

qPCR analysis of p53 expression in HFL-1 cells expressing endogenous p53, following p53+2nt siRNA transfection is presented in Figure 1b as % of the control p53 expression in HFL-1 cells.

A western blot analysis of p53 expression in 5Fu treated p53-wt HCT116 cells, following p53+2nt siRNA transfection is presented in Figure 1c.

Transfection efficiency of monomer, tandem and RNAstar molecules (FACS using FAM-labeled molecules).

The results in Tables 4-5 below represent the comparison of transfection efficiency of the tested molecules in 293 cells.

Table 4 - Experiment1

20Nm siRNA	% of transfected cells	FAM intensity	5nM siRNA	% of transfected cells	FAM intensity
TGASEII+ 2nt	74%	80		44%	30
HNOEL+2 nt	80%	59		41%	29
SeqO2	72%	33		60%	34
SeqO2b	72%	32		70.5%	44
SeqO2c	75%	34		71.5%	41

Table 5 – Experiment 2

20Nm siRNA	% of transfected cells	FAM intensity	5nM siRNA	% of transfected cells	FAM intensity
P53+2nt	72%	90		43%	35
TGASEII+ 2nt	64%	50		26%	26
HNOEL+2 nt	61%	64		21%	27
SeqO2b	80%	85		60%	48
RNAstar	65%	53		50%	40

HNOEL monomer and tandem molecules activity on HNOEL gene expression:

The results of a western blot analysis of HNOEL expression in 293 cells expressing exogenous human HNOEL cDNA, following HNOEL+2nt and tandem siRNA transfection are presented in Figure 2.

TGASEII monomer and tandem molecules activity on TGASEII gene expression:

The results of a qPCR analysis of TGASEII expression in NRK49 cells expressing exogenous rat TGASEII cDNA, following TGASE+2nt and tandem siRNA transfection is presented in Table 6 as % of control TGASEII expression in NRK49 cells.

Table 6

SiRNA concentration	**20nM**	**5nM**	**20nM**	**5nM**
NRK49 CELLS	100%			
PTEN-Cy3	116%			
MR3	60%			
HNOEL	95%	136%	123%	40%
TGASEII	46%	61%	56%	91%
SeqO2	37%	39%	37%	62%
SeqO2b	28%	36%	14%	24%
SeqO2c	36%	43%	33%	58%

RNAstar molecule, activity on p53, TGASEII and HNOEL genes expression:

The results of a qPCR analysis of TGASEII, HNOEL and p53 expression in HFL-1 cells expressing endogenous genes, following RNAstar transfection are presented in Tables 7-8 as % of control TGASEII, HNOEL or p53 expression in HFL-1 cells.

Table 7 – Experiment 1

	p53 gene	**TGaseII gene**	**HNOEL gene**
HFL-1 cells	100%	100%	100%
PTEN	130%	82%	110%
GFPsi	111%	71%	102%
P53+2nt_20nM	28%	104%	119%
5nM	33%	70%	100%
0.5nM	87%	71%	103%

HNOEL+2nt_20nM	105%	91%	65%
5nM	113%	98%	91%
0.5nM	131%	108%	108%
TGASEII+2nt_20nM	88%	96%	84%
5nM	156%	111%	119%
0.5nM	114%	92%	105%
RNAstar_20nM	125%	104%	99%
5nM	136%	106%	115%
0.5nM	81%	108%	112%

Table 8 – Experiment 2

	P53 gene	**TGASEII gene**	**HNOEL gene**
PTEN 20nM	100%	100%	100%
GFPsi_20nM	177%	109%	124%
P53+2nt_20nM	54%	14.9%	9.7%
5nM	25.7%	9.7%	7%
TGASEII+2nt_20nM	117%	35%	97%
5nM	26%	72%	57%
HNOEL+2nt_20nM	95%	77%	59%
5nM	110%	85%	66%
TGASE/HNOEL+2nt_20nM	89%	32%	32%
5nM	49%	32%	30%
TGASEII/HNOEL/P53+2nt_20nM	14%	27%	49%
5nM	44%	69%	80%
RNAstar_20nM	61%	34%	40%
5nM	163%	115%	96%

Effect of the RNAstar molecule on p53 gene expression::

The results of a Western blot analysis of p53 expression in 5Fu treated HCT116 cells, following RNAstar transfection are presented in Figure 3.

The transfection efficiency of RNA star molecule in HFL-1 cells is presented in Table 9.

Table 9

% of transfected cells	20nM	5nM
SeqO2b	36%	20%
RNAstar	15%	14%

Comparison between the activity of co-transfected HNOEL/TGASEII monomers and tandem molecules:

The results of a qPCR analysis of HNOEL and TGASEII expression in HFL-1 cells following transfection of tandem SeqO2b or SeqO2b/2 siRNA molecules and co transfection of HNOEL and TGASEII monomers (blunt and +2nt molecules) are presented in Tables 10-11 as % of control expression (TGASEII or HNOEL) in HFL-1 cells.

Table 10 – Experiment 1

	TGaseII gene	HNOEL gene
HFL-1 cells	100%	100%
PTEN	108%	147%
GFPsi	128%	122%
SeqO2b	28%	42%
TGaseII	38%	137%
HNOEL	90%	28%
TGaseII/HNOEL	20%	20%
TGaseII+2nt	44%	98%
HNOEL+2nt	86%	67%
TGaseII/HNOEL+2nt	83%	82%

(20nM concentration for all tested oligos)

Table 11 – Experiment 2

	TGaseII gene	HNOEL gene
PTEN_20nM	100%	100%

GFPsi_20nM	109%	124%
SeqO2b_20nM	32%	43%
5nM	15%	25%
SeqO2b/2_20nM	11%	8%
5nM	13%	16%
TGaseII_20nM	83%	42%
5nM	66%	59%
HNOEL_20nM	16%	58%
5nM	14%	15%
TGaseII/HNOEL_20nM	96%	9%
5nM	88%	7%
TGaseII+2nt_20nM	35%	97%
5nM	72%	57%
HNOEL+2nt_20nM	77%	59%
5nM	85%	65%
TGaseII/HNOEL+2nt_20nM	32%	32%
5nM	32%	30%

Effect of tandem molecules on TGase and HNOEL expression:

The results of a qPCR analysis of HNOEL and TGASEII expression in HFL-1 cells following transfection with tandem variant molecules (SeqO1, SeqO3, SeqO2b, SeqO2b/2) are presented in Figure 4 a + b (a = HNOEL, b = TGASEII) as % of control TGASEII or HNOEL expression in HFL-1 cells.

Effect of RNAstar molecule on p53, TGASEII and HNOEL gene expression:

The results of a qPCR analysis of TGASEII, HNOEL and p53 expression in HFL-1 cells expressing endogenous genes, following RNAstar transfection are presented in Tables 12-13 as % of control TGASEII, HNOEL or p53 expression in HFL-1 cells.

Table 12 – Experiment 1

	P53 gene	TGASEII gene	HNOEL gene
HFL-1 cells	100%	100%	100%
PTEN	130%	82%	110%
GFPsi	111%	71%	102%
P53+2nt_20nM	28%	104%	119%
5nM	33%	70%	100%
0.5nM	87%	71%	103%
HNOEL+2nt_20nM	105%	91%	65%
5nM	113%	98%	91%
0.5nM	131%	108%	108%
TGASEII+2nt_20nM	88%	96%	84%
5nM	156%	111%	119%
0.5nM	114%	92%	105%
RNAstar_20nM	125%	104%	99%
5nM	136%	106%	115%
0.5nM	81%	108%	112%

Table 13 – Experiment 2

	P53 gene	TGASEII gene	HNOEL gene
PTEN 20nM	100%	100%	100%
GFPsi_20nM	177%	109%	124%
P53+2nt_20nM	54%	14.9%	9.7%
5nM	25.7%	9.7%	7%
TGASEII+2nt_20nM	117%	35%	97%
5nM	26%	72%	57%
HNOEL+2nt_20nM	95%	77%	59%
5nM	110%	85%	66%
TGASE/HNOEL+2nt_20nM	89%	32%	32%
5nM	49%	32%	30%

TGASEII/HNOEL/P53+2nt_20nM	14%	27%	49%
5nM	44%	69%	80%
RNAstar_20nM	61%	34%	40%
5nM	163%	115%	96%

p53 blunt and p53+2nt monomers activity on TGASEII and HNOEL expression:

The results of a qPCR analysis of TGASEII, and HNOEL expression in HFL-1 cells expressing endogenous genes, following transfection by a "single" p53 siRNA molecule are presented in Figure 5 as % of TGASEII and HNOEL expression in HFL-1 cells.

All of the above results demonstrate the efficacy of the tandem molecules of the present invention in inhibiting the endogenous genes which they target.

Example 5

Additional experimental Results with RNAstar

The activity of the RNAstar gapped molecules was examined 72h following transfection. The control active siRNAs chosen for the assay were MRH2 (HNOEL), HMRG1 (TGASEII), QH1 (P53) and SeqO2b_SeqO2b/2 (TGASEII and HNOEL) siRNA molecules, which have been found to be active in down-regulating their target genes by the assignee of the present invention. PTEN siRNA and GFP siRNA were used as negative controls.

The following cell lines were used in the study:

Cell line	**Tested genes**	**Species tested gene**	**Expression type**	**Activity observation**
HFL1	TGaseII, p53, HNOEL	Human	Endogenous	qPCR
293	HNOEL	Human	Exogenous	qPCR/Western blot
NRK49	TGase II	Rat	Exogenous	qPCR/Western

				blot
HCT116	p53	Human	Endogenous, 5FU induced	Western blot

The different variants tested and the genes they target are presented in Figure 6; underlined nucleotides were modified, but it is envisaged that unmodified nucleotides may also be used.

Results

A) qPCR analysis of HNOEL expression in HFL-1 cells expressing endogenous human HNOEL gene following transfection with gapped RNAstar:

The data in Table 14 demonstrate residual (% of PTEN) human HNOEL expression in HFL-1 cells.

Table 14

siRNA concentration	0.5nM	5nM	20nM
PTEN-Cy3			100%
SiGFP			199%
HNOEL monomer	111%	144%	129%
SeqO2b/2 (positive Control)	104%	37%	16%
Complex-p53/HNOEL/TGASEII siRNA monomers	115%	70%	82%
RNAstar	39%	33%	30%
Ig/4 (HNOEL)	89%	207%	181%
Ig/5 (gapped RNAstar)	149%	126%	63%
Ig/6 (gapped RNAstar)	115%	37%	41%
Ig/7 (gapped RNAstar)			14%

B) qPCR analysis of TGASEII expression in HFL-1 cells expressing endogenous human TGASEII gene following transfection with gapped RNAstar:

The data in Table 15 demonstrate residual (% of PTEN) human TGASEII expression in HFL-1 cells.

Table 15

siRNA concentration	0.5nM	5nM	20nM
PTEN-Cy3			100%
siGFP			131%
TGASEII monomer	144%	134%	113%
SeqO2b/2 (positive Control)	137%	145%	103%
Complex-p53/HNOEL/TGASEII siRNA monomers	164%	88%	93%
RNAstar	63%	89%	62%
Ig/2 (TGASEII)	134%	157%	113%
Ig/5 (gapped RNAstar)	137%	83%	38%
Ig/6 (gapped RNAstar)		28%	53%
Ig/7 (gapped RNAstar)			50%

C) qPCR analysis of p53 expression in HFL-1 cells expressing endogenous human p53 gene following transfection with gapped RNAstar:

The data in Table 16 demonstrate residual (% of PTEN) human p53 expression in HFL-1 cells.

Table 16

siRNA concentration	0.5nM	5nM	20nM
PTEN-Cy3			100%
siGFP			127%
Tp53 monomer	37%	24%	15%
Complex-p53/HNOEL/TGASEII siRNA monomers	89%	66%	93%

RNAstar		92%	92%
Ig/3 (p53)	97%	121%	81%
Ig/5 (gapped RNAstar)	82%	43%	23%
Ig/6 (gapped RNAstar)	97%	52%	76%
Ig/7 (gapped RNAstar)			43%

D) qPCR analysis of TGASEII expression in NRK49 cells expressing exogenous rat TGASEII gene following transfection with gapped RNAstar:

The data in Table 17 demonstrate residual (% of Control) rat TGASEII expression in NRK49 cells.

Table 17

siRNA concentration	5nM			20nM		
	Exp 1	Exp2	Average	Exp 1	Exp2	Average
PTEN-Cy3				82%	109%	95.5 ±19
SiGFP				105%	75%	90 ±21
TGASEII monomer	64%	75%	69.5±7.7	87%	84%	85.5 ±2
SeqO2b		57%	57		22%	22
Complex-p53/HNOEL/TGASEII siRNA monomers	21%	73%	47±37	22%	49%	35.5 ±19
RNAstar	52%	52%	52±0	66%	60%	63 ±4
Ig/2 (TGASEII)	94%		94	65%		65
Ig/5 (gapped RNAstar)	53%	77%	65±17	55%	64%	59.5 ±6
Ig/6 (gapped RNAstar)	77%	58%	67.5±13	61%	89%	75 ±20
Ig/7 (gapped RNAstar)	16%	33%	24.5 ±12	17%	32%	24.5 ±10

E) qPCR analysis of HNOEL expression in NRK49 cells expressing endogenous rat HNOEL gene following transfection with gapped RNAstar:

The data in Table 18 demonstrate residual (% of Control) rat HNOEL expression in NRK49 cells.

Table 18

siRNA concentration	5nM			20nM		
	Exp 1	Exp2	Average	Exp 1	Exp2	Average
PTEN-Cy3				57%	69%	63 ±8
siGFP				119%	102%	110 ±12
HNOEL monomer	108%	72%	90 ±25	114%	88%	101 ±18
SEQO2B		85%	85		91%	91
Complex-p53/HNOEL/TGASEII siRNA monomers	69%	147%	108 ±55	62%	132%	97 ±49
RNAstar	74%	180%	127 ±75	154%	156%	155 ±1
Ig/5 (gapped RNAstar)	57%	200%	128.5 ±101	32%	123%	77.5 ±64
Ig/6 (gapped RNAstar)	44%	140%	92 ±69	20%	122%	71 ±72
Ig/7 (gapped RNAstar)	33%	146%	89.5 ±79	39%	173%	106 ±94

F) qPCR analysis of HNOEL expression in 293 cells expressing exogenous human HNOEL gene following transfection with gapped RNAstar:

The data in Table 19 demonstrate residual (% of Control) human HNOEL expression in 293 cells.

Table 19

siRNA concentration	5nM			20nM		
	Exp 1	Exp2	Average	Exp 1	Exp2	Average
PTEN-Cy3				101%	64%	82.5 ±26

siGFP				118%	86%	102 ±22
HNOEL monomer	122%	89%	105.5 ±23	87%	115%	101 ±20
SeqO2b		52%	52		22%	22
Complex-p53/HNOEL/TGASEII siRNA monomers	71%	81%	76 ±7	53%	110%	81.5 ±40
RNAstar	86%	72%	79 ±10	45%	87%	66 ±29
Ig/5 (gapped RNAstar)	64%	47%	55.5 ±12	49%	34%	41.5 ±10
Ig/6 (gapped RNAstar)	77%	50%	63.5 ±19	70%	45%	57.5 ±17
Ig/7 (gapped RNAstar)	103%	63%	83 ±28	86%	50%	68 ±25

The results of the corresponding Western blots appear in Figure 8.

Conclusions

The activity of RNAstar molecule on the transcription level of three target genes (p53, HNOEL and TGASEII) was examined. 60% inhibition of HNOEL expression, 40-50% inhibition of TGASEII expression, and a little inhibition of p53 expression were observed.

The Ig/5 variant is significantly more active than Ig/6 and Ig/7 variants on p53 gene; the Ig/6 and Ig/7 variants displayed more activity on TGASE and HNOEL genes as compared to the Ig/5 variant; Further, the RNAstar gapped variants appear significantly more active than the non-gapped RNAstar molecule against the three particular targets tested.

In general, the variants tested were active in inhibiting the expression of the target genes; the degree of inhibition varies between each variant / target gene combination. Thus all variants are potentially active against any given gene. Table 20 sums up all experiments conducted.

Table 20

Exp No	Exp1	Exp2	Exp3	Exp4	Exp5	Exp5	Exp1	Exp2	Exp3	Exp5	Exp1	Exp2
Tested cells	HFL-1 qPCR	NRK49 qPCR	293 qPCR	293 Western	293 qPCR	NRK49 qPCR	HFL-1 qPCR	NRK49 qPCR	NRK49 Western	NRK49 qPCR	HFL-1 qPCR	HCT116 Western
SeqO2b					+++	±				+++		
RNAstar	+++	-	++		+	-	+	++		++	-	-
Ig/5	+ -	+++	++	+	++±	-	+	++	-	++	+++	+++
Ig/6	+++	+++	+	++	++±	-	+++	+	++	++	+	+-
Ig/7	+++	+++	-	++	++±	-	++	+++	+++	+++	++	-

Example 6

Additional Experimental Results with Tandem structures

Procedure

1. General

1.1 $2x10^5$ HFL-1 cells expressing endogenous TGASEII and HNOEL genes were seeded per well in 6- well plate (70-80% confluent).

1.2 24h subsequently, cells were transfected with siRNA oligos using the lipofectamine 2000 reagent (Invitrogene) at a final concentration of 10nM and 20nM .

PTEN-Cy3 oligos were used as a positive control for transfection.

GFPsi molecules were used as a negative control for siRNA activity.

1.3 72h after transfection cells were harvested and RNA was extracted from the cells.

1.4 Transfection efficiency was tested by fluorescent microscopy.

2. Sample preparation:

For each transfected well:

2.1 Dilute 3ul lipofectamine 2000 reagent in 250ul serum free medium, and incubate for 5min at RT.

2.2 Dilute siRNA molecules as mentioned below:

2.3 Oligos:

- PTEN Cy3 stock $1.5x10^6$nM (dilute 1:150 to have final concentration of 10uM with PBS)
- Monomers/tandem stock 100uM (dilute 1:10 to have final concentration of 10uM with PBS)

Oligos	No of wells	Final volume	SiRNA stock 10uM
20nM (1:500)	2	4ml	500ul medium+8ul siRNA
10nM (1:1000)	2	4ml	500ul medium+4ul siRNA
GFPsi 40nM (1:250)	2	4ml	500ul medium+16ul siRNA

2.4 Combine lipofectamine 2000 Reagent with siRNA (1:1 volume), mix gently and incubate at RT for 20min

3. **Transfection**

3.1 At this time replace cell medium with 1.5ml fresh growth medium (containing serum).

3.2 Add lipofectamine/siRNA complex onto cells (500ul per well), and rock the plate back and forth (2ml final volume in each well)

Incubate cells at 37^0C in a CO2 incubator. (medium can be replaced 6 or 24h after transfection).

Structure of siRNA variants used

Variant Ib/2 (SeqO2b/2)

```
Seq02b/2-s5'      agagcgagaugaucuggaarUsrUagagaagaucuacguguua      3'
TGas/2+HNas/23'   ucucgcucuacuagaccuu      ucucuucuagaugcacaau      5'
```

Variant IIa/1 (RNaseH)

Seq02b/3-s 5′ agagcgagaugaucuggaa**AAAAAAAAA**agagaagaucuacguguua 3′
Seq02b/3-as 3′ ucucgcucuacuagaccuu**uuuuuuuuu**ucucuucuagaugcacaau 5′

Variant IIa/2 (RNaseH-control)

Seq02b/3-s 5′ agagcgagaugaucuggaa**AAAAAAAAA**agagaagaucuacguguua 3′
TGas/2+HNas/2 3′ ucucgcucuacuagaccuu ucucuucuagaugcacaau 5′

Variant IIIa/1 (RNase)

Seq02b/4-s 5′ agagcgagaugaucuggaa**CUCUCUCUC**agagaagaucuacguguua 3′
TGas/2+HNas/2 3′ ucucgcucuacuagaccuu ucucuucuagaugcacaau 5′

Variant IIIa/1 (Reductase)

Seq02b/5-s 5′ agagcgagaugaucuggaa**--SS--**agagaagaucuacguguua 3′
TGas/2+HNas/2 3′ ucucgcucuacuagaccuu ucucuucuagaugcacaau 5′

Variant Ib/3

Seq02b/2-s5′ agagcgagaugaucuggaa agagaagaucuacguguua 3′
TGas/2+HNas/2 3′ ucucgcucuacuagaccuu**rUsrU**ucucuucuagaugcacaau 5′

Note that in the above structures linkers are presented in bold, while modified nucleotides are underlined.

Results

The results are presented in Figures 9 and 10 as residual HNOEL or TGASEII expression in HFL-1 cells. Results are an average of 3 independent experiments.

As shown in the Figures:

All tested molecules showed activity. Of the four tested tandem molecules (IIa/1_RNaseH, IIIa/1_RNase, IIIa/1_Reductase and IIa/1_sense gap), variant IIIa/1 reductase showed the highest activity against the target genes examined - approximately, 60% and 80% inhibition in HNOEL expression following transfection of 10nM and 20nM, respectively, and 40% inhibition in TGASEII expression following transfection of 20nM.

Thus, as indicated above for the RNAstar structure, the degree of inhibition varies between each variant / target gene combination. Thus all variants are potentially active against any given gene.

Example 7

Pharmacology and drug delivery

The nucleotide sequences of the present invention can be delivered either directly or with viral or non-viral vectors. When delivered directly the sequences are generally rendered nuclease resistant. Alternatively the sequences can be incorporated into expression cassettes or constructs such that the sequence is expressed in the cell as discussed herein below. Generally the construct contains the proper regulatory sequence or promoter to allow the sequence to be expressed in the targeted cell.

The compounds or pharmaceutical compositions of the present invention are administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the disease to be treated, the site and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners.

The pharmaceutically "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve improvement including but not limited to improved survival rate or more rapid recovery, or improvement or elimination of symptoms and other indicators as are selected as appropriate measures by those skilled in the art.

The treatment generally has a length proportional to the length of the disease process and drug effectiveness and the patient species being treated. It is noted that humans are treated generally longer than the mice or other experimental animals exemplified herein.

The compounds of the present invention can be administered by any of the conventional routes of administration. It should be noted that the compound can be administered as the compound or as pharmaceutically acceptable salt and can be administered alone or as an active ingredient in combination with pharmaceutically acceptable carriers, solvents, diluents, excipients, adjuvants

and vehicles. The compounds can be administered orally, subcutaneously or parenterally including intravenous, intraarterial, intramuscular, intraperitoneally, and intranasal administration as well as intrathecal and infusion techniques. Implants of the compounds are also useful. Liquid forms may be prepared for injection, the term including subcutaneous, transdermal, intravenous, intramuscular, intrathecal, and other parental routes of administration. The liquid compositions include aqueous solutions, with and without organic cosolvents, aqueous or oil suspensions, emulsions with edible oils, as well as similar pharmaceutical vehicles. In addition, under certain circumstances the compositions for use in the novel treatments of the present invention may be formed as aerosols, for intranasal and like administration. The patient being treated is a warm-blooded animal and, in particular, mammals including man. The pharmaceutically acceptable carriers, solvents, diluents, excipients, adjuvants and vehicles as well as implant carriers generally refer to inert, non-toxic solid or liquid fillers, diluents or encapsulating material not reacting with the active ingredients of the invention.

When administering the compound of the present invention parenterally, it is generally formulated in a unit dosage injectable form (solution, suspension, emulsion). The pharmaceutical formulations suitable for injection include sterile aqueous solutions or dispersions and sterile powders for reconstitution into sterile injectable solutions or dispersions. The carrier can be a solvent or dispersing medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, and an oil, especially a vegetable oil and a lipid and suitable mixtures thereof.

Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Nonaqueous vehicles such a cottonseed oil, sesame oil, olive oil, soybean oil, corn oil, sunflower oil, or peanut oil and esters, such as isopropyl myristate, can also be used as solvent systems for compound compositions. Additionally, various additives which enhance the stability, sterility, and isotonicity of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example,

parabens, chlorobutanol, phenol, sorbic acid, and the like. In many cases, it is desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the present invention, however, any vehicle, diluent, or additive used has to be compatible with the compounds.

Sterile injectable solutions can be prepared by incorporating the compounds utilized in practicing the present invention in the required amount of the appropriate solvent with several of the other ingredients, as desired.

A pharmacological formulation of the present invention can be administered to the patient in an injectable formulation containing any compatible carrier, such as various vehicle, adjuvants, additives, and diluents; or the compounds utilized in the present invention can be administered parenterally to the patient in the form of slow-release subcutaneous implants or targeted delivery systems such as monoclonal antibodies, vectored delivery, iontophoretic, polymer matrices, liposomes, and microspheres. Examples of delivery systems useful in the present invention include U. S. Patent Nos. 5,225,182; 5,169,383; 5,167,616; 4,959,217; 4,925,678; 4,487,603; 4,486,194; 4,447,233; 4,447,224; 4,439,196; and 4,475,196. Many other such implants, delivery systems, and modules are well known to those skilled in the art.

A pharmacological formulation of the compound utilized in the present invention can be administered orally to the patient. Conventional methods such as administering the compound in tablets, suspensions, solutions, emulsions, capsules, powders, syrups and the like are usable. Known techniques which deliver it orally or intravenously and retain the biological activity are preferred. In one embodiment, the compound of the present invention can be administered initially by intravenous injection to bring blood levels to a suitable level. The patient's levels are then maintained by an oral dosage form, although other forms of administration, dependent upon the patient's condition and as indicated above, can be used.

In general, the active dose of compound for humans is in the range of from 1ng/kg to about 20-100 mg/kg body weight per day, preferably about 0.01 mg to about 2-10 mg/kg body weight per

day, in a regimen of one dose per day or twice or three or more times per day for a period of 1-2 weeks or longer, preferably for 24-to 48 hrs or by continuous infusion during a period of 1-2 weeks or longer.

Administration of compounds of the present invention to the eye

The compounds of the present invention can be administered to the eye topically or in the form of an injection, such as an intravitreal injection, a sub-retinal injection or a bilateral injection. Further information on administration of the compounds of the present invention can be found in Tolentino et al., *Retina* 24 (2004) 132-138; Reich et al., *Molecular vision* 9 (2003) 210-216.

Pulmonary administration of compounds of the present invention

The therapeutic compositions of the present invention are preferably administered into the lung by inhalation of an aerosol containing such composition / compound, or by intranasal or intratracheal instillation of said compositions. Formulating the compositions in liposomes may benefit absorption. Additionally, the compositions may include a PFC liquid such as perflubron, and the compositions may be formulated as a complex of the compounds of the invention with polyethylemeimine (PEI).

For further information on pulmonary delivery of pharmaceutical compositions see Weiss et al., *Human gene therapy* 10:2287-2293 (1999); Densmore et al., *Molecular therapy* 1:180-188 (1999); Gautam et al., *Molecular therapy* 3:551-556 (2001); and Shahiwala & Misra, *AAPS PharmSciTech* 5 (2004). Additionally, respiratory formulations for siRNA are described in U.S. patent application No. 2004/0063654 of Davis et el.

Administration of compounds of the present invention to the ear

A preferred administration mode is directly to the affected portion of the ear or vestibule, topically as by implant for example, and, preferably to the affected hair cells or their supporting cells, so as to direct the active molecules to the source and minimize its side effects. A preferred administration mode is a topical delivery of the inhibitor(s) onto the round window membrane of the cochlea. Such a method of administration of other compounds is disclosed for example in Tanaka et al. (Hear Res. 2003 Mar;177(1-2):21-31).

Additional modes of administration to the ear are by administration of liquid drops to the ear canal, delivery to the scala tympani chamber of the inner ear by transtympanic injection, or provision as a diffusible member of a cochlear hearing implant.

In the treatment of pressure sores or other wounds, the administration of the pharmaceutical composition is preferably by topical application to the damaged area, but the compositions may also be administered systemically.

Additional formulations for improved delivery of the compounds of the present invention can include non-formulated compounds, compounds covalently bound to cholesterol, and compounds bound to targeting antibodies (Song et al., Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors, Nat Biotechnol. 2005 Jun;23(6):709-17).

CLAIMS

1. An oligonucleotide comprising consecutive nucleotides wherein a first segment of such nucleotides encodes a first inhibitory RNA molecule, a second segment of such nucleotides encodes a second inhibitory RNA molecule, and a third segment of such nucleotides encodes a third inhibitory RNA molecule.

2. The oligonucleotide of claim 1 wherein each of the first, the second and the third segment comprises one strand of a double stranded RNA and the first, second and third segments are joined together by a linker.

3. The oligonucleotide of claim 1 having the general structure:

5′	oligo1 (sense)	**LINKER A**	Oligo2 (sense)	3′
3′	oligo1 (antisense)	**LINKER B**	Oligo3 (sense)	5′
3′	oligo3 (antisense)	**LINKER C**	oligo2 (antisense)	5′

wherein one or more of linker A, linker B, or linker C is present.

4. The oligonucleotide of claim 1 having the general structure:

5′	oligo1 (sense)	**LINKER A**	Oligo2 (antisense)	3′
3′	oligo1 (antisense)	**LINKER B**	Oligo3 (sense)	5′
3′	oligo3 (antisense)	**LINKER C**	oligo2 (sense)	5′

wherein one or more of linker A, linker B, or linker C is present.

5. The oligonucleotide of claim 1 having the general structure:

5′	oligo1 (sense)	**LINKER A**	oligo3 (antisense)	3′
3′	oligo1 (antisense)	**LINKER B**	oligo2 (sense)	5′
5′	oligo3 (sense)	**LINKER C**	oligo2 (antisense)	3′

wherein one or more of linker A, linker B, or linker C is present.

6. The oligonucleotide of any one of claims 3-5 wherein each of linker A, linker B and linker C is present and each linker is identical to each other.

7. The oligonucleotide of claim 6 wherein each linker is a hexaethylenglycol linker.

8. The oligonucleotide of claim 6 wherein each linker is a nucleotide linker.

9. An oligonucleotide comprising consecutive nucleotides wherein a first segment of such nucleotides encodes a first inhibitory RNA molecule and a second segment of such nucleotides encodes a second inhibitory RNA molecule.

10. The oligonucleotide of claim 9 wherein each of the first and the second segment comprise one strand of a double stranded RNA and the first and second segments are joined together by a single stranded RNA linker.

11. The oligonucleotide of claim 9 wherein each of the first and the second segment comprise one strand of a double stranded RNA and the first and second segments are joined together by a single stranded DNA linker.

12. The oligonucleotide of claim 9 wherein each of the first and the second segment comprise one strand of a double stranded RNA and the first and second segments are joined together by a linker which comprises a disulfide bond.

13. The oligonucleotide of claim 9 wherein each of the first and the second segment comprise one strand of a double stranded RNA and the first and second segments are joined together by a linker which comprises a peptide bond.

14. The oligonucleotide of claim 9 wherein each of the first and the second segment comprise one strand of a double stranded RNA and the first and second segments are joined together by a double stranded RNA linker.

15. The oligonucleotide of claim 9 wherein each of the first and the second segment comprise one strand of a double stranded RNA and the first and second segments are joined together by a double stranded DNA linker.

16. The oligonucleotide of claim 9 wherein each of the first and the second segment comprise one strand of a double stranded RNA and the first and second segments are joined together by a linker which comprises a partially single stranded and partially double stranded RNA.

17. The oligonucleotide of claim 9 wherein each of the first and the second segment comprise one strand of a double stranded RNA and the first and second segments are joined together by a linker which comprises a partially single stranded and partially double stranded DNA.

18. The oligonucleotide of any one of claims 9-17 further wherein the oligonucleotide comprises modification at the 2' position of one or more sugars.

19. The oligonucleotide of claim 18 where the 2' modifications are 2'Omethyl and/or 2'fluoro substitutions.

20. The oligonucleotide of claim 18 where the 2' modifications are on alternating nucleotides.

21. The oligonucleotide of claim 9 having the general structure:

```
5'  oligo1 (sense)      LINKER A  oligo2 (sense)      3'
3'  oligo1 (antisense)  LINKER B  oligo2 (antisense)  5'
```

wherein either linker A or linker B is present or both linkers A and B are present.

22. The oligonucleotide of claim 9 having the general structure:

```
5'  oligo1 (antisense)  LINKER A  oligo2 (antisense)    3'
3'  oligo1 (sense)      LINKER B  oligo2 (sense)        5'
```

wherein either linker A or linker B is present or both linkers A and B are present.

23. The oligonucleotide of claim 9 having the general structure:

```
5'  oligo1 (sense)      LINKER A  oligo1 (antisense)    3'
3'  oligo2 (antisense)  LINKER B  oligo2 (sense)        5'
```

wherein either linker A or linker B is present or both linkers A and B are present.

24. The oligonucleotide of claim 9 having the general structure:

```
5'  oligo1 (antisense)  LINKER A  oligo1 (sense)        3'
3'  oligo2 (sense)      LINKER B  oligo2 (antisense)    5'
```

wherein either linker A or linker B is present or both linkers A and B are present.

25. The oligonucleotide of claim 9 having the general structure:

```
5'  oligo1 (sense)      LINKER A  Oligo2 (antisense)    3'
3'  oligo2 (sense)      LINKER B  Oligo1 (antisense)    5'
```

wherein either linker A or linker B is present or both linkers A and B are present.

26. The oligonucleotide of claim 9 having the general structure:

```
5'  oligo1 (antisense)  LINKER A  Oligo2 (sense)        3'
3'  oligo2 (antisense)  LINKER B  Oligo1 (sense)        5'
```

wherein either linker A or linker B is present or both linkers A and B are present.

27. The oligonucleotide of claim 21 having the structure:

```
5'  oligo1 (sense)       dTsdTuu       oligo2 (sense)       3'
3'  oligo1 (antisense)   (gap) dTsdT   oligo2 (antisense)   5'
```

28. The oligonucleotide of claim 21 having the structure:

```
5'  oligo1 (sense)       rUsrU     oligo2 (sense)       3'
3'  oligo1 (antisense)   (gap)     oligo2 (antisense)   5'
```

29. The oligonucleotide of claim 21 having the structure:

```
5'  oligo1 (sense)       dTsdTaa   oligo2 (sense)       3'
3'  oligo1 (antisense)   aadTsdT   oligo2 (antisense)   5'
```

30. The oligonucleotide of claim 21 having the structure:

```
5'  oligo1 (sense)       dTsdT     oligo2 (sense)       3'
3'  oligo1 (antisense)   dTsdT     oligo2 (antisense)   5'
```

31. The oligonucleotide of claim 21 having the structure:

```
5'  oligo1 (sense)       dTsdTuu   oligo2 (sense)       3'
3'  oligo1 (antisense)   uudTsdT   oligo2 (antisense)   5'
```

32. The oligonucleotide of claim 21 having the structure:

```
5'  oligo1 (sense)       Xn      oligo2 (sense)       3'
3'  oligo1 (antisense)   (gap)   oligo2 (antisense)   5'
```

wherein X_n = a single stranded poly RNA linker wherein n is an integer from 2-50 inclusive.

33. The oligonucleotide of claim 21 having the structure:

```
5'  oligo1 (sense)       Yn      oligo2 (sense)       3'
3'  oligo1 (antisense)   (gap)   oligo2 (antisense)   5'
```

wherein the linker Y_n = a single stranded polyDNA linker wherein n is an integer from 2-50 inclusive.

34. The oligonucleotide of claim 21 having the structure:

```
5'  oligo1 (sense)       --ss--     Oligo2 (sense)       3'
3'  oligo1 (antisense)   (gap)      Oligo2 (antisense)   5'
```

wherein the linker designated --ss-- is a di-sulfide linker.

35. The oligonucleotide of claim 21 having the structure:

```
5'  oligo1 (sense)       1-10 a.a.  Oligo2 (sense)       3'
3'  oligo1 (antisense)   (gap)      Oligo2 (antisense)   5'
```

wherein the linker designated 1-10 a.a. is an amino acid linker.

36. A vector comprising the oligonucleotide of any one of claims 1-35.

37. A vector which upon transcription gives rise to the oligonucleotide of any one of claims 1-35.

38. A pharmaceutical composition comprising the oligonucleotide of any one of claims 1-35 or the vector of either claim 36 or claim 37 and a pharmaceutically acceptable carrier.

Figure 1 (a)

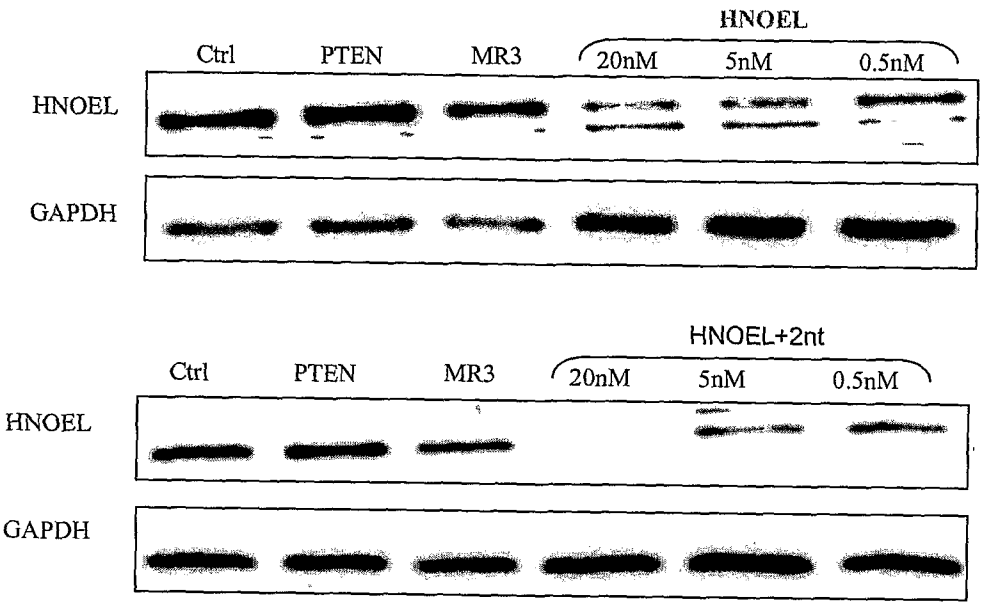


Figure 1 (b)

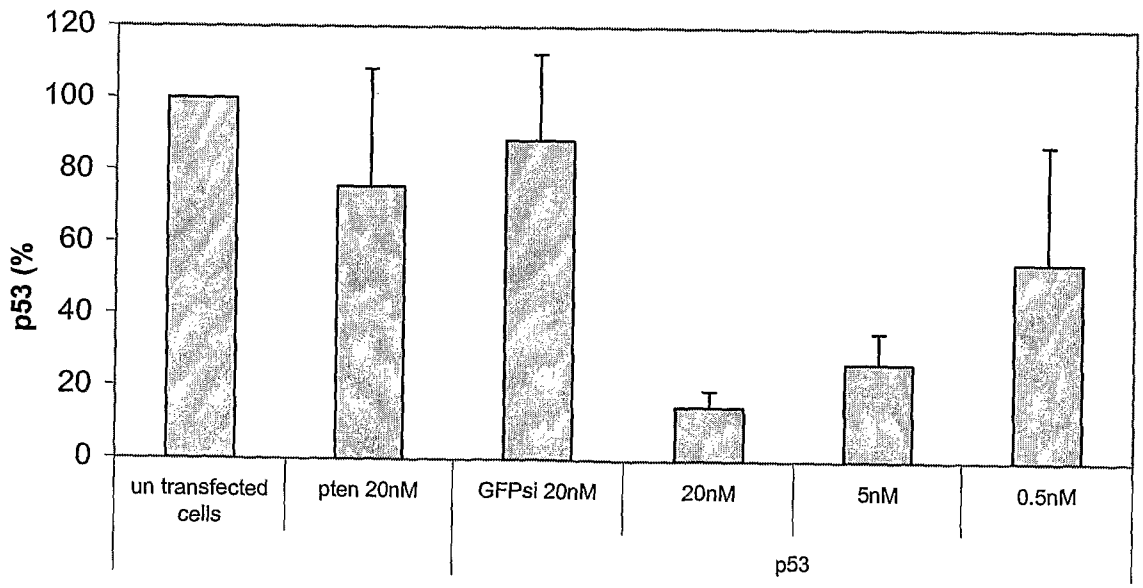


Figure 1 (c)

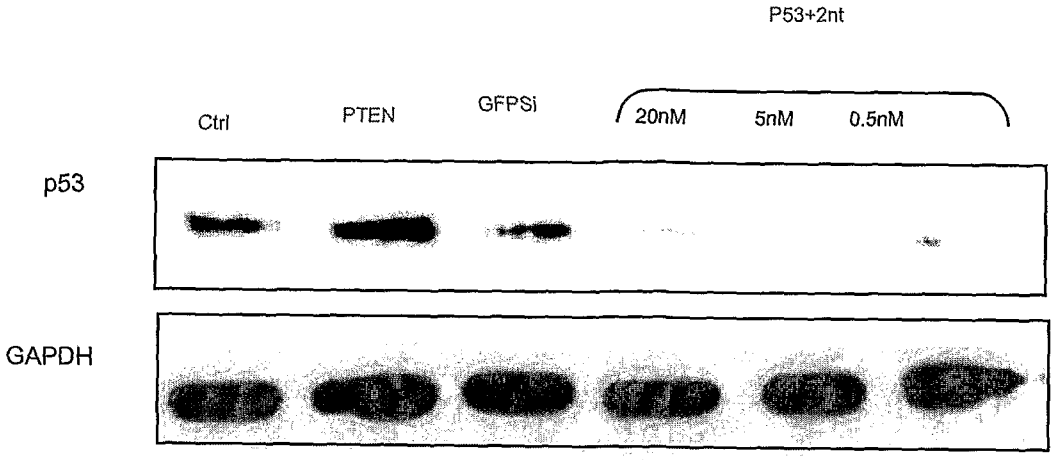


Figure 2

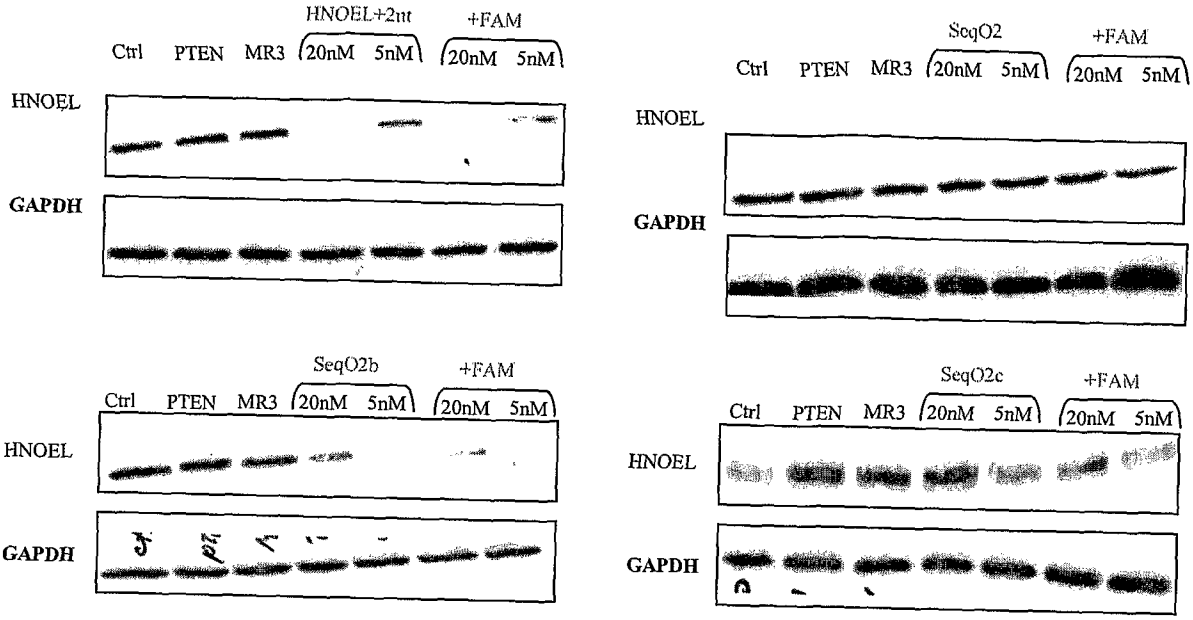

HNOEL+2nt
+FAM
Ctrl
PTEN
MR3
20nM
5nM
20nM
5nM
HNOEL
GAPDH
SeqO2
+FAM
Ctrl
PTEN
MR3
20nM
5nM
20nM
5nM
HNOEL
GAPDH
SeqO2b
+FAM
Ctrl
PTEN
MR3
20nM
5nM
20nM
5nM
HNOEL
GAPDH
SeqO2c
+FAM
Ctrl
PTEN
MR3
20nM
5nM
20nM
5nM
HNOEL
GAPDH

Figure 3

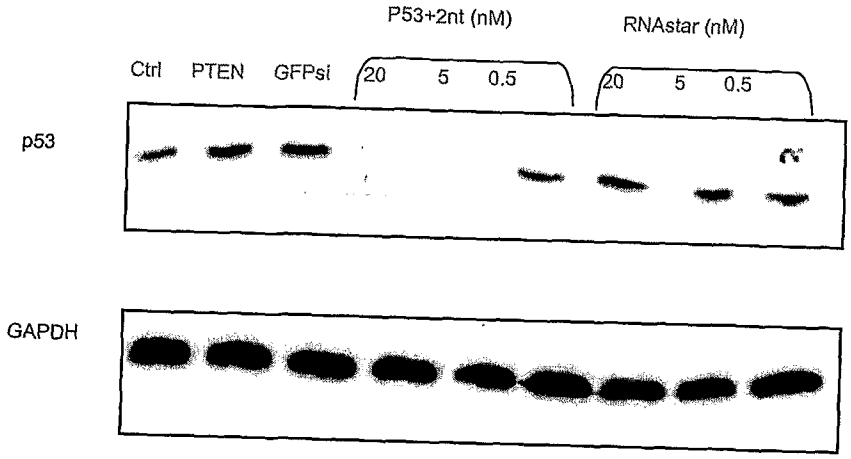


Figure 4 (a)

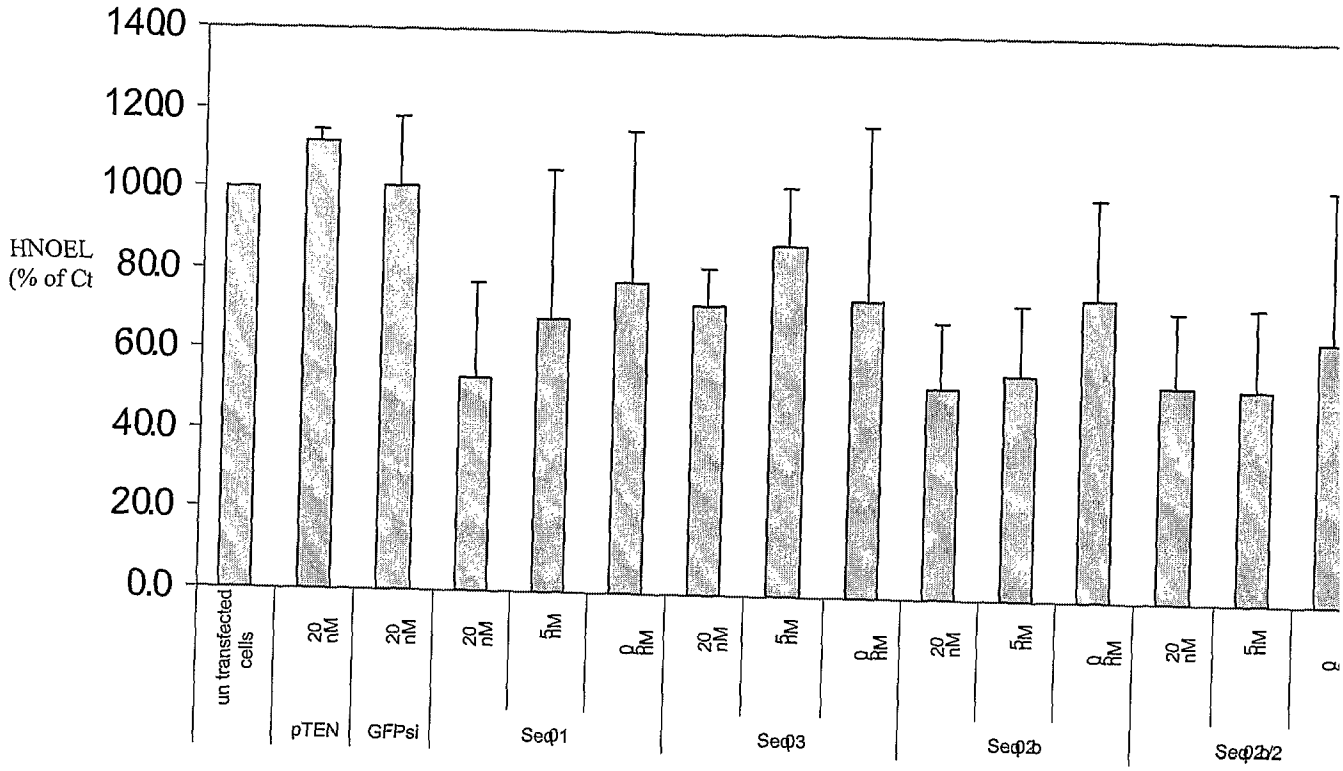

1400
1200
1000
800
600
400
200
0.0
HNOEL
(% of Ct
un transfected cells
20 nM
pTEN
20 nM
GFPsi
20 nM
5 nM
Seφ1
20 nM
5 nM
Seφ3
20 nM
5 nM
Seφ2b
20 nM
5 nM
Seφ2b/2

Figure 4 (b)

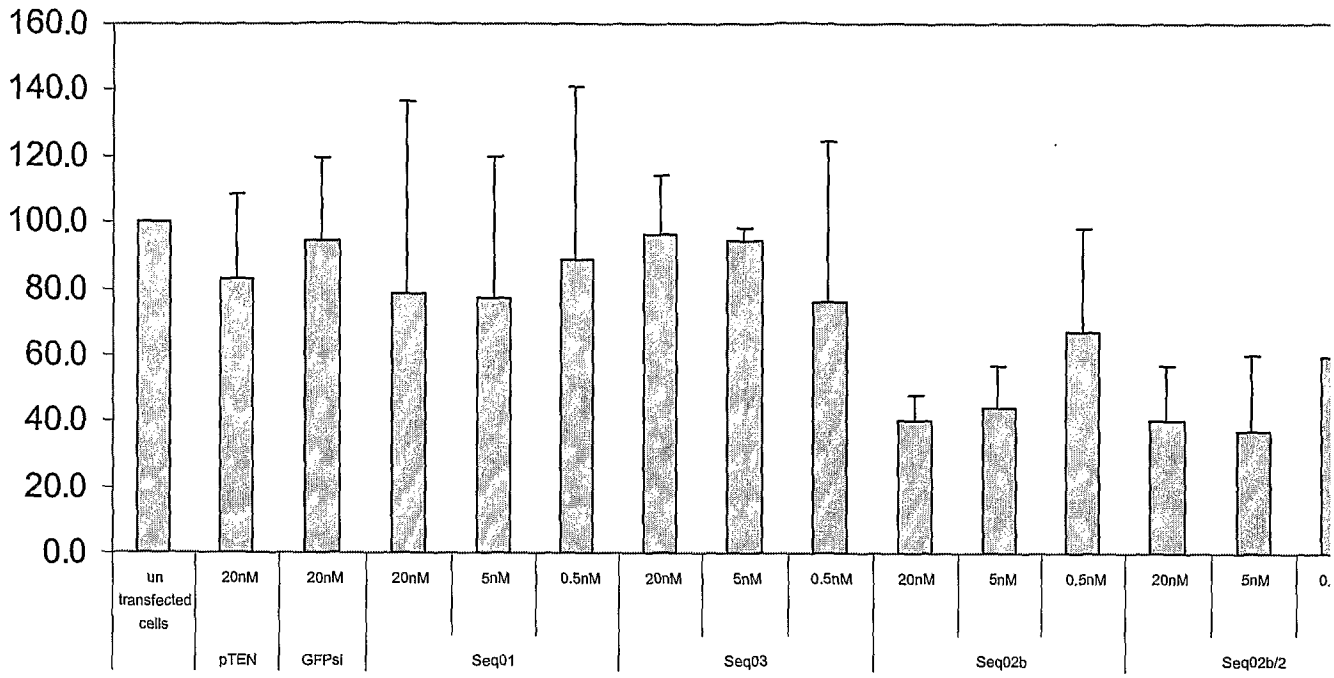

160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
un transfected cells
20nM
pTEN
20nM
GFPsi
20nM
5nM
0.5nM
Seq01
20nM
5nM
0.5nM
Seq03
20nM
5nM
0.5nM
Seq02b
20nM
5nM
Seq02b/2

Figure 5

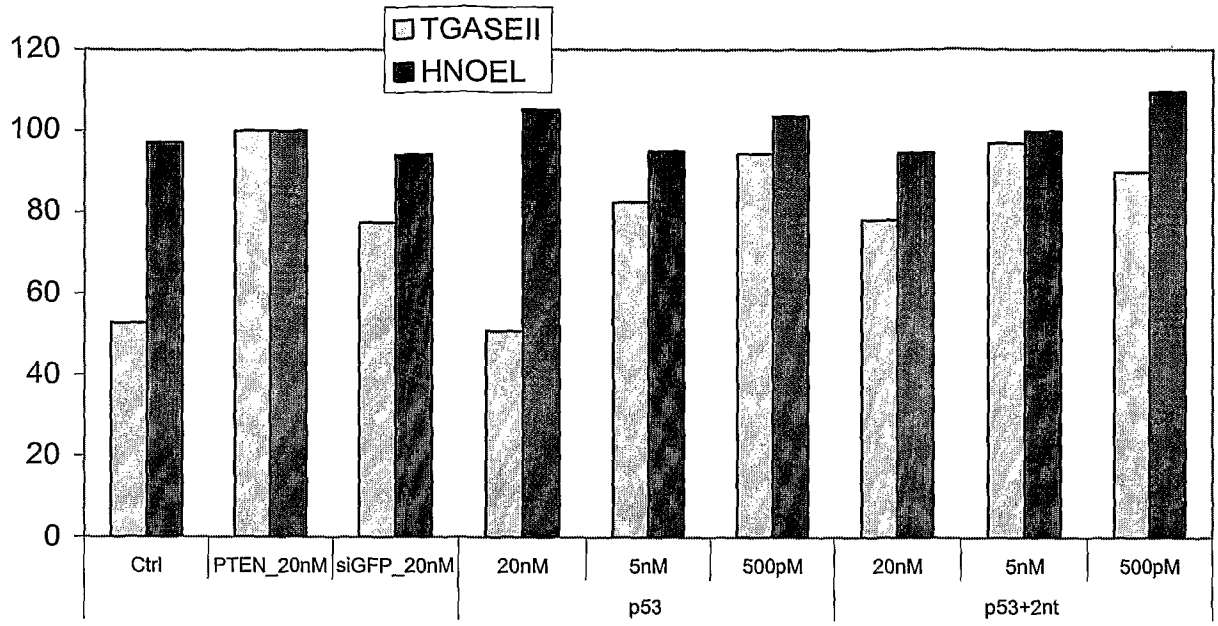

TGASEII
HNOEL
120
100
80
60
40
20
0
Ctrl
PTEN_20nM
siGFP_20nM
20nM
5nM
500pM
20nM
5nM
500pM
p53
p53+2nt

Figure 6 (a) **Gapped RNAstar Variants**

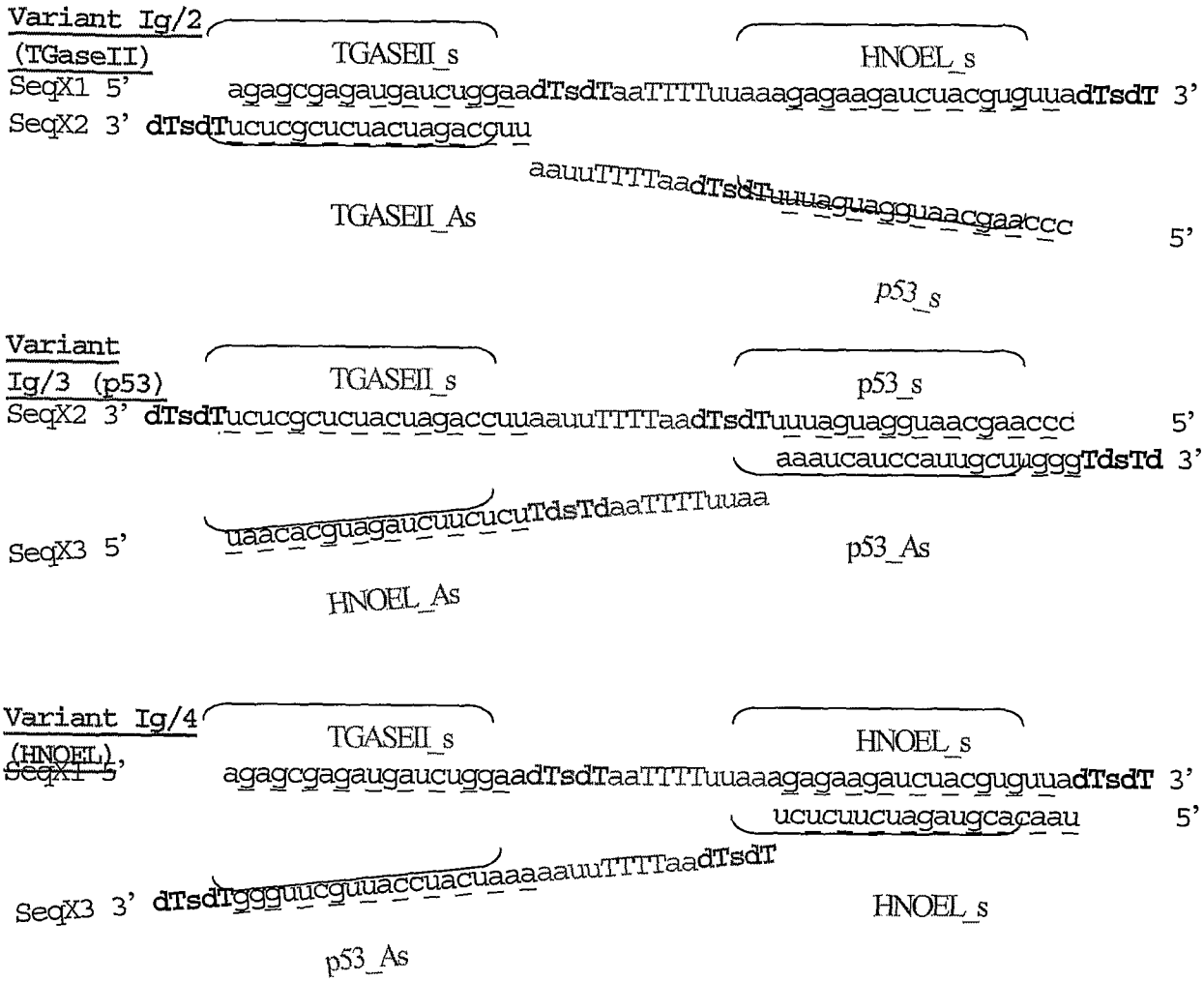


Figure 6 (b)

Variant Ig/5

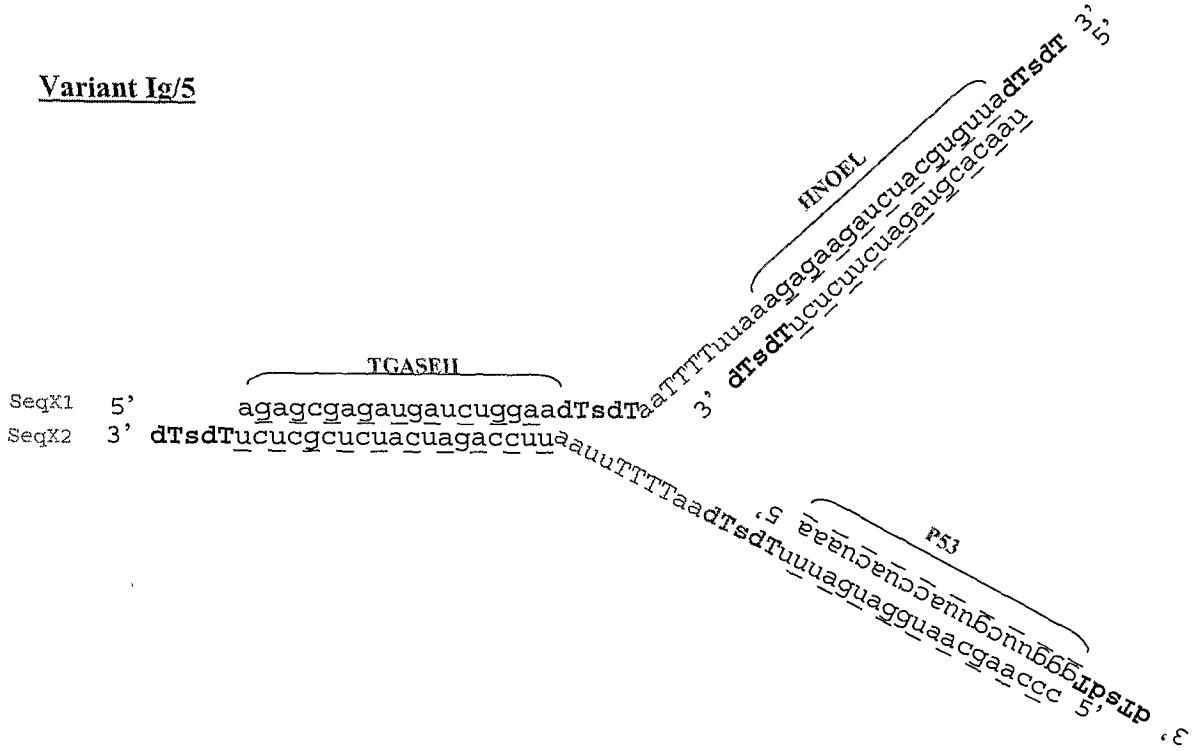


Figure 6 (c)

Variant Ig/6

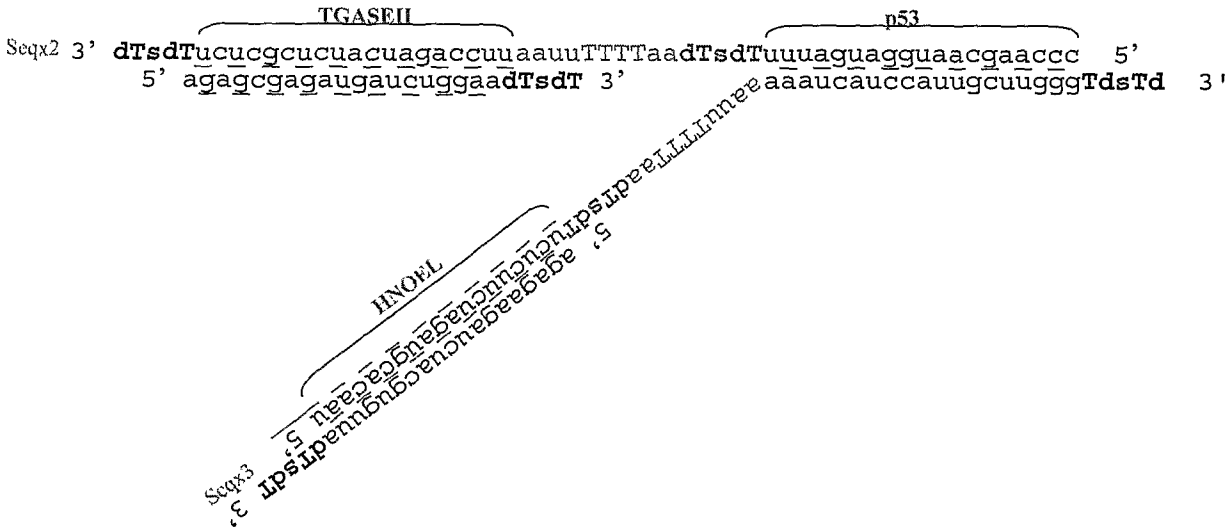


Figure 6 (d)

Variant Ig/7

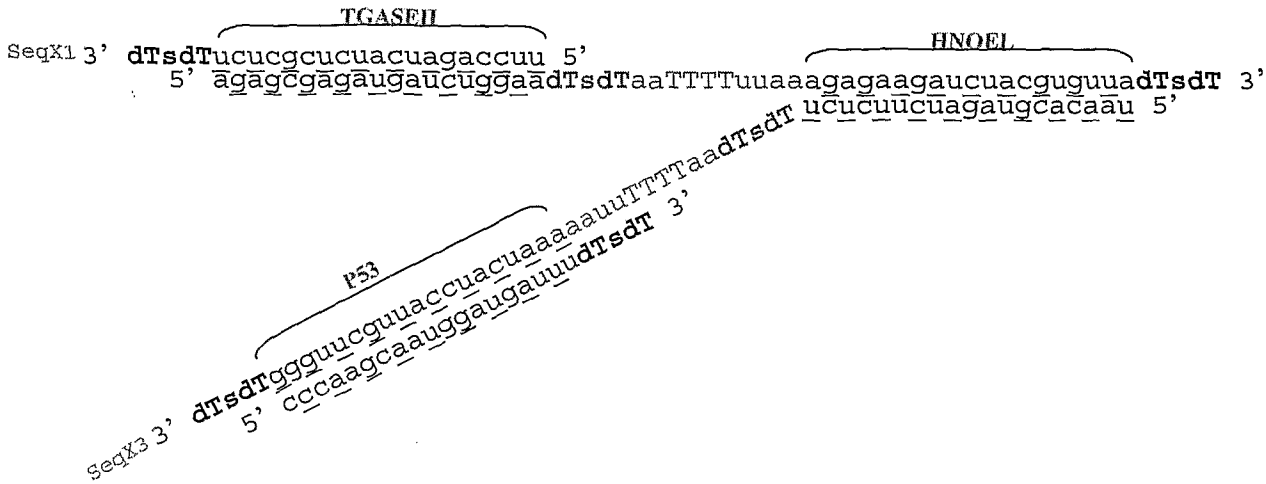
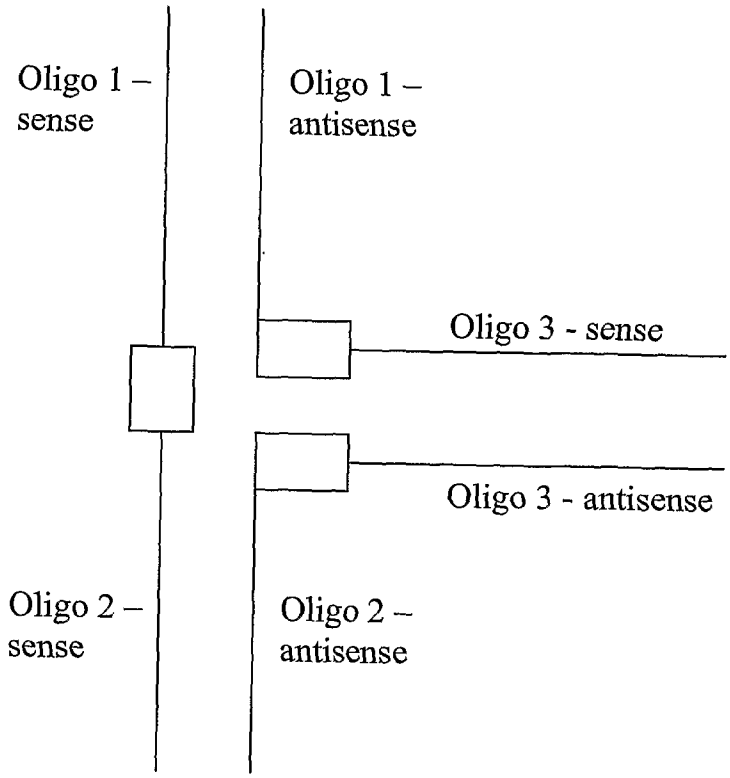


Figure 7
Exemplary structure of RNAstar



A = linker A;
B = linker B;
C = linker C.

Figure 8 (a)

A) Western blot analysis of HNOEL expression in 293 cells expressing exogenous human HNOEL cDNA, 72h following gapped RNAstar transfection

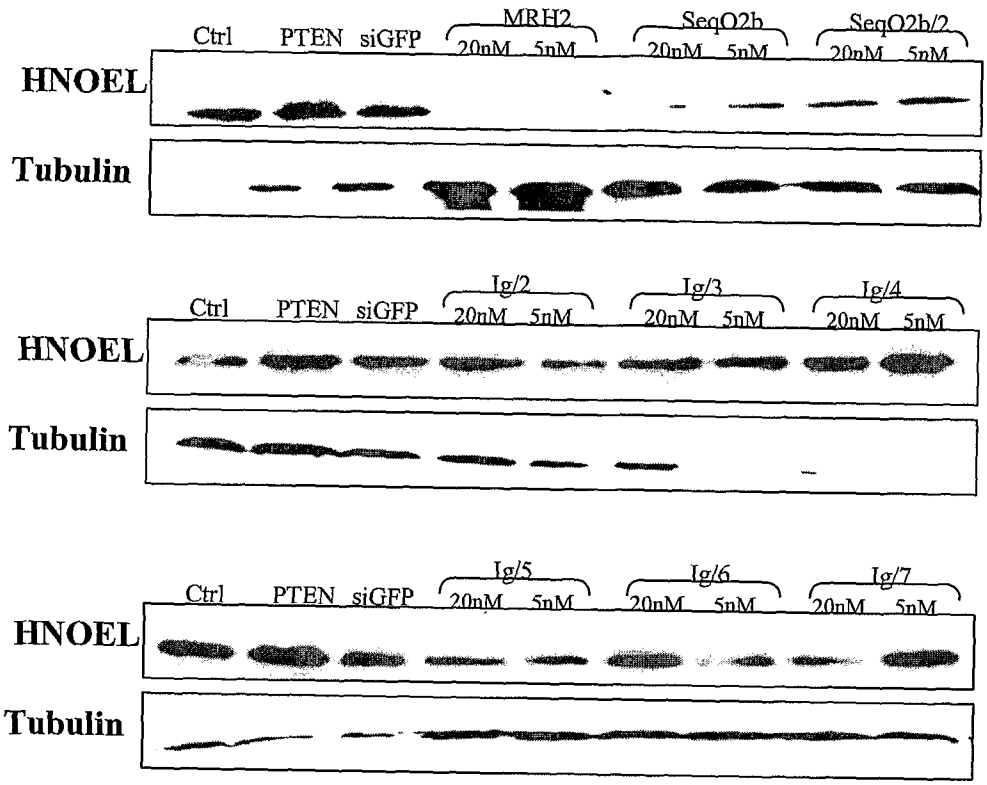


Figure 8 (b)

B) Western blot analysis of TGASEII expression in NRK49 cells expressing exogenous rat TGASEII cDNA, 72h following gapped RNAstar transfection

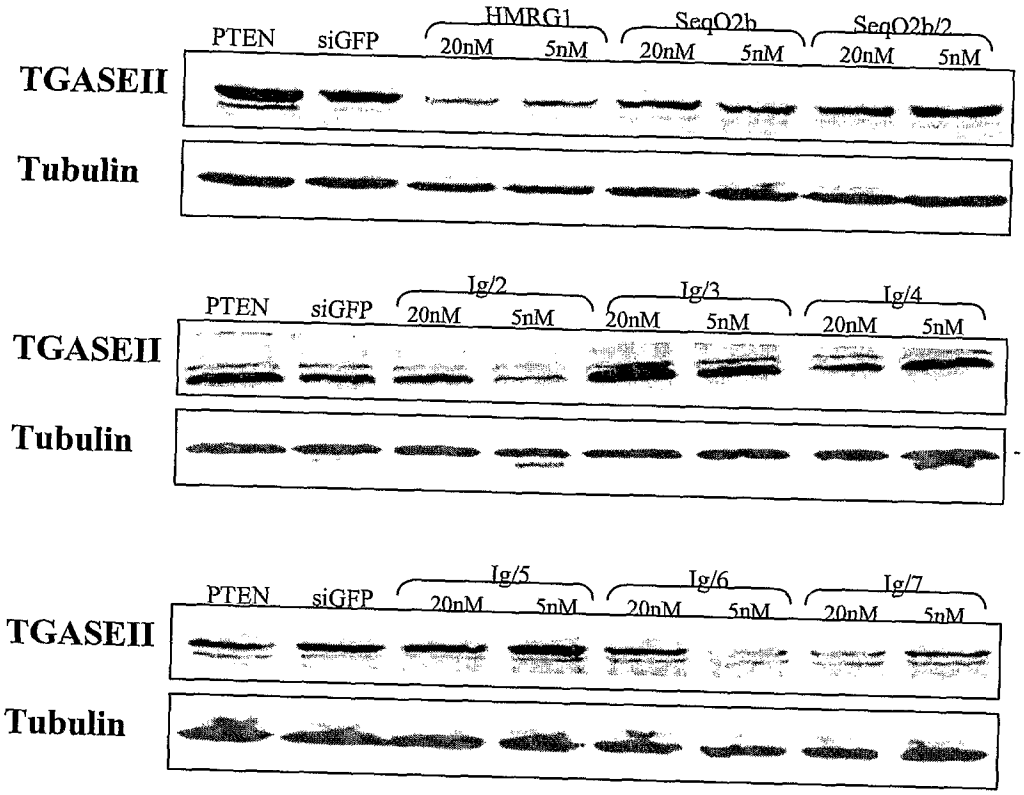


Figure 8 (c)

C) Western blot analysis of p53 expression in 5Fu treated HCT116 cells, 72h following gapped RNAstar transfection

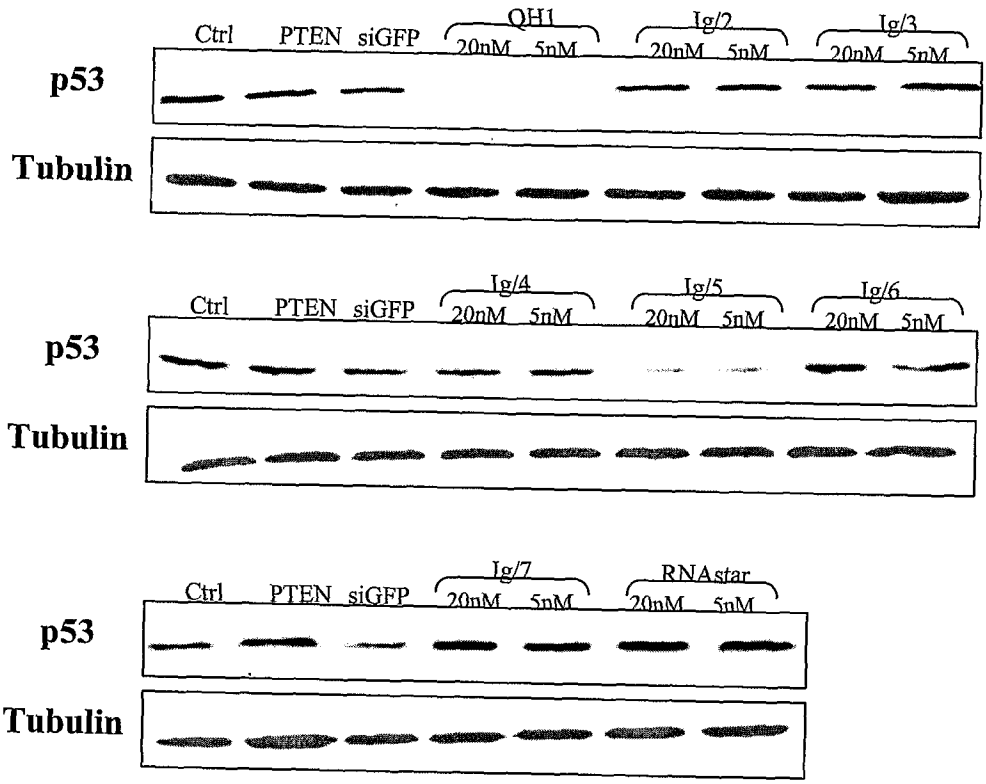


Figure 9

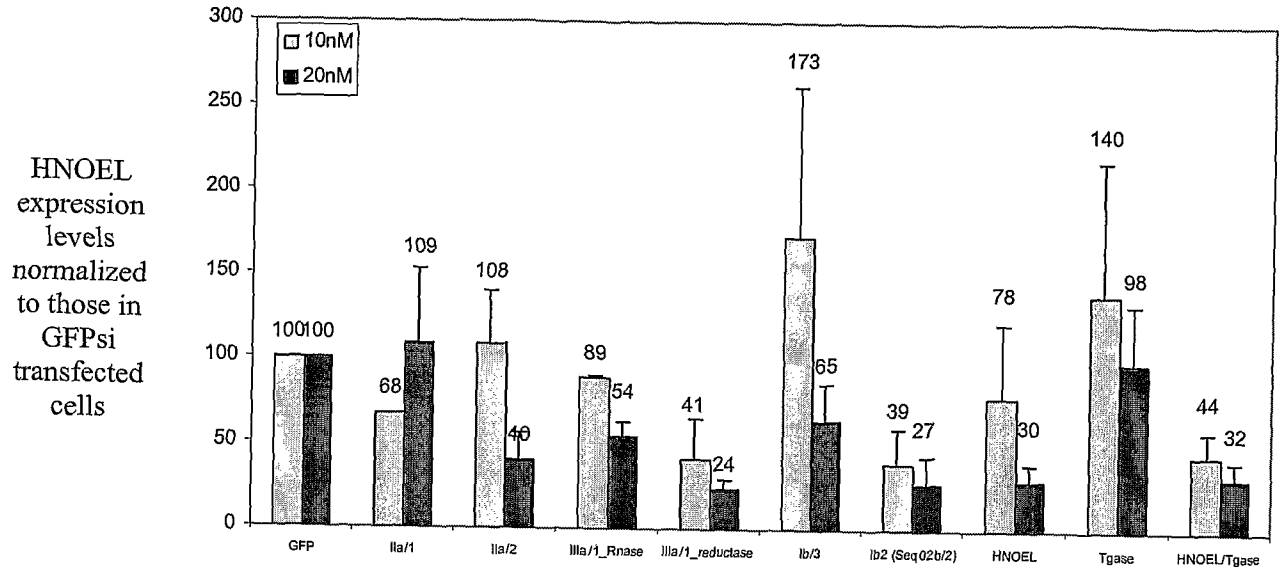
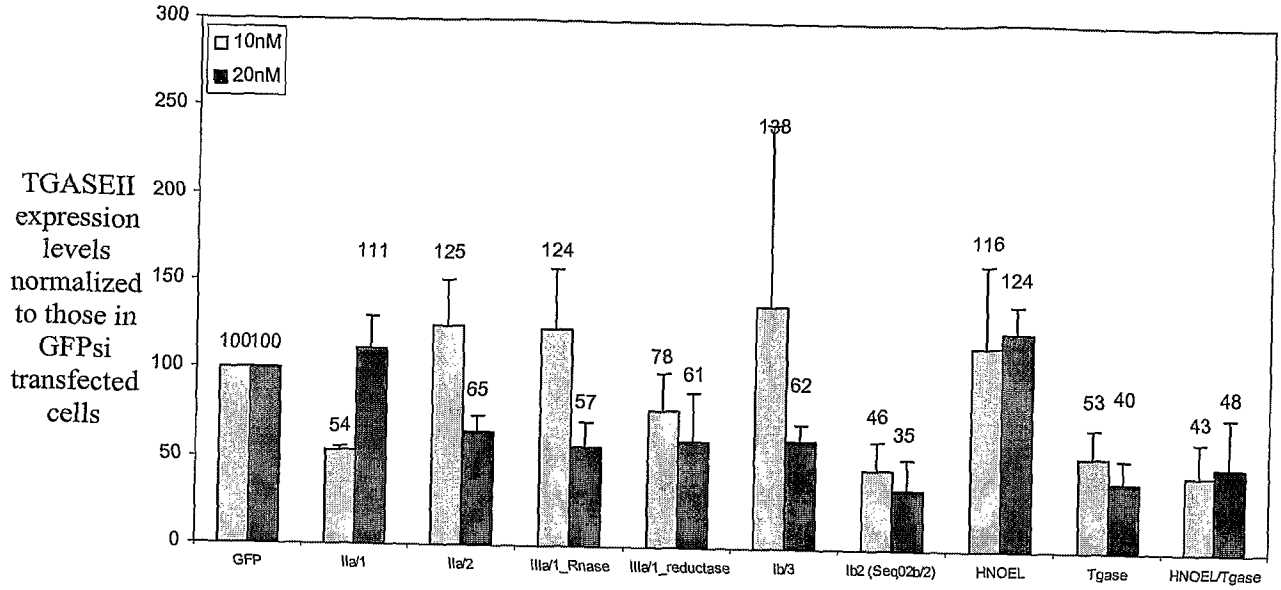

HNOEL expression levels normalized to those in GFPsi transfected cells
10nM
20nM
300
250
200
150
100
50
0
100
100
68
109
108
40
89
54
41
24
173
65
39
27
78
30
140
98
44
32
GFP
IIa/1
IIa/2
IIIa/1_Rnase
IIIa/1_reductase
Ib/3
Ib2 (Seq02b/2)
HNOEL
Tgase
HNOEL/Tgase

Figure 10


TGASEII expression levels normalized to those in GFPsi transfected cells
10nM
20nM
300
250
200
150
100
50
0
100100
54
111
125
65
124
57
78
61
138
62
46
35
116
124
53
40
43
48
GFP
IIa/1
IIa/2
IIIa/1_Rnase
IIIa/1_reductase
Ib/3
Ib2 (Seq02b/2)
HNOEL
Tgase
HNOEL/Tgase