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(54) Title: MULTITARGETING INTERFERING RNAS AND METHODS OF THEIR USE AND DESIGN

(57) Abstract: Interfering RNA molecules are now designed and produced with specificity for multiple binding sequences present in distinct genetic contexts in one or more pre-selected target RNA molecules and are used to modulate expression of the target sequences. Such a multitargeting interfering RNA approach provides a powerful tool for gene regulation.

MULTITARGETING INTERFERING RNAS AND METHODS OF THEIR USE AND DESIGN

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of US Provisional Patent Application Nos. 60/738,441 filed November 21, 2005 and 60/738,640 filed November 21, 2005, respectively, which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The present invention concerns methods and reagents useful in modulating gene expression. Particularly, the invention relates to modulating gene expression using a multitargeting interfering RNA molecule that targets multiple target sites on one or more pre-selected RNA molecules.

BACKGROUND OF THE INVENTION

RNA interference (RNAi) is a diverse, evolutionarily conserved mechanism in eukaryotic cells, which inhibits the transcription and translation of target genes in a sequence-specific manner. It is now known that single and double-stranded RNA can modulate expression of or modify processing of target RNA molecules by a number of mechanisms. Some such mechanisms tolerate variation in the amount of sequence complementarity required between the modulatory (or interfering) RNA and the target RNA. Certain microRNAs can translationally repress target mRNA having as little as 6 nucleotides of complementarity with the microRNA. The development of RNA interfering agents, for example, using double-stranded RNA to repress expression of disease-related genes is currently an area of intense research activity.

Double-stranded RNA of 19-23 bases in length is recognized by an RNA interference silencing complex (RISC) into which an effector strand (or "guide strand") of the RNA is loaded. This guide strand acts as a template for the recognition and destruction of highly complementary sequences present in the transcriptome. Alternatively, through the recognition and binding of RNA sequences of lower complementarity, interfering RNAs may induce translational repression without mRNA degradation. Such translational repression appears to be a mechanism of action

of endogenous microRNAs, a group of short non-coding RNAs involved in differentiation and development.

Efforts at implementing interfering RNAs therapeutically thus far have focused on producing specific double stranded RNAs, each with complete complementarity to a particular target transcript. Such double-stranded RNAs (dsRNAs) are potentially effective where a single suitable target can be identified, however, dsRNAs, particularly those designed against one target, may have at least two categories of off-target side effects that need to be avoided or minimized. Undesirable side effects can arise through the triggering of innate immune response pathways (e.g. Toll-like Receptor 3, 7, and 8, and the so-called interferon response) and through inadvertent inhibition of protein expression from related or unrelated transcripts (either by RNA degradation, translational repression or other mechanisms). Some bioinformatic and/or experimental approaches have been developed to try to minimize off-target effects. Algorithms for *in silico* hybridization are known, and others have been developed for predicting target accessibility and loading bias in an effort to eliminate or minimize side-effects while maintaining effectiveness.

Several double-stranded RNA molecules for potentially treating human diseases of viral and nonviral origin are in various stages of development. The diseases include Age-related Macular Degeneration, Amyotrophic Lateral Sclerosis (ALS), and Respiratory Syncytial Virus (RSV) infection. These RNA molecules, however, are designed to target only a single site in an RNA sequence. Although RNA interference may be useful and potent in obtaining knock-down of specific gene products, many diseases involve complex interactions between ontologically-unrelated gene products. Multiple putative targets can be identified for a single disease. Attempts to confirm that inhibiting these targets one by one is therapeutically valuable have been disappointing. Indeed, obtaining therapeutic effectiveness is proving to be extremely challenging, probably because of multiple levels of redundancy in most signaling pathways. For example, many disorders, such as cancer, type 2 diabetes, and atherosclerosis, feature multiple biochemical abnormalities. In addition, some putative targets may be subject to enhanced mutation rates, thereby negating the effects of interfering RNAs on any such target.

For example, therapeutic approaches to viral infections continue to be major challenges in agriculture, as well as in animal and human health. The nature of the replication of viruses makes them highly plastic, "moving targets" therapeutically – capable of altering structure,

infectivity, and host profile. The recent emergence of viruses such as Severe Acute Respiratory Syndrome ("SARS") and Avian Influenza Virus ("bird flu") exemplify these challenges. Even well-described viruses such as those involved in Acquired Immunodeficiency Syndrome or AIDS (e.g. Human Immunodeficiency Viruses, HIV-1 and HIV-2), continue to defy efforts at treatment and vaccination because of on-going viral mutation and evolution.

Furthermore, although nucleic acid therapeutics such as interfering RNAs are candidates for viral therapy, in part because modern rapid gene sequencing techniques allow viral genome sequences to be determined even before any encoded functions can be assessed, the error-prone replication of viruses, particularly RNA viruses, means that substantial genomic diversity can arise rapidly in an infected population. Thus far, strategies for the development of nucleic acid therapeutics have largely centered on the targeting of highly-conserved regions of the viral genome. It is unclear whether these constructs are efficient at treating viral infection or preventing emergence of resistant viral clones.

Therapeutic approaches that involve the design and use of one interfering RNA for control of several key "drivers" of the disease are thus desirable. Therefore, there is a need for interfering RNAs which can target multiple pre-selected target sequences within one or more target genes to modulate expression of the targets. Methods for the design and for making such therapeutic multitargeting interfering RNAs are also needed. Antiviral interfering RNAs that can be developed rapidly upon the isolation and identification of new viral pathogens and that can be used to help slow, or even prevent, the emergence of new, resistant isotypes are also needed.

SUMMARY OF THE INVENTION

Interfering RNA molecules are now designed and produced with specificity for multiple binding sequences present in distinct genetic contexts in one or more pre-selected target RNA molecules and are used to decrease expression of the target sequences.

In a first embodiment, this invention relates to a multitargeting interfering RNA molecule comprising a guide strand of the Formula (I):

5'-p-XSY-3'

wherein p consists of a terminal phosphate group that is independently present or absent;wherein S consists of a first nucleotide sequence of a length of about 5 to about 20 nucleotides that is at least partially complementary to a first portion of each of at least two binding sequences present in distinct genetic contexts in one or more pre-selected target RNA molecules; wherein X is absent or consists of a second nucleotide sequence; wherein Y is absent or consists of a third nucleotide sequence, provided that X and Y are not absent simultaneously; wherein XSY is at least partially complementary to each of said binding sequences to allow a stable interaction therewith. Preferably S is completely complementary to the first portion of each of at least two binding sequences and also preferably, the first portion of each of at least two binding sequences is a seed sequence. X can consist of one or two nucleotides and Y can independently be at least partially complementary to a second portion of each of the binding sequences, said second portion is adjacent to and connected with the 5'-end of said first portion of the binding sequences. Also preferably, S is of a length of about 8 to about 15 nucleotides. XSY is preferably of a length of about 17 to about 25 nucleotides. Preferably, the multitargeting interfering RNA molecules of this invention further comprise a passenger strand that is at least partially complementary to the guide strand to allow formation of a stable duplex between the passenger strand and the guide strand and these RNA molecules preferably include one or more terminal overhangs and these overhangs preferably are of between 1 to 5 nucleotides. Preferably the passenger strand and the guide strand are completely complementary to each other. It is possible for the multitargeting interfering RNA molecules of this invention to target binding sequences present in distinct genetic contexts in one or alternatively in at least 2 pre-selected target RNA molecules. Preferably at least one of the pre-selected target RNA molecules is a non-coding RNA molecule. Alternatively, at least one of the pre-selected target RNA molecules can be a messenger RNA molecule and preferably one or more of the pre-selected target RNA molecules are involved in a disease or disorder. Preferably, the disease is a human disease. Also preferably, in the multitargeting interfering RNA molecules of this invention, at least one of the binding sequences is present in the 3'-untranslated region (3'UTR) of a messenger RNA molecule.

In this embodiment, preferably one or more of the pre-selected target RNA molecules encode a protein of a class selected from the group consisting of receptors, cytokines, transcription

factors, regulatory proteins, signaling proteins, cytoskeletal proteins, transporters, enzymes, hormones, and antigens. Preferably, the one or more of the pre-selected target RNA molecules encode a protein selected from the group consisting of ICAM-1, VEGF-A, MCP-1, IL-8, VEGF-B, IGF-1, Gluc6p, Inppl1, bFGF, PlGF, VEGF-C, VEGF-D, β-catenin, κ-ras-B, κ-ras-A, EGFR,Bcl-2, presenilin-1, BACE-1, MALAT-1, BIC, TGFβ, and TNF alpha. Also preferably, the multitargeting interfering RNA molecule decreases expression of any combination of VEGF-A, κ-ras and Bcl-2 in an expression system or alternatively decreases expression of both MALAT-1 and BIC in an expression system or still alternatively decreases expression of both ICAM-1 and VEGF-A in an expression system. Other multitargeting interfering RNA molecules decrease expression of both TGFβ and IL-8 in an expression system or alternatively decrease expression of both IL-8 and MCP-1 in an expression system. The multitargeting interfering RNA molecules of this invention can also further be manufactured to comprise a guide strand that forms stable interactions with at least two binding sequences present in distinct genetic contexts in one or more pre-selected target RNA molecules.

Preferably one or more of the pre-selected target RNA molecules is viral RNA. The virus is preferably selected from the group consisting of a human immunodeficiency virus (HIV), a hepatitis C virus (HCV), an influenza virus, a rhinovirus, and a severe acute respiratory syndrome (SARS) virus. Where the virus is HIV, one or more of the pre-selected target RNA molecules preferably encode an essential protein selected from the group consisting of GAG, POL, VIF, VPR, TAT, NEF, REV, VPU and ENV. Where the virus is HCV, one or more of the other preselected RNA molecules encodes TNFα.

In the multitargeting interfering RNA molecules of this invention, the molecules preferably comprise at least one modified ribonucleotide, universal base, acyclic nucleotide, abasic nucleotide, non-ribonucleotide or combinations thereof. In other aspects of this embodiment, S consists essentially of a nucleotide sequence selected from the group consisting of:

UAUGUGGGUGGG (SEQ ID NO: 1), UGUUUUUG (SEQ ID NO: 2), ACCCCGUCUCU (SEQ ID NO: 5), AGCUGCA (SEQ ID NO: 7), AAACAAUGGAAUG (SEQ ID NO: 8), GGUAGGUGGGUGGG (SEQ ID NO: 10), CUGCUUGAU (SEQ ID NO: 12), UCCUUUCCA (SEQ ID NO: 13), UUUUUCUUU (SEQ ID NO: 14), UUCUGAUGUUU

(SEQ ID NO: 15), UCUUCCUCUAU (SEQ ID NO: 16), UGGUAGCUGAA (SEQ ID NO: 17), CUUUGGUUCCU (SEQ ID NO: 18), CUACUAAUGCU (SEQ ID NO: 19), UCCUGCUUGAU (SEQ ID NO: 20), AUUCUUUAGUU (SEQ ID NO: 21), CCAUCUUCCUG (SEQ ID NO: 22), CCUCCAAUUCC (SEQ ID NO: 23), CUAAUACUGUA (SEQ ID NO: 24), UUCUGUUAGUG (SEQ ID NO: 25), GCUGCUUGAUG (SEQ ID NO: 26), ACAUUGUACUG (SEQ ID NO: 27), UGAUAUUUCUC (SEQ ID NO: 28), AACAGCAGUUG (SEQ ID NO: 29), GUGCUGAUAUU (SEQ ID NO: 30), CCCAUCUCCAC (SEQ ID NO: 31), UAUUGGUAUUA (SEQ ID NO: 32), CAAAUUGUUCU (SEQ ID NO: 33), UACUAUUAAAC (SEQ ID NO: 34), GCCUAUCAUAU (SEQ ID NO:58), UGGUGCCUGCU (SEQ ID NO: 59), AAUUAAUAUGGC (SEQ ID NO: 60), CCCUCUGGGCU (SEQ ID NO: 61), UUCUUCCUCAU (SEQ ID NO: 62), UAUUUAUACAGA (SEQ ID NO: 63), CACCAAAAUUC (SEQ ID NO: 64), UGAGUNNGAACAUU (SEQ ID NO: 72) where N is any base, CUCCAGG (SEQ ID NO: 74), UCAGUGGG (SEQ ID NO: 76), UCCUCACAGGG (SEQ ID NO: 78), GUGCUCAUGGUG (SEQ ID NO: 79), CCUGGAGCCCUG (SEQ ID NO: 80), UCUCAGCUCCAC (SEQ ID NO: 81), ACCCUCGCACC (SEQ ID NO: 86), GUGUUGAAG (SEQ ID NO: 88), UUCCACAAC (SEQ ID NO: 90), UCCACUGUC (SEQ ID NO: 92), CAGAAUAG (SEQ ID NO: 93), AACUCUCUA (SEQ ID NO: 94) and CGUGAAGAC (SEQ ID NO: 98).

In other aspects, S consists essentially of a nucleotide sequence selected from the group consisting of: UAUGUGGGUGGG (SEQ ID NO: 1), UCCUCACAGGG (SEQ ID NO: 78), GUGUUGAAG (SEQ ID NO: 88), UUCCACAAC (SEQ ID NO: 90), AACUCUCUA (SEQ ID NO: 94) and CGUGAAGAC (SEQ ID NO: 98). Preferably, S consists essentially of a nucleotide sequence of 6 or more contiguous bases contained within any of the sequences selected from the group consisting of: UAUGUGGGUGGG (SEQ ID NO: 1), UCCUCACAGGG (SEQ ID NO: 78), GUGUUGAAG (SEQ ID NO: 88), UUCCACAAC (SEQ ID NO: 90), AACUCUCUA (SEQ ID NO: 94) and CGUGAAGAC (SEQ ID NO: 98).

In yet other aspects, the multitargeting interfering RNA molecule consists essentially of:

5' UUCCUCACAGGGCAGUGAUUC 3' (SEQ ID NO: 122)
3'UUAAAGAGUGUCCCGUCACUA 5', (SEQ ID NO: 124)

5' UACAAAUCUACUUCAACAUUU 3' (SEQ ID NO: 131)
3'GUAUGUUUAGAUGAAGUUGUG 5', (SEQ ID NO: 132)

or

5' AACAUAUGUUCUUCAACAUUU 3' (SEQ ID NO: 133)
3'GUUUGUAUACAAGAAGUUGUG 5', (SEQ ID NO: 134)

Other exemplary multitargeting interfering RNA molecules include:

5' UAUGUGGGUGGGUGAGUCUAA 3' (SEQ ID NO: 100)
3'UUAUACACCCACCCACUCAGA 5', (SEQ ID NO: 101)

5' UGUUUUGUUGUUACAUAUGAC 3' (SEQ ID NO: 102)
3'UUACAAAACAACAAUGUAUAC 5', (SEQ ID NO: 103)

5' UAUGUGGGUGGGGUGUCUCUA 3' (SEQ ID NO: 104)
3'UUAUACACCCACCCCACAGAG 5', (SEQ ID NO: 105)

5' UAUGUGGGUCGGGUGGUCUAA 3' (SEQ ID NO: 106)
3'UUAUACACCCACCCCACCAGA 5', (SEQ ID NO: 107)

5' UAUGUGGGUGGGGUGGUGUCU 3' (SEQ ID NO: 108)
3'UUAUACACCCACCCCACCACA 5', (SEQ ID NO: 109)

5' UAUGUGGGUGGGGUGAGUGUCU 3' (SEQ ID NO: 110)
3'UUAUACACCCACCCACUCACA 5', (SEQ ID NO: 111)

5' CUCACCCACCCACAUACAUUU 3' (SEQ ID NO: 112)
3'CUGAGUGGGUGGGUGUAUGUA 5', (SEQ ID NO: 113)

5' UCACCCACCCACAUACAUAUU 3' (SEQ ID NO: 114)
3'UGAGUGGGUGGGUGUAUGUAU 5', (SEQ ID NO: 115)

5' UCACCCACCCACAUACAUUUU 3' (SEQ ID NO: 116)
3'UGAGUGGGUGGGUGUAUGUAA 5', (SEQ ID NO: 117)

5' UAUGUGGGUGGGUCAGUCUA 3' (SEQ ID NO: 118)
3'UAUACACCCACCCACUCAGA 5', (SEQ ID NO: 119)

5' GGGUUUACCAGGAAGAUGGUU 3' (SEQ ID NO: 120)
3'UACCCAAAUGGUCCUUCUACC 5', (SEQ ID NO: 121)

5' UUCCUCACAGGGCAGUGAUUC 3' (SEQ ID NO: 122)
3'UUAAGGAGUGUCCCGUCACUA 5', (SEQ ID NO: 123)

5' UUCCUCACAGGGCAGUGGUUC 3' (SEQ ID NO: 125)
3'UUAAGGAGUGUCCCGUCACCA 5', (SEQ ID NO: 126)

5' CCCGGACCCUUACAGAGUUUU 3' (SEQ ID NO: 127)
3'ACGGGCCUGGGAAUCUCUCAA 5', (SEQ ID NO: 128)

5' UACCCUCGCACCGAUCUCCCAA 3' (SEQ ID NO: 129)
3'UUAUGGGAGCGUGGCUAGAGGG 5', (SEQ ID NO: 130)

5' UUCCACAACACAAGCUGUGUU 3' (SEQ ID NO: 135)
3'UUAACGUGUUGUGUUCGACAC 5', (SEQ ID NO: 136)

5' GGACCCUUAGAGAGUUUCAUU 3' (SEQ ID NO: 137)
3'GGCCUGGGAAUCUCUCAAAGU 5', (SEQ ID NO: 138)

5' UUCGUGAAGACGGUGGGCCGA 3' (SEQ ID NO: 139)
3'dTdTAAGCACUUCUGCCACCCGG 5', (SEQ ID NO: 140)

or

5' AGACUCACCCACCCAGAUAUU 3' (SEQ ID NO: 141)
3'AAUCUCAGUGGGUGGGUCUAU 5' (SEQ ID NO: 142)

Yet others include:

5' UAUGUGGGUGGGUGAGUCUAA 3' (SEQ ID NO: 100)
3' UUAUACACCCACCCACUCAGA 5', (SEQ ID NO: 101)

5' GGACCCUUAGAGAGUUUCAUU 3' (SEQ ID NO: 137)
3' GGCCUCGGAAUCUCUCAAAGU 5', (SEQ ID NO: 138)

or

5' UUCGUGAAGACGGUGGGCCGA 3' (SEQ ID NO: 139)
3' dTdTAAGCACUUCUGCCACCCGG 5', (SEQ ID NO: 140)

Preferably the above multitargeting interfering RNA molecules also include at least one modified ribonucleotide, universal base, acyclic nucleotide, abasic nucleotide and non-ribonucleotide, overhang variation or a combination thereof.

In another aspect of this invention, the invention relates to a biological system comprising the multitargeting interfering RNA molecules of this invention and those preferred biological systems include a virus, a microbe, a cell, a plant, or an animal. Vectors comprising a nucleotide sequence that encodes the multitargeting interfering RNA molecules of this invention are also contemplated. Preferred vectors are viral vectors. Preferred viral vectors are selected from the group consisting of an adeno-associated virus, a retrovirus, an adenovirus, a lentivirus, and an alphavirus. The invention also relates to cells comprising the vectors of this invention.

The multitargeting interfering RNA molecules of this invention can also be short hairpin RNA molecules.

The invention further relates to pharmaceutical compositions comprising the multitargeting interfering RNA molecules of this invention and an acceptable carrier. Alternatively, the composition can include a vector comprising the RNA molecule and an acceptable carrier.

The invention further relates to methods of using the multitargeting interfering RNA molecules of this invention. In a preferred method for using the multitargeting interfering RNA molecules of this invention, the method includes inducing RNA interference in a biological system, comprising the step of introducing a multitargeting interfering RNA molecule of this invention into the biological system. More specifically, the invention relates to methods of inducing RNA interference in a biological system, comprising the steps of: (a) selecting one or more target RNA molecules; (b) designing a multitargeting interfering RNA molecule comprising a guide strand that can form stable interactions with at least two binding sequences present in distinct genetic contexts in the set of one or more target RNA molecules; (c) producing the multitargeting interfering RNA molecule; and (d) administering the multitargeting interfering RNA molecule into the biological system, whereby the guide strand of the multitargeting interfering RNA molecule forms stable interactions with the binding sequences present in distinct genetic contexts in the target RNA molecules, and thus induces RNA

interference of the target RNA molecules. Preferably the biological system is a virus, a microbe, a cell, a plant, or an animal. Preferred animals include rats, mice, monkeys, and humans. Also preferably the target RNA molecules comprise RNA molecules that are involved in a disease or disorder of the biological system or are selected from the biological system. Alternatively, the target RNA molecules comprises one or more RNA molecules selected from a second biological system that is infectious to the biological system or where the target RNA molecules are selected from both a first biological system and a second biological system that is infectious to the first biological system. For example, the target RNA molecules can comprise one or more RNA molecules selected from an animal or a plant and one or more RNA molecules selected from a microbe or a virus that is infectious to the animal or the plant. As a more specific example, the target RNA molecules can comprise one or more RNA molecules selected from a human and one or more RNA molecules selected from a virus selected from the group consisting of a human immunodeficiency virus (HIV), a hepatitis C virus (HCV), an influenza virus, a rhinovirus, and a severe acute respiratory syndrome (SARS) virus. The target RNA molecues can also be RNA molecules encoding a protein of a class of proteins, including, without limitation, receptors, cytokines, transcription factors, regulatory proteins, signaling proteins, cytoskeletal proteins, transporters, enzymes, hormones, and antigens. For example groups of proteins can include ICAM-1, VEGF-A, MCP-1, IL-8, VEGF-B, IGF-1, Gluc6p, Inppl1, bFGF, PlGF, VEGF-C, VEGF-D, β-catenin, κ-ras-B, κ-ras-A, EGFR, Bcl-2, presenilin-1, BACE-1, MALAT-1, BIC, TGFβ, and TNF alpha. Preferred combinations include, for example, ICAM-1 and VEGF-A, one or more viral RNA molecules such as human immunodeficiency virus (HIV), a hepatitis C virus (HCV), an influenza virus, a rhinovirus, and a severe acute respiratory syndrome (SARS) virus or an essential protein for HIV selected from the group consisting of GAG, POL, VIF, VPR, TAT, NEF, REV, VPU and ENV. The target RNA molecules can also be selected from human proteins including, for example, TNFalpha, LEDGF(p75), BAF, CCR5, CXCR4, furin, NFkB, STAT1. Specific combinations include a virus protein and a human protein associated with the disease caused by that virus. So, as one example, preselected target RNA molecules can comprise Hepatitis C Virus (HCV) and one or more of the other preselected RNA molecules encodes TNFα.

This invention also relates to methods for treating a disease or condition in a subject, the method comprising the steps of: (a) selecting one or more target RNA molecules, wherein the modulation in expression of the target RNA molecules is potentially therapeutic for the treatment of the disease or condition; (b) designing a multitargeting interfering RNA molecule comprising a guide strand that can form stable interactions with at least two binding sequences present in distinct genetic contexts in the one or more target RNA molecules; (c) producing the multitargeting interfering RNA molecule; and (d) administering the multitargeting interfering RNA molecule into the subject, whereby the guide strand of the multitargeting interfering RNA molecule forms stable interactions with the binding sequences present in distinct genetic contexts in the one or more target RNA molecules, and thus induces RNA interference and modulation of expression of the target RNA molecules.

The invention also relates to methods for designing a multitargeting interfering RNA molecule, comprising the steps of: a) selecting one or more target RNA molecules, wherein modulation in expression of the target RNA molecules is desired; b) obtaining at least one nucleotide sequence for each of the target RNA molecules; c) selecting a seed sequence of 6 nucleotides or more, wherein said seed sequence occurs in at least two distinct genetic contexts in nucleotide sequences identified in step b) for the target RNA molecules; d) selecting at least two binding sequences, wherein each of binding sequences comprises the seed sequence, and the binding sequences are present in distinct genetic contexts in the target molecules; and e) designing a multitargeting interfering RNA molecule having a guide strand that shares a substantial degree of complementarity with each of the at least two binding sequences to allow stable interaction therewith. Preferably the method further comprises designing a passenger strand that is at least partially complementary to the guide strand to allow formation of a stable duplex between the passenger strand and the guide strand.

In yet another method for designing a multitargeting interfering RNA molecule, the method comprises the steps of: a) selecting one or more target RNA molecules, wherein modulation in expression of the target RNA molecules is desired; b) identifying at least one nucleotide sequence for each of the target RNA molecules; c) selecting a length, n, in nucleotides, for a seed sequence; wherein n = about 6 or more; d) generating a collection of candidate seed sequences of the length n from each nucleotide sequence identified in step b),

wherein each candidate seed sequence occurs at least once in nucleotide sequences obtained in step b); c) determining the genetic context of each of the candidate seed sequences in each nucleotide sequence obtained in step b), by collecting, for each occurrence of the candidate seed sequence, a desired amount of the 5' and 3' flanking sequence; f) selecting a seed sequence of the length *n* from the candidate seed sequences, wherein the seed sequence occurs at least in two distinct genetic contexts in nucleotide sequences identified in step b); g) selecting a consensus target sequence, wherein said consensus target sequence comprises the seed sequence and a desired consensus sequence for the sequence flanking either one or both of the 5' and 3' ends of the seed; and h) designing a multitargeting interfering RNA molecule comprising a guide strand that shares a substantial degree of complementarity with the consensus target sequence to allow stable interaction therewith. Preferably the step of generating a collection of candidate seed sequences comprises the steps of beginning at a terminus, sequentially observing the nucleotide sequence using a window size of *n* and stepping along the nucleotide sequence with a step size of 1. Also preferably, the step of selecting seed sequences comprises the step of discarding any sequence of the length *n* that i) is composed of a consecutive string of 5 or more identical single nucleotides; ii) is composed of only adenosine and uracil; iii) is predicted to occur with an unacceptably high frequency in the non-target transcriptome of interest; iv) is predicted to have a propensity to undesirably modulate the expression or activity of one or more cellular component; or v) is any combination of i) to iv). Optionally, steps c) to g) can be repeated with a new value of *n*. The multitargeting interfering RNA molecules, once designed can then be tested in an expression system.

In preferred methods for designing a multitargeting interfering RNA molecule, the step of selecting a consensus target sequence further comprises the step of discarding any sequence that is composed of only a single base, is composed only of A and U, has a consecutive string of 5 or more bases which are C, is G/C rich at the 3' end, is predicted to occur with unacceptable frequency in the non-target transcriptome of interest; or any combination thereof. Also preferably the passenger strand is designed so that it is at least partially complementary to the guide strand to allow formation of a stable duplex between the passenger strand and the guide strand. Once the multitargeting interfering RNA molecule is designed, it is contemplated that it can be modified. Such modifying methods are also contemplated within the scope of the invention and a preferred method comprises modifying the multitargeting interfering RNA

molecule comprising the steps alone or in combination of i) improving the incorporation of the guide strand of the multitargeting interfering RNA molecule into the RNA induced silencing complex (RISC); ii) increasing or decreasing the modulation of the expression of at least one target RNA molecule; iii) decreasing stress or inflammatory response when the multitargeting interfering RNA molecule is administered into a subject; iv) altering the half life of the multitargeting RNA molecule in an expression system.

The invention futher relates to a method of designing a full length multitargeting interfering RNA from a seed sequence, comprising the steps of: a) deducing the sequence of the complete complement of the seed sequence; b) generating permutations for the extension of the complete complement of the seed sequence to the desired length *n*; c) creating a collection of putative guide strand sequences, each of which comprises the sequence of the complete complement of the seed sequence and one of the permutations generated in step b); d) using RNAhybrid to determine the binding pattern and the minimum free energy (mfe) of the putative guide strand sequences created in step c) against all the target sequences comprising the seed sequence; e) discarding putative guide strand sequences where i) there is a contiguous run of 5 or more G residues; and ii) the Load Bias is < 1.2; and f) selecting a guide strand sequence of the length *n* for a multitargeting interfering RNA sequence from the list of the remaining putative guide strand sequences based on their Relative Activity Score. The method preferably can additionally comprise the steps of producing the multitargeting interfering RNA comprising the guide strand sequence and testing the multitargeting interfering RNA in an expression system.

In yet another aspect, the invention relates to a method of making a multitargeting interfering RNA molecule, comprising the steps of: i) designing a multitargeting interfering RNA molecule having a guide strand that can form stable interactions with at least two binding sequences present in distinct genetic contexts in a set of pre-selected target RNA molecules; and ii) producing the multitargeting interfering RNA molecule.

It is also contemplated within the scope of this invention that the invention further comprises a pharmaceutical composition comprising a therapeutically effective amount of one or more multitargeting interfering RNA molecules together with a pharmaceutically acceptable carrier.

Other aspects, features and advantages of the invention will be apparent from the following disclosure, including the detailed description of the invention and its preferred embodiments and the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: A flow-chart highlighting aspects of an exemplary design process for multitargeting interfering RNAs (CODEMIRs or VIROMIRs).

Figure 2: Graph showing cytotoxicity of control siRNA and CODEMIRs at a final concentration of 40 nM in ARPE-19 cells in culture medium at 48 hrs post-transfection. A: mock transfected cells (Lipofectamine2000 alone); B: irrelevant siRNA control; C: VEGF-specific siRNA; D: ICAM-specific siRNA; E: CODEMIR-1; F: CODEMIR-2.

Figure 3: Graph showing VEGF (closed bars) and ICAM (open bars) protein expression as a percent of control (untransfected) cells in cells treated with various siRNAs and CODEMIRs. A: Unstimulated cells; B: Untransfected, stimulated cells; C: Irrelevant control siRNA; D: ICAM-specific siRNA; E: VEGF-specific siRNA; F: CODEMIR-1; G: CODEMIR-2.

Figure 4: Comparison of CODEMIR 1 activity with that of a naturally-occurring microRNA with some sequence similarity. A: Untransfected cells; B: Irrelevant siRNA; C: CODEMIR-1; D: synthetic miR-299 (CODEMIR-84).

Figure 5: Example of a VIROMIR targeting two sites in the same target RNA, in this case, the HIV genome.

Figure 6: Tolerance of CODEMIRs for mismatches at the 5' extremity for activity against VEGF (closed bars) and ICAM-1 (open bars) expression. A: Untransfected cells; B: Irrelevant siRNA; C: ICAM- and VEGF-specific siRNAs; D: CODEMIR-13; E: CODEMIR-14; F: CODEMIR-15. The presence of a single mismatch at the 5'extremity of the seed did not significantly reduce the activities of CODEMIRs -14 and -15 relative to the fully matched seed of CODEMIR-13 (see Table 8-2 for sequences).

Figure 7. Production of p24 HIV capsid protein in HEK-293 cells co-transfected with pNL4.3 plasmid and 67 ng of either pSIL vector (control) or pSIL vector encoding sequences for shRNA approximating VM004, VM006 or VM010. A: Control (empty) vector; B: VM004 shRNA construct; C: VM006 shRNA construct; D: VM010 shRNA construct.

Figure 8. Survival of HCT116 cells with and without serum withdrawal following transfection with 40 nM CC014-21 and siRNA controls. A: Mock-transfected cells; B: Irrelevant control siRNA (siGC47); C: cytotoxic transfection siRNA control (siTOX); D: Bcl-2-specific siRNA; E: Kras-specific siRNA; F: VEGF-specific siRNA (PVE); G: CC014; H: CC015; I: CC016; J: CC017; K: CC018; L: CC019; M: CC020 and N: CC021.

Figure 9. Abundance of K-Ras in HCT116 cells following transfection with 40 nM CC014-21 as measured by Western and normalized to beta-actin. A: Mock-transfected cells; B: CC014; C: CC015; D: CC016; E: CC017; F: CC018; G: CC019; H: CC020; I: CC021; J: Kras-specific siRNA.

Figure 10. Secretion of VEGF by HCT116 cells 48 h post-transfection with 40 nM CC014-21. VEGF in the cell medium was measured by ELISA. PVE is a VEGF-specific siRNA active in several species (human and rodent). A: Mock-transfected cells; B: Irrelevant control siRNA; C: VEGF-specific siRNA (PVE); D: Kras-specific siRNA; E: Bcl-2-specific siRNA; F: CC014; G: CC015; H: CC016; I: CC017; J: CC018; K: CC019; L: CC020; M: CC021.

Figure 11. Positivity for Annexin V (early apoptosis) and Propidium Iodide (necrosis/late apoptosis) staining of HCT116 cells 48 hr following transfection with 40 nM CC014-18 and controls. A: Untreated cells; B: Mock-transfected cells; C: Irrelevant control siRNA (siGC47); D: cytotoxic transfection control siRNA (siTOX); E: VEGF-specific siRNA; F: Bcl-2-specific siRNA; G: Kras-specific siRNA; H: CC014; I: CC015; J: CC016; K: CC017 and L: CC018.

Figure 12. Colony forming ability of HCT116 cells following transfection with 40 nM siRNAs, CC015 and CC018-21. A: Untreated cells; B: Irrelevant control siRNA C: VEGF-specific siRNA; D: Kras-specific siRNA; E: Bcl-2-specific siRNA; F: CC015; G: CC018; H: CC019; I: CC020 and J: CC021.

Figure 13. VEGF Secretion by ARPE-19 cells transfected with 40nM 2'-F modified CODEMIR-1 analogs. VEGF production was measured by ELISA of the cell culture supernatant. Each point represents the mean of triplicate wells; error bars indicate standard deviation of the mean. A: Untransfected cells; B: Mock-transfected cells; C: Irrelevant siRNA control; D: CODEMIR-1; E: CODEMIR-33; F: CODEMIR-87; G: CODEMIR-92; H: CODEMIR-144; I: CODEMIR-145; J: CODEMIR-165; K: CODEMIR-166 and L: CODEMIR-167.

Figure 14. Guide strand terminal modifications of CODEMIR-1 tested in vitro (see Figure 15 for data), "Oligo" refers to the active guide strand of CODEMIR-1. A: CODEMIR-146; B: CODEMIR-147; C: CODEMIR-148; D: CODEMIR-149; E: CODEMIR-150; F: CODEMIR-151; G: CODEMIR-152; H: CODEMIR-153; I: CODEMIR-154; J: CODEMIR-155 and K: CODEMIR-156.

Figure 15. VEGF secretion by ARPE-19 cells transfected with 10nM of each terminal conjugated variant of CODEMIR-1. VEGF in cell culture supernatant was measured by ELISA. Each point represents the mean of triplicate wells; error bars indicate standard deviation of the mean. A: Untransfected cells; B: Mock-transfected cells; C: Irrelevant siRNA control; D: CODEMIR-1; E: CODEMIR-146; F: CODEMIR-147; G: CODEMIR-148; H: CODEMIR-149; I: CODEMIR-150; J: CODEMIR-151; K: CODEMIR-152; L: CODEMIR-153; M: CODEMIR-154; N: CODEMIR-155 and O: CODEMIR-156.

Figure 16. Time course of VEGF suppression by CODEMIR-1. Cells were transfected once on day 0 with 40 nM dsRNA and repeatedly stimulated with deferroxamine. Supernatant was collected at the indicated time points and assayed by ELISA. Error bars indicate standard deviation of the mean. Squares: Untransfected cells; Triangles: Mock-transfected; Inverted triangles: Irrelevant siRNA control and Diamonds: CODEMIR-1.

Figure 17. Stability of chemically modified variants of CODEMIR-1 in human serum: 100nM Duplex RNA was incubated at 37°C in 10% human AB serum, with RNA concentration monitored using Oligreen dye. Each point is the mean of triplicate samples. Error bars (in many cases smaller than symbols) indicate standard deviation of the mean. Solid squares: CODEMIR-1; Triangles: CODEMIR-33; Inverted triangles: CODEMIR-87; Diamonds: CODEMIR-92; Circles: CODEMIR-144 and Open squares: CODEMIR-145.

Figure 18. VEGF (closed bars) and ICAM (open bars) secretion by ARPE-19 cells transfected with chemically modified variants of CODEMIR-1. ARPE-19 cells were transfected with 40nM duplex RNA and VEGF or ICAM secretion was assayed 48 hours post-transfection by ELISA. Each bar represents the mean of triplicate samples. Error bars indicate standard deviation of the mean. In 1 (top panel) - A: Untransfected cells; B: Mock-transfected cells; C: Irrelevant siRNA control; D: CODEMIR-1; E: CODEMIR-33; F: CODEMIR-87 and G: CODEMIR-92. In 2 (bottom panel) - A: Untransfected cells; B: Mock-transfected cells; C:

Irrelevant siRNA control; D: CODEMIR-1; E: CODEMIR-87; F: CODEMIR-144; G: CODEMIR-33 and H: CODEMIR-145.

Figure 19. Effect of mismatches in the seed region of CODEMIR-1 upon VEGF (open bars) and ICAM (closed bars) suppressive activity. ARPE-19 cells were transfected with 40nM duplex RNA and VEGF (ELISA) or ICAM (FACS) assayed and expressed relative to results obtained with untransfected cells. Expression was assayed 48 hours post-transfection. Each bar represents the mean of triplicate samples. Error bars indicate standard deviation of the mean. A: Untransfected cells; B: Mock-transfected; C: Irrelevant siRNA control; D: CODEMIR-1; E: CODEMIR-122; F: CODEMIR-123 and G: CODEMIR-124.

Figure 20. Schematic illustration of the design of all 32 variants of CODEMIR-1 that are directly aligned to the VEGF and ICAM mRNAs. The sequence alignment of two target sequences is shown. A potential guide strand (CODEMIR) is shown below the sequence alignment. Because of the lack of total consensus between the two targets, two possible bases can be used at some positions (alternate bases at mismatch positions are shown in the row indicated with an arrow). Note the possible use of a U to match and wobble-pair the two target sequences, respectively.

Figure 21. Screening 32 variants of CODEMIR-1 for VEGF suppressive activity. ARPE-19 cells were transfected with 40nM of the indicated RNA duplex, and VEGF secretion was measured by ELISA 48 hours post-transfection. The guide strand of each CODEMIR is shown in the 5' to 3' direction. Each bar represents the mean of triplicate samples. Error bars indicate standard deviation of the mean. A: Untransfected; B: Irrelevant siRNA control; C: CODEMIR-1; D: CODEMIR-52; E: CODEMIR-53; F: CODEMIR-54; G: CODEMIR-55; H: CODEMIR-56; I: CODEMIR-57; J: CODEMIR-58; K: CODEMIR-59; L: CODEMIR-60; M: CODEMIR-61; N: CODEMIR-62; O: CODEMIR-63; P: CODEMIR-64; Q: CODEMIR-65; R: CODEMIR-66; S: CODEMIR-67; T: CODEMIR-68; U: CODEMIR-69; V: CODEMIR-70; W: CODEMIR-71; X: CODEMIR-72; Y: CODEMIR-73; Z: CODEMIR-74; AA: CODEMIR-75; BB: CODEMIR-76; CC: CODEMIR-77; DD: CODEMIR-78; EE: CODEMIR-79; FF: CODEMIR-80; GG: CODEMIR-81; HH: CODEMIR-82; II: CODEMIR-83 and JJ: CODEMIR-84.

Figure 22. Relationship between target complementarity, length of 5' complementarity and VEGF suppression for 32 variants of CODEMIR-1. Open squares: CODEMIR-1 variants with 12 bases of contiguous complementarity to the VEGF binding sequence; Triangles:

CODEMIR-1 variants with 14 bases of contiguous complementarity; Inverted triangles: CODEMIR-1 variants with 17 bases of contiguous complementarity; Open diamonds: CODEMIR-1 variants with 18 bases of contiguous complementarity; Circles: CODEMIR-1 variants with 19 bases of contiguous complementarity and Solid squares: CODEMIR-1 variant (VAIC) with 21 bases of contiguous complementarity.

Figure 23. Screening 31 variants of CODEMIR-1 for suppression of ICAM production. ARPE-19 cells were transfected with 40nM of the indicated RNA duplex, and sICAM secretion was measured by ELISA 48 hours post-transfection. The guide strand of each CODEMIR is shown in the 5' to 3' direction. Each bar represents the mean of triplicate samples. Error bars indicate standard deviation of the mean. A: Untransfected; B: Irrelevant siRNA control; C: CODEMIR-1; D: CODEMIR-52; E: CODEMIR-53; F: CODEMIR-54; G: CODEMIR-55; H: CODEMIR-56; I: CODEMIR-57; J: CODEMIR-58; K: CODEMIR-59; L: CODEMIR-60; M: CODEMIR-61; N: CODEMIR-62; O: CODEMIR-63; P: CODEMIR-64; Q: CODEMIR-65; R: CODEMIR-66; S: CODEMIR-67; T: CODEMIR-68; U: CODEMIR-69; V: CODEMIR-70; W: CODEMIR-71; X: CODEMIR-72; Y: CODEMIR-73; Z: CODEMIR-74; AA: CODEMIR-75; BB: CODEMIR-76; CC: CODEMIR-77; DD: CODEMIR-78; EE: CODEMIR-79; FF: CODEMIR-80; GG: CODEMIR-81; HH: CODEMIR-82; II: CODEMIR-83 and JJ: ICAM-specific siRNA.

Figure 24. Comparison of CODEMIR-1 variants with (CODEMIR-56 and CODEMIR-76) and without (CODEMIR-120 and CODEMIR-121) 7 G motifs. ARPE-19 cells were transfected with 40nM duplex RNA and VEGF (open bars) or ICAM (closed bars) expression was assayed 48 hours post-transfection. Each bar represents the mean of triplicate samples. Error bars indicate standard deviation of the mean. A: Untransfected; B: Mock-transfected cells; C: Irrelevant siRNA control; D: VEGF- and ICAM-specific siRNAs; E: CODEMIR-56; F: CODEMIR-76; G: CODEMIR-120 and H: CODEMIR-121.

Figure 25. VEGF and ICAM expression in ARPE-19 cells after transfection with LNA and inosine containing CODEMIRs. ARPE-19 cells were transfected with 40nM duplex RNA and VEGF (closed bars) or ICAM (open bars) expression was assayed 48 hours post-transfection. Each bar represents the mean of triplicate samples. Error bars indicate standard deviation of the mean. A: Untransfected; B: Mock-transfected cells; C: Irrelevant siRNA

control; D: CODEMIR-1; E: CODEMIR-99; F: CODEMIR-100; G: CODEMIR-101 and H: CODEMIR-102.

Figure 26. Comparison of VEGF suppressive activity of CODEMIRs containing inosine bases or mismatches at positions 13 and/or 15 of the guide strand. ARPE-19 cells were transfected with 10nM duplex RNA and VEGF (ELISA) expression was assayed 48 hours post-transfection. Each bar represents the mean of triplicate samples. Error bars indicate standard deviation of the mean. A: Untransfected; B: Mock-transfected cells; C: Irrelevant siRNA control; D: CODEMIR-1; E: CODEMIR-68; F: CODEMIR-69; G: CODEMIR-70; H: CODEMIR-71; I: CODEMIR-100; J: CODEMIR-101 and K: CODEMIR-102.

Figure 27. VEGF production by ARPE-19 cells after transfection with variants of CODEMIR-1 containing asymmetric bulges. ARPE-19 cells were transfected with 40nM of the indicated RNA duplex, and VEGF secretion was measured by ELISA 48 hours post-transfection. Each bar represents the mean of triplicate samples. Error bars indicate standard deviation of the mean. A: Untransfected; B: Mock-transfected cells; C: Irrelevant siRNA control; D: CODEMIR-1; E: CODEMIR-104; F: CODEMIR-105; G: CODEMIR-106 and H: CODEMIR-107.

Figure 28. Screening multiple seed sites in the VEGF 3' UTR. ARPE-19 cells were transfected with 40nM of the indicated RNA duplex, and VEGF secretion was measured by ELISA 48 hours post-transfection. Each bar represents the mean of triplicate samples. Error bars indicate standard deviation of the mean. A: Untransfected; B: Mock-transfected cells; C: Irrelevant siRNA control; D: CODEMIR-1; E: CODEMIR-108; F: CODEMIR-109; G: CODEMIR-110; H: CODEMIR-111; I: CODEMIR-112; J: CODEMIR-113; K: CODEMIR-114; L: CODEMIR-115; M: CODEMIR-116; N: CODEMIR-117; O: CODEMIR-118 and P: CODEMIR-119.

Figure 29. Design of shRNA based on CODEMIR-1. Sequence shown is 5' to 3' (upper strand). Sequences in bold indicate the predicted active CODEMIR-1 duplex.

Figure 30. Effect of CODEMIR-1 hairpins on VEGF and ICAM-1 expression. ARPE-19 cells were transfected with 2 μg/ml of each hairpin plasmid. VEGF (A) and ICAM (B) expression was determined 48 hours post-transfection. The open bars indicate results obtained with the "empty" vector control and the closed bars are those obtained with the shRNA constructed to approximate CODEMIR-1. Each bar represents the mean of triplicate samples.

Error bars indicate standard deviation of the mean. A significant effect of the shRNA approximating CODEMIR-1 was found for both targets (* = $p<0.001$; ** = $p<0.05$).

Figure 31. Efficacy of a 1 bp overhang (CODEMIR-24) and "blunt-ended" variant (CODEMIR-25) of CODEMIR-1 against VEGF (closed bars) and ICAM-1 (open bars). A: Untransfected; B: Mock-transfected cells; C: Irrelevant siRNA control; D: ICAM- and VEGF-specific siRNAs; E: CODEMIR-1; F: CODEMIR-24 and G: CODEMIR-25.

DETAILED DESCRIPTION

Various publications, articles and patents are cited or described in the background and throughout the specification; each of these references is herein incorporated by reference in its entirety. Discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is for the purpose of providing context for the present invention. Such discussion is not an admission that any or all of these matters form part of the prior art with respect to any inventions disclosed or claimed.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention pertains. In this invention, certain terms are used frequently, which shall have the meanings set forth as follows. These terms may also be explained in greater detail later in the specification.

The following are abbreviations that are at times used in this specification:

bp = base pair

cDNA = complementary DNA

CODEMIR = COmputationally-DEsigned, Multi-targeting Interfering RNA

kb = kilobase; 1000 base pairs

kDa = kilodalton; 1000 dalton

mRNA = messenger RNA

miRNA = microRNA

ncRNA = non-coding RNA

nt = nucleotide

PAGE = polyacrylamide gel electrophoresis

PCR = polymerase chain reaction

RISC = RNA interference silencing complex

RNAi = RNA interference

SDS = sodium dodecyl sulfate

siRNA = short interfering RNA

shRNA = short hairpin RNA

SNPs = single nucleotide polymorphisms

UTR = untranslated region

VIROMIR = multitargeting interfering RNA preferentially targeted to viral targets

It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a cell" is a reference to one or more cells and includes equivalents thereof known to those skilled in the art and so forth.

An "activity", a "biological activity", or a "functional activity" of a polypeptide or nucleic acid refers to an activity exerted by a polypeptide or nucleic acid molecule as determined *in vivo* or *in vitro*, according to standard techniques. Such activities can be a direct activity, such as an association with or an enzymatic activity on a second protein, or an indirect activity, such as a cellular signaling activity mediated by interaction of a protein with a second protein.

"Biological system" is meant, material, in a purified or unpurified form, from biological sources, including but not limited to human, animal, plant, insect, microbial, viral or other sources, wherein the system comprises the components required for biologic activity (e.g., inhibition of gene expression). The term "biological system" includes, for example, a cell, a virus, a microbe, an organism, an animal, or a plant.

A "cell" means an autonomous self-replicating unit that may constitute an organism (in the case of unicellular organisms) or be a sub unit of multicellular organisms in which individual cells may be specialized and/or differentiated for particular functions. A cell can be prokaryotic or eukaryotic, including bacterial cells such as *E. coli*, fungal cells such as yeast, bird cell, mammalian cells such as cell lines of human, bovine, porcine, monkey, sheep, apes, swine, dog, cat, and rodent origin, and insect cells such as *Drosophila* and silkworm derived cell lines, or plant cells. The cell can be of somatic or germ line origin, totipotent or hybrid, dividing or non-dividing. The cell can also be derived from or can comprise a gamete or embryo, a stem cell, or a fully differentiated cell. It is further understood that the term "cell" refers not only to the particular subject cell, but also to the progeny or potential progeny of such a cell. Because

certain modifications can occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

The term "complementary" or "complementarity"as used herein with respect to polynucleotides or oligonucleotides (which terms are used interchangeably herein) refers to a measure of the ability of individual strands of such poly- or oligonucleotides to associate with each other. Two major fundamental interactions in RNA are stacking and hydrogen bonding. Both contribute to free-energy changes for associations of oligoribonucleotides. The RNA-RNA interactions include the standard Watson-Crick pairing (A opposite U, and G opposite C) and the non-Watson-Crick pairing (including but not limited to the interaction through the Hoogsteen edge and/or sugar edge) (see e.g., Leontis et al., 2002, Nucleic Acids Research, 30: 3497-3531).

The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of the association between the nucleic acid strands. "Complementarity" between two nucleic acid sequences corresponds to free-energy changes for helix formation. Thus, determination of binding free energies for nucleic acid molecules is useful for predicting the three-dimensional structures of RNAs and for interpreting RNA-RNA associations. e.g., RNAi activity or inhibition of gene expression or formation of double stranded oligonucleotides. Such determination can be made using methods known in the art (see, e.g., Turner et al., 1987, Cold Spring Harb Symp Quant Biol. 52:123-33; Frier et al., 1986, Proc. Nat. Acad. Sci. USA 83:9373- 9377; Turner et al., 1987, J. Am. Chem. Soc. 109:3783-3785).

As the skilled artisan will appreciate, complementarity, where present, can be partial, for example where at least one or more nucleic acid bases between strands can pair according to the canonical base pairing rules. For example, the sequences 5'-CTGACAATCG-3', 5'-CGAAAGTCAG-3' are partially complementary (also referred to herein as "incompletely complementary") to each other. "Partial complementarity" or "partially complementary" as used herein indicates that only a percentage of the contiguous residues of a nucleic acid sequence can form Watson-Crick base pairing with the same number of contiguous residues in a second nucleic acid sequence in an anti-parallel fashion. For example, 5, 6, 7, 8, 9, or 10 nucleotides out of a total of 10 nucleotides in the first oligonucleotide forming Watson-Crick base pairing with a second nucleic acid sequence having 10 nucleotides represents 50%, 60%, 70%, 80%, 90%, and 100% complementarity respectively.

Complementarity can also be total where each and every nucleic acid base of one strand is capable of forming hydrogen bonds according to the canonical base pairing rules, with a corresponding base in another, antiparallel strand. For example, the sequences 5'-CTGACAATCG-3' and 5'-CGATTGTCAG-3' are totally complementary (also referred to herein as "completely complementary") to each other. As used herein "complete complementarity" or "completely complementary" indicates that all the contiguous residues of a nucleic acid sequence can form Watson-Crick base pairing with the same number of contiguous residues in a second nucleic acid sequence in an anti-parallel fashion.

The skilled artisan will appreciate that where there are no bases that can adequately base pair with corresponding contiguous residues in an antiparallel strand, the two strands would be considered to have no complementarity. In certain embodiments herein, at least portions of two antiparallel strands will have no complementarity. In certain embodiments such portions may comprise even a majority of the length of the two strands.

In addition to the foregoing, the skilled artisan will appreciate that in strands of equal length that are completely complementary, all sections of those strands are completely complementary to each other. Strands which are not of equal length, i.e. present in a nucleotide duplex having one or both ends not being blunt, may be considered by those of skill in the art to be completely complementary, however there will be one or more bases in the overhanging end or ends ("overhangs") which do not have corresponding bases in the opposing strand with which to base pair. In the case of strands that are incompletely or partially complementary, it is to be understood that there may be portions or sections of the strands wherein there are several or even many contiguous bases which are completely complementary to each other, and other portions of the incompletely complementary strands which have less than complete complementarity - i.e. those sections are only partially complementary to each other.

The percentage of complementarity between a first nucleotide sequence and a second nucleotide sequence can be evaluated by sequence identity or similarity between the first nucleotide sequence and the complement of the second nucleotide sequence. A nucleotide sequence that is X% complementary to a second nucleotide sequence is X% identical to the complement of the second nucleotide sequence. The "complement of a nucleotide sequence" is completely complementary to the nucleotide sequence, whose sequence is readily deducible from the nucleotide sequence using the rules of Watson-Crick base pairing.

"Sequence identity or similarity", as known in the art, is the relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, identity also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case can be, as determined by the match between strings of such sequences. To determine the percent identity or similarity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same or similar amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical or similar at that position. The percent identity or similarity between the two sequences is a function of the number of identical or similar positions shared by the sequences (i.e., % identity = number of identical positions/total number of positions (e.g., overlapping positions) x 100). In one embodiment, the two sequences are the same length.

Both identity and similarity can be readily calculated. Methods commonly employed to determine identity or similarity between sequences include, but are not limited to those disclosed in Carillo et al, (1988), *SIAM J. Applied Math.* 48, 1073. Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in computer programs.

A non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin et al., (1990), *Proc. Natl. Acad. Sci.* USA 87:2264-2268, modified as in Karlin et al., (1993), *Proc. Natl. Acad. Sci.* USA 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al., (1990), *J Mol. Biol* 215:403-410. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997), *Nucleic Acids Res.* 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. Additionally, the FASTA method (Atschul et al., (1990), *J. Molec. Biol.* 215, 403), can also be used.

Another non-limiting example of a mathematical algorithm useful for the comparison of sequences is the algorithm of Myers et al, (1988), *CABIOS* 4:11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0).

In an embodiment, the percent identity between two sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package. The GCG GAP program aligns two complete sequences to maximize the number of matches and minimizes the number of gaps.

In another embodiment, the percent identity between two sequences is determined using the local homology algorithm of Smith and Waterman (*J Mol Biol.* 1981, 147(1):195-7), which has been incorporated into the BestFit program in the GCG software package. The BestFit program makes an optimal alignment of the best segment of similarity between two sequences. Optimal alignments are found by inserting gaps to maximize the number of matches.

Nucleotide sequences that share a substantial degree of complementarity will form a stable interaction with each other. As used herein, the term "stable interaction" with respect to two nucleotide sequences indicates that the two nucleotide sequences have the natural tendency to interact with each other to form a double stranded molecule. Two nucleotide sequences can form a stable interaction with each other within a wide range of sequence complementarity. In general, the higher the complementarity the stronger or the more stable the interaction. Different strengths of interactions may be required for different processes. For example, the strength of interaction for the purpose of forming a stable nucleotide sequence duplex in vitro may be different from that for the purpose of forming a stable interaction between an iRNA and a binding sequence in vivo. The strength of interaction can be readily determined experimentally or predicted with appropriate software by a person skilled in the art.

Hybridization can be used to test whether two polynucleotides are substantially complementary to each other and to measure how stable the interaction is. Polynucleotides that share a sufficient degree of complementarity will hybridize to each other under various hybridization conditions. In one embodiment, polynucleotides that share a high degree of complementarity thus form a strong stable interaction and will hybridize to each other under stringent hybridization conditions. "Stringent hybridization conditions" has the meaning known in the art, as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second

Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989). An exemplary stringent hybridization condition comprises hybridization in 6x sodium chloride/sodium citrate (SSC) at about 45 °C, followed by one or more washes in 0.2x SSC and 0.1% SDS at 50 - 65 °C.

As used herein the term "mismatch" refers to a nucleotide of either strand of two interacting strands having no corresponding nucleotide on the corresponding strand or a nucleotide of either strand of two interacting strands having a corresponding nucleotide on the corresponding strand that is non-complementary.

As used herein, a "match" refers to a complementary pairing of nucleotides.

As used herein, the term "expression system" refers to any in vivo or in vitro system that can be used to evaluate the expression of a target RNA molecule and or the RNAi activity of a multitargeting RNA molecule of the invention. In particular embodiments, the "expression system" comprises one or more target RNA molecules, a multitargeting interfering RNA molecule targeting the one or more target RNA molecules, and a cell or any type of in vitro expression system known to a person skilled in the art that allows expression of the target RNA molecules and RNAi.

As used herein, the term "RNA" includes any molecule comprising at least one ribonucleotide residue, including those possessing one or more natural nucleotides of the following bases: adenine, cytosine, guanine, and uracil; abbreviated A, C, G, and U, respectively, modified ribonucleotides, universal base, acyclic nucleotide, abasic nucleotide and non-ribonucleotides. "Ribonucleotide" means a nucleotide with a hydroxyl group at the 2' position of a p- D-ribo-furanose moiety.

Modified ribonucleotides include, for example 2'deoxy, 2'deoxy-2'-fluoro, 2'O-methyl, 2'O-methoxyethyl, 4'thio or locked nucleic acid (LNA) ribonucleotides. Also contemplated herein is the use of various types of ribonucleotide analogues, and RNA with internucleotide linkage (backbone) modifications. Modified internucleotide linkages include for example, phosphorothioate-modified, and even inverted linkages (i.e. 3'-3' or 5'-5'). Preferred ribonucleotide analogues include sugar-modified, and nucleobase-modified ribonucleotides, as well as combinations thereof. In preferred sugar-modified ribonucleotides the 2' - OH-group is replaced by a substituent selected from H, OR, R, halo, SH, SR, NH_2, NHR, NR_2 or ON, wherein R is C1-C6 alkyl, alkenyl or alkynyl and halo is F, Cl, Br, or I. In preferred backbone-modified ribonucleotides the phosphoester group connecting to adjacent ribonucleotides is replaced by a

modified group, e.g. a phosphorothioate group. Any or all of the above modifications may be combined. In addition, the 5'termini can be OH, phosphate, diphosphate or triphosphate. Nucleobase-modified ribonucleotides, i.e. ribonucleotides wherein the naturally-occurring nucleobase is replaced with a non-naturally occurring nucleobase instead, for example, uridines or cytidines modified at the 5-position (e.g. 5-(2-amino)propyl uridine, and 5-bromo uridine); adenosines and guanosines modified at the 8-position (e.g. 8-bromo guanosine); deaza nucleotides (e.g. 7-deaza-adenosine); O- and N-alkylated nucleotides (e.g. N6-methyl adenosine) are also contemplated for use herein.

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

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

As used herein with respect to the listing of RNA sequences, the bases thymidine ("T") and uridine ("U") are frequently interchangeable depending on the source of the sequence information (DNA or RNA). Therefore, in disclosure of target sequences, seed sequences, candidate seeds, consensus target sequences, target RNA binding sites, and the like, the base "T" is fully interchangeable with the base "U". However, with respect to specific disclosures of the interfering RNA molecules of the invention, it is to be understood that for such sequences the use of the base "U" cannot be generally substituted with "T" in a functional manner. It is however known in the art that certain occurrences of the base "U" in RNA molecules can be substituted with "T" without substantially deleterious effect on functionality. For example, the substitution of T for U in overhangs, such as UU overhangs at the 3' end is known to be silent, or at a minimum, acceptable, and thus is permissible in the interfering RNA sequences provided herein. Thus, it is contemplated that the skilled artisan will appreciate how to vary even the specific interfering RNA sequences disclosed herein to arrive at other structurally-related and

functionally-equivalent structures that are within the scope of the instant invention and the appended claims.

A "target RNA molecule" or a "pre-selected target RNA molecule" as used herein refers to any RNA molecule whose expression or activity is desired to be modulated, for example decreased, by an interfering RNA molecule of the invention in an expression system. A "target RNA molecule" can be a messenger RNA (mRNA) molecule that encodes a polypeptide of interest. A messenger RNA molecule typically includes a coding region and non-coding regions preceding ("5'UTR") and following ("3'UTR") the coding region. A "target RNA molecule" can also be a non-coding RNA (ncRNA), such as small temporal RNA (stRNA), micro RNA (miRNA), small nuclear RNA (snRNA), short interfering RNA (siRNA), small nucleolar RNA (snoRNA), ribosomal RNA (rRNA), transfer RNA (tRNA) and precursor RNAs thereof. Such non-coding RNAs can also serve as target RNA molecules because ncRNA is involved in functional or regulatory cellular processes. Aberrant ncRNA activity leading to disease can therefore be modulated by multitargeting interfering RNA molecules of the invention. The target RNA can further be the genome of a virus, for example a RNA virus, or a replicative intermediate of any virus at any stage, as well as any combination of these.

The "target RNA molecule" can be a RNA molecule that is endogenous to a biological system, or a RNA molecule that is exogenous to the biological system, such as a RNA molecule of a pathogen, for example a virus, which is present in a cell after infection thereof. A cell containing the target RNA can be derived from or contained in any organism, for example a plant, animal, protozoan, virus, bacterium, or fungus. Non-limiting examples of plants include monocots, dicots, or gymnosperms. Non-limiting examples of animals include vertebrates or invertebrates. Non-limiting examples of fungi include molds or yeasts.

A "target RNA molecule" as used herein may include any variants or polymorphism of a desired RNA molecule. Most genes are polymorphic in that a low but nevertheless significant rate of sequence variability occurs in a gene among individuals of the same species. Thus, a RNA molecule may correlate with multiple sequence entries, each of which represents a variant or a polymorphism of the RNA molecule. In designing any gene suppression tool there is the risk that the selected binding sequence(s) used in the computer-based design may contain relatively infrequent alleles. As a result, the active sequence designed might be expected to provide the required benefit in only a small proportion of individuals. The frequency, nature and

position of most variants (often referred to as single nucleotide polymorphisms (SNPs)) are easily accessible to those trained in the art. In this respect, sequences within a target molecule that are known to be highly polymorphic can be avoided in the selection of binding sequences during the bioinformatic screen. Alternatively, a limitless number of sequences available for any particular target may be used in the design stages of an interfering RNA of the invention to make sure that the targeted binding sequence is present in the majority of allelic variants, with the exception of the situation in which targeting of the allelic variant is desired (that is, when the allelic variant itself is implicated in the disease of interest).

A "target RNA molecule" comprises at least one targeted binding sequence that is sufficiently complementary to the guide sequence of an interfering RNA molecule of the invention to allow a stable interaction of the binding sequence with the guide sequence. The targeted binding sequence can be refined to include any part of the transcript sequence (eg. 5'UTR, ORF, 3'UTR) based on the desired effect. For example, translational repression is a frequent mechanism operating in the 3'UTR (eg. as for microRNA). Thus, the targeted binding sequence can include sequences in the 3' UTR for effective translational repression.

The "targeted binding sequence", "binding sequence", or "target sequence" shall all mean a portion of a target RNA molecule sequence comprising a seed sequence and the sequence flanking either one or both ends of the seed, said binding sequence is predicted to form a stable interaction with the guide strand of a multitargeting interfering RNA of the invention based on the complementarity between the guide strand and the binding sequence.

As used herein, the term "non-target transcriptome" or "non-targeted transcriptome" indicates the transcriptome aside from the targeted RNA molecules. For example, when a multitargeting interfering RNA is designed to target a viral RNA, the non-targeted transcriptome is that of the host. When a multitargeting interfering RNA is designed to target a given RNA in a biological system, the non-targeted transcriptome is the transcriptome of the biological system aside from the targeted given RNA.

As used herein the term "seed" or "seed sequence" or "seed region sequence" refers to a sequence of at least about 6 contiguous nucleotides present in a target RNA that is completely complementary to a portion of the guide strand of an interfering RNA. Although 6 or more contiguous bases are preferred, the expression "about 6" refers to the fact that windows of at least 5 or more contiguous bases or more can provide useful candidates in some cases and can

ultimately lead to the design of useful interfering RNAs. Thus, all such seed sequences are contemplated within the scope of the instant invention.

"Conservation or conserved" indicates the extent to which a specific sequence, such as the seed sequence, is found to be represented in a group of related target sequences, regardless of the genetic context of the specific sequence.

"Genetic context" refers to the flanking sequences that surround a specific identified sequence and that are sufficiently long to enable one of average skill in the art to determine its position within a genome or RNA molecule relative to sequence annotations or other markers in common use.

As used herein, the term "interfering RNA" is used to indicate single or double stranded RNA molecules that modulate the presence, processing, transcription, translation, or half-life of a target RNA molecule, for example by mediating RNA interference ("RNAi"), in a sequence-specific manner. As used herein, the term "RNA interference" or "RNAi" is meant to be equivalent to other terms used to describe sequence specific RNA interference, such as post-transcriptional gene silencing, translational inhibition, or epigenetics. This includes, for example, RISC-mediated degradation or translational repression, as well as transcriptional silencing, altered RNA editing, competition for binding to regulatory proteins, and alterations of mRNA splicing. It also encompasses degradation and/or inactivation of the target RNA by other processes known in the art, including but not limited to nonsense-mediated decay, and translocation to P bodies. Thus, the interfering RNAs provided herein (e.g. CODEMIRs and VIROMIRs) may exert their functional effect via any of the foregoing mechanisms alone, or in combination with one or more other means of RNA modulation known in the art. The interfering RNAs provided herein can be used to manipulate or alter the genotype or phenotype of an organism or cell, by intervening in cellular processes such as genetic imprinting, transcription, translation, or nucleic acid processing (e.g., transamination, methylation, etc.).

The term "interfering RNA" is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi, for example short interfering RNA (siRNA), double-stranded RNA (dsRNA), microRNA (miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering modified oligonucleotide, chemically-modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), and others.

The "interfering RNA" can be, for example, a double-stranded polynucleotide molecule comprising self-complementary sense and antisense strand. The "sense" also named "passenger" strand is required for presentation of the "antisense" also named "guide", "guiding", or "target-complementary" strand to the RISC. The guide strand is retained in the active RISC complex and guides the RISC to the target nucleotide sequence by means of complementary base-pairing, which in turn results in RNAi. The relative thermodynamic characteristics of the 5' termini of the two strands of a double-stranded interfering RNA determine which strand will serve the function of a passenger or a guide strand during RNAi. Indeed, the asymmetric RISC formation can be defined by the relative thermodynamic strength of the first four nucleotide-pairs of the 5' termini of an interfering RNA calculated by the nearest-neighbor methods. Hutvagner (2005), *FEBS Letters* 579: 5850-5857. Thus, in designing an interfering RNA of the invention, the guide strand can be pre-determined by the 5' termini thermodynamic characteristics.

In an interfering RNA of the invention, the guide strand can have a sequence completely complementary to one or more but not all binding sequences present in the one or more target RNA molecules. It can also be partially complementary to a binding sequence present in a target RNA molecule, so long as the complementarity is sufficient for the formation of a stable interaction between the guide strand and the binding sequence on the target molecule. The "passenger strand" can be completely or partially complementary to the guide strand, so long as the complementarity is sufficient for the formation of a stable interaction between the guide strand and the passenger strand. Thus, the passenger strand can be completely or partially identical to the binding sequence on a target molecule. Both the passenger strand and the guide strand can be modified and refined to enhance some aspect of the function of the interfering RNA molecule of the invention. For example, various pharmacophores, dyes, markers, ligands, conjugates, antibodies, antigens, polymers, peptides and other molecules can be conveniently linked to the molecules of the invention. The interfering RNA can further comprise a terminal phosphate group, such as a 5'- phosphate or 5',3'- diphosphate. These may be of use to improve cell uptake, stability, tissue targeting or any combination thereof.

The "interfering RNA" can be assembled from two separate oligonucleotides, where one oligonucleotide is the sense strand and the other is the antisense strand. The "interfering RNA" can also be assembled from a single oligonucleotide, where the self-complementary sense and antisense regions of the interfering RNA are linked by means of a nucleic acid based or non-

nucleic acid-based linker(s). The "interfering RNA" can be a polynucleotide with a duplex, asymmetric duplex, hairpin or asymmetric hairpin secondary structure, having self-complementary sense and antisense regions. The "interfering RNA" can also be a single-stranded polynucleotide having one or more loop structures and a stem comprising self-complementary regions (e.g. short hairpin RNA, shRNA), wherein the polynucleotide can be processed either in vivo or in vitro to generate one or more double stranded interfering RNA molecules capable of mediating RNA inactivation. The cleavage of the self-paired region or regions of the single strand RNA to generate double-stranded RNA can occur *in vitro* or *in vivo*, both of which are contemplated for use herein.

The "interfering RNA" can also be a single stranded polynucleotide having nucleotide sequence complementary to a nucleotide sequence in a target nucleic acid molecule or a portion thereof (i.e., the guide strand), for example, where such interfering RNA molecule does not require the presence within the molecule of nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof (i.e., the passenger strand).

As used herein, the term "interfering RNA" need not be limited to those molecules containing only RNA, but further encompasses those possessing one or more modified ribonucleotides and non- nucleotides, such as those described supra.

The term "interfering RNA" includes double- stranded RNA, single-stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the multitargeting interfering RNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in the RNA molecules of the instant invention can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

The interfering RNA of the invention, also termed "multitargeting interfering RNA" is an interfering RNA having a guide strand that can form stable interactions with at least two binding sites present in distinct genetic contexts on one or more target RNA molecules. Examples of the multitargeting interfering RNA include CODEMIRs, COmputationally-DEsigned, Multi-

targeting Interfering RNAs, and VIROMIRs, where these multitargeting interfering RNA molecules are preferentially targeted to viral targets.

"Sequence" means the linear order in which monomers occur in a polymer, for example, the order of amino acids in a polypeptide or the order of nucleotides in a polynucleotide.

A "subject" as used herein, refers to an organism to which the nucleic acid molecules of the invention can be administered. A subject can be an animal or a plant, preferably a mammal, most preferably a human, who has been the object of treatment, observation or experiment, or any cell thereof.

The term "therapeutically effective amount" as used herein, means that amount of active compound or pharmaceutical agent that elicits the biological or medicinal response in a subject that is being sought by a researcher, veterinarian, medical doctor or other clinician, which includes preventing, ameliorating or alleviating the symptoms of the disease or disorder being treated. Methods are known in the art for determining therapeutically effective doses for the instant pharmaceutical composition.

A "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be inserted. Another type of vector is a viral vector, wherein additional DNA segments can be inserted. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e. g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors, expression vectors, are capable of directing the expression of genes to which they are operably linked.

As used herein, "modulate (or modulation of) the expression of an RNA molecule" means any RNA interference mediated regulation of the level and/or biological activity of the RNA molecule. It includes any RNAi-related transcriptional or post-transcriptional gene silencing, such as by cleaving, destabilizing the target RNA molecule or preventing RNA translation. In one embodiment, the term "modulate" can mean "inhibit," but the use of the word "modulate" is not limited to this definition. The modulation of the target RNA molecule is determined in a suitable expression system, for example *in vivo*, in one or more suitable cells, or

in an acellular or *in vitro* expression system such as are known in the art. Routine methods for measuring parameters of the transcription, translation, or other aspects of expression relating to RNA molecules are known in the art, and any such measurements are suitable for use herein.

By "inhibit", "down-regulate", "reduce", or "decrease" as with respect to a target RNA or its expression it is meant that the expression of the gene or level and/or biological activity of target RNA molecules is reduced below that observed in the absence of the nucleic acid molecules (e.g., multitargeting interfering RNA) of the invention. In one embodiment, inhibition, down-regulation or reduction with a multitargeting interfering RNA molecule is greater than that observed in the presence of an inactive or attenuated molecule. In another embodiment, inhibition, down- regulation, or reduction with a multitargeting interfering RNA molecule is greater than that observed in the presence of, for example, multitargeting interfering RNA molecule with scrambled sequence or with mismatches. In another embodiment, inhibition, down-regulation, or reduction of gene expression with a nucleic acid molecule of the instant invention is greater in the presence of the nucleic acid molecule than in its absence.

"Inhibit", "down-regulate", "reduce", or "decrease" as with respect to a target RNA or its expression encompasses, for example, reduction of the amount or rate of transcription or translation of a target RNA, reduction of the amount or rate of activity of the target RNA, and/or a combination of the foregoing in a selected expression system. The skilled artisan will appreciate that a decrease in the total amount of transcription, the rate of transcription, the total amount of translation, or the rate of translation, or even the activity of an encoded gene product are indicative of such a decrease. The "activity" of an RNA refers to any detectable effect the RNA may have in a cell or expression system, including for example, any effect on transcription, such as enhancing or suppressing transcription of itself or another RNA molecule. The measurement of a "decrease" in expression or the determination of the activity of a given RNA can be performed *in vitro* or *in vivo*, in any system known or developed for such purposes, or adaptable thereto. Preferably the measurement of a "decrease" in expression by a particular interfering RNA is made relative to a control, for example, in which no interfering RNA is used. In some comparative embodiments such measurement is made relative to a control in which some other interfering RNA or combination of interfering RNAs is used. Most preferably a change, such as the decrease is statistically significant based on a generally accepted test of statistical significance. However, because of the large number of possible measures and the need

for the ability to rapidly screen candidate interfering RNAs, it is contemplated herein that a given RNA need only show an arithmetic decrease in one such *in vitro* or *in vivo* assay to be considered to show a "decrease in expression" as used herein.

More particularly, the biological modulating activity of the multitargeting interfering RNA is not limited to, or necessarily reliant on, degradation or translational repression by conventional RISC protein complexes involved in siRNA and microRNA gene-silencing, respectively. Indeed, short double-stranded and single-stranded RNA have been shown to have other possible sequence-specific roles via alternative mechanisms. For example, short double-stranded RNA (dsRNA) species may act as modulatory effectors of differentiation/cell activity, possibly through binding to regulatory proteins (Kuwabara, T., et al., (2004), *Cell*, 116: 779-93). Alternatively, dsRNA may lead to the degradation of mRNA through the involvement of AU-rich element (ARE)-binding proteins (Jing, Q., et al., (2005), *Cell*, 120: 623-34). Further, dsRNA may also induce epigenetic transcriptional silencing (Morris, K.V., et al., (2004) *Science*, 305: 1289-89). Processing of mRNA can also be altered through A to I editing and modified splicing.

As used herein, "palindrome" or "palindromic sequence" means a nucleic acid sequence that is completely complementary to a second nucleotide sequence that is identical to the nucleic acid sequence, e.g., UGGCCA. The term also includes a nucleic acid molecule comprising two nucleotide sequences that are palindromic sequences.

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

The present invention provides a multitargeting interfering RNA molecule comprising a guide strand that forms stable interactions with at least two binding sequences present in distinct genetic contexts in one or more pre-selected target RNA molecules. In one general aspect, the

present invention provides a multitargeting interfering RNA molecule comprising a guide strand of the Formula (I):

5'-p-XSY-3' (Formula I)

In Formula (I), p consists of a terminal phosphate group that can be present or absent from the 5'-end of the guide strand. Any terminal phosphate group known to a person skilled in the art can be used. Such phosphate groups include, but are not limited to, monophosphate, diphosphate, triphosphate, cyclic phosphate or to a chemical derivative of phosphate such as a phosphate ester linkage.

In Formula (I), S consists of a first nucleotide sequence of a length of about 5 to about 20 nucleotides that is at least partially, preferably completely, complementary to a first portion of each of at least two binding sequences present in distinct genetic contexts in one or more pre-selected target RNA molecules. In particular embodiments, S has a length of about 6 to about 15 nucleotides, such as a length of 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 nucleotides that are at least partially, preferably completely, complementary to the first portion of the at least two binding sequences. In one embodiment, S is completely complementary to a seed sequence of each of one, two, three, four, five, or more distinct binding sequences present in distinct genetic contexts in one or more pre-selected target RNA molecules. The skilled artisan will appreciate that the at least two distinct binding sequences may be on the same target RNA molecule, or they can be on different RNA molecules.

In certain embodiments, S consists essentially of a nucleotide sequence selected from the group consisting of:

5' UAUGUGGGUGGG (SEQ ID NO: 1) 3', UGUUUUG (SEQ ID NO: 2), ACCCCGUCUCU (SEQ ID NO: 5), AGCUGCA (SEQ ID NO: 7), AAACAAUGGAAUG (SEQ ID NO: 8), GGUAGGUGGGUGGG (SEQ ID NO: 10), CUGCUUGAU (SEQ ID NO: 12), UCCUUUCCA (SEQ ID NO: 13), UUUUUCUUU (SEQ ID NO: 14), UUCUGAUGUUU (SEQ ID NO: 15), UCUUCCUCUAU (SEQ ID NO: 16), UGGUAGCUGAA (SEQ ID NO: 17), CUUUGGUUCCU (SEQ ID NO: 18), CUACUAAUGCU (SEQ ID NO: 19), UCCUGCUUGAU (SEQ ID NO: 20), AUUCUUUAGUU (SEQ ID NO: 21), CCAUCUUCCUG (SEQ ID NO: 22), CCUCCAAUUCC (SEQ ID NO: 23), CUAAUACUGUA (SEQ ID NO: 24), UUCUGUUAGUG

(SEQ ID NO: 25), GCUGCUUGAUG (SEQ ID NO: 26), ACAUUGUACUG (SEQ ID NO: 27), UGAUAUUUCUC (SEQ ID NO: 28), AACAGCAGUUG (SEQ ID NO: 29), GUGCUGAUAUU (SEQ ID NO: 30), CCCAUCUCCAC (SEQ ID NO: 31), UAUUGGUAUUA (SEQ ID NO: 32), CAAAUUGUUCU (SEQ ID NO: 33), UACUAUUAAAC (SEQ ID NO: 34), GCCUAUCAUAU (SEQ ID NO:58), UGGUGCCUGCU (SEQ ID NO: 59), AAUUAAUAUGGC (SEQ ID NO: 60), CCCUCUGGGCU (SEQ ID NO: 61), UUCUUCCUCAU (SEQ ID NO: 62), UAUUUAUACAGA (SEQ ID NO: 63), CACCAAAAUUC (SEQ ID NO: 64), UGAGUNNGAACAUU (SEQ ID NO: 72) where N is any base, CUCCAGG (SEQ ID NO: 74), UCAGUGGG (SEQ ID NO: 76), UCCUCACAGGG (SEQ ID NO: 78), GUGCUCAUGGUG (SEQ ID NO: 79), CCUGGAGCCCUG (SEQ ID NO: 80), UCUCAGCUCCAC (SEQ ID NO: 81), ACCCUCGCACC (SEQ ID NO: 86), GUGUUGAAG (SEQ ID NO: 88), UUCCACAAC (SEQ ID NO: 90), UCCACUGUC (SEQ ID NO: 92), CAGAAUAG (SEQ ID NO: 93), AACUCUCUA (SEQ ID NO: 94) and CGUGAAGAC (SEQ ID NO: 98).

In certain preferred embodiments, S consists essentially of a nucleotide sequence selected from the group consisting of: UAUGUGGGUGGG (SEQ ID NO: 1), UCCUCACAGGG (SEQ ID NO: 78), GUGUUGAAG (SEQ ID NO: 88), UUCCACAAC (SEQ ID NO: 90), AACUCUCUA (SEQ ID NO: 94) and CGUGAAGAC (SEQ ID NO: 98).

In other embodiments, S consists essentially of a nucleotide sequence of 6 or more contiguous bases contained within any of the sequences selected from the group consisting of: UAUGUGGGUGGG (SEQ ID NO: 1), UCCUCACAGGG (SEQ ID NO: 78), GUGUUGAAG (SEQ ID NO: 88), UUCCACAAC (SEQ ID NO: 90), AACUCUCUA (SEQ ID NO: 94) and CGUGAAGAC (SEQ ID NO: 98).

In certain embodiments, S is partially complementary to a first portion of at least two distinct binding sequences present in distinct genetic contexts in one or more pre-selected target RNA molecules, such as 6 of 7, 7 of 8, 8 of 9, 9 of 10, 10 of 11, 11 of 12, 12 of 13, 13 of 14, 14 of 15, or 15 of 16 consecutive nucleotides of S are completely complementary to the first portion of at least two target RNA binding sequences. In other embodiments, S and the first portion of the distinct binding sequences have lesser overall complementarity such as 10 of 12, 11 of 13, 12 of 14, 13 of 15, or 14 of 16 nucleotides of complete complementarity.

In Formula (I), X is absent or consists of a second nucleotide sequence. In particular embodiments, X consists of one or two nucleotides.

In Formula (I), Y is absent or consists of a third nucleotide sequence, provided that X and Y are not absent simultaneously. Y has complementarity that ranges from complete to nonexistent with respect to a second portion of each of the at least two distinct binding sequences, where the second portion is adjacent to and connected with the 5'-end of the first portion of the binding sequences. In one embodiment, Y is at least partially complementary to a second portion of at least one binding sequence, thus allowing the guide strand to have improved interaction with the at least one binding sequence. Preferably, Y provides optimal or desired binding to each of the second portions of the distinct binding sequences by comprising a consensus-like sequence to which these second portions can bind. This aspect of having a region of less than complete complementarity in the guide strand is particularly useful in certain embodiments, for example, by providing an area of some consensus between distinct binding sequences.

In particular embodiments of the invention, by combining in the guide strand, S with complete complementarity to a seed portion of each binding sequence, and Y, that is incompletely complementary to a second portion of each binding sequence, the overall nucleotide sequence of XSY is such that it is at least partially complementary to each of the distinct binding sequences to allow a stable interaction with each of the binding sequences, thus providing multitargeting interfering RNA of any target molecules comprising the binding sequences. In some embodiments XSY may be fully complementary to at least one of the distinct binding sequences. In other embodiments, XSY is partially complementary to both distinct binding sequences.

The multitargeting interfering RNA can comprise both a guide strand of formula (I) described supra and a passenger strand that is at least partially complementary to the guide strand to allow formation of stable duplexes between the passenger strand and the guide strand. The passenger strand and the guide strand can be completely complementary to each other. The passenger strand and the guide strand can have the same or different length. In an embodiment of the present invention, each strand of a multitargeting interfering RNA molecule of the invention is independently about 17 to about 25 nucleotides in length, in specific embodiments about 17, 18, 19, 20, 21, 22, 23, 24, and 25 nucleotides in length. Using shorter length

interfering RNA molecules without the need for the generation of multiple active sequences through processing of RNA by enzymes such as Dicer and RNaseIII, provides advantages, for example, in reduction of cost, manufacturing, and chance of off-target effects.

The interaction between the passenger strand and the guide strand can be adjusted to improve loading of the guide strand into the cellular RISC complex (Khvorova et al. (2003) Cell, 115: 209-16; Schwarz et al. (2003) Cell, 115: 199-208), or to otherwise improve the functional aspects of the multitargeting interfering RNA. The skilled artisan will appreciate that there are routine methods for altering the strength and other properties of the base paired strands through the addition, deletion, or substitution of one or more bases in either strand of the synthetic duplex. In particular as one example, these strategies can be applied to the design of the extremities of the duplex to ensure that the predicted thermodynamics of the duplex are conducive to the loading of the desired strand. These strategies are well known to persons skilled in the art.

It is also contemplated herein that a substantially double-stranded RNA molecule comprises a single-stranded RNA molecule with, for example, a hairpin loop or similar secondary structure that allows the molecule to self-pair to form at least a region of double-stranded nucleic acid comprising the guide strand of Formula (I).

The skilled artisan will appreciate that the double-stranded RNA molecules provide certain advantages for use in therapeutic applications. Although blunt-ended molecules are disclosed herein for certain embodiments, in various other embodiments, overhangs, for example of 1-5 nucleotides, are present at either or both termini. In some embodiments, the overhangs are 2 or 3 bases in length. Presently preferred overhangs include 3'-UU overhangs in certain embodiments. Other overhangs exemplified for use herein include, but are not limited to, 3'-AA, 3'-CA, 3'-AU, 3'-UC, 3'-CU, 3'-UG, 3'-CC, 3'-UA, 3'-U, and 3'-A. Still other either 5'-, or more preferably 3'-, overhangs of various lengths and compositions are contemplated for use herein on the RNA molecules provided.

In certain embodiments at least one target RNA molecule is an mRNA. More specifically, in some embodiments at least one target encodes a receptor, cytokine, transcription factor, regulatory protein, signaling protein, cytoskeletal protein, transporter, enzyme, hormone, or antigen. As such, the potential range of protein targets in the cell is not limited, however the skilled artisan will appreciate that certain targets are more likely to be of value in a particular

disease state or process. In addition, the skilled artisan will appreciate that target RNA molecules, whether coding or regulatory, originating from a pathogen (e.g. a virus) are useful with the multitargeting RNAs and methods provided herein.

In one embodiment, at least one of the binding sequences is in the 3' UTR of an mRNA. In embodiments featuring multitargeting of different RNA molecules, preferably the target RNAs are not solely splice variants of a single gene, nor solely isoforms of each other. In other embodiments where it is vital or preferred to modulate some or all such splice variants or isoforms, the multiple targets may encompass such sequences.

The inclusion of one target or more targets does not preclude the use of, or intention for, a particular interfering RNA to target another selected target. Such targeting of any additional RNA target molecules may result in less, equal, or greater effect in an expression system. Notwithstanding the foregoing, the multitargeting interfering RNAs of the instant invention are preferably screened for off-target effects, especially those that are likely. For example, reviewing the potential binding to the entire transcriptome, or as much as of it as is known at the time provides a useful approach to such screening. For example, where a genome has been completely sequenced, the skilled artisan will appreciate that the entire transcriptome can be conveniently screened for likely off-target effects. In cases for which local delivery of multitargeting interfering RNA is anticipated, specialized tissue-specific transcriptomes (eg retina for ocular applications) may be more relevant because non-target transcripts that are identified through bioinformatic approaches from the complete transcriptome may actually not be present in the tissue into which the multitargeting interfering RNA is applied.

In one embodiment, the guide strand of a multitargeting interfering RNA of the invention forms stable interactions with at least two targeted binding sequences present in distinct genetic contexts on a single target RNA molecule, thus modulating the expression or activity of the RNA molecule. Targeting multiple binding sites on a single target RNA molecule with a single guide strand can provide more effective RNAi of the target RNA molecule. This approach is particularly useful for the modulation of virus gene expression where the mutation rate is high.

In another embodiment, the guide strand of a multitargeting interfering RNA of the invention forms stable interactions with at least two distinct binding sequences present in distinct genetic contexts on multiple pre-selected target RNA molecules, thus modulating the expression

or activity of multiple pre-selected target RNA molecules. Targeting multiple target RNA molecules with a single guide strand represents an alternative to the prototypical one-drug, one-target approach. In considering the complexity of biological systems, designing a drug selective for multiple targets will lead to new and more effective medications for a variety of diseases and disorders.

In specific embodiments, RNA molecules that are involved in a disease or disorder of a biological system are pre-selected and targeted by a multitargeting interfering RNA molecule of the invention. The biological system can be, for example, a plant, or an animal such as a rat, a mouse, a dog, a pig, a monkey, and a human. The pre-selected target RNA molecules can, for example, encode a protein of a class selected from the group consisting of receptors, cytokines, transcription factors, regulatory proteins, signaling proteins, cytoskeletal proteins, transporters, enzymes, hormones, and antigens. The pre-selected target RNA molecules can, for example, encode a protein selected from the group consisting of ICAM-1, VEGF-A, MCP-1, IL-8, VEGF-B, IGF-1, Gluc6p, Inppl1, bFGF, PlGF, VEGF-C, VEGF-D, β-catenin, κ-ras-B, κ-ras-A, EGFR, Bcl-2, presenilin-1, BACE-1, MALAT-1, BIC, TGFβ and TNF alpha. Therefore, the multitargeting interfering RNA molecule of the invention can, for example, decrease expression of any combination of ICAM-1, VEGF-B, VEGF-C, VEGF-D, IL-8, bFGF, PlGF, MCP-1 and IGF-1, any combination of ICAM-1, VEGF-A and IGF-1, any combination of β-catenin, κ-ras, and EGFR, both ICAM-1 and VEGF-A, both presenilin-1 and one or more isoforms of BACE-1, both VEGF-A and bFGF, any combination of VEGF-A, ICAM-1, PlGF, and IGF-1, any combination of VEGF-A, κ-ras, EGFR and Bcl-2, both MALAT-1 and BIC, both TGFβ and IL-8 , both IL-8 and MCP-1, any combination of VEGF-A, Bcl-2 and κ-ras, or both Gluc6p and Inppl1, in a biological system, such as an animal.

The pre-selected target RNA molecule can also encode a protein, including an essential protein, for a virus. Such essential proteins can be a protein, for example, that is involved in the replication, transcription, translation, or packaging activity of the virus. Exemplary essential proteins for a HIV virus are GAG, POL, VIF, VPR, TAT, NEF, REV, VPU and ENV, all of which can be a pre-selected target molecule of the invention. The multitargeting interfering RNA of the invention can be used to modulate RNA expression in viruses, including but not limited to, a human immunodeficiency virus (HIV), a hepatitis C virus (HCV), an influenza

virus, a rhinovirus, and a severe acute respiratory syndrome (SARS) virus or a combination thereof.

In some embodiments, the multitargeting interfering RNA of the invention are designed to target one or more target RNA molecules in a first biological system and one or more target molecules in a second biological system that is infectious to the first biological system. In particular embodiments, the multitargeting interfering RNA of the invention are designed to target one or more host RNA molecules and one or more RNA molecules of a virus or a pathogen for the host. Therefore, the multitargeting interfering RNA molecule of the invention can, for example, decrease expression of TNF alpha and modulate expression of a hepatitis C virus (HCV).

In particular embodiments of the invention, specific multitargeting interfering RNA molecules are provided which are functional against specific targets. These multitargeting interfering RNA molecules are useful for modulating expression of RNA, for example, their intended target RNA molecules, and data supporting the activity are also provided herein in the working examples.

In one embodiment, synthetic multitargeting interfering RNA molecules comprising the sequence UAUGUGGGUGGG (SEQ ID NO: 1), or UGUUUUG (SEQ ID NO: 2), are provided. In one embodiment the molecules are double stranded in at least the region comprising the recited sequence. Preferably the molecules decrease expression of both VEGF-A and ICAM-1 in an expression system. In another embodiment, the multitargeting interfering RNA molecules decrease expression of at least one target RNA molecule comprising the sequence CCCACCCACAUA (SEQ ID NO: 3), or CAAAACA (SEQ ID NO: 4). More preferably they target multiple sites on two or more RNAs.

Multitargeting interfering RNA molecules comprising the sequence ACCCCGUCUCU (SEQ ID NO: 5) are also provided herein. In one embodiment the molecules are double stranded in at least the region comprising the recited sequence. Preferably, the multitargeting interfering RNAs decrease expression of any combination of ICAM-1, VEGF-A and IGF-1 in an expression system. In another embodiment, in an expression system, the multitargeting interfering RNA molecules decrease expression of at least one target RNA molecule comprising the sequence

AGACACGGCGU (SEQ ID NO: 6). More preferably they target multiple sites on two or more RNAs.

Also provided herein are multitargeting interfering RNA molecules comprising the sequence AGCUGCA (SEQ ID NO: 7). In one embodiment the molecules are double stranded in at least the region comprising the recited sequence. The multitargeting interfering RNAs preferably decrease expression of any combination of ICAM-1, VEGF-B, VEGF-C, VEGF-D, IL-8, bFGF, PlGF, MCP-1 and IGF-1 in an expression system. In one embodiment, in an expression system the multitargeting interfering RNA molecules modulate expression of at least one target RNA molecule comprising the sequence UGCAGCU. More preferably they target multiple sites on two or more RNAs.

In another embodiment, multitargeting interfering RNA molecules comprising the sequence AAACAAUGGAAUG (SEQ ID NO: 8) are provided. In one embodiment the molecules are double stranded in at least the region comprising the recited sequence. The multitargeting interfering RNAs preferably modulate expression of any combination of κ-ras, EGFR and β catenin in an expression system. Preferably in an expression system, the multitargeting interfering RNA molecules modulate expression of at least one target RNA molecule comprising the sequence CAUUCCAUUGUUU (SEQ ID NO: 9). More preferably they target multiple sites on two or more RNAs.

Also provided herein are multitargeting interfering RNA molecules comprising the sequence GGUAGGUGGGUGGG (SEQ ID NO: 10). In one embodiment the molecules are double stranded in at least the region comprising the recited sequence. The multitargeting interfering RNAs preferably modulate expression of gluc6p and Inppl1 in an expression system. In an expression system, the multitargeting interfering molecules preferably modulate expression of at least one target RNA molecule comprising the sequence CCCACCCACCUACC (SEQ ID NO: 11). More preferably they target either multiple sites on a single RNA or multiple sites on two or more RNAs.

Also provided herein are multitargeting interfering RNA molecules comprising the sequence CUGCUUGAU (SEQ ID NO: 12), UCCUUUCCA (SEQ ID NO: 13), UUUUUCUUU (SEQ ID NO: 14), UUCUGAUGUUU (SEQ ID NO: 15), UCUUCCUCUAU (SEQ ID NO: 16), UGGUAGCUGAA (SEQ ID NO: 17), CUUUGGUUCCU (SEQ ID NO: 18), CUACUAAUGCU (SEQ ID NO: 19), UCCUGCUUGAU (SEQ ID NO: 20),

AUUCUUUAGUU (SEQ ID NO: 21), CCAUCUUCCUG (SEQ ID NO: 22), CCUCCAAUUCC (SEQ ID NO: 23), CUAAUACUGUA (SEQ ID NO: 24), UUCUGUUAGUG (SEQ ID NO: 25), GCUGCUUGAUG (SEQ ID NO: 26), ACAUUGUACUG (SEQ ID NO: 27), UGAUAUUUCUC (SEQ ID NO: 28), AACAGCAGUUG (SEQ ID NO: 29), GUGCUGAUAUU (SEQ ID NO: 30), CCCAUCUCCAC (SEQ ID NO: 31), UAUUGGUAUUA (SEQ ID NO: 32), CAAAUUGUUCU (SEQ ID NO: 33), or UACUAUUAAAC (SEQ ID NO: 34). In one embodiment the molecules are double stranded in at least the region comprising the recited sequence. In an expression system, the multitargeting interfering RNAs preferably modulate expression of at least one RNA encoded by an HIV genome. More preferably the multitargeting interfering molecules modulate expression of at least two RNAs so encoded. In another embodiment the multitargeting interfering RNA molecules modulate expression of at least three, or even four, HIV RNAs. Preferably, the multitargeting interfering RNA molecules decrease expression of at least one target RNA molecule comprising the sequence AUCAAGCAG (SEQ ID NO: 35), UGGAAAGGA (SEQ ID NO: 36), AAAGAAAAA (SEQ ID NO: 37), AAACAUCAGAA (SEQ ID NO: 38), AUAGAGGAAGA (SEQ ID NO: 39), UUCAGCUACCA (SEQ ID NO: 40), AGGAACCAAAG (SEQ ID NO: 41), AGCAUUAGUAG (SEQ ID NO: 42), AUCAAGCAGGA (SEQ ID NO: 43), AACUAAAGAAU (SEQ ID NO: 44), CAGGAAGAUGG (SEQ ID NO: 45), GGAAUUGGAGG (SEQ ID NO: 46), UACAGUAUUAG (SEQ ID NO: 47), CACUAACAGAA (SEQ ID NO: 48), CAUCAAGCAGC (SEQ ID NO: 49), CAGUACAAUGU (SEQ ID NO: 50), GAGAAAUAUCA (SEQ ID NO: 51), CAACUGCUGUU (SEQ ID NO: 52), AAUAUCAGCAC (SEQ ID NO: 53), GUGGAGAUGGG (SEQ ID NO: 54), UAAUACCAAUA (SEQ ID NO: 55), AGAACAAUUUG (SEQ ID NO: 56) or GUUUAAUAGUA (SEQ ID NO: 57), when measured in a selected expression system. More preferably they target multiple sites on two or more RNAs.

In another embodiment, multitargeting interfering RNA molecules comprising the sequence GCCUAUCAUAU (SEQ ID NO: 58), UGGUGCCUGCU (SEQ ID NO: 59), AAUUAAUAUGGC (SEQ ID NO: 60), CCCUCUGGGCU (SEQ ID NO: 61), UUCUUCCUCAU (SEQ ID NO: 62), UAUUUAUACAGA (SEQ ID NO: 63), or CACCAAAAUUC (SEQ ID NO: 64) are provided. In one embodiment the molecules are

double stranded in at least the region comprising the recited sequence. Preferably the multitargeting interfering RNAs modulate expression of presenilin-1 and one or more isoforms of BACE-1 in an expression system. Also preferred are such multitargeting interfering RNA molecules that in an expression system modulate expression of at least one target RNA molecule comprising the sequence AUAUGAUAGGC (SEQ ID NO: 65), AGCAGGCACCA (SEQ ID NO: 66), GCCAUAUUAAUU (SEQ ID NO: 67), AGCCCAGAGGG (SEQ ID NO: 68), AUGAGGAAGAA (SEQ ID NO: 69), UCUGUAUAAAUA (SEQ ID NO: 70), or GAAUUUUGGUG (SEQ ID NO: 71). More preferably they target multiple sites on two or more RNAs.

Also provided herein are multitargeting interfering RNA molecules comprising the sequence UGAGUNNGAACAUU (SEQ ID NO: 72), where N is any base. In one embodiment the molecules are double stranded in at least the region comprising the recited sequence. In a preferred embodiment, the multitargeting interfering RNAs modulate expression of VEGF-A and bFGF in an expression system. The multitargeting interfering RNA molecules preferably modulate expression in an expression system of at least one target RNA molecule comprising the sequence AAUGUUCVVACUCA (SEQ ID NO: 73) where VV is CC or AG. More preferably they target multiple sites on two or more RNAs.

Also provided herein are multitargeting interfering RNA molecules comprising the sequence CUCCAGG (SEQ ID NO: 74). In one embodiment the molecules are double stranded in at least the region comprising the recited sequence. These multitargeting interfering RNAs, in one embodiment, modulate expression of any combination of VEGF-A, ICAM-1, PlGF and IGF-1 in an expression system. The multitargeting interfering RNA molecules preferably modulate expression, in an expression system, of at least one target RNA molecule comprising the sequence CCUGGAG (SEQ ID NO: 75). More preferably they target multiple sites on two or more RNAs.

In another embodiment, multitargeting interfering RNA molecules comprising the sequence UCAGUGGG (SEQ ID NO: 76) are provided herein. In one embodiment the molecules are double stranded in at least the region comprising the recited sequence. Preferably, the multitargeting interfering RNAs modulate expression of any combination of VEGF-A, K-ras, EGFR and bcl 2 in an expression system. Also preferred are those multitargeting interfering RNA molecules that modulate expression of at least one target RNA molecule comprising the

sequence CCCACUGA (SEQ ID NO: 77) in such an expression system. More preferably they target multiple sites on two or more RNAs.

In yet another embodiment, provided are multitargeting interfering RNA molecules comprising the sequence UCCUCACAGGG (SEQ ID NO: 78), GUGCUCAUGGUG (SEQ ID NO: 79), CCUGGAGCCCUG (SEQ ID NO: 80), or UCUCAGCUCCAC (SEQ ID NO: 81). In one embodiment the molecules are double stranded in at least the region comprising the recited sequence. The multitargeting interfering RNAs preferably decrease expression of TNFα and at least one RNA encoded by the HCV genome in an expression system. The multitargeting interfering RNA molecules preferably decrease expression of at least one target RNA molecule comprising the sequence CCCUGUGAGGA (SEQ ID NO: 82), CACCAUGAGCAC (SEQ ID NO: 83), CAGGGCUCCAGG (SEQ ID NO: 84), or GUGGAGCUGAGA (SEQ ID NO: 85) in an expression system. More preferably they target multiple sites on two or more RNAs.

Also provided herein are multitargeting interfering RNA molecules comprising the sequence ACCCUCGCACC (SEQ ID NO: 86). In one embodiment the molecules are double stranded in at least the region comprising the recited sequence. The multitargeting interfering RNAs preferably modulate expression of MALAT-1 and BIC in an expression system. In an expression system, the multitargeting interfering molecules preferably modulate expression of at least one target RNA molecule comprising the sequence GGUGCGAGGGU (SEQ ID NO: 87). More preferably they target either multiple sites on a single RNA or multiple sites on two or more RNAs.

In yet another embodiment, provided are multitargeting interfering RNA molecules comprising the sequence GUGUUGAAG (SEQ ID NO: 88). In one embodiment the molecules are double stranded in at least the region comprising the recited sequence. The multitargeting interfering RNAs preferably modulate expression of TGFβ and IL-8 in an expression system. In an expression system, the multitargeting interfering molecules preferably modulate expression of at least one target RNA molecule comprising the sequence CUUCAACAC (SEQ ID NO: 89). More preferably they target either multiple sites on a single RNA or multiple sites on two or more RNAs.

In another embodiment, provided are multitargeting interfering RNA molecules comprising the sequence UUCCACAAC (SEQ ID NO: 90). In one embodiment the molecules are double stranded in at least the region comprising the recited sequence. The multitargeting

interfering RNAs preferably modulate expression of IL-8 and MCP-1 in an expression system. In an expression system, the multitargeting interfering molecules preferably modulate expression of at least one target RNA molecule comprising the sequence GUUGUGGAA (SEQ ID NO: 91). More preferably they target either multiple sites on a single RNA or multiple sites on two or more RNAs.

Also provided herein are multitargeting interfering RNA molecules comprising the sequences UCCACUGUC (SEQ ID NO: 92), CAGAAUAG (SEQ ID NO: 93) or AACUCUCUA (SEQ ID NO: 94). In one embodiment the molecules are double stranded in at least the region comprising the recited sequence. These multitargeting interfering RNAs, in one embodiment, modulate expression of any combination of VEGF-A, Bcl-2 and κ-Ras in an expression system. The multitargeting interfering RNA molecules preferably modulate expression, in an expression system, of at least one target RNA molecule comprising the sequences GACAGUGGA (SEQ ID NO: 95), CUAUUCUG (SEQ ID NO: 96) or UAGAGAGUU (SEQ ID NO: 97). More preferably they target multiple sites on two or more RNAs.

In another embodiment, provided are multitargeting interfering RNA molecules comprising the sequence CGUGAAGAC (SEQ ID NO: 98). In one embodiment the molecules are double stranded in at least the region comprising the recited sequence. The multitargeting interfering RNAs preferably modulate expression of HCV in an expression system. In an expression system, the multitargeting interfering molecules preferably modulate expression of at least one target RNA molecule comprising the sequence GUCUUCACG (SEQ ID NO: 99). More preferably they target either multiple sites on a single RNA or multiple sites on two or more RNAs.

It will be understood by one skilled in the art that these exemplary seeds, and their complete complements, also subsume any number of shorter seeds and their complete complements, respectively, and that these are envisaged as part of the invention. For example, the 12-base seed: CCCACCCACAUA (SEQ ID NO: 3) comprises further two 11-base, three 10-base, four 9-base, five 8-base, six 7-base and seven 6-base seeds, all of which could be used in the design of useful multitargeting interfering RNA.

Also provided herein are multitargeting RNA duplexes consisting essentially of:

5' UAUGUGGGUGGGGUCAGUCUAA 3' (SEQ ID NO: 100)
3'UUAUACACCCACCCACUCAGA 5', (SEQ ID NO: 101)

5' UGUUUUGUUGUUACAUAUGAC 3' (SEQ ID NO: 102)
3'UUACAAAACAACAAUGUAUAC 5', (SEQ ID NO: 103)

5' UAUGUGGGUGGGGUCUCUCUA 3' (SEQ ID NO: 104)
3'UUAUACACCCACCCCACAGAG 5', (SEQ ID NO: 105)

5' UAUGUCCGUGGGGUGGUCUAA 3' (SEQ ID NO: 106)
3'UUAUACACCCACCCCACCAGA 5', (SEQ ID NO: 107)

5' UAUGUGGGUCCGGUGGUGUCU 3' (SEQ ID NO: 108)
3'UUAUACACCCACCCCACCACA 5', (SEQ ID NO: 109)

5' UAUGUGGGUGGCUGAGUGUCU 3' (SEQ ID NO: 110)
3'UUAUACACCCACCCACUCACA 5', (SEQ ID NO: 111)

5' CUCACCCACCCACAUACAUUU 3' (SEQ ID NO: 112)
3'CUGAGUGGGUCCGUGUAUGUA 5', (SEQ ID NO: 113)

5' UCACCCACCCACAUACAUAUU 3' (SEQ ID NO: 114)
3'UGAGUGGGUGGGUGUAUGUAU 5', (SEQ ID NO: 115)

5' UCACCCACCCACAUACAUUUU 3' (SEQ ID NO: 116)
3'UGAGUCCCUGGGUGUAUGUAA 5', (SEQ ID NO: 117)

5' UAUGUGGGUGCGUGAGUCUA 3' (SEQ ID NO: 118)
3'UAUACACCCACCCACUCAGA 5', (SEQ ID NO: 119)

5' GGGUUUACCAGGAAGAUGGUU 3' (SEQ ID NO: 120)
3' UACCCAAAUGGUCCUUCUACC 5', (SEQ ID NO: 121)

5' UUCCUCACAGGGCAGUGAUUC 3' (SEQ ID NO: 122)
3' UUAAGGAGUGUCCCGUCACUA 5', (SEQ ID NO: 123)

5' UUCCUCACAGGGCAGUGAUUC 3' (SEQ ID NO: 122)
3' UUAAAGAGUGUCCCGUCACUA 5', (SEQ ID NO: 124)

5' UUCCUCACAGGGCAGUGGUUC 3' (SEQ ID NO: 125)
3' UUAAGGAGUGUCCCGUCACCA 5', (SEQ ID NO: 126)

5' CCCGGACCCUUAGAGAGUUUU 3' (SEQ ID NO: 127)
3' ACGGGCCUGGGAAUCUCUCAA 5', (SEQ ID NO: 128)

5' UACCCUCGCACCGAUCUCCCAA 3' (SEQ ID NO: 129)
3' UUAUGGGAGCGUGGCUAGAGGG 5', (SEQ ID NO: 130)

5' UACAAAUCUACUUCAACAUUU 3' (SEQ ID NO: 131)
3' GUAUGUUUAGAUGAAGUUGUG 5', (SEQ ID NO: 132)

5' AACAUAUGUUCUUCAACAUUU 3' (SEQ ID NO: 133)
3' GUUUGUAUACAAGAAGUUGUG 5', (SEQ ID NO: 134)

5' UUCCACAACACAAGCUGUGUU 3' (SEQ ID NO: 135)
3' UUAAGGUGUUGUGUUCGACAC 5', (SEQ ID NO: 136)

5' CGACCCUUAGAGAGUUUCAUU 3' (SEQ ID NO: 137)
3' GGCCUGGGAAUCUCUCAAAGU 5', (SEQ ID NO: 138)

5' UUCGUGAAGACGGUGGGCCGA 3' (SEQ ID NO: 139)
3' dTdTAAGCACUUCUGCCACCCGG 5', (SEQ ID NO: 140)
or
5' AGACUCACCCACCCAGAUAUU 3' (SEQ ID NO: 141)
3' AAUCUGAGUGGGUGGGUCUAU 5' (SEQ ID NO: 142)

Such molecules, the skilled artisan will appreciate, can target multiple sites on a single RNA or multiple sites on two or more RNAs and are useful to decrease the expression of such one or preferably two or more such targeted RNAs in an expression system.

In some embodiments, a given multitargeting interfering RNA will be more effective at modulating expression of one of several target RNAs than another. In other cases, the multitargeting interfering RNA will similarly affect all targets in one or more expression systems. Various factors can be responsible for causing variations in silencing or RNAi efficiency: (i) asymmetry of assembly of the RISC causing the passenger strand to enter more efficiently into the RISC than the guide strand; (ii) inaccessibility of the targeted segment on the target RNA molecule; (iii) a high degree of off-target activity by the interfering RNA; (iv) sequence-dependent variations for natural processing of RNA, and (v) the balance of the structural and kinetic effects described in (i) to (iv). *See* Hossbach et al. (2006), *RNA Biology* 3: 82-89. In designing a multitargeting interfering RNA molecule of the invention, special attention can be given to each of the listed factors to increase or decrease the RNAi efficiency on a given target RNA molecule.

Another general aspect of the invention is a method for designing a multitargeting interfering RNA. The method of the invention includes various means leading to a multitargeting interfering RNA that effectively targets distinct binding sequences present in distinct genetic contexts in one or more pre-selected target RNA molecules. In one embodiment, a multitargeting interfering RNA can be designed by visual or computational inspection of the sequences of the target molecules, for example, by comparing target sequences and identifying sequences of length *n* which occur in the one or more target sequences. Alternatively, all possible sequences of a pre-selected length *n* can be generated by virtue of each permutation possible for each nucleotide position to a given length (4^n) and then examining for their occurrence in the target sequences.

In preferred embodiments, the methods provided herein design interfering RNA molecules, either single or duplex in nature, which have at least one region (S) of complete complementarity to a first portion (a seed) of the distinct binding sequences, and in certain embodiments, further comprises a second region (Y) of at least partial complementarity to a second portion of one or more distinct binding sequences. In other embodiments, the region Y can have complete complementarity to a second portion of one or more distinct binding

sequences, and in yet others there may be no complementarity between Y and a second portion of one or more distinct binding sequences.

In one embodiment, a method of designing a multitargeting interfering RNA comprises the steps of a) selecting one or more target RNA molecules, wherein the modulation in expression of the one or more target RNA molecules is desired; b) obtaining at least one nucleotide sequence for each of the one or more target RNA molecules; c) selecting a seed sequence of 6 nucleotides or more, said seed sequence occurs in distinct genetic contexts in the target RNA molecules; d) selecting at least two distinct binding sequences, each of which comprises the seed sequence and is present in distinct genetic contexts in the target molecules; e) designing a multitargeting interfering RNA molecule having a guide strand that shares a substantial degree of complementarity with each of the at least two binding sequences to allow stable interaction therewith.

In one embodiment, the method comprises designing a passenger strand that is at least partially complementary to the guide strand to allow formation of a stable duplex between the passenger strand and the guide strand.

In another embodiment, the method of the invention comprises the steps of: a) selecting one or more target RNA molecules, wherein the modulation in expression of the target RNA molecules is desired; b) obtaining at least one nucleotide sequence for each of the target RNA molecules; c) selecting a length, *n*, in nucleotides, for a seed sequence; wherein n = about 6 or more; d) generating a collection of candidate seed sequences of the length *n* from each of the nucleotide sequences obtained in step b), wherein each candidate seed sequence occurs at least once in nucleotide sequences obtained in step b); e) determining the genetic context of each of the candidate seed sequences in nucleotide sequences obtained in step b), by collecting, for each occurrence of the candidate seed sequence, a desired amount of the 5' and 3' flanking sequence; f) selecting a seed sequence of the length *n* from the candidate seed sequences, wherein the seed sequence occurs in at least two distinct genetic contexts in nucleotide sequences obtained in step b); g) selecting a consensus target sequence, wherein said consensus target sequence comprises the seed sequence and a desired consensus sequence for the sequence flanking either one or both of the 5' and 3' ends of the seed; and h) designing a multitargeting interfering RNA molecule that comprises a guide strand that shares a substantial degree of complementarity with the consensus target sequence to allow stable interaction therewith.

In one embodiment, the method comprises designing a passenger strand that is at least partially complementary to the guide strand to allow formation of a stable duplex between the passenger strand and the guide strand.

One of skill in the art would recognise that several iterations of various steps of the methods can be performed. For example, in some embodiments, the method further comprises repeating steps c) to h) with a different value of *n*. In other embodiments, the method may comprise repeating steps f) to h) for a different seed sequence. In yet other embodiments, the method may comprise selecting a different consensus target sequence based around a particular seed and flanking sequence. The skilled person will recognise that various combinations of the above are also envisaged.

In other embodiments, the method of the invention further comprises the steps of making the multitargeting interfering RNA molecule and testing it in an expression system.

Preferred target RNA molecules are strategically-selected molecules, for example viral or host RNAs involved in disease processes, viral genomes, particularly those of clinical significance, and the like. A detailed discussion of target RNA is provided above and applies equally to this and other aspects of the invention, as if set out in its entirety here. The basis for the selection of a target RNA molecule will be appreciated by those of skill in the art. Preferred target RNAs are those involved in diseases or disorders one wishes to control by the administration of the multitargeting interfering RNA

The step of obtaining the sequences for the selected target is conducted by obtaining sequences from publicly available sources, such as the databases provided by the National Center For Biotechnology Information (NCBI) (through the National Institutes of Health (NIH) in the United States), the European Molecular Biology Laboratories (through the European Bioinformatics Institute throughout Europe) available on the World-Wide Web, or proprietary sources such as fee-based databases and the like. Sequences can also be obtained by direct determination. This may be desirable where a clinical isolate or an unknown gene is involved or of interest, for example, in a disease process. Either complete or incomplete sequences of a target RNA molecule can be used for the design of multitargeting interfering RNA of the invention.

Also provided herein are methods wherein a plurality of independent target nucleotide sequences are obtained in step b) for each of one or more target RNA molecules selected in step

a). The databases described above frequently have multiple sequences available for particular targets. This is especially true where genetic variation is naturally higher, for example with viral sequences. In various embodiments, the plurality of target nucleotide sequences represents strain variation, allelic variation, mutation, or multiple species. The number of such a plurality of sequences may range from several or a low multiple, to numerous - for example dozens or even hundreds or thousands of sequences for a given target. It is especially possible to have such numbers of sequences when working with viral sequences.

The sequences chosen can be further limited based on additional desirable or undesirable features such as areas of low sequence complexity, poor sequence quality, or those that contain artefacts relating to cloning or sequencing such as inclusion of vector-related sequences. Furthermore, regions with extensive inaccessible secondary structure could be filtered out at this stage. Indeed, Luo and Chang have demonstrated that siRNA targeting accessible regions of mRNA structure such as loops were more likely to be effective than those aligned with stems (Luo & Chang, (2004), *Biochem. Biophys. Res. Commun.*, 318: 303-10). The sequences chosen, however, need not be limited to 3'UTR sequences or regions of low secondary structure (as illustrated in some of the specific examples.)

The step of selecting a length of *n* nucleotide bases for a seed sequence is preferably an iterative process that does not require any particular basis or logic at first glance – i.e. the starting seed length may be any number of bases above about 5. The longer the length that is chosen for a seed, the less likely it will appear in a portion of the target RNA, e.g. in a target RNA binding sequence. The shorter the seed sequence length, the more frequently it will occur as would be expected. Preferably, an iterative process is used to find the preferred sequences for candidate seeds as described below. Thus, after a particular value for *n* is used to identify candidate seeds of length *n*, another value (e.g. *n+1, n-1*) will be used and the process can be repeated to identify candidate seed sequences of length *n+1*, *n-1* and so on. In one embodiment, the first scan through target sequences will begin with any seed length (e.g. n=9) and subsequent rounds of searching will either increase or decrease the seed length (e.g. based on the number of seeds returned in previous scans). A person of ordinary skill in the art will recognize that the number of candidate seeds will increase as the length of the seed is decreased.

In a situation where a plurality of sequences are available for particular target RNA molecule (eg viral isolates), it will be appreciated by one of skill in the art that the totality of the

sequences can be searched for candidate seeds. However, candidate seeds may in some cases be found only in a proportion of the sequences for the RNA molecule. In these situations, it may be desirable to prioritise those seeds which occur in a larger proportion of the sequences.

The seeds can be selected from a pool of "candidate seeds," also referred to herein as "seed candidates." Seed candidates include sequences of a particularly desired or selected length present at least once in each of the target molecules. The candidate seeds are preferably generated by computer, for example by moving stepwise along a target sequence with a "window" (expressed in terms of a fixed number of contiguous nucleotides) of the desired or selected seed length. Preferably each step is a single base progression, thus generating a "moving window" of selected length through which the target sequence is sequentially viewed. Other step distances are contemplated, however, the skilled artisan will appreciate that only a step of one nucleotide will allow the generation of all possible seed sequences.

When there is only one target RNA molecule, the pool of candidate seeds includes any sequences of the selected length from the "moving window" scan of the target RNA molecule. When there are multiple target RNA molecules, the pool of candidate seeds includes those sequences of the selected length from the "moving window" scan of the target RNA molecules, which occur at least once in each of the target RNA molecules. Therefore when there are multiple target RNA molecules, step (d) above comprises two substeps: (i) generating a collection of nucleotide sequences of the selected length *n* from each nucleotide sequence obtained in step b); and (ii) selecting candidate seed sequences of the length *n* from the collections of nucleotide sequences, wherein each candidate seed sequence occurs at least once in the nucleotide sequences obtained in step b).

As used herein, the term "distribution" in the context of a seed or candidate seed means the overall average frequency, or number of occurrences, of a seed or candidate seed within a nucleotide sequence of interest. For example, Example 13 (below) has the following pattern of occurrence for a 9 base seed in HCV isolates of genotype 1a/1b:

4 times in genome: 5/155 isolates

3 times in genome: 68/155 isolates

2 times in genome: 50/155 isolates

1 time in genome: 31/155 isolates

0 times in genome: 1/155 isolates

This equates to a "distribution" of 2.29. In comparison, the "conservation" (see below) would be 154/155 (=99%).

In one embodiment, the method further comprises the step of discarding those candidate seed sequences that do not occur with at least a predetermined minimum average rate of occurrences within the sequences obtained for a target RNA molecule. This step allows the method to take into account the average distribution of the candidate seed sequences within sequences for the same target RNA.

In another embodiment, the method further comprises the step of discarding those candidate seed sequences that do not occur within at least a predetermined minimum percentage of the target nucleotide sequences obtained for a target nucleotide molecule (that is, the extent of conservation). This step allows the method to more fully take into account the frequency with which a particular candidate seed occurs across multiple target sequences obtained for a target RNA. The multitargeting function of preferred RNAs made by the methods provided herein is enhanced by the higher representation of a candidate seed sequence both within and across such sets of target sequences.

Preferably the method ultimately chosen will include one or more of these steps, or all of them as needed. For example, in one embodiment, the method further comprises the step of discarding any candidate seed sequence that: is composed of only a single base, is composed only of A and U, has a consecutive string of 5 or more bases which are C, is predicted to have a propensity to undesirably modulate the expression or activity of one or more cellular components, is predicted to occur with unacceptable frequency in the non-target transcriptome of interest; or any combination thereof. In the situation in which a double stranded multitargeting interfering RNA is to be designed, candidate seed sequences may also be discarded if they contain sequences predicted to have a propensity to activate a cellular sensor of foreign nucleic acid, have a consecutive string of 5 or more bases which are G, or a combination thereof.

Seeds then are selected from the pool of candidate sequences as the ones that are present in two different genetic contexts in the one or more target sequences. Genetic contexts are determined by collecting, for each occurrence of the candidate seed sequence, a desired amount of the 5' and 3' flanking sequence.

In an exemplary process of making a multitargeting interfering RNA of the invention, a seed sequence is used to generate one or more "consensus target sequences", which comprises the seed sequence and a desired consensus sequence for the sequence flanking either one or both of the 5' and 3' ends of the seed. The term "consensus target sequence" does not suggest that there is only one best sequence approximating multiple binding sequences on target molecule(s), rather a population of one or more alternative sequences may all be consensus target sequences. The "consensus sequence for the sequence flanking either one or both of the 5' and 3' ends of the seed" is readily derived from the examination of the genetic context of seed sequence in each of the target molecules by visual inspection, or through the use of bioinformatic tools or calculations. While the seed sequence portion of a consensus target sequence will usually have complete identity to a corresponding portion in each of targeted binding sites, the consensus sequence for the sequence flanking either one or both of the 5' and 3' ends of the seed need not be completely identical to the sequence flanking the 5' and 3' ends of the seed of some but not all of the sequences of the target molecules. However, it may be identical to some, all or none of the sequences.

Preferably, when a double stranded molecule is to be designed, the consensus target sequence does not include any sequence that is predicted to have a propensity to undesirably modulate the expression or activity of one or more cellular components.

Consensus target sequences may be determined by eye or by algorithm. For example, a computer algorithm can be used to score all possible permutations of paired nucleotides in the positions in which the sequences are different between the at least two targets. This is particularly useful when the targets have some identity beyond the seed, but for which an alignment by eye proves difficult. This method can be used to determine the consensus target sequence, or alternatively, the antisense strand of the candidate multitargeting interfering RNA directly. When designing the antisense strand directly, the algorithm scores wobbles (G:U, U:G), other non-canonical pairs (eg A:C, C:A) and remaining mismatches with increasingly large negative penalty terms, respectively. These penalty terms are adjusted for their position within the multitargeting interfering RNA, with those placed at the 3' extremity incurring lower fractional penalties. Penalties are also adjusted in the presence of multiple contiguous

mismatches or wobbles. Those proposed multitargeting interfering RNAs with the lowest aggregate penalty scores for all targets are prioritized for further evaluation.

A further alternative approach that is particularly useful when there is little identity between the target sequences outside of the seed sequence or when a large number of target sequences need to be considered (eg when large numbers of nucleotide sequences for viral isolates are screened) is to generate all possible permutations of the extension from the complement of the seed in the 3' direction to a required length, thereby generating the putative SY of Formula (I) and hybridising each putative SY against the target sequences of interest *in silico* to determine those which demonstrate the most favourable properties in terms of hybridisation to the target and preferential strand loading.

In most cases, when a duplex molecule is being designed, the overhangs, if required, are considered as part of the hybridization process. Hybridization is typically examined from a thermodynamic perspective using RNAhybrid software (Rehmsmeier et al., 2004, RNA, 10: 1507-17) or similar algorithm.

In one embodiment, the invention provides an algorithm for determination of the guide strand of a length *n* for a multitargeting interfering RNA comprising a seed sequence.

1) Definition of terms
   a. **Load Bias:** Scoring A or U as "1" and G or C as "2" in each candidate guide strand:
      i. Multiply the A-U/G-C score by the relevant scaling factor in the Table below
      ii. sum the products for the residues at positions 1-5.
      iii. sum the products for the residues at positions n to n-4.
      iv. divide the total at (iii) by the total at (ii)

5' end of the guide strand			3' end of the guide strand (excepting overhangs)	
Residue position	Scaling factor		Residue position	Scaling factor
1	5		n-4	1
2	4		n-3	2
3	3		n-2	3
4	2		n-1	4
5	1		n	5

b. **Activity Score:** For each target $T_1...T_n$, calculate the product of the minimum free energy (mfe) of the binding of the guide strand and the number of contiguous bases complementary to the target at the 5' end of the guide strand. (r-5'). The product of these scores is the Activity Score.

$T_1(mfe*r\text{-}5')*T_2(mfe*r\text{-}5')*...T_n(mfe*r\text{-}5')$

c. **Maximum Activity Score:** For each target $T_1...T_n$ calculate the product of the mfe of the binding of that guide strand which is completely complementary to the target and the length of the guide strand (for example: 21 bases as shown below). The product of these scores is the Maximum Activity Score .

$T_1(mfe*21)*T_2(mfe*21)*...T_n(mfe*21)$

d. **Relative Activity Score:** The Activity Score for an interfering RNA divided by the Maximum Activity Score for the targets.

2) Starting with the complement of the seed sequence, generate all possible permutations for the extension of the complement of the seed to a length of *n*, for example 21 bases, thus creating a complete list of putative guide strands

3) Use RNAhybrid to determine the binding pattern and minimum free energy of all the putative guide strands against all the target sequences which contain the seed sequence;

4) Discard all putative guide strands where

a. There is a contiguous run of 5 or more G residues

b. The Load Bias is < 1.2

5) Rank the remaining putative guide strands by their Relative Activity Score and select a guide strand sequence of the length *n* for a multitargeting interfering RNA sequence from the list of the remaining putative guide strand sequences based on their Relative Activity Score.

Consider a certain number or fraction (eg the top 1%) of the putative guide strands for additional steps, such as overhangs, chemical modifications etc, ynthesis and determination of biological activity.

In a particular embodiment, a simple algorithm that may be useful in the case of a fully complementary duplex is the following:

Scoring A or U as "1" and G or C as "2" in the guide strand:

i. multiply the A-U/G-C score by the relevant scaling factor in the Table A below;

ii. sum the products for the residues at positions 1-5;

iii. sum the products for the residues at positions n-4 to n (excepting overhangs if present);

iv. divide the total at (iii) by the total at (ii); and

v. scores greater than 1.2 for the guide strand indicate likely favourable loading of that strand.

Table A

5' end of guide strand			3' end of guide strand	
Residue position	Scaling factor		Residue position	Scaling factor
1	5		n-4	1
2	4		n-3	2
3	3		n-2	3
4	2		n-1	4
5	1		n	5

n= number of nucleotides involved in forming the duplex (excluding overhangs if present).

It will be appreciated by one skilled in the art that in the case of a double stranded multitargeting interfering RNA, ensuring maximum strand loading of the desired guide strand is beneficial not only with respect to the potency of the molecule but also will greatly reduce inadvertent off-target effects resulting from incorporation of the passenger strand into RISC.

Sequences demonstrating strong binding (typically having mean free energies of <-20 kcal/mol) are of particular interest for the multitargeting interfering RNA. As before, one skilled in the art will appreciate that this method, used to design the active multitargeting interfering RNA can, with slight modification, be used to design suitable consensus target sequences, which are then converted to putative SY sequences. Regardless of the flow path of design, the candidate SY sequences are then prioritized for testing not only on this basis but also taking into account other features that may be important for the functionality of the multitargeting interfering RNA (by, for example, use of appropriate penalty terms). This may involve discarding those putative SY sequences which are composed of only a single base, are composed only of A and U, are predicted to be involved in substantial intramolecular base pairing, have a consecutive string of 5 or more bases which are G, are predicted to occur with unacceptable frequency in the antiparallel orientation in the non-target transcriptome of interest; are predicted to have a propensity to activate a cellular sensor of foreign nucleic acid, or any combination thereof. In some cases, the addition of one or two nucleotides to the 5' end of the putative SY (ie X) is considered. This is particularly relevant when an otherwise useful SY sequence is G/C rich at the 5' end and this is predicted to disfavor loading in the case of a double stranded multitargeting interfering RNA. The addition of one or two A/U nucleotides to the 5' extremity of the putative SY sequence, will most likely improve loading. Another situation that necessitates this addition includes, but is not limited to, when there is a need for the double stranded multitargeting interfering RNA to be able to support target cleavage. In most cases, cleavage requires the active strand to be complementary to the target spanning the site bounded by the 10 and 11^{th} nucleotides. When the seed is of insufficient length (eg n=9), the addition of two additional nucleotides to the 5' end of SY will shift S along sufficiently to fulfill this requirement. Because multitargeting interfering RNA in most cases tolerate mismatches at

positions 1 and 2, the addition of this additional region X, which need not be complementary to the at least two target sequences, further increases the flexibility of design.

In the case of a double stranded multitargeting interfering RNA of the invention, a further embodiment comprises the step of discarding seeds that are extremely G/C rich at their 3' extremity. A G/C rich 3' extremity of the seed may be undesirable as the resulting matching guide strand could be disadvantaged from a loading point of view, depending on the G/C content of the 3' end of the guide strand (excluding the overhangs). Whilst this may not be preferable, strategies can be used to overcome the predicted poor loading bias when seeds are G/C rich at their 3' end. These include, but are not limited to, the use of an A/U-rich extension in the 5' end of the guide strand, i.e. "X" in Formula (I), or the selection of G/C rich termini in the design of the 3' end of the guide strand i.e. "Y" in Formula (I). Also, the substitution of U for C in the corresponding passenger strand by virtue of wobble base pairing will at least partially rectify strand loading when there is a G near the 5' terminus of the guide strand. Finally, one skilled in the art will appreciate that modifications that disfavor strand loading could be used on the passenger strand to further enhance the loading of the desired strand. Such modifications also include manipulation of the length and composition of the overhangs. Alternatively, chemical modifications that increase the binding energy of RNA duplexes such as LNA, 2'-O-methyl and 2'-methoxyethyl can be used for bases positioned at the 5' end of the passenger strand so as to favor loading of the guide strand.

Various steps can optionally be added individually or in combination. Such steps are used to further the rational process of designing the multitargeting interfering RNAs - such as to reduce the number of sequences unlikely to work for the intended purpose, to increase the effectiveness of the RNAs, to reduce off target effects and the like. Many of these steps can be automated, or require only a limited amount of input from an operator, though the use of bioinformatic computer systems, which as the skilled artisan will appreciate, will facilitate the methods.

Similar to the situation with antisense, for which it is now recognized that there are specific sequences that have a high propensity to activate cellular sensors of foreign DNA, other receptors may detect particular RNA sequences and produce stress responses (for example, see Sioud, M. (2005), *J Mol Biol* 348, 1079-1090. Specific "motifs" associated with increased

inflammatory responses (Hornung, V. et al. (2005) *Nat Med* 11, 263-270; Judge, A.D. et al. (2005) *Nat Med* **11**, 263-270) could be easily excluded.

DNA sequences with stretches of contiguous guanosines are known to produce additional effects not related to targeting of mRNA. Although the situation in the case of RNA is less clear, most manufacturers recommend not selecting dsRNA duplexes containing long runs of guanosines (G) for their experiments. It was found in this invention that greater than 4 consecutive G greatly reduced the activity of the corresponding CODEMIR (see Example 17). Therefore, many seeds could be eliminated if they contain 5 or more contiguous C. One skilled in the art will recognise that the presence of 5 or more Cs in a seed will correspond to 5 or more Gs in the active portion of the RNA molecule of the invention. However, we also consider that it is desirable to further exclude the occurrence of 5 or more Gs in the passenger strand when the molecule is double stranded, therefore the occurrence of 5 or more contiguous Gs in the seed in combination with the consensus target sequence is also considered to be undesirable when a duplex molecule is to be constructed.

A further embodiment to excluding seeds specifically related to duplex molecules of the invention applies to the presence of a G/C rich region at the 3' end and to the presence of 5 or more contiguous Gs in the seed or consensus target sequence (which includes the seed).

In another embodiment, the method further comprises the step of discarding any consensus target sequence that: is composed of only a single base, is composed only of A and U, , has a consecutive string of 5 or more bases which are C, is G/C rich at the 3' end, is predicted to occur with unacceptable frequency in the non-target transcriptome of interest; or any combination thereof. In the situation in which a double stranded multitargeting interfering RNA is to be designed, consensus target sequences may also be discarded if they contain sequences predicted to have a propensity to activate a cellular sensor of foreign nucleic acid, if they are predicted to be involved in intramolecular base pairing, have a consecutive string of 5 or more bases which are G, or a combination thereof.

Like candidate seeds and seeds, consensus target sequences can be used as intermediates in the design of a multitargeting interfering RNA of the invention. In particular, the consensus target sequences are used to design a strand of the corresponding multitargeting interfering RNA The consensus target sequences are at least substantially the complement of a "guide strand" of a candidate multitargeting interfering RNA. Preferably they are the complete complement of a

"guide strand" of a candidate multitargeting interfering RNA. The consensus target sequence is at least possibly, but not necessarily, identical to the "passenger strand" of the corresponding candidate multitargeting interfering RNA, when these are double stranded. They may be non-identical because "passenger strands" are frequently further refined to optimize RISC loading and other functionality through the use of sequence modifications, for example, end modifications, such as inclusion of overhangs (non-blunt ends, e.g. 3'-UU), and the incorporation of mismatches and wobble bases. Such modifications will be understood by those of skill in the art.

Scanning the consensus target sequences against a non-target transcriptome of interest for prediction of off-target effects, and eliminating any sequence predicted to have unacceptable off-target effects on a non-target transcriptome of interest are also useful ways of reducing the number of consensus target sequences, and any of the foregoing may be added as a step in the process. This is performed by searching for similar sequences using, for example, BLAST software. An alternative, but not necessarily equivalent procedure includes the in silico hybridization of the complement of the consensus target sequence against the transcriptome using, for example, RNAhybrid or equivalent software. In practice, it is prudent to routinely screen specific designed multitargeting interfering RNAs, e.g. CODEMIRs, VIROMIRs and the like, for cytotoxicity, due to unforeseen, but problematic, off-target effects.

Any undesirable properties for such a therapeutic RNA, as would be understood by those of skill in the art, can be used as a basis on which to discard candidate seed sequences, consensus binding sites or proposed multitargeting interfering RNA.

The RNA molecules obtained from the basic method outlined above can be modified and often will be to improve their properties. The methods then can further comprise the step of modifying a multitargeting interfering RNA duplex designed in step h) to improve actual or predicted loading into a RISC complex, to improve activity against at least one target RNA molecule, to decrease stress or inflammatory response when administered in a host cell; to alter half-life in an expression system, or any combination thereof.

In certain embodiments, the designed multitargeting interfering RNA molecule can be modified, for example, i) to improve the incorporation of the guide strand of the multitargeting interfering RNA molecule into the RNA induced silencing complex (RISC); ii) to increase or decrease the modulation of the expression of at least one target RNA molecule; iii) to decrease

stress or inflammatory response when the multitargeting interfering RNA molecule is administered into a subject; or iv) any combination of i) to iii).

The skilled artisan will understand how to modify the RNA molecules either in the laboratory, or preferably *in silico*. In preferred embodiments the modifying step comprises one or more of altering, deleting, or introducing one or more nucleotide bases to create at least one mismatched base pair, wobble base pair, or terminal overhang, or to increase RISC mediated processing. Techniques for doing so are known in the art. Preferably the modifications are at least initially performed *in silico*, and the effects of such modifications can be readily tested against experimental parameters to determine which offer improved properties of the interfering RNA products.

In a presently preferred embodiment, the methods, through to the step of *actually* making an RNA, are conducted entirely *in silico*, or by visual inspection and determination. In one embodiment the method further comprises the step of choosing a new value for the seed length, *n*, and repeating each of the remaining steps. It is clear that the method can be iterative and the benefits of computers for such purposes are well known.Also provided herein are methods that further comprise the step of actually making and testing at least one designed interfering RNA in a suitable cellular expression system. This will be necessary so as to identify those interfering RNA that have the required or sufficient activity against the target RNA molecules or that produce the required phenotype in the model system (eg death of cancer cell, inhibition of angiogenesis, suppression of lesion formation, accelerated wound healing etc).

As will be appreciated, large numbers of seeds and thereby potential multitargeting interfering RNAs can be generated using the above methodology. While the rules above can be used to filter potential candidates based on undesirable properties, one skilled in the art will appreciate that with access to high throughput screening methodologies as well as recent improvements in fidelity, cost and access to RNA synthesis that testing of hundreds to thousands of candidates can be easily performed to further assist in the development of active multitargeting interfering RNAs. Thus, it is occasionally preferable to screen significant numbers of candidates as opposed to prioritising a few candidates solely on the basis of algorithmic design. A combination of careful in silico design along with biological testing of candidates can be used to identify candidates with superior activity in an efficient manner.

Screens that can be considered for the high throughput assessment of candidates include reporter assays, multiplexed ELISAs, viral replicon systems, dot-blot assays, RT-PCR etc.

Candidate multitargeting interfering RNA are routinely synthesized as double-stranded RNA molecules with 19 bp of complementarity and 3' two nucleotide overhangs. For the guide strand (the strand with complementarity to the target RNAs and which is predicted to be incorporated into RISC), the two nucleotide overhangs are routinely designed to be complementary to the target RNAs, although dTdT or UU overhangs may also suit. The passenger strand (complementary to the guide strand) can be usually designed to include a 3' two nucleotide UU overhang. However, other types and lengths of overhangs could be considered, as could "blunt-ended" duplexes. Candidate multitargeting interfering RNA can also be single-stranded molecules.

When produced by an expression system such as a vector or plasmid, it is possible to assemble multiple multitargeting interfering RNAs into a single therapeutic product. Skilled artisans will realize that multiple multitargeting interfering RNAs can be co-expressed by several strategies, including but not limited to, expression of individual multitargeting interfering RNAs from multiple expression vectors (plasmid or viral), expression from multiple expression cassettes contained within a single vector, with each expression cassette containing a promoter, a single multitargeting interfering RNA and terminator. Multiple multitargeting interfering RNAs can also be generated through a single polycistronic transcript, which contains a series of multitargeting interfering RNAs.

The multiple multitargeting interfering RNAs can be expressed sequentially (sense / intervening loop / antisense) or expressed with the sense sequence of each multitargeting interfering RNA sequentially linked 5' to 3', joined directly or with intervening loop / spacer sequence, followed by the antisense sequence of each multitargeting interfering RNAs sequentially linked 5' to 3'.

In the first instance, multitargeting interfering RNA are typically tested in cell culture using an appropriate cell line representative of the targeted tissue. Some non-limiting specific conditions used are outlined in the specific examples. Multitargeting interfering RNA that modulate target RNA expression or activity can then be studied further. Specifically, semi-quantitative RT-PCR for the target RNA may be performed to establish whether repression is likely to be mediated by RNA degradation. In general, cells are transfected with the

multitargeting interfering RNA at a concentration of 5-40 nM in the culture medium and after 48 hours, are washed, trypsinized and harvested for total RNA using a RNeasy kit (Qiagen). RT-PCR is then performed using primer sets specific for the target RNAs.

Proteomic and microarray experiments may be used to assess off-target effects. Likewise, to select active multitargeting interfering RNA with little propensity for activation of innate immune response, analysis of markers of IFN-response (eg STAT1, IFNb, IL-8, phosphoEIF etc) can be performed on treated cells.

Preferably, the candidate multitargeting interfering RNA are tested for non-specific toxic effects by, for example, direct assays of cell toxicity. Alternatively, in some cases such as cancer, cytotoxicity is the desired outcome and may reflect the successful repression of key oncogenic signaling pathways. Multitargeting interfering RNA are additionally assessed for their ability to repress the production of specific target proteins. Multitargeting interfering RNA demonstrating efficacy in this respect are then assessed for additional evidence of off-target effects, including arrest of non-target protein production and activation of Protein Kinase R mediated responses.

The RNA molecule may be expressed from transcription units inserted into vectors. The vector may be a recombinant DNA or RNA vector, and includes DNA plasmids or viral vectors. The viral vectors expressing the multitargeting interfering RNA molecules can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, lentivirus or alphavirus.

Preferably the vector is an expression vector suitable for expression in a mammalian cell. Methods which are well known to those skilled in the art can be used to construct expression vectors containing a sequence which encodes the multi target RNA molecule. These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination or genetic recombination. Such techniques are described in Sambrook et al (1989) Molecular Cloning, A laboratory manual, Cold Spring Harbor Press, Plainview N.Y. and Asubel F M et al (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York N.Y. Suitable routes of administration of the pharmaceutical composition of the present invention may, for example, include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, intravenous and subcutaneous injections.

Alternatively, the pharmaceutical composition may be administered in a local rather than systemic manner, for example, via injection of the pharmaceutical composition directly into a target organ or tissue, such as intramedullary, intrathecal, direct intraventricular, intraperitoneal, or intraocular injections, often in a depot or sustained release formulation. Intravesicular instillation and intranasal/inhalation delivery are other possible means of local administration as is direct application to the skin or affected area. *Ex vivo* applications are also envisaged. Furthermore, the pharmaceutical composition of the present invention may be delivered in a targeted delivery system, for example, in a liposome coated with target cell-specific antibody. The liposomes will be targeted to and taken up selectively by the target cell. Other delivery strategies include, but are not limited to, dendrimers, polymers, nanoparticles and ligand conjugates of the RNA.

The multitargeting interfering RNA molecules of the invention can be added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues ex vivo, or in vivo through injection, infusion pump or stent, with or without their incorporation in biopolymers.

In another aspect, the invention provides biological systems containing one or more multitargeting interfering RNA molecule of this invention. The biological system can be, for example, a virus, a microbe, a plant, an animal, or a cell. The invention also provides a vector comprising a nucleotide sequence that encodes the multitargeting interfering RNA molecule of the invention. In particular embodiment, the vector is viral, from example, derived from a virus selected from the group consisting of an adeno-associated virus, a retrovirus, an adenovirus, a lentivirus, and an alphavirus. The multitargeting interfering RNA can be short a hairpin RNA molecule, which can be expressed from a vector of the invention. The invention further provides a pharmaceutical composition comprising a multitargeting interfering RNA molecule of the invention and an acceptable carrier. In particular embodiments, the pharmaceutical composition comprises a vector for a multitargeting interfering RNA molecule of the invention.

In another general aspect, the present invention provides a method of inducing RNA interference in a biological system, which comprises the step of introducing a multitargeting interfering RNA molecule of the invention into the biological system.

In a particular embodiment, the present invention provides a method of inducing RNA interference in a biological system, comprising the steps of: (a) selecting a set of target RNA molecules; (b) designing a multitargeting interfering RNA molecule comprising a guide strand that can form stable interactions with at least two binding sequences present in distinct genetic contexts in the set of target RNA molecules; (c) producing the multitargeting interfering RNA molecule; and (d) administering the multitargeting interfering RNA molecule into the biological system, whereby the guide strand of the multitargeting interfering RNA molecule forms stable interactions with the binding sequences present in distinct genetic contexts in the set of target RNA molecules, and thus induces RNA interference of the target RNA molecules.

In another particular embodiment, the present invention provides a method of treating a disease or condition in a subject, the method comprising the steps of: (a) selecting a set of target RNA molecules, wherein the modulation in expression of the target RNA molecules is potentially therapeutic for the treatment of the disease or condition; (b) designing a multitargeting interfering RNA molecule comprising a guide strand that can form stable interactions with at least two binding sequences present in distinct genetic contexts in the set of target RNA molecules; (c) producing the multitargeting interfering RNA molecule; (d) administering the multitargeting interfering RNA molecule into the subject, whereby the guide strand of the multitargeting interfering RNA molecule forms stable interactions with the binding sequences present in distinct genetic contexts in the set of target RNA molecules, and thus induces modulation of expression of the target RNA molecules.

One skilled in the art will recognize that these design steps can be performed in a different order to produce an equivalent final product. Also, one skilled in the art will recognize that some steps can be substituted with alternative procedures that are broadly equivalent.

The target RNA molecule or molecules can be selected, for example, from any RNA in a cell or virus. A viral genome, or even multiple viral genomes, for example two or more related or unrelated viruses, can also be conveniently targeted. The useful or desirable targets for any disease or related process may be identified by any of one or more means including, for example, projected or validated drug targets from the literature, including the patent literature, or from target discovery processes. The skilled artisan will understand and appreciate how to select

useful or desirable targets. The target genes are then prioritized based on evidence supporting a key role for their products in the disease process of interest.

In some cases, specific attention may need to be paid to the accuracy and/or relevance of the sequence to the disease of interest. For example, in targeting cancer, it is advisable to avoid mutational "hot-spots". It is also to be noted that the sequence used need not be the complete sequence. Also, selective targeting of a specific splice variant or isoform may be desired and the target sequence used in the design of multitargeting interfering RNAs may need to be restricted to that predominantly present only in the diseased tissue that is targeted by the multitargeting interfering RNA of the invention.

The use of single- or double-stranded RNA compounds that can target multiple sites within a viral genome, for example, viral RNA targets, is also provided herein. The multitargeting interfering RNA molecules that target multiple sites in the genome of one or multiple isolates of a virus are sometimes referred herein as "VIROMIRs". Targeting repeated sequence elements in viral genomes is an attractive approach for viral therapy. Such multitargeting is calculated to create a formidable hurdle to the emergence of resistant clones, which would require multiple, simultaneous, mutations. Also, multiple sites can be chosen to maximize coverage of sequence variations across a range of viral isolates. Elements can be identified computationally that are present in a pre-selected percentage of isolates, such as a majority or even the totality of known isolates, thereby ensuring maximal therapeutic benefit. Alternatively, isolates of greatest actual or projected clinical significance can be preferentially targeted. The design process can also facilitate development, manufacture, and ultimately administration of the therapeutic compounds. The additional targeting of one or more host proteins or other intermediates of the pathway involved in the pathogenesis of the viral disease can also be designed. Bacterial infection and any other disease caused by a pathogen can be targeted by a similar approach.

The RNA compounds of the present invention can be used to treat or prevent diseases in plants, animals and in particular humans. The RNA compounds can be either cell-expressed into the relevant plant, animal or human cell to derive the required effect or be administered as a chemically synthesized compound directly or indirectly by means of a delivery molecule or device. The RNA compounds of the present invention can be used to treat or minimise pest attack on plants and animals. The pests may be vertebrate or invertebrate.

The present invention may be used for treatment or prevention of a disease state resulting from expression of the target genes. Disease states include, but are not limited to, autoimmune diseases, inherited diseases, cancer, or infection by a pathogen. Treatment would include prevention or amelioration of any symptom or clinical indication associated with the disease. In a preferred embodiment, the disease state is cancer (eg colorectal adenocarcinoma), diabetes, diabetic retinopathy, age related macular degeneration (AMD), psoriasis, HIV infection, HCV infection or Alzheimer's disease. The current invention therefore encompasses, but is not limited to, the concept of treating these diseases with multitargeting interfering RNAs. As can be readily appreciated, the same principles can be applied to the treatment of all other complex diseases.

The selection of target sequences for the multitargeting interfering RNAs will be determined by the disease(s) against which the therapeutic is desired. The target RNA sequence may include mRNA, and noncoding RNA and may be encoded either by the host or infectious agent or both. The target RNA set for any one multitargeting interfering RNA can be selected from any of these types and may represent any combination of gene or gene products including but not limited to: receptors, cytokines, transcription factors, viral genes, bacterial genes, plant genes, insect genes, yeast and fungal genes, regulatory and/or signalling proteins, non-coding RNA, cytoskeletal proteins, transporters, enzymes, hormones, antigens, hypothetical proteins and proteins of unknown function.

Additionally, targeting of different isolates of a pathogenic agent is envisaged. The multiple target sites can then be chosen to maximise coverage of sequence variations across a range of isolates.

The modulation of the target RNA molecule is determined in a suitable expression system, for example *in vivo*, in one or more suitable cells, or in an acellular or *in vitro* expression system such as are known in the art. Routine methods for measuring parameters of the transcription, translation, or other aspects of expression relating to RNA molecules are known in the art, and any such measurements are suitable for use herein.

The multitargeting RNAs in accordance with various aspects of the invention are useful to modulate the expression of one or more target RNA in an expression system. More preferably, they are used to reduce expression of one or more target RNA. Such decrease can occur directly or indirectly by any mechanism known in the art, or which is yet to be discovered,

for the decrease of RNA expression as defined herein by an RNA. In some embodiments, they may completely eliminate expression of the one or more RNA targets. In some embodiments, a given RNA will be more effective at modulating expression of one of several target RNAs than another. In other cases, the RNA may similarly affect all targets in one or more expression systems.

The multitargeting RNAs provided herein are of particular value in the treatment of complex multigenic diseases in which single gene-specific therapeutics may be at a disadvantage because of the multiple redundancies in pathophysiologic pathways. The current invention enables a conscious and calculated approach in which multiple or key proteins or pathways, such as signaling pathways or molecules, can be targeted with a single agent to generate greatly increased therapeutic potential.

In some cases, the targets of interest may be at least partially controlled by a common "master regulator," for example, an upstream pleiotropic factor. Such common regulators are often transcription factors. For example, IL-8 and MCP-1 could theoretically be down-regulated by targeting the nuclear factor, NFkappaB. However, by way of example, NFkappaB is also a factor involved in the survival of Retinal Pigmented Epithelial cells (RPE), particularly in times of stress. Thus, the indiscriminate down-regulation of such a cell-survival factor would likely lead to the undesirable consequence of increased death of RPE cells in diseased eyes. Rather than identifying upstream pleiotropic controllers as potential targets with the concomitant risk of negatively impacting a desirable pathway or process, the novel approach disclosed herein is amenable to the modulation of multiple specific targets of interest without having to indiscriminately modulate common upstream factors.

An additional aspect of the multitargeting interfering RNAs provided herein is applicable to the treatment of diseases characterized by cellular heterogeneity. For example, in solid tumours, the presence of mutated genes and activated pathways may vary widely within the same tumour, between tumours in the same patient, as well as between tumours of a similar histology in different patients. The development of an RNA molecule active against several key pathways may derive synergistic activity against cells reliant on several of these targeted pathways. However, activity against a greater proportion of the tumour cells will also be likely because of the "multi-targeted" nature of the RNA molecule. Furthermore, targeting of several key

pathways will "cover" or allow treatment of more of the patient population. Hence, improved clinical outcomes are likely with treatment with the RNA molecules provided herein.

In certain embodiments, for example where RISC is involved in the mechanism of action, the targeting of multiple disease-related transcripts with a single multitargeting interfering RNA makes optimal use of available RISC, in contrast to the administration of multiple siRNA molecules, which could saturate the available intracellular machinery.

Targeting multiple sites within the same RNA target sequence is also envisioned for the interfering RNAs provided herein, *i.e.* the multitargeting aspect is not limited to multiple targets within multiple target RNA molecules. Many human diseases, including cancer and viral infections, are characterized by RNA targets exhibiting high mutation rates. This increases the likelihood of resistance to nucleic acid therapeutics arising in these diseases, due to mutation of the target RNA. Targeting multiple sites within the target RNA decreases the likelihood of such resistance arising, since at least two simultaneous mutations would be required for resistance. Therefore in certain embodiments, the multi-targeting approach used with multitargeting interfering RNAs can be directed to the generation of multiple hits against a single target RNA molecule, for example, to prevent escape mutants. Targeting of multiple sites within the same transcript (for example, with RNA viruses) may also produce synergistic effects on the inhibition of viral growth. Further, employing a mechanism or mechanisms requiring only partial complementarity with the target RNA molecule can decrease the possibility of developing resistance through single point mutation.

This invention will be better understood by reference to the examples that follow. Those skilled in the art will readily appreciate that these examples are only illustrative of the invention and not limiting.

Example 1

Selection of CODEMIRs for Diabetic Retinopathy (DR)

CODEMIRs suitable for therapy for DR were sought. In the disease state, VEGF-A and ICAM-1 are likely drivers of the loss of integrity of the blood-retinal barrier, which loss leads for example to diabetic macular edema, a prelude to angiogenesis and DR. Therefore, VEGF-A and

ICAM-1 were selected as targets for the design of CODEMIRs. Such CODEMIRs were also of therapeutic interest for treatment of psoriasis and other conditions characterized by mononuclear cell infiltration and angiogenesis.

Transcript sequences corresponding to the 3' untranslated regions (3' UTRs) of VEGF-A and ICAM-1 were used to search for a suitable seed of at least 6 contiguous bases using a searching algorithm combined with a database. Publicly available sequences related to VEGF-A and ICAM-1 obtained from the Ensembl database were used to perform the initial analysis (see Table 1-1). (Ensembl is a joint project between the European Molecular Biology Laboratory(EMBL)/European Bioinformatics Institute (EBI) and the Wellcome Trust Sanger Institute (WTSI)). The database and related tools are publicly available on the World Wide Web at the Ensembl website.

Table 1-1: Ensembl Transcript IDs of selected human target sequences (Ensembl database, release 33)

BFGF	ENST00000264498
IGF-1	ENST00000337514
MCP-1	ENST00000225831
VEGF-A	ENST00000356655
VEGF-B	ENST00000309422
VEGF-C	ENST00000280193
VEGF-D	ENST00000297904
ICAM-1	ENST00000264832
IL-8	ENST00000307407
PIGF	ENST00000256315

A pool of all possible candidate seeds of length 6 or greater was generated using the specified length as window and moving sequentially along the sequence in a stepwise fashion by advancing the window one base at a time. Low complexity seeds were eliminated. The pool of candidate seed sequences was further restricted to those for which at least three contiguous bases were predicted to bind to an unpaired region in at least 50% of optimal and suboptimal folded structures. Optimal and suboptimal (within ~1 kcal/mol of the optimal fold energy) folded

structures were determined using the Vienna RNA package (Hofacker, (2003). *Nucleic Acids Res.*, 31: 3429-31).

Two different seeds were selected that optimally fulfilled the selection criteria. The first seed was selected on the basis of its length of 12 nucleotides. There were also several smaller seeds recorded. One 7 nt seed was in a genetic context that favored the design of the consensus target sequence by virtue of additional, albeit discontinuous, regions of further identity between the two target sequences near the seed. The two seeds were used to generate two sets of consensus target sequences. The consensus target sequences for the first seed were determined by optical alignment of the two target sequences and determining the likely effect various base changes would have on the binding of the complementary sequence to both target RNA. In the case of the second seed, a permutation analysis was favoured. In this case, the two target sequences differ at only seven positions over the 14 bases that are 5' to the seed. Because the extreme tail of the multitargeting interfering RNA has less requirement for complementarity, only two possible terminal triplets were considered for the 5'end of the consensus target sequence; namely TAT and GTC. This reduced by four the number of possible permutations. Systematic generation of all remaining 32 possible consensus target sequences ($32 = 2^5$) was followed by in silico hybridization of the 21 base complementary sequences to the two targets. The sequence providing the best overall hybridization to the two targets was designated CODEMIR-2. The consensus target sequences identified for each of the two seeds are listed in Table 1-2.

Table 1-2: Exemplary Seed Sequences and Consensus Target Sequences for the design of multitargeting interfering RNAs, e.g. CODEMIRs targeting VEGF-A and ICAM-1 (all 5' to 3'). *[1,2] These consensus target sequences were used to design CODEMIR1 and CODEMIR2, respectively.

Seed Sequence	Consensus Target Sequences
CCCACCCACATA (SEQ ID NO: 143)	TAGAGACACCCCACCCACATA (SEQ ID NO: 144)
	TTAGACCACCCCACCCACATA (SEQ ID NO: 145)
	AGACACCACCCCACCCACATA (SEQ ID NO: 146)
	TTAGACTCACCCACCCACATA * (SEQ ID NO: 147)
	AGACACTCACCCACCCACATA (SEQ ID NO: 148)

Seed Sequence	Consensus Target Sequences
CAAAACA (SEQ ID NO: 4)	TATATGTGTAGCATCAAAACA (SEQ ID NO: 149)
	GTCATGTGTAGCATCAAAACA (SEQ ID NO: 150)
	TATATGTGTAGCAACAAAACA (SEQ ID NO: 151)
	GTCATGTGTAGCAACAAAACA (SEQ ID NO: 152)
	TATATGTGTAGAATCAAAACA (SEQ ID NO: 153)
	GTCATGTGTAGAATCAAAACA (SEQ ID NO: 154)
	TATATGTGTAGAAACAAAACA (SEQ ID NO: 155)
	GTCATGTGTAGAAACAAAACA (SEQ ID NO: 156)
	TATATGTGTAACATCAAAACA (SEQ ID NO: 157)
	GTCATGTGTAACATCAAAACA (SEQ ID NO: 158)
	TATATGTGTAACAACAAAACA (SEQ ID NO: 159)
	GTCATGTGTAACAACAAAACA (SEQ ID NO: 160)
	TATATGTGTAAAATCAAAACA (SEQ ID NO: 161)
	GTCATGTGTAAAATCAAAACA (SEQ ID NO: 162)
	TATATGTGTAAAAACAAAACA (SEQ ID NO: 163)
	GTCATGTGTAAAAACAAAACA (SEQ ID NO: 164)
	TATATATGTAGCATCAAAACA (SEQ ID NO: 165)
	GTCATATGTAGCATCAAAACA (SEQ ID NO: 166)
	TATATATGTAGCAACAAAACA (SEQ ID NO: 167)
	GTCATATGTAGCAACAAAACA (SEQ ID NO: 168)
	TATATATGTAGAATCAAAACA (SEQ ID NO: 169)
	GTCATATGTAGAATCAAAACA (SEQ ID NO: 170)
	TATATATGTAGAAACAAAACA (SEQ ID NO: 171)
	GTCATATGTAGAAACAAAACA (SEQ ID NO: 172)
	TATATATGTAACATCAAAACA (SEQ ID NO: 173)
	GTCATATGTAACATCAAAACA (SEQ ID NO: 174)
	TATATATGTAACAACAAAACA (SEQ ID NO: 175)
	GTCATATGTAACAACAAAACA *[2] (SEQ ID NO: 176)
	TATATATGTAAAATCAAAACA (SEQ ID NO: 177)
	GTCATATGTAAAATCAAAACA (SEQ ID NO: 178)
	TATATATGTAAAAACAAAACA (SEQ ID NO: 179)
	GTCATATGTAAAAACAAAACA (SEQ ID NO: 180)

Only several exemplary sequences are listed in Table 1-2 because, as mentioned above, additional consensus target sequences with partial identity to VEGF-A and ICAM-1 could have been readily generated, particularly for the second, shorter seed. In Figure 1, five consensus target sequences for the 12 nt seed are shown. The predicted hybridization of the CODEMIR guide strands to the target VEGF-A and ICAM-1 transcripts is also shown. CODEMIRs targeting VEGF-A and ICAM-1 were designed using the methods provided. Consensus target sequences (VAIC1-01 to –05) were derived by aligning the target mRNA sequences 5' of the seed region to generate sites of 21 nucleotides in length. Candidate CODEMIR guide strand sequences complementary to these consensus target sequences were determined, and hybridization between the CODEMIR guide sequences and the targets was predicted by *in silico* modeling. Based on the results, the consensus target sequence, VAIC1-04, was used to generate CODEMIR 1 (*Figures 2 and 3*).

Candidate CODEMIRs were synthesized as double-stranded RNA molecules with 19 bp of complementarity and 3' overhangs of two nucleotides in length. The two nucleotide overhangs for the guide strands were designed to be complementary to the consensus target sequences. The complementary passenger strand was designed to include a two nucleotide 3'-UU overhang.

Two CODEMIRs were selected for more extensive testing, one each designed from the 12 nt seed and the 7 nt seed. The sequences of the guide strands and their predicted hybridization to the targeted VEGF-A and ICAM-1 3' UTRs are shown in Table 1-3. It can be seen that even with a seed sequence only 7 nt in length, using the approach provided herein, CODEMIRs (for example CODEMIR2, Table 1-3) can be designed to achieve significant hybridization with at least two different targets. The degree of 3' identity that the CODEMIRs have with the intended targets is evident.

Table 1-3: **Exemplary CODEMIRs multitargeting both VEGF-A and ICAM-1.**

	CODEMIR-1	CODEMIR-2
Guide sequence (5' to 3')	UAUGUGGGUGGGUGAGUCUAA (SEQ ID NO: 100)	UGUUUUGUUGUUACAUAUGAC (SEQ ID NO: 102)
CODEMIR	5' UAUGUGGGUGGGUGAGUCUAA 3' (SEQ ID NO: 100) 3'UUAUACACCCACCCACUCAGA 5' (SEQ ID NO: 101)	5' UGUUUUGUUGUCACAUAUGAC 3' 3'UUACAAAACAACAAUGUAUAC 5' top strand (SEQ ID NO: 102) bottom strand (SEQ ID NO: 103)
VEGF-A binding (VEGF-A = upper strand) Top strand 5' to 3' Bottom 3' to 5'	G A C UAGAC **CACCCACCCACAUA** (SEQ ID NO: 181) AUCUG **GUGGGUGGGUGUAU** A A (SEQ ID NO: 100)	U A A UUAUAUGUAA **AACAAAACA** (SEQ ID NO: 182) AGUAUACAUU **UUGUUUUGU** C G (SEQ ID NO: 102)
ICAM-1 binding (ICAM-1 = upper strand) Top strand 5' to 3' Bottom 3' to 5'	G CCAC C UUAG CUC **CCCACCCACAUA** (SEQ ID NO: 183) AAUC GAG **GGGUGGGUGUAU** U U (SEQ ID NO: 100)	A U C CAUGUGUAGCA **CAAAACA** (SEQ ID NO: 184) GUAUACAUUGU **GUUUUGU** CA U (SEQ ID NO: 102)

Representations of the predicted binding of CODEMIR -1 and CODEMIR -2 to each of the targets. The likely mismatches and bulges are shown. The seeds in the consensus target sites are shown in **bold** font.

Testing CODEMIRs in a Human Cell Culture System Producing VEGF-A and ICAM-1

Cells and Culturing: RPE (retinal pigmented epithelium) cells in culture were used to screen the anti-angiogenic CODEMIRs designed, as described above. The human cell line, ARPE-19, was used. ARPE-19 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 10 mM glutamine. For ELISA detection of secreted proteins of interest, or *in situ* cell surface antigen immunostaining, ARPE cells were seeded at 4 x 10^3 cells per well in a 96 well tissue culture plate. For FACS analysis, ARPE-19 cells were seeded at 2.5 x 10^4 cells per well in a 24 well tissue culture plate. Cells were transfected 24 hours after seeding using lipofectamine (InVitrogen) at a ratio of 1 microL lipofectamine per 20 pmol of CODEMIR RNA duplex or control siRNA. In most studies, medium was replaced 24 hours after transfection at which time deferroxamine (130 μM) or IL-1β (1 ng/mL) was added for the VEGF-A and ICAM-1 experiments, respectively. Experiments were performed in triplicate and repeated at least twice.

Assays for VEGF-A and ICAM-1: The ARPE-19 cells were assayed to confirm production of both VEGF-A and ICAM-1. VEGF-A was assayed in the supernatant using a commercially available ELISA assay (R&D Systems) according to the manufacturer's instructions. Cell surface ICAM-1 was assayed either by immunostaining followed by fluorescence activated cell sorting (FACS), by *in situ* immunostaining of cell-surface ICAM-1 in 96 well plates using colorimetric detection, or alternatively by ELISA of cell lysates using a commercial ICAM ELISA kit (R&D systems).

Cytotoxity and Off-Target Effects: Apart from being a source of proangiogenic factors, RPE cells are critical to the survival of the neurosensory photoreceptor cells. Hence, the CODEMIRs were screened for cytotoxic effects in ARPE-19 cells to enable selection of CODEMIRs without significant cytotoxicity. CODEMIRs were transfected into ARPE-19 cells, typically at final concentrations of 1-100 nM in the culture medium. Cell survival was measured 48 hours after transfection using the Cell Titer Blue Assay, which measures cellular respiration.

Activity of the CODEMIRs against multiple RNA targets: The CODEMIRs were assessed for their ability to repress the production of each of the target proteins. Specific siRNAs individually targeting either VEGF-A or ICAM-1 were used as single target comparative controls. (See e.g. Table 1-4).

An siRNA which targeted neither VEGF nor ICAM (siRNA CONTROL) was used as a non-targeting control (Table 1-4)

Table 1-4: Sequences of comparative and non-targeting control siRNA (guide strand).

	Control guide strand sequence (5' to 3')
siRNA CONTROL	GUCUGCGAUCGCAUACAAU dTdT (SEQ ID NO: 185)
siICAM-1	UAGAGGUACGUGCUGAGGC dTdT (SEQ ID NO: 186)
siVEGF	GUGCUGGCCUUGGUGAGGU dTdT (SEQ ID NO: 187)

Results: CODEMIR -1 and CODEMIR -2, as well as comparative and non-targeting control siRNAs were evaluated for cytotoxicity in ARPE-19 cells. All were found to have negligible toxicity over the concentration range of 0 - 40 nM (Figure 2) when transfected over a period of 48-72 hours. None of the CODEMIRs tested showed any appreciable toxicity at concentrations less than about 50 nM. Individual designed CODEMIRs were routinely screened for potential cytotoxicity due to off-target effects.

The supernatants from the cell culture 48 hours after changing of medium (plus deferroxamine in the case of VEGF-A and IL-1β for ICAM-1) were assayed for VEGF-A and ICAM-1 and cells harvested. Figure 3 shows protein suppression by CODEMIRs multitargeting both VEGF-A and ICAM-1. ARPE-19 cells were treated with a siRNA not targeted to either VEGF or ICAM (*siRNA Control*), or RNAs specific for VEGF-A (*siVEGF*), ICAM-1 (*siICAM*), or CODEMIR-1 or -2, each at a concentration of 40nM. VEGF-A (as determined by ELISA) and cell-surface ICAM-1 (as determined by FACS) proteins were assayed 48 hrs after treatment and expressed as a percentage of those from untransfected control cells. In experiments measuring VEGF-A secretion, cells were stimulated with the hypoxia mimic deferroxamine (130 μM) 24 hrs post-transfection, whereas IL-1β was added at 1 ng/mL in the corresponding experiment for ICAM-1. Data from unstimulated and stimulated but untransfected cells are shown for comparison. Depicted results are composite data from several experiments. The comparative controls each significantly reduced the expression of their respective targets (ANOVA and Dunnett's multiple comparison test

versus control siRNA, $p<0.01$), without reducing the other, non-targeted protein. In fact, increased ICAM-1 expression was observed with siVEGF (ANOVA and Dunnett's multiple comparison test versus control siRNA, $p<0.01$).

CODEMIR-1 profoundly repressed VEGF-A secretion to an extent that exceeded the comparative siRNA control at the same concentration. The same CODEMIR also suppressed ICAM-1 expression in a comparable manner to the ICAM-1 comparative control. CODEMIR -2 also repressed both ICAM-1 and VEGF-A to a lesser, but nevertheless significant extent (ANOVA and Dunnett's multiple comparison test versus control siRNA, $p<0.01$). An siRNA duplex targeting the exact CODEMIR1 site in VEGF-A produced only slightly greater knock-down of VEGF-A secretion (data not shown). Thus, a lack of total complementarity in the interfering RNA molecules provided herein is not significantly deleterious, and the methods provided herein of designing individual CODEMIRs each of which target multiple RNAs, can be used to generate such interfering molecules.

The activity of CODEMIR-1 and CODEMIR-2 was assessed, in terms of VEGF mRNA expression, by RT-PCR. Comparative controls were analysed alongside; siVEGF (a comparative control siRNA targeting a different site in VEGF) and siCONTROL (a non-targeting control siRNA) and, finally, a sample not containing DNA (water control). VAIC, a multitargeting interfering RNA designed to be fully complementary to the target binding sequence of CODEMIR-1 was also examined. Two RT-PCR reactions were run on samples of RNA prepared from ARPE-19 cells. Specifically, ARPE-19 cells were transfected with Lipofectamine 2000 and 40nM of the respective RNA treatment, incubated for 24 hrs, then treated with 130 microM deferroxamine for an additional 24 hrs before harvest and extraction of RNA. In one reaction, the PCR product was a 266 nt PCR amplicon from part of the Open Reading Frame (ORF) of VEGF. In the second reaction, the amplified amplicon (243 nt) is located in the 3'UTR of VEGF. To control for loading, PCR amplification of a GAPDH "house-keeping" transcript was also determined. The relative intensities of the GAPDH bands were equal in all treatment lanes. Both VEGF amplicons were significantly reduced in intensity for the ARPE-19 cells transfected with either CODEMIR-1 or siVAIC. The comparative siVEGF control had visibly reduced intensity for the ORF amplicon but not the 3'UTR amplicon. Overall, these results indicate that CODEMIR-1 induced substantial mRNA degradation, whereas CODEMIR-2 resulted in less VEGF mRNA degradation. Without limiting aspects of the invention to any particular theory of operation, it is at least noteworthy that CODEMIR-1 has a relatively high degree of complementarity to the VEGF mRNA target, including the central base pairs adjacent to the RISC cleavage site, while

CODEMIR2 has a central mismatch with the VEGF target mRNA. Thus, CODEMIRs with less than complete complementarity to a given target RNA sequence, may induce mRNA degradation, for example by RISC processing, as an additional means of modulation, or as an alternative to other mechanisms, such as repressing translation of the target

It will be appreciated by one skilled in the art that multitargeting interfering RNA molecules (CODEMIRs) will comprise the sequence corresponding to the complement of the seed. In this example of RNA molecules for altering expression of VEGF-A and ICAM-1, the complementary sequences are UAUGUGGGUGGG (SEQ ID NO: 1) and UGUUUUG (SEQ ID NO: 2) for CODEMIRs 1 and 2 respectively.

Additional CODEMIRs targeting both VEGF and ICAM were designed based on each of the consensus target sequences shown in Figure 1. Each of the CODEMIRS so designed was also significantly active in modulating the target RNAs (CODEMIR1 previously described, other data not shown). The active CODEMIRs are as follows:

5' UAUGUGGGUGGGGUGUCUCUA 3' (SEQ ID NO: 104)
3' UUAUACACCCACCCCACAGAG 5' (SEQ ID NO: 105)

5' UAUGUGGGUCGGGUGGUCUAA 3' (SEQ ID NO: 106)
3' UUAUACACCCACCCCACCAGA 5' (SEQ ID NO: 107)

5' UAUGUGGGUGCGGUGGUGUCU 3' (SEQ ID NO: 108)
3' UUAUACACCCACCCCACCACA 5' (SEQ ID NO: 109)

5' UAUGUGGGUGGGUGAGUGUCU 3' (SEQ ID NO: 110)
3' UUAUACACCCACCCACUCACA 5' (SEQ ID NO: 111)

Variants of CODEMIR-1 with altered overhang lengths were also tested in the ARPE system and found to have significant activity, although CODEMIR-25 had negligible activity on ICAM-1 (data not shown). The sequences of these two CODEMIRs are as follows:

CODEMIR-24
5' UAUGUGGGUCGGUGAGUCUA 3' (SEQ ID NO: 118)
3' UAUACACCCACCCACUCAGA 5' (SEQ ID NO: 119)

CODEMIR-25

5' UAUGUGGGUGGGGUGAGUCU 3' (SEQ ID NO: 188)

3'. AUACACCCACCCCACUCAGA 5' (SEQ ID NO: 189)

Example 2

Comparison of CODEMIR-1 activity with that of a naturally occurring microRNA with some homology

After CODEMIR-1 was designed for multitargeting of both VEGF-A and ICAM-1 targets, according to the methods provided herein (see e.g., Example 1), it was noted that CODEMIR-1 has some homology with the naturally occurring human microRNA miR-299.

Processing of miR-299 is predicted to produce two active strands, miR-299-5p and miR-299-3p. As can be seen below, miR-299-3p has a region with 12 of 15 of the same bases as the guide strand of CODEMIR-1, and the first 8 of those bases are identical. That region of CODEMIR-1 corresponds to the complement of the seed sequence used in its design. Although the two sequences share this homology, they have different central and 3' tail regions (see below).

```
CODEMIR-1  1      UAUGUGGGUGGGGUGAGUCUAA   21  (SEQ ID NO: 100)
                  ||||||||  ||| |
hsa-miR-299-3p 1  UAUGUGGGAUGGUAAACCGCUU 22  (SEQ ID NO: 190)
```

Little is known about the activity of miR-299. No targets for miR-299-3p or miR-299-5p are reported in the Tarbase database, a central repository of validated interactions between mRNAs and miRNAs available on the World Wide Web at the University of Pennsylvania's DNA & Protein Analysis Lab (DIANA Lab) world wide website site at diana.pcbi.upenn.edu/tarbase.html. After further investigation, no published studies of the activity of miR-299-3p could be located. Thus, a comparison was made of the activity of CODEMIR-1 and miR-299 against each of the targets, VEGF-A and ICAM-1, in an expression system.

As can be seen in Figure 4, when compared to an RNA duplex negative control not specific for the targets tested, a RNA duplex comprising the predicted mature strands of miR-299 demonstrated some VEGF suppressive activity, although less than that obtained with

CODEMIR-1. The miR-299 had no significant activity against ICAM-1 (data not shown). CODEMIR-1, therefore, has a markedly different activity profile from the naturally occurring miR-299. Despite some similarities in structure to CODEMIR-1, miR-299 did not have any demonstrable activity with respect to the second RNA (ICAM-1) which CODEMIR-1 was specifically designed to target. This shows the effectiveness of using a rational design process to obtain a functional multitargeting interfering RNA, and the advantages that can be gained, even over naturally occurring sequences that have homology to the seed region used in the design process.

Example 3

Further exemplification of multitargeting of angiogenic factors

More complex angiogenic phenotypes than diabetic retinopathy are found in advanced cancer and in Age-related Macular Degeneration. With Age-related Macular Degeneration (AMD), it is currently thought that accumulation of partially phagocytosed remnants in and below the Retinal Pigmented Epithelial (RPE) layer causes cellular stresses that lead to the production of angiogenic cytokines or chemokines by the RPE cells (eg VEGF-A, IL-8, MCP-1). Additional proteins expressed on the membrane surfaces of RPE cells may further drive this process (e.g. ICAM-1). Overall, the angiogenic factors involved in AMD include: VEGF-A, VEGF-B, IGF-1, MCP-1, IL-8, ICAM-1, bFGF, and PlGF. Indeed, this multitude of pro-angiogenic cytokines acting in combination is analogous to the angiogenesis seen in advanced cancer. In order to increase the number of angiogenic factors that could be covered by a CODEMIR, the full mRNA transcripts derived from the Ensembl database (see Table 1-1) for VEGF-A, ICAM-1 and IGF-1 were used in the search of suitable seeds as previously outlined. A seed consisting of 11 bp present in all 3 transcripts was identified (Table 3-1). Consensus target sequences were derived as described above (Table 3-1). Guide strand sequences for CODEMIRs targeting these sites were determined, and the predicted binding of these CODEMIRs to the 3 target sequences was assessed using RNAhybrid software. Because the 3' seed region contains 4 contiguous G bases, the loading bias is unlikely to be favorable. As shown in Table 3-2, the high duplex binding energy at the 5'end of the guide strand can be reduced by the judicious introduction of mismatched base pairings by modification of the passenger strand.

Table 3-1: Target sequences aligned with candidate CODEMIR consensus target sequences for targeting VEGF-A, ICAM-1 and IGF-1

	Target Site Sequences (5' to 3')
VEGF-A	AAGTCGAGGAAGAG**AGAGACGGGGTCAGAG** (SEQ ID NO: 191)
ICAM-1	TTTTTTTTTTTTCC**AGAGACGGGGTCTCGC** (SEQ ID NO: 192)
IGF-1	TTTGGATTTTTAAT**AGAGACGGGGTTTTAC** (SEQ ID NO: 193)
	Consensus target Sequences
	TGGTTAAC**AGAGACGGGGTCA** (SEQ ID NO: 542)
	TGGTTAAC**AGAGACGGGGTCT** (SEQ ID NO: 194)
	ATGGTTAAC**AGAGACGGGGTA** (SEQ ID NO: 195)
	GATGGTTAAC**AGAGACGGGGT** (SEQ ID NO: 196)
	GGTTAAC**AGAGACGGGGTCTA** (SEQ ID NO: 197)
	GGTTAAC**AGAGACGGGGTCTT** (SEQ ID NO: 198)
	GGGTAAC**AGAGACGGGGTCTA** (SEQ ID NO: 199)
	GGGTAAC**AGAGACGGGGTCTT** (SEQ ID NO: 200)

Table 3-2: Alteration of guide strand 5' terminal stability by introduction of mismatched base pairings through modification of the passenger strand.
NOTE: Underlining in this table indicates the seed, which in some instances has been modified (bolding).

CODEMIR duplex (lower strand = guide, 3' to 5')		mfe*
UGGUUAACAGAGACGGGCUUU	(SEQ ID NO: 201)	-12.2
CUACCAAUUGUCUCUGCCCCA	(SEQ ID NO: 202)	
UGGUUAACAGAGACGGGAUUU	(SEQ ID NO: 203)	-9.7
CUACCAAUUGUCUCUGCCCCA	(SEQ ID NO: 202)	
UGGUUAACAGAGACGGAGUUU	(SEQ ID NO: 204)	-6.4
CUACCAAUUGUCUCUGCCCCA	(SEQ ID NO: 202)	
UGGUUAACAGAGACGAAGUUU	(SEQ ID NO: 205)	-6.1
CUACCAAUUGUCUCUGCCCCA	(SEQ ID NO: 202)	
UGGUUAACAGAGACAGGGUUU	(SEQ ID NO: 206)	-9.4
CUACCAAUUGUCUCUGCCCCA	(SEQ ID NO: 202)	
UGGUUAACAGAGACGGAAUUU	(SEQ ID NO: 207)	-6.4
CUACCAAUUGUCUCUGCCCCA	(SEQ ID NO: 202)	
UGGUUAACAGAGACGAAGUUU	(SEQ ID NO: 208)	-3.1
CUACCAAUUGUCUCUGCCCCA	(SEQ ID NO: 202)	
UGGUUAACAGAGACAAGGUUU	(SEQ ID NO: 209)	-6.1
CUACCAAUUGUCUCUGCCCCA	(SEQ ID NO: 202)	

*Minimum free energy of terminal (5' of the guide) pentamer (kcal/mol)

Alternatively, a broader coverage of the angiogenic factors can be derived. For example, the 7 nt seed TGCAGCT (SEQ ID NO: 210) can be used to construct CODEMIRs targeting simultaneously: VEGF-B, -C, -D, IL-8, bFGF, PlGF, MCP-1, ICAM-1 and IGF-1.

It will be appreciated by one skilled in the art that multitargeting interfering RNA molecules (CODEMIRs) will comprise the sequence corresponding to the complement of the seed. Accordingly, in the example of an RNA molecule for altering expression of any combination of ICAM-1, VEGF-A and IGF-1, the complementary sequence to the seed in Table 3-1 (AGAGACGGGGT) (SEQ ID NO: 211) is ACCCCGUCUCU (SEQ ID NO: 5).

In the example of an RNA molecule for altering expression of any combination of ICAM-1, VEGF-B, VEGF-C, VEGF-D, IL-8, bFGF, PlGF, MCP-1 and IGF-1, the complementary sequence to the seed above (TGCAGCT) (SEQ ID NO: 210) is AGCUGCA (SEQ ID NO: 7).

Example 4

CODEMIRs for metabolic disorders

CODEMIRs may also be suitable for the treatment of complex metabolic diseases such as type 2 diabetes. Two potential gene targets for the treatment of this disease are glucose-6-phosphatase and Inppl1. Full transcript sequences were examined for the presence of common candidate seeds. In this case, a seed of 14 nt identity (CCCACCCACCTACC) (SEQ ID NO: 212) was identified (top of Table 4-1). Candidate CODEMIRs were then designed, via the intermediate of consensus target sites that are shown in Table 4-1.

It will be appreciated by one skilled in the art that multitargeting interfering RNA molecules (CODEMIRs) will comprise the sequence corresponding to the complement of the seed. In this example, these complementary sequences will comprise GGUAGGUGGGUGGG (SEQ ID NO: 10).

Table 4-1 - Target sequences aligned with CODEMIR consensus target sites for targeting Gluc6p and Inppl1.

	Sequences (5' to 3')
Gluc6p	CAGAGTATTTCTGCCCCACCCACCTACCCCCCAAAAA (SEQ ID NO: 213)
Inppl1	GAGCAGATCTCCTTCCCCACCCACCTACCGCTATGAGC (SEQ ID NO: 214)
	Candidate "consensus target sites"
	CTCCGCCCCACCCACCTACCA (SEQ ID NO: 215)
	CTCCTCCCCACCCACCTACCA (SEQ ID NO: 216)
	TCTCCGCCCCACCCACCTACC (SEQ ID NO: 217)
	TCTCCTCCCCACCCACCTACC (SEQ ID NO: 218)

Example 5

Multitargeting of cancer-related gene products

CODEMIRs can also be applied to target multiple unrelated cancer genes in order to more competently control the tumour phenotype. By way of example, inappropriate activation of β-catenin, K-Ras and EGFR is found in many advanced colorectal adenocarcinomas. Simultaneous targeting of these three genes was sought with a CODEMIR. The full-length transcripts for all three genes were used in the search for candidate seeds. In the case of K-ras, both alternative transcripts (a and b) were included. A 13 bp seed (CAUUCCAUUGUUU) (SEQ ID NO: 9) was found and appropriate candidate CODEMIRs identified. Some alternative CODEMIR consensus target sequences are listed in Table 5-1.

CODEMIRs targeting this seed and which comprise a complement to this seed may be of interest for the treatment of colorectal and other cancer, especially those in which altered β-catenin, K-ras and/or EGFR signaling contributes to the malignant phenotype.

It will be appreciated by one skilled in the art that multitargeting interfering RNA molecules (CODEMIRs) will comprise the sequence corresponding to the complement of the seed. In this example, the complementary sequence is AAACAAUGGAAUG (SEQ ID NO: 8).

Table 5-1 – Target site sequences aligned with candidate CODEMIR consensus sequences for the targeting of *β-catenin*, *K-ras-B*, *K-ras-A* and *EGFR*.

	Target Site Sequences (5' to 3')	
beta-Catenin	CAGAGGACUAAAUAC**CAUUCCAUUGUUU**GUGCAG	(SEQ ID NO: 219)
K-ras A	CUGGUAACAGUAAUA**CAUUCCAUUGUUU**UAGUAA	(SEQ ID NO: 220)
K-ras-B	CUGGUAACAGUAAUA**CAUUCCAUUGUUU**UAGUAA	(SEQ ID NO: 220)
EGFR	GACUUGUUUGUCUUC**CAUUCCAUUGUUU**UGAAAC	(SEQ ID NO: 221)
	Candidate consensus target sequences	
	AUAAUC**CAUUCCAUUGUUU**UA	(SEQ ID NO: 222)
	AUAAUA**CAUUCCAUUGUUU**UA	(SEQ ID NO: 223)
	AUAUUC**CAUUCCAUUGUUU**UA	(SEQ ID NO: 224)
	AUAUUA**CAUUCCAUUGUUU**UA	(SEQ ID NO: 225)
	GUAAUC**CAUUCCAUUGUUU**UA	(SEQ ID NO: 226)
	GUAAUA**CAUUCCAUUGUUU**UA	(SEQ ID NO: 227)
	GUAUUC**CAUUCCAUUGUUU**UA	(SEQ ID NO: 228)
	GUAUUA**CAUUCCAUUGUUU**UA	(SEQ ID NO: 229)
	CUAUAAUC**CAUUCCAUUGUUU**	(SEQ ID NO: 230)
	CUAUAAUA**CAUUCCAUUGUUU**	(SEQ ID NO: 231)
	CUAUAUUC**CAUUCCAUUGUUU**	(SEQ ID NO: 232)
	CUAUAUUA**CAUUCCAUUGUUU**	(SEQ ID NO: 233)
	CUGUAAUC**CAUUCCAUUGUUU**	(SEQ ID NO: 234)
	CUGUAAUA**CAUUCCAUUGUUU**	(SEQ ID NO: 235)
	CUGUAUUC**CAUUCCAUUGUUU**	(SEQ ID NO: 236)
	CUGUAUUA**CAUUCCAUUGUUU**	(SEQ ID NO: 237)
	CAAUAAUC**CAUUCCAUUGUUU**	(SEQ ID NO: 238)
	CAAUAAUA**CAUUCCAUUGUUU**	(SEQ ID NO: 239)
	CAAUAUUC**CAUUCCAUUGUUU**	(SEQ ID NO: 240)
	CAAUAUUA**CAUUCCAUUGUUU**	(SEQ ID NO: 241)
	CAGUAAUC**CAUUCCAUUGUUU**	(SEQ ID NO: 242)
	CAGUAAUA**CAUUCCAUUGUUU**	(SEQ ID NO: 243)
	CAGUAUUC**CAUUCCAUUGUUU**	(SEQ ID NO: 244)
	CAGUAUUA**CAUUCCAUUGUUU**	(SEQ ID NO: 245)

Example 6

Targeting of multiple sites within the HIV genome

The CODEMIR approach can be used to target proteins of interest that are likely to be mutated in chronic forms of disease. Mutations may be particularly prevalent in cancer and viral disease in which drug-resistant forms often evolve. In this example, VIROMIRs were designed to target multiple sites in the Human Immunodeficiency Virus (HIV). The requirement for simultaneous mutation at several sites, in order to overcome the effects of such a VIROMIR, is likely to provide a high genetic hurdle to the emergence of resistant viral clones or quasispecies. The genome of the HXB2 strain of HIV I serotype B (GenBank Accession K03455) was used as the principal sequence of interest and was examined with bioinformatics methods detailed elsewhere in this application to find seeds occurring at more than one location. All HIV I clade B isolates in the LANL database as of 1 August 2005 which contain full sequences for any of the GAG, ENV, POL, TAT, VIF, VPR, VPU and NEF genes were used in these analyses.

Three 9-base seeds were found to occur in 4 genetic contexts in this reference sequence. These were: ATCAAGCAG (SEQ ID NO: 246), TGGAAAGGA (SEQ ID NO: 247) and AAAGAAAAA (SEQ ID NO: 37)

In the population of Clade B isolates described above, the first seed (ATCAAGCAG) (SEQ ID NO: 246), which is of greater interest because of its greater base complexity, was found to be present in 91%, 76%, 78% and 74% of the isolates with respect to the GAG, POL, VIF and ENV genetic contexts of this seed, respectively. This is shown below as the seed flanked by the genetic context at each site.

AAACACAGTGGGGGGAC**ATCAAGCAG**CCATCCAAATGTTAAAAG (SEQ ID NO: 248) GAG 91%
CCTGTTGGTGGCCGGGAA**TCAAGCAG**GAATTTGGAATTCCCTAC (SEQ ID NO: 249) POL 76%
TAGCCCTAGGTGTGAATA**TCAAGCAG**CACATAACAAGGTAGGAT (SEQ ID NO: 250) VIF 78%
AACTCACAGTCTGGGGGC**ATCAAGCAG**CTCCAGGCAAGAATCCTG (SEQ ID NO: 251) ENV 74%

The bolded regions correspond to independent occurrences of the seed. These regions have been annotated to the genes GAG, POL, VIF, and ENV.

An additional twenty 11-base seeds occurred at two sites within the reference HIV genome. There were no seeds of a length of 10 bases that were not subsumed in the 11 base seeds. These seeds are listed below, each with its genetic context, the HIV gene in which it occurs, and its rate of occurrence in that genetic context.

1
AGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAAACATCAGAAGCTGTAGACAAATACTGGGACAGCTACAACCATCCCTT (SEQ ID NO: 252) GAG 52%
ACAACATCTGTTGAGGTGGGGACTTACCACACCAGACAAAAAACATCAGAAAGAACCTCCATTCCTTTGGATGGGTTATGAACTCCATC (SEQ ID NO: 253) POL 84%

2
AAGGATAGAGATAAAAGACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGAAAAAAGCACAGCAAGCAGCAGC (SEQ ID NO: 254) GAG 81%
AACAGGGACTAATTAGGAAAAGCAGGATATGTTAACTAATAGAGGAAGACAAAAAGTTGTCACCCTAACTGACACAACAAATCAGAAG (SEQ ID NO: 255) POL 23%

3
AGGCAAGAGTTTGGCTGAAGCAATGAGCCAATAACAAATTCAGCTACCATAATGATGCAGAGAGGCAATTTTAGGAACCAAGAAAGA (SEQ ID NO: 256) GAG 31%
CTTGGCACTACCTAACCAGGACGATCTGCGGAGCCTGTGCCTCTTCAGCTACCACCGCTTGAGAGACTTACTCTTGATTGTAACGAGGATTGT (SEQ ID NO: 257) REV 42%

4
AACAAATTCAGCTACCATAATGATGCAGAGAGGCAATTTTAGGAACCAAGAAAGATTGTTAAGTGTGTCAATTGTGGCAAAGAAGGGCA (SEQ ID NO: 258) GAG 38%
ACCCAGGGATTAAAGTAAGGCAATTATGTAAACTCCTTAGAGGAACCAAAGCACTAACAGAAGTAATACCACTAACAGAAGAAGCAGAGC (SEQ ID NO: 259) POL 12%

5
CAAAAGTTAACAATGGCCATTGACAGAAGAAAAATAAAAGCATTAGTAGAAATTGTACAGAGATGGAAAAGGAAGGGAAAATTTCAA (SEQ ID NO: 260) POL 68%
TAGTACATGTAACGCAACCTATACCAATAGTAGCAATAGTAGCATTAGTAGTAGCAATAGTAGCAATTGTGTGGTCCATAGTAA (SEQ ID NO: 261) VPU 72%

6
CACCGGTGCTACGGTTAGGGCCGCCTGTTGGTGGGCGGGAATCAAGCAGGAATTTGGAATTCCCTACAATCCCCAAAGTCAAGGAGTAGT (SEQ ID NO: 262) POL 75%
AAGCCTTATTAGGACACATAGTTAGCCCTAGGTGTGAATATCAAGCAGGACATAACAAGGTAGGATCTCTACAATACTTGGCACTAGCA (SEQ ID NO: 263) VIF 78%

7
CAGGGAAAGAATAGTAGACATAATAGCAACAGACATACAAACTAAAGAATTACAAAAAATTCAAAATTTTCGGGTTT (SEQ ID NO: 264) POL 52%
CTCAAATGTGGTGAATCTCCTACAGTATTGGAGTCAGGAACTAAAGAATAGTGCTGTTAGCTTGCTCAATGCCACAGCCATAGCAGTA (SEQ ID NO: 265) REV 68%

8
ACAGGAGCAGATGATACAGTATTAGAAGAAATGAGTTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTTATCAAA (SEQ ID NO: 266) POL 95%
GAAACAGGGCAGGAAAGCAGCATATTTTCTTTTAAAATTAGCCAGGAAGATGGCCAGTAAAAACAATACATACTGACAATGGCAGCAATTTC (SEQ ID NO: 267) POL 95%

9

AATGAGTTTGCCAGGAAGATGGAAACCAAAAATGATAGGG**GGAATTGGAGG**TTTTATCAAAGTAAGACAGTATGATCAGATACTCATAGA (SEQ ID NO: 268) POL 98%
AAAGGAAAAGGTCTACCTGGCATGGGTACCAGCACACAAA**GGAATTGGAGG**AAATGAACAAGTAGATAAATTAGTCAGTGCTGGAATCAG (SEQ ID NO: 269) POL 92%

10

GGCAACTAAAGGAAGCTCTATTAGATACAGGAGCAGATGA**TACAGTATTAG**AAGAAATGAGTTTGCCAGGAAGATGGAAACCAAAAATGA (SEQ ID NO: 270) POL 80%
ATCAGATACTCATAGAAATCTGTGGACATAAAGCTATAGG**TACAGTATTAG**TAGGACCTACACCTGTCAACATAATTGGAAGAAATCTGT (SEQ ID NO: 271) POL 92%

11

AAAGTAAGGCAATTATGTAAACTCCTTAGAGGAACCAAAG**CACTAACAGAA**GTAATACCACTAACAGAAGAAGCAGAGCTAGAACTGGC (SEQ ID NO: 272) POL 85%
AAACTCCTTAGAGGAACCAAAGCACTAACAGAAGTAATAC**CACTAACAGAA**GAAGCAGAGCTAGAACTGGCAGAAAACAGAGAGATTCT (SEQ ID NO: 273) POL 85%

12

CCCACAAGATTTAAACACCATGCTAAACACAGTGGGGGGGA**CATCAAGCAGC**CATGCAAATGTTAAAAGAGACCATCAATGAGGAAGCTGC (SEQ ID NO: 274) GAG 89%
TTGAGGCGCAACAGCATCTGTTGCAACTCACAGTCTGGGGC**CATCAAGCAGC**TCCAGGCAAGAATCCTGGCTGTGGAAAGATACCTAAAGG (SEQ ID NO: 275) ENV 74%

13

CATACCTAGTATAAACAATGAGACACCAGGGATTAGATAT**CAGTACAATGT**GCTTCCACAGGGATGGAAAGGATCACCAGCAATATTCCA (SEQ ID NO: 276) POL 86%
AAGACGTTCAATGGAACAGGACCATGTACAAATGTCAGCAC**AGTACAATGT**ACACATGGAATTAGGCCAGTAGTATCAACTCAACTGCTG (SEQ ID NO: 277) ENV 84%

14

ACTATTTTTAGATGGAATAGATAAGGCCCAAGATGAACAT**GAGAAATATCA**CAGTAATTGGAGAGCAATGGCTAGTGATTTTAACCTGCC (SEQ ID NO: 278) POL 61%
CTAATAGAAAGAGCAGAAGACAGTGGCAATGAGAGTGAAG**GAGAAATATCA**GCACCTGTGGAGATGGGGGTGGAGATGGGGCACCATGCT (SEQ ID NO: 279) ENV 3%

15

TACTTGGGCAGGAGTGGAAGCCATAATAAGAATTCTGCAAC**AACTGCTGTT**TACCCATTTTCAGAATTGGGTGTCGACATAGCAGAATAG (SEQ ID NO: 280) VPR 77%
AGTACAATGTACACATGGAATTAGGCCAGTAGTATCAACT**CAACTGCTGTT**AAATGGCAGTCTAGCAGAAGAAGAGGTAGTAATTAGATC (SEQ ID NO: 281) ENV 61%

16

TAGAAAGAGCAGAAGACAGTGGCAATGAGAGTGAAGGAGA**AATATCAGCAC**TTGTGGAGATGGGGGTGGAGATGGGGCACCATGCTCCTTG (SEQ ID NO: 282) ENV 2%
AATGATAATGGAGAAAGGAGAGATAAAAAACTGCTCTTTC**AATATCAGCAC**AAGCATAAGAGGTAAGGTGCAGAAAGAATATGCATTTTTT (SEQ ID NO: 283) ENV 3%

17

AGACAGTGGCAATGAGAGTGAAGGAGAAATATCAGCACTTGTGGAGATGGGGGTGGAGATGGGGCACCATGCTCCTTGGGATGTTGATGA (SEQ ID NO: 284) ENV 57%

TGAGAGTGAAGGAGAAATATCAGCACTTGTGGAGATGGGGGTGGAGATGGGGCACCATGCTCCTTGGGATGTTGATGATCTGTAGTGCTA (SEQ ID NO: 285) ENV 10%

18

TCCTGTGTTAGTTTAAAGTGCACTGATTTGAAGAATGATACTAATACCAATAGTAGTAGCGGGAGAATGATAATGGAGAAAGGAGAGATAA (SEQ ID NO: 286) ENV 16%

AAGGTGCAGAAAGAAATATGCATTTTTTTATAAACTTGATATAATACCAATAGATAATGATACTACCAGCTATAAGTTGACAAGTTGTAAC (SEQ ID NO: 287) ENV 1%

19

AATGGAATAACACACTTTAAAACAGATAGCTAGCAAATTAAGAGAACAATTTGGAAATAATAAACAATAATCTTTAAGCAATCCTCAGGAG (SEQ ID NO: 288) ENV 63%

GTACAGGCCAGACAACTATTGTCTGGTATAGTGCAGCAGCAGAACAATTTGCTGAGGGCTATTGAGGCGCAACAGCATCTGTTGCAACTC (SEQ ID NO: 289) ENV 20%

20

ATTGTGGAGGGGAATTTTTCTACTGTAATTCAACACAACTGTTTAATAGTACTTGGAAGGGTCAAAGA (SEQ ID NO: 290) ENV 79%

TTTTCTACTGTAATTCAACACAACTGTTTAATAGTACTTGGAAGGGTCAAATAACACTGAAGGAAGTG (SEQ ID NO: 291) ENV 20%

Ultimately, an effective RNA therapeutic of the invention should provide broad coverage of the affected population and it is obviously desirable to target sequences that are highly represented in this patient population. Therefore, of the seeds presented above, those with undesirably low rates of occurrence in their specific genetic contexts in the Clade B isolates available from the LANL Database as defined previously were removed from present consideration. For the purposes of this example, undesirably low rates of occurrence in their specific genetic contexts was defined as <50% of the Clade B isolates.

In order to prioritize and test candidate VIROMIRs, it is important to have screening methods that are compatible with the intended target sequence. The pNL4.3 assay is widely used in the field of HIV research as a valuable, validated screen for drugs active in HIV and was used by us to test candidate VIROMIRs. However, there are some differences between the sequences of the HIV component of the pNL4.3 plasmid and that of the reference HIV strain (K03455) used in the design of the VIROMIRs. Therefore, comparison of the sequences of the reference strain and the sequence of the pNL4.3 plasmid was carried out and only the designed VIROMIRs from the above-detailed steps with conserved seed sites present in the plasmid were selected. VIROMIRs corresponding to seeds #1 and #12 from the previous list of 11-base seeds were excluded on this basis. However, one skilled in the art will realize that these 2 VIROMIRs may be of use therapeutically and that the decision here was simply based on compatibility with the testing system chosen. Other testing systems such as viral challenge assays, fusion reporters, viral pseudoparticles among others, each representing any multitude of therapeutically relevant or irrelevant sequences could equally be considered.

There were then nine 11-base seeds for consideration and, of these, Seed 6 (POL/VIF; see above) was chosen as an example of the design of a VIROMIR to target multiple sites in a viral genome. A number of consensus target sequences for the two seed 6 occurrences were derived (Table 6-1), candidate VIROMIR guide sequences were identified, and the predicted hybridization of these sequences to the HIV target sites was assessed using RNAhybrid software. The predicted VIROMIR RNA duplexes were further analyzed with respect to factors likely to generate the desired strand loading bias. An example of a CODEMIR guide strand targeting seed site 6 in the HIV genome is shown in Figure 5.

Table 6-1: Target site sequences and aligned candidate CODEMIR consensus target sequences for targeting two sites in the HIV genome.

	Target Site Sequences (5' to 3')
Target site 1	TCGTGGGCGGCAATCAAGCAGGAATTTG (SEQ ID NO: 292)
Target site 2	CTACGTGTGAATATCAAGCAGGACATAA (SEQ ID NO: 293)
	Candidate consensus target sequences
	TTGTGCGAAAATCAAGCAGGA (SEQ ID NO: 294)
	TTGTGCGAATATCAAGCAGGA (SEQ ID NO: 295)
	TTGGGCGAAAATCAAGCAGGA (SEQ ID NO: 296)
	TTGGGCGAATATCAAGCAGGA (SEQ ID NO: 297)
	TCGTGCGAAAATCAAGCAGGA (SEQ ID NO: 298)
	TCGTGCGAATATCAAGCAGGA (SEQ ID NO: 299)
	TCGGGCGAAAATCAAGCAGGA (SEQ ID NO: 300)
	TCGGGCGAATATCAAGCAGGA (SEQ ID NO: 301)
	GTGTGCGAAAATCAAGCAGGA (SEQ ID NO: 302)
	GTGTGCGAATATCAAGCAGGA (SEQ ID NO: 303)
	GTGGGCGAAAATCAAGCAGGA (SEQ ID NO: 304)
	GTGGGCGAATATCAAGCAGGA (SEQ ID NO: 305)
	GGGTGCGAAAATCAAGCAGGA (SEQ ID NO: 306)
	GGGTGCGAATATCAAGCAGGA (SEQ ID NO: 307)
	GCGGGCGAAAATCAAGCAGGA (SEQ ID NO: 308)
	GCGGGCGAATATCAAGCAGGA (SEQ ID NO: 309)
	ATGGGCGAAAATCAAGCAGGA (SEQ ID NO: 310)

Consensus target sequences were similarly designed for the one selected 9-base seed and the other eight 11-base seeds. As appreciated by one skilled in the art and as outlined in other examples of the invention and Table 6-1, there are many possible consensus target sequences, although only 1 such sequence in each case was used here. The guide strands were generated as the complements of these consensus target sequences as indicated above. The corresponding passenger strands were designed to be the complement of the guide strand, minus the first 2 bases at the 5'-extremity and with a 3'-extremity extension of UU, thereby generating dual 2-base overhangs at each 3' extremity.

The 10 VIROMIR candidates were thus:

VM001

5' GUGGGCGAACAUCAAGCAGUU 3' (SEQ ID NO: 311)
3' GGCACCCCGCUUGUAGUUCGUC 5' (SEQ ID NO: 312)

VM002

5' GCAAUAAAAGCAUUAGUAGUU 3' (SEQ ID NO: 313)
3' AUCGUUAUUUUCGUAAUCAUC 5' (SEQ ID NO: 314)

VM003

5' GCGCGAAAAUCAAGCAGGAUU 3' (SEQ ID NO: 315)
3' UACCCGCUUUUAGUUCGUCCU 5' (SEQ ID NO: 316)

VM004

5' GAGUCAGAAACUAAAGAAUUU 3' (SEQ ID NO: 317)
3' GUCUCAGUCUUUGAUUUCUUA 5' (SEQ ID NO: 318)

VM005

5' GCAAUAGAUACAGUAUUAGUU 3' (SEQ ID NO: 319)
3' UUCGUUAUCUAUGUCAUAAUC 5' (SEQ ID NO: 320)

VM006

5' GGGUUUACCAGGAAGAUCGUU 3' (SEQ ID NO: 120)
3' UACCCAAAUGGUCCUUCUACC 5' (SEQ ID NO: 121)

VM007

5' CGCUAAAAGCAAUUGGAGGUU 3' (SEQ ID NO: 321)
3' UUGCGAUUUUCCUUAACCUCC 5' (SEQ ID NO: 322)

VM008

5' CGUGAUAACAGUACAAUGUUU 3' (SEQ ID NO: 323)
3' UUGCACUAUUGUCAUGUUACA 5' (SEQ ID NO: 324)

VM009

5' AGCCAUAGCACUAACAGAAUU 3' (SEQ ID NO: 325)
3' CUUCGCUAUCGUGAUUGUCUU 5' (SEQ ID NO: 326)

VM010

5' UUCCACCUCAACUGCCUGUUUU 3' (SEQ ID NO: 327)
3' UUAAGGUGGAGUUGACGACAA 5' (SEQ ID NO: 328)

As an added precaution, the predicted hybridization of the ten guide strand sequences of these VIROMIRs to the pNL4.3 target sequence was assessed using RNAhybrid to ensure appropriate binding as predicted based on the presence of the seed. In some cases, this binding analysis identified other possible seed-based binding interactions between the candidate guide strands and other sites on the HIV genome.

HIV generally causes chronic infection with *in vivo* viral reservoirs. Consequently, VIROMIRs targeting HIV are most likely to be therapeutically effective as cell-expressed short hairpin RNAs (shRNAs) rather than as synthetic RNA duplexes because of a need for continued therapeutic cover to prevent re-emergence from latent sites.

Three representative VIROMIRs were selected (VM004, VM006 and VM010) for expression as shRNA. Contiguous DNA sequences corresponding to: BamHI restriction site, G initiator, VIROMIR passenger, Xho loop sequence (ACTCGAGA), VIROMIR guide strand, polIII terminator and HindIII restriction site were assembled and prepared as dsDNA. They were then cloned into a pSIL vector under the control of a H1 promoter. By way of example, the double-stranded DNA insert designed to encode an shRNA VIROMIR approximating VM006 is shown below (loop sequence in parentheses and terminator italicized):

5'GATCCGATGGGTTTACCAGCAAGATGG(ACTCGAGA)CCATCTTGCTGGTAAACCCATTTTTTTGGAA -3' (SEQ ID NO: 329)

3' GCTACCCAAATGGTCGTTCTACC(TGAGCTCT)GGTAGAAGGACCATTTGGGTAAAAAAACCTTTCGA-5' (SEQ ID NO: 330)

One skilled in the art will appreciate that when transcribed, the encoded RNA folds into a hairpin structure, which is modified by the cellular Drosha and Dicer proteins to generate active VIROMIR RNA duplex(es). The skilled artisan will also recognize that a number of variations of the design of the shRNA construct could be considered. These include but are not limited to: length, sequence and orientation of the shRNA duplex components (guide strand, passenger strand, precursors), length and sequence of the loop, choice of promoter, initiator and terminator sequences as well as the cloning strategies used to assemble the final construct.

Each of these shRNA contructs was tested in HEK-293 cells by co-transfecting with the pNL4.3 plasmid. Specifically, HEK-293 cells were seeded at density of 2 x 10^5 cells in 1 ml Optimem medium / well in a 12-well plate. Cells were transfected 24 hr later with 200µL DNA: Lipofectamine mix (200ng pNL4.3 plasmid, 67ng VIROMIR pSIL construct in 100µL complexed with 2.7µL Lipofectamine 2000 in Optimem). After changing the medium 24 hours later, the production of p24 was assayed by collection of the supernatant after a further 24 hours of incubation.

The production of p24 was expressed as a percentage of the production from cells transfected with the empty control plasmid. As shown in Figure 7, HIV was suppressed by

60% in the case of the shRNA form of VM006, whereas the other two constructs had no detectable activity.

It will be appreciated by one skilled in the art that multitargeting interfering RNA molecules will comprise the sequence corresponding to the complement of the seed. In this example, these complementary sequences will comprise CUGCUUGAU (SEQ ID NO: 12), UCCUUUCCA (SEQ ID NO: 13), UUUUUCUUU (SEQ ID NO: 14), UUCUGAUGUUU (SEQ ID NO: 15), UCUUCCUCUAU (SEQ ID NO: 16), UGGUAGCUGAA (SEQ ID NO: 17), CUUUGGUUCCU (SEQ ID NO: 18), CUACUAAUGCU (SEQ ID NO: 19), UCCUGCUUGAU (SEQ ID NO: 20), AUUCUUUAGUU (SEQ ID NO: 21), CCAUCUUCCUG (SEQ ID NO: 22), CCUCCAAUUCC (SEQ ID NO: 23), CUAAUACUGUA (SEQ ID NO: 24), UUCUGUUAGUG (SEQ ID NO: 25), GCUGCUUGAUG (SEQ ID NO: 26), ACAUUGUACUG (SEQ ID NO: 27), UGAUAUUUCUC (SEQ ID NO: 28), AACAGCAGUUG (SEQ ID NO: 29), GUGCUGAUAUU (SEQ ID NO: 30), CCCAUCUCCAC (SEQ ID NO: 31), UAUUGGUAUUA (SEQ ID NO: 32), CAAAUUGUUCU (SEQ ID NO: 33), UACUAUUAAAC (SEQ ID NO: 34)

Example 7

Multitargeting of gene products implicated in Alzheimer's disease

In some cases it may be beneficial to include multiple transcript variants corresponding to at least one of the targets of interest. For example, down-regulation of presenilin-1 and the four variant isoforms of BACE-1 could be therapeutically advantageous for the treatment of Alzheimer's disease. In this case, examination of the five corresponding sequences yields several candidate seeds of 11 and 12 bp identity including: ATATGATAGGC (SEQ ID NO: 331), AGCAGGCACCA (SEQ ID NO: 66), GCCATATTAATT (SEQ ID NO: 332), AGCCCAGAGGG (SEQ ID NO: 68), ATGAGGAAGAA (SEQ ID NO: 333), TCTGTATAAATA (SEQ ID NO: 334) and GAATTTTGGTG (SEQ ID NO: 335). From these, those that have appropriate secondary characteristics (strand loading bias, continued partial identity, sequence complexity etc) may be of specific interest for the design of CODEMIRs useful in the treatment of Alzheimer's disease.

It will be appreciated by one skilled in the art that multitargeting interfering RNA molecules (CODEMIRs) will comprise the sequence corresponding to the complement of the seed. In this example, these complementary sequences are GCCUAUCAUAU (SEQ ID NO: 58), UGGUGCCUGCU (SEQ ID NO: 59), AAUUAAUAUGGC (SEQ ID NO: 60), CCCUCUGGGCU (SEQ ID NO: 61), UUCUUCCUCAU (SEQ ID NO: 62), UAUUUAUACAGA (SEQ ID NO: 63), and CACCAAAAUUC (SEQ ID NO: 64).

Example 8

Use of wobbles and mismatches in the design of CODEMIRs

The length of the seed may, in some cases be reduced to enable the requisite multi-targeting to occur. However, in these cases, it is advisable to limit the selection of these short seeds to those that have regions of further identity juxtaposed near the identified seed. For example, VEGF-A and bFGF targeting could be achieved through the design of a CODEMIR guide strand that would be predicted to bind to **AATGTTCCCACTCA** (VEGF-A) (SEQ ID NO: 336) and **AATGTTCAGACTCA** (bFGF) (SEQ ID NO: 337). In this instance, this further region of identity may compensate for the short ACTCA (SEQ ID NO: 338) seed.

Alternatively, the seed could include mismatches that would correspond to wobble base-pairing between the target and the CODEMIR guide strand. In this situation, G:U wobble base pairing can be utilized to design the 5' region of a CODEMIR guide strand with predicted binding to several target transcripts. In Table 8-1, the 5'region of a CODEMIR targeting VEGF-A, ICAM-1, PlGF and IGF-1 is derived from a suitable seed (CCTGGAG) (SEQ ID NO: 339) in the corresponding target mRNA.

Table 8-1. Use of wobble-base pairing compatible mis-matches

Gene of interest	Target sequence (5' – 3')	
VEGF-A	AGTCCTGGAG (SEQ ID NO: 340)	
ICAM-1	AACCCTGGAG (SEQ ID NO: 341)	
PlGF	GGCCCTGGAG (SEQ ID NO: 342)	
IGF-1	GGTCCTGGAG (SEQ ID NO: 343)	
	Consensus	CODEMIR 5'region
	RRYCCTGGAG* (SEQ ID NO: 344)	5'CUCCAGGGUU 3' (SEQ ID NO: 345)

*Y indicates pyrimidine and R purine

Further cases of tolerated mismatches in CODEMIRs have been examined in the context of the first base of the seed. Tolerability of a mismatch in this position would greatly enhance the multi-targeting nature of CODEMIRs. For example, in a situation in which two targets share the same seed, but a third target has a mismatch in the first seed position, maintaining activity in spite of this mismatch against the third target enhances the repertoire of CODEMIR activity. Flexibility in this position also enables modification of the 5'terminus of the CODEMIR guide strand with the goal of modulating strand-loading bias, which may also impact on activity. The efficacy of CODEMIRs with a 5' mismatch of the guide strand with the target was investigated with CODEMIRs 13 to 15 (Table 8-2). These results, shown in Figure 6, indicate that CODEMIRs with a single mismatch retain activity against the target.

Table 8-2 – Examples of CODEMIRs targeting VEGF and ICAM-1 containing CODEMIR-target mismatches (bold face) at the 5' end of the guide strand

CODEMIR (Guide strand on bottom 3' to 5')	VEGF binding*	ICAM-1 binding*
CODEMIR-13	5'A A A 3'	5' C C 3'
5'CUCACCCACCCACACAUACAU 3' (SEQ ID NO: 112)	GAC CACCCACCCACAUACAU (SEQ ID NO: 346)	CUC CCCACCCACACAUACAU (SEQ ID NO: 347)
3'CUGAGUGGGUGGGCGUGUAUGUA 5' (SEQ ID NO: 113)	CUG GUGGGUGGGCGUGUAUGUA	GAG GGGUGGGCGCAUGUA
	3' A 5' (SEQ ID NO: 113)	3'CU U 5' (SEQ ID NO: 113)
CODEMIR-14	5' A C 3'	5'C U 3'
5'UCACCCACCCACAUACAUAU 3' (SEQ ID NO: 114)	CACCCACCCACAUACAUA (SEQ ID NO: 348)	CUC CCCACCCACAUACAU (SEQ ID NO: 347)
3'UGAGUGGGUGGGUGUAUGUAC 5' (SEQ ID NO: 115)	GUGGGUGGGUGUAUGUAC	GAG GGGUGGGUGUAUGUA
	3'UGA 5' (SEQ ID NO: 115)	3'U U U 5' (SEQ ID NO: 115)
CODEMIR-15	5' A A 3'	5'C C 3'
3'UCACCCACCCACAUACAUAU 3' (SEQ ID NO: 116)	CACCCACCCACAUACAU (SEQ ID NO: 349)	CUC CCCACCCACAUACAUC (SEQ ID NO: 350)
3'UGAGUGGGUGGGUGUAUGUAA 5' (SEQ ID NO: 117)	GUGGGUGGGUGUAUGUA	GAG GGGUGGGUGUAUGUAA
	3'UGA A 5' (SEQ ID NO: 117)	3'U C 5' (SEQ ID NO: 117)

* Upper strand = target mRNA, lower strand = CODEMIR guide. Predicted mismatches are shown in bold font.

It will be appreciated by one skilled in the art that multitargeting interfering RNA molecules (CODEMIRs) will comprise the sequence corresponding to the complement of the seed. In the example of an RNA molecule for altering expression of VEGF-A and bFGF, a guide strand could comprise UGAGUNNGAACAUU (SEQ ID NO: 72) where N is any base. In the example of an RNA molecule for altering expression of any combination of VEGF-A, ICAM-1, PlGF and IGF-1, the complementary sequence to the seed CCUGGAG (SEQ ID NO: 75) comprises CUCCAGG (SEQ ID NO: 74).

Example 9

Co-suppression of virus and disease-implicated host protein

In the case of infectious diseases, CODEMIRs can also be utilized to target both the genome of the infectious agent and one or more key host "drivers" of the disease. For example, TNF-alpha is considered a major disease-associated factor in Hepatitis C Virus infection and its sequelae. Analysis of the genome of HCV and the TNF-alpha mRNA sequence was used to identify seeds consisting of: CCCTGTGAGGA (SEQ ID NO: 351), CACCATGAGCAC (SEQ ID NO: 352), CAGGGCTCCAGG (SEQ ID NO: 353) and, GTGGAGCTGAGA (SEQ ID NO: 354).

1. The first seed, CCCTGTGAGGA (SEQ ID NO: 351) was found to be highly conserved (92%) in the 155 available sequences for genotype 1a/1b isolates and, therefore, an attractive target sequence from a therapeutic point of view.

In terms of genetic context, the seed is in the 5'NTR of HCV and the ORF of TNFalpha

CGGCGACACTCCACCATGAATCACTCCCCTGTGAGGAACTACTGTCTTCACGCAGAAAGC (SEQ ID NO: 355) HCV 43

TGCCCAGGTCTACTTTGGGATCATTGCCCTGTGAGGAGGACGAACATCCAACCTTCCCAA (SEQ ID NO: 356) TNFa 864

A consensus target site is, possibly:

5'-GAATCACTGCCCTGTGAGGAA-3' (SEQ ID NO: 357)
and duplex:

5'- UUCCUCACAGGGCAGUGAUUC-3' (SEQ ID NO: 122)
3'-UUAAGGAGUGUCCCGUCACUA-5' (SEQ ID NO: 123)

with predicted binding:

```
HCV    5' U        C            C 3'
           GAAUCACU CCCUGUGAGGAA      (SEQ ID NO: 358)
           CUUAGUGA GGGACACUCCUU
guide  3'          C              5' (SEQ ID NO: 122)
mfe: -38.8 kcal/mol

TNFa   5' G                       G 3'
           GGAUCAUUGCCCUGUGAGGAG      (SEQ ID NO: 359)
           CUUAGUGACGGGCACACUCCUU
guide  3'                         5' (SEQ ID NO: 122)
mfe: -42.6 kcal/mol
```

One skilled in the art would recognize that this interfering RNA could be further modified to improve its strand loading bias and stability. This could be achieved through the use of modified bases (eg LNA, 2'-F or 2'-O-methyl) in the 3' duplex section of the guide strand (would also likely improve stability), for example:

```
5'-  UUCCUCACAGGGCAGUGAUUC-3' (SEQ ID NO: 122)
3'-UUAAGGAGUGUCCCGUCACUA-5'(SEQ ID NO: 123)
```

in which the bolding indicates LNA-modified bases. Alternatively, judicious introduction of mismatches such as, for example:

```
5'-  UUCCUCACAGGGCAGUGAUUC-3' (SEQ ID NO: 122)
3'-UUAAAGAGUGUCCCGUCACUA-5' (SEQ ID NO: 124)
```

in which the bolding indicates a mismatched base in the non-active passenger strand could be used. Alternatively, one could consider substitution of bases in the 3' duplex part of the guide strand and corresponding changes in the passenger strand such as:

```
5'-  UUCCUCACAGGGCAGUGGUUC-3' (SEQ ID NO: 125)
3'-UUAAGGAGUGUCCCGUCACCA-5' (SEQ ID NO: 126)
```

in which the bolding indicates the changed bases. The change in the 3' end of the guide strand is possibly associated with minimal change in activity or hybridization given the G:U wobble that would occur with the target binding sequences as shown below:

```
HCV     5' U         C              C 3'
            GAAUCACU CCCUGUGAGGAA      (SEQ ID NO: 358)
            CUUGGUGA GGGACACUCCUU
guide   3'           C                5' (SEQ ID NO: 125)

TNFa    5' G                        G 3'
            GGAUCAUUGCCCCUGUGAGGAC     (SEQ ID NO: 359)
            CUUGGUCACGGGACACUCCUU
guide   3'                           5' (SEQ ID NO: 125)
```

It will be appreciated by one skilled in the art that multitargeting interfering RNA molecules (CODEMIRs) will comprise the sequence corresponding to the complement of the seed. In this example, these complementary sequences are UCCUCACAGGG (SEQ ID NO: 78), GUGCUCAUGGUG (SEQ ID NO: 79), CCUGGAGCCCUG (SEQ ID NO: 80) and UCUCAGCUCCAC (SEQ ID NO: 81).

Example 10

Simultaneous multitargeting of tumour and stromal factors important in cancer progression.

An extension of this concept can be considered in the case of some diseases in which several tissue "compartments" act together in augmenting the deleterious effects of the disease. Examples of such a situation would encompass, for example, the role of the stromal cells in cancer. In this situation, blocking the secretion of paracrine factors (eg growth factors such as VEGF) by the stroma would be advantageous. Indeed, targeting the neovasculature simultaneously with cytotoxic chemotherapy has significant clinical benefit in the case of colorectal cancer. Some of these factors also function in an autocrine fashion and cancer cells produce, for example, VEGF and other growth factors. Also, the simultaneous down-regulation of an anti-apoptotic protein (eg bcl-2, bcl-X_L) might permit greater anti-cancer activity. Therefore, a CODEMIR targeting VEGF-A, K-ras, EGFR and bcl-2 would be of considerable interest. An example of a seed common to all these targets was identified as: CCCACTGA. In this example, the complementary sequence is UCAGUGGG (SEQ ID NO: 76).

Seeds common to the mRNA sequences of the more limited sub-set of VEGF, Bcl-2 and K-Ras were identified as: GACAGTGGA, CTATTCTG and TAGAGAGTT. The

sequences complementary to these seeds are: UCCACUGUC (SEQ ID NO: 92), CAGAAUAG (SEQ ID NO: 93) and AACUCUCUA (SEQ ID NO: 94).

A set of 8 CODEMIRs (CC014-CC021) were designed from the latter three seeds. In this early and preliminary experiment, the design of the CODEMIRs did not necessarily follow the guidelines eventually developed. For example, the positioning of the seed-binding region was not necessarily constrained to the region proximal to the 5' end of the guide strand. The predicted binding to all three targets is shown in Table 10-1.

According to eventual design guidelines, one of these CODEMIRs (CC014) would be predicted to be sub-optimal based on reduced likelihood of loading because of its G/C rich 5' terminus of the guide strand relative to the passenger strand. CC015 was of particular interest for the targeting of K-ras, because of the presence of 2 binding sites featuring the seed. All of these CODEMIRs were synthesized and tested against all three targets as part of the evaluation.

The two colon cancer cell lines HCT116 and SW480 were used for the evaluation of anticancer CODEMIRs. Cells were plated at 4000 cells per well of a 96-well plate. Transfection was performed 24 hours later using 0.2 – 0.3 µL Lipofectamine2000 per well and sufficient dsRNA to yield a final concentration of 40 nM. Transfection efficiency was evaluated by measuring the effect of siTOX (a cytotoxic siRNA from Dharmacon) on cellular survival relative to mock and inactive controls using the Cell Titer Blue assay (CTB).

All cancer CODEMIRs were then screened for effects on cellular survival using this assay. Briefly, 8 h post-transfection, the serum-containing medium was removed and replaced with OptiMEM (serum-free) as we found this to improve the dynamic window of the assay in separating specific (siTOX) versus non-specific (siGC47) cell death. Different times for serum withdrawal and recovery were tested. The optimum protocol appeared to be withdrawal of serum for 16 h followed by a 48 h recovery period in 10 % FCS (fetal calf serum). Survival was measured 72 hr post-transfection. This assay was used to measure the effects of all cancer CODEMIRs on the survival of HCT116 cells (Figure 8). Transfection with siRNA specifically designed to target K-Ras or Bcl-2 had greater effects on the survival of serum-starved cells as compared to non-starved cells. Transfection with a VEGF-specific siRNA with activity in several species (human, rodent), PVE, did not affect the survival of HCT116 cells under either of these conditions, relative to a non-targeting siRNA, siGC47. Of the cancer CODEMIRs, CC015, in particular, reduced cellular survival under both conditions.

Table 10-1. Cancer CODEMIRs and predicted hybridization to target mRNA

Name	Duplex (top strand 5' to 3', bottom strand 3' to 5')	(a) Predicted target hybridization	
CC014	Passenger CAAAAAUGACAGUGGACGAUU GGGUUUUUACUGUCACCUGCU Guide Top strand (SEQ ID NO: 360) Bottom strand (SEQ ID NO: 361)	**KRAS** 5' U A 3' AAAAU**GACAGUGGA** UUUUACUGUCACCU guide 3' GGGU GCU 5'	(SEQ ID NO: 362) (SEQ ID NO: 361)
		BCL-2 5' G U 3' CAAGAGU**GACAGUGGAU** GUUUUUACUGUCACCUG guide 3' GG CU 5'	(SEQ ID NO: 363) (SEQ ID NO: 361)
		VEGF 5' C CCGGC CG C 3' CCCGG GG **GACAGUGGA**CG GGGUU UU CUGUCACCUGC guide 3' UA U	(SEQ ID NO: 364) (SEQ ID NO: 361)
CC015	Passenger GGACCCUUAGAGAGUUUCAUU GGCCUGGGAAUCUCUCAAAGU Guide Top strand (SEQ ID NO: 137) Bottom strand (SEQ ID NO: 138)	**KRAS** 5' G A GUGUGAACCUUUGAGCUUUCA C 3' **#1** CC GGCCCU **UAGAGAGUU**UCA GG CUGGGA AUCUCUCAAAGU guide 3' C 5'	(SEQ ID NO: 365) (SEQ ID NO: 138)
		KRAS 5' GA CU A C 3' **#2** G UUC **UAGAGAGUU**UCA C GGG AUCUCUCAAAGU guide 3' GG CU A 5'	(SEQ ID NO: 366) (SEQ ID NO: 138)
		BCL-2 5' U G 3' GACCUU**UAGAGAGUU** CUGGGAAUCUCUCAA guide 3' GGC AGU 5'	(SEQ ID NO: 367) (SEQ ID NO: 138)

		VEGF 5' C AGA U 3' CCGGA UAGAGAGUUUUA GGCCU AAUCUCUCAAGU guide 3' GGG 5'	(SEQ ID NO: 368) (SEQ ID NO: 138)
CC016	Passenger ACCUUAGAGAGUUUCACAU CCUGGGAAUCUCUCAAGUGU Guide Top strand (SEQ ID NO: 369) Bottom strand (SEQ ID NO: 370)	KRAS 5' A UGAGCUUUCA G 3' ACCUU UAGAGAGUUUCACA UGGGA AUCUCUCAAGUGU guide 3' CC 5' ECL-2 5' U GCU U 3' GACCUUUAGAGAGUU UUACG CUGGGAAUCUCUCAA AGUGU guide 3' C 5' VEGF 5' A GGAAGA U 3' GCCC UAGAGAGUUUUAU UGGG AAUCUCUCAAGUG guide 3' CC U 5'	(SEQ ID NO: 371) (SEQ ID NO: 370) (SEQ ID NO: 372) (SEQ ID NO: 370) (SEQ ID NO: 373) (SEQ ID NO: 370)
CC017	Passenger AUCCCUAUUCUGUUCUUUAUU CGUAGGGAUAAGACAAGAAAU Guide Top strand (SEQ ID NO: 374) Bottom strand (SEQ ID NO: 375)	KRAS 5' U G U 3' CAUCCCUAUUCUGU UUUUA GUAGGGAUAAGACA GAAAU guide 3' C A 5' BCL-2 5' A A A 3' UCCUAUUCUG UCUU GGGAUAAGAC AGAA guide 3' CGUA A AU 5' VEGF 5' A G U 3' CAUU CUAUUCUGUUUUUA GUAG GAUAAGACAAGAAAU guide 3' C G 5'	(SEQ ID NO: 376) (SEQ ID NO: 375) (SEQ ID NO: 377) (SEQ ID NO: 375) (SEQ ID NO: 378) (SEQ ID NO: 375)

CC018	Passenger CAUCCCUAUUCUGUUCUUAUU CUGUAGGGAUAAGACAAGAAU Guide Top Strand (SEQ ID NO: 379) Bottom strand (SEQ ID NO: 380)	**KRAS** 5' U G U 3' CAUCC**CUAUUCUGU** UUUUA (SEQ ID NO: 376) GUAGGGAUAAGACA AGAAU guide 3' CU 5' (SEQ ID NO: 380) **BCL-2** 5' A A U 3' UU**CUAUUCUG** UCUUA (SEQ ID NO: 381) GGGAUAAGAC AGAAU guide 3' CUGUA A 5' (SEQ ID NO: 380) **VEGF** 5' A G U 3' GACAUU **CUAUUCUG**UUUUU (SEQ ID NO: 382) CUGUAG GAUAAGACAAGAA guide 3' G U 5' (SEQ ID NO: 380)
CC019	Passenger CCCGGACCCUUAGAGAGUUUU ACGGGCCUGGGAAUCUCUCAA Guide Top strand (SEQ ID NO: 127) Bottom strand (SEQ ID NO: 128)	**KRAS** 5' G U UGUGA UGAGCUUUCA U 3' GCCC G ACCUU **UAGAGAGUU** (SEQ ID NO: 383) CGGG C UGGGA AUCUCUCAA guide 3' A C 5' (SEQ ID NO: 128) **BCL-2** 5' G UU G 3' GU G ACCUU**UAGAGAGUU** (SEQ ID NO: 384) CG C UGGGAAUCUCUCAA guide 3' A GG C 5' (SEQ ID NO: 128) **VEGF** 5' A AGA U 3' GCCCGGA **UUAGAGAGUU** (SEQ ID NO: 385) CGGGCCU AAUCUCUCAA guide 3' A GGG 5' (SEQ ID NO: 128)
CC020	Passenger CCGGACCCUUAGAGAGUUUUU CGGGCCUGGGAAUCUCUCAAA Guide Top strand (SEQ ID NO: 386) Bottom strand (SEQ ID NO: 387)	**KRAS** 5' G U UGUGA UGAGCUUUCA C 3' GCCC G ACCUU **UAGAGAGUUU** (SEQ ID NO: 388) CGGG C UGGGA AUCUCUCAAA guide 3' C 5' (SEQ ID NO: 387) **BCL-2** 5' G UU G 3' GU G ACCUU**UAGAGAGUU** (SEQ ID NO: 384) CG C UGGGAAUCUCUCAA guide 3' GG C A 5' (SEQ ID NO: 387)

CC02	Top strand (SEQ ID NO: 543) Bottom strand (SEQ ID NO: 389)	Passenger CGGACCCUUAGAGAGUUCUU GGGCCUGGGAAUCUCUCAAG Guide	**VEGF** 5' A AGA U 3' GCCCGGA **UUAGAGAGUUU** CGGGCCU AAUCUCUCAAA guide 3' GGG 5' (SEQ ID NO: 385) **KRAS** 5' C U UGA UGAGCUUUCA A 3' CCUG G ACCUU **UAGAGAGUUUC** GGGC C UGGGA AUCUCUCAAAG guide 3' 5' (SEQ ID NO: 390) **BCL-2** 5' U G 3' GACCUU**UAGAGAGUU** CUGGGAAUCUCUCAA guide 3' GGGC AG 5' (SEQ ID NO: 389) **VEGF** 5' G AGA A 3' CCCGGA **UUAGAGAGUUUU** GGGCCU AAUCUCUCAAAG guide 3' GGG 5' (SEQ ID NO: 389)

The abundance of Bcl-2 in HCT116 cells was measured by ELISA (R&D systems) and by Western. The signal for this protein was relatively weak and appreciable knock-down could only be detected for HCT-116 cells transfected with a Bcl-2-specific siRNA (siBcl2) when measured by Western blotting. No impact of any of the CODEMIRs or even siBcl2 was detected using the ELISA assay.

The abundance of K-Ras following transfection with CODEMIRs CC014-CC021 was measured in HCT116 cell extracts by Western (Figure 9). CC015 reduced K-Ras to a similar level as a K-ras-specific siRNA (siKRas) while CC020 and CC021 also had some effect (Figure 9).

VEGF production by HCT-116 cells was measured using the same VEGF ELISA (R&D systems) used to measure the effects of CODEMIR-1 (AM001) in ARPE cells. HCT116 cells produce moderate levels of VEGF (~200 pg/mL in 48hrs when seeded at 4000 cells/well) as measured by ELISA. CODEMIRs CC015, CC019, CC020 and CC021 all resulted in a decrease in the production of VEGF by HCT116 cells. The most marked effect was caused by CC019 which resulted in a ~ 55 % reduction as compared to untransfected cells (Figure 10). CC015, which also had suppressive activity against K-ras, reduced VEGF by ~ 20%.

Annexin V and PI staining followed by FACS analysis was used to analyze apoptosis in HCT116 cells following transfection with various siRNAs and CODEMIRs. Cells that are positive for Annexin V and negative for Propidium Iodide (PI) are considered to be cells undergoing early apoptosis. This assay was used to determine the number of dead (PI positive) and apoptotic cells following transfection with several cancer CODEMIRs into HCT116 (Figure 11) and SW480 (data not shown) cells. SiTOX, siBcl2 and siKRas each caused a marked increase in the Annexin V binding in HCT116 cells while another VEGF-specific siRNA only caused a slight increase (Figure 11). Transfection of HCT116 cells with CC015 resulted in Annexin V binding similar to that for the KRas and Bcl2-specific siRNAs. CC015 also increased Annexin V binding in SW480 cells, as did transfection with siKRas (data not shown).

A Caspase 3/7 activation assay (FACS staining with caspase substrate) was performed to gain more insight into the mode of apoptosis in HCT116 cells following transfection with CC015. There was no noticeable increase in caspase activation following transfection with siKRas, siTOX or CC015 (data not shown).

Soft agar-based anchorage independent growth assays are of interest because it has been demonstrated that the ability of a cell to form a colony in agar is linked to its ability to form a tumour *in vivo*. A conventional soft-agar assay was performed in which the cells were transfected and harvested 24 h later, at which point cells from each treatment were counted and re-plated in a 6-well plate in 0.4 % low-melting agarose. Seven days post-transfection the colonies that formed were counted under a microscope. With HCT116 cells, CC015 was very effective at reducing colony formation to an even greater extent than siKRas and siBcl-2 while CC018, CC019, CC020 and CC021 had no apparent effect on colony formation relative to a non-targeting siRNA, siGC47 (Figure 12). In SW480 cells, transfection with CC015 also markedly reduced the ability of the cells to form colonies while CC019, CC020 and CC021 had no effect (data not shown).

Overall, we found that CC015, which was designed using a seed found in the mRNA of Bcl-2, K-ras and VEGF, was able to suppress K-ras and to a lesser extent VEGF. In keeping with our experiments performed with CODEMIR-1, however, it was clear that having the seed-binding region of the guide strand upstream of 3 mismatched bases, could not be expected to lead to suppression of Bcl-2. Likewise, the positioning of the seed-binding region of CC016-18 in relation to this target was sub-optimal. However, technical issues with measuring Bcl-2 mean that we cannot rule out a possible effect on Bcl-2. The cancer CODEMIR CC015 was found to induce cell death in HCT-116 and SW480 cells but not the non-cancer cell line ARPE-19 (data not shown). CC015 also markedly inhibited colony formation in soft-agar. Table 10-2 summarizes the effects of CC015 in HCT116 and SW480 cells.

It will be appreciated by one skilled in the art that multitargeting interfering RNA molecules (CODEMIRs) will comprise the sequence corresponding to the complement of the seed. In this example, these complementary sequences are UCCACUGUC (SEQ ID NO: 92), CAGAAUAG (SEQ ID NO: 93) and AACUCUCUA (SEQ ID NO: 94).

Table 10-2. Summary of the effects of CC015 on cancer cells.

	HCT116	SW480
Survival (in serum)	Decreased	Decreased
Survival (serum withdrawal)	Decreased survival	Not tested
Annexin V staining	Increased	Increased
PI staining	Increased	Increased
Colony forming assay	Decreased	Decreased
VEGF secretion	Reduced (15-20 %)	Not determined
K-Ras abundance	Reduced (40 %)	Not determined
Bcl-2 abundance	No change	Not determined

Example 11

Multitargeting when at least one target is a non-coding RNA

Although we present above many examples relating to targeting mRNA encoding protein targets, the concept is equally applicable to non-coding RNAs. In cancer and other diseases, non-coding RNA such as MALAT-1, BIC, PCGEM1, DD3 and BC200 have been shown to be associated with more aggressive progression and worse patient outcomes. Targeting seeds common to some or all of these could be considered in the design of CODEMIRs. Other non-coding RNA (eg. such as microRNA, pre- and pri-microRNA, snoRNA etc) can equally be considered.

In the case of a CODEMIR designed to target two cancer-related non-coding RNA molecules, MALAT-1 and BIC, an exemplary seed sequence is: GGTGCGAGGGT (SEQ ID NO: 393)

When viewed in the relationship to the genetic context of this seed in the two target RNA sequences:

AACGTGGCAGGGACGCCGCGGGACTTC GGTGCGAGGGT CACGGCCGCGTTAACTGGC (MALAT-1) (SEQ ID NO: 394)

CAAGTAGGGTACGCACTTTGCCGGATT GGTGCGAGGGT ACTGGGTGAGTCGCCTACT (BIC) (SEQ ID NO: 395)

it is apparent that a suitable consensus target site can easily be generated with the methods provided elsewhere in the invention. Because of the G/C-rich region near the 3' end of the seed, the 5'end of the active (guide) strand of the duplex multitargeting interfering RNA would need to be shifted away from this G/C region, although the presence of a G/C-region in the 5' extension of the seed (with the exception of the desired overhangs) would assist in mitigating these effects. A candidate consensus sequence could be (seed underlined and bolded):

5'-TTGGGAGATC**GGTGCGAGGGT**A-3' (SEQ ID NO: 396)

with the resulting duplex being, for example:

5'-UACCCUCGCACCGAUCUCCCAA-3' (SEQ ID NO: 129) (guide)
3'-UUAUGGGAGCGUGGCUAGAGGG-5' (SEQ ID NO: 130)
(passenger)

The guide strand of this CODEMIR has predicted binding to the two targets (RNAhybrid) as follows:

```
MALAT-1 5'    C       CU              C 3'
              GGGGGA  UCGGUGCGAGGGU      (SEQ ID NO: 397)
              CCCUCU  AGCCACGCUCCCA
guide  3'  AA                       U 5' (SEQ ID NO: 129)
mfe: -43.6 kcal/mol

BIC   5'  U                         G 3'
          UUGGGGGAUUGCUGCGAGGCUA      (SEQ ID NO: 398)
          AACCCUCUAGCCACGCUCCCAU
guide  3'                           5' (SEQ ID NO: 129)
mfe: -48.4 kcal/mol
```

It will be appreciated by one skilled in the art that multitargeting interfering RNA molecules will comprise the sequence corresponding to the complement of the seed. In this example, this complementary sequence is: ACCCUCGCACC (SEQ ID NO: 86).

Example 12

CODEMIRs for the treatment or prophylaxis of pulmonary fibrosis

Pulmonary fibrosis is frequently observed as a sequelae of lung injury (smoke inhalation, pneumonia, trauma etc). It also occurs without a known causative factor in idiopathic pulmonary fibrosis, which is progressive and almost universally fatal. Regardless of the etiology, it appears that the transforming growth factor β (TGFβ) axis is a major signaling pathway in pulmonary fibrosis, although other factors such as Connective Tissue Growth Factor (CTGF), IL-8 and MCP-1 are likely to play a role. CODEMIRs targeting TGFb, IL-8 and MCP-1 were sought. The 3'UTR of these mRNA were used in this search as described in other examples. A seed common to IL-8 and TGFb was identified as CUUCAACAC (SEQ ID NO: 89). Two consensus target sequences were designed by eye from the aligned mRNA sequences for these two targets. As shown in Table 12-1, the bias of complementarity for the two targets was reversed for PF007 and PF008. That is, the consensus target sequence was in one case made more similar to IL-8 (PF007) and in the second was more similar to TGF-β (PF008). This aspect of design of the multitargeting interfering RNA of the invention will be seen by one skilled in the art to enable the titration of the activity of the multitargeting interfering RNA against the one or more target RNA. In this instance, PF007 and PF008 had similar activity against TGFb with ~50% reduction of TGFb secretion by A549 cells when assayed by ELISA 48 hours post transfection with 40 nM dsRNA and lipofectamine. In contrast, IL-8 secretion was suppressed by 80% and 35% with PF008 and PF007, respectively. Thus these two CODEMIRs would be expected to be of potential utility for the treatment of pulmonary fibrosis. They could also be further improved to increase strand loading. Given that the 5' terminal base of the guide strand is a G, the corresponding base used in the passenger strand was a U to provide weaker wobble-base pairing. However, one skilled in the art would realize that additional modification could be envisaged which is exemplified but not limited to the inclusion of an additional C or G to the 3'end of the guide strand, an additional A or U to the 5'end of the guide strand, or both. Matching these in the corresponding passenger strand would improve further the loading bias without necessarily any deleterious impact on their functional activity.

Table 12-1. CODEMIRs targeting pulmonary fibrosis targets (TGFβ, and IL-8).

	Duplex	Target Binding (RNA hybrid)
PF007	Passenger UACAAAUCUACUUCAACAUUU (SEQ ID NO: 131) GUAUGUUUAGAUGAAGUUGUG (SEQ ID NO: 132) Guide	TGFB1 5' G U G U A 3' CAUAUA AU U CUUCAACAC (SEQ ID NO: 399) GUAUGU UA A GAAGUUGUG PF007 3' U G U 5' (SEQ ID NO: 132) IL-8 5' A U 3' GCAAAUCUACUUCAACAC (SEQ ID NO: 400) UGUUUAGAUGAAGUUGUG PF007 3' GUA 5' (SEQ ID NO: 132)
PF008	Passenger AACAUAUGUUCUUCAACAUUU (SEQ ID NO: 133) GUUUGUAUACAAGAAGUUGUG (SEQ ID NO: 134) Guide	TGFB1 5' G U A 3' CA AUAUAUGUUCUUCAACAC (SEQ ID NO: 399) GU UGUAUACAAGAAGUUGUG PF008 3' U 5' (SEQ ID NO: 134) IL-8 5' U A CUA U 3' CAAGCA AU CUUCAACAC (SEQ ID NO: 544) GUUUGU UA GAAGUUGUG PF008 3' A CAA 5' (SEQ ID NO: 134)

Other active CODEMIRs were found including PF018 which targets the seed of GUUGUGGAA in IL-8 and MCP-1. This CODEMIR, with the guide sequence UUCCACAACACAAGCUGUGUU (SEQ ID NO: 135), suppressed IL-8 and MCP-1 secretion by 45% and 60%, respectively. The CODEMIR PF018 duplex is as follows:

5'-UUCCACAACACAAGCUGUGUU-3' (SEQ ID NO: 135) (guide strand)
3'-UUAAGGUGUUGUGUUCGACAC-5' (SEQ ID NO: 136) (passenger strand)

It will be appreciated by one skilled in the art that multitargeting interfering RNA molecules will comprise the sequence corresponding to the complement of the seed. In this example, these complementary sequences are GUGUUGAAG (SEQ ID NO: 88) and UUCCACAAC (SEQ ID NO: 90).

Example 13

CODEMIRs for the treatment of HCV

All possible 6, 7, 8, 9, 10, 11 and 12mer seeds present at least twice in at least one of the 155 clade 1a and 1b HCV genome sequences available from the LANL database have been generated.

These break down as:

seed length	number of seeds
6	4012
7	10683
8	11352
9	5942
10	2267
11	725
12	236

This is a total of 35217 sequences

One of the seeds from the set identified was: 5'-GTCTTCACG-3' (SEQ ID NO: 401). This seed was selected on the basis of its high conservation in HCV 1a/1b genotype sequences. Indeed it was found at least once in the sequences of 154 of 155 1a/1b isolates (conservation of 99%). When examined against other genotypes (1a-6a), it was found at least once in 97% of isolates. The other important feature of this seed is its high distribution,

occurring more than once in most sequences. This distribution, relative to 155 isolates was as follows:

4 times in genome: 5/155 isolates

3 times in genome: 68/155 isolates

2 times in genome: 50/155 isolates

1 time in genome: 31/155 isolates

A further selection criterion was that this seed occurs only rarely in the 3'UTR portion of the human transcriptome, indicating that this seed would be unlikely to generate broad non-specific effects to the host tissue.

This seed, however, is G/C rich at the 3' extremity, such that a complementary guide sequence would, in the context of a double stranded multitargeting interfering RNA, be unlikely to load if the guide strand were to be completely complementary to this sequence and the 3' extremity of the passenger strand was not similarly G/C rich.

The genetic contexts of the seed in a representative isolate shown below were used to devise a strategy to adjust the loading bias.

1 ATCACTCCCCTGTGAGGAACTACT **GTCTTCACG** CAGAAAGCGTCTAGC (SEQ ID NO: 402)
2 ATGGAGACCACTATGCGGTCTCCG **GTCTTCACG** CACAACTCATCTCCC (SEQ ID NO: 403)
3 GATCACCTGGAGTTCTGGGAGAGC **GTCTTCACG** GCCCTCACCCACATA (SEQ ID NO: 404)
4 CAGGACGATGCGGCGAGCCTACGA **GTCTTCACG** GACGCTATGACTAGG (SEQ ID NO: 405)

Through examination of the genetic context, it is clear that the complementary sequence (S according to claim definition) could be extended in the 5'direction with the addition of UU, thereby generating a candidate XS sequence: 5'-UUCGUGAAGAC-3' (SEQ ID NO: 406)

All possible 21 bp sequences that contain this candidate XS sequence were generated. Each putative full length XSY sequence was then tested for its load bias, to favour those XSY sequences that would, in the context of a double stranded multitargeting interfering RNA, be likely to have the guide strand loaded into the RISC complex. This was accomplished by examining the base composition for the 5 bases at the 5' terminus and the 5 bases at the 3' terminus of each XSY sequence and scoring them according to the following table:

	Position from the 5' or 3' terminus				
Base	1st	2nd	3rd	4th	5th
G/C	10	8	6	4	2
A/U	5	4	3	2	1

All putative full-length XSY sequences for which the ratio of the sum of the scores of the 5 bases at the 3' terminus relative to the 5 bases at the 5' terminus was less than 1.2 were

discarded. In addition, any XSY sequence that contained a contiguous run of 5 or more G nucleotides was also discarded. The resulting initial set of XSY sequences contained 422848 sequences. The RNAhybrid program [Hofacker, (2003), *Nucleic Acids Res.*, 31: 3429-31] was then used to determine the binding pattern for each of these 422848 sequences against each of the 4 genetic contexts in a representative HCV strain (Genbank accession AB049092). The RNAhybrid analysis required an exact binding of the XS sequence (UUCGUGAAGAC) (SEQ ID NO: 406) to each genetic context for positions 3 to 11. For each of the binding patterns, the minimum free energy (mfe) and maximum length of contiguous completely complementary sequence were used to generate a relative binding score according to the following algorithm:

Score = (mfe of XSY sequence x length of contiguous complementarity x 100) / (mfe of the completely complementary sequence at that genetic context x 21)

The average score for each XSY sequence across the 4 genetic contexts was then determined. From the 422848 sequences, the 183 potential XSY sequences that satisfied the criteria of being in the top 100 average scores or having a score of > 40 in at least 3 of the four genetic contexts were considered for further analysis. The RNAhybrid and scoring analysis described above was used to analyse the binding patterns of the 183 selected XSY sequences against the sequences of the 4 genetic contexts of all 155 clade 1a/1b isolates (620 sequences). In this analysis an exact binding of the XS sequence (UUCGUGAAGAC) (SEQ ID NO: 406) was not required.

Using the criterion of a score >= 50, XSY sequences were selected which gave the highest number of scores >= 50 across the 620 genetic contexts. In this case, there were 9 XSY sequences which each gave a score of >= 50 in 147 of the 620 genetic contexts. These 9 XSY sequences are:

UUCGUGAAGACGGUGGGCCGA (SEQ ID NO: 139)
UUCGUGAAGACGGUGGGCCG**G** (SEQ ID NO: 407)
UUCGUGAAGACGGUGGGCCG**C** (SEQ ID NO: 408)
UUCGUGAAGACGGUGGGCCG**U** (SEQ ID NO: 409)
UUCGUGAAGACGGU**A**GCCCGA (SEQ ID NO: 410)
UUCGUGAAGACGGU**A**GGCCG**G** (SEQ ID NO: 411)
UUCGUGAAGACGGU**A**GGCCG**C** (SEQ ID NO: 412)
UUCGUGAAGACGGU**A**GGCCG**U** (SEQ ID NO: 413)
UUCGUGAAGAC**A**GUGGGCCG**C** (SEQ ID NO: 414)
UUCGUGAAGAC**A**GU**A**GGCCG**C** (SEQ ID NO: 415)

All 9 XSY sequences had very similar binding patterns at each genetic context. Columns 2 and 3 shown below in Table 13-2 represent the six possible binding patterns for the 9 XSY sequences to the four genetic contexts.

Table 13-2. Possible binding patterns to four genetic contexts of HCV seed

Genetic Context	Binding patterns (top strand is target 5'-3', guide strand at bottom 3'-5')	
1	5' A AA C-3, GG CUACUGUCUUCACG (SEQ ID NO: 416) CC GRUGRCAGAAGUGC 3'NG G UU-5' (SEQ ID NO: 417)	
2	U C G C GCGGUCU C GUCUUCACGGA (SEQ ID NO: 418) YGCCGGR G CAGAAGUGCUU U R (SEQ ID NO: 419)	UG C G C CGGUCU C GUCUUCACGGA (SEQ ID NO: 418) GCCGGR G CAGAAGUGCUU R U R (SEQ ID NO: 420)
3	G AGA G GG GC GUCUUCACGG (SEQ ID NO: 421) CC UG CAGAAGUGCU NG GGR R U (SEQ ID NO: 417)	
4	G A GA G GCG GCCUAC GUCUUCACGGA (SEQ ID NO: 422) YGC CGGRUG CAGAAGUGCUU R (SEQ ID NO: 419)	GG A GA G CG GCCUAC GUCUUCACGGA (SEQ ID NO: 422) GC CGGRUG CAGAAGUGCUU R R (SEQ ID NO: 420)

(N = any of A, G, C, U. R = either of A, G. Y = either of C, T.)

The further construction of a VIROMIR is shown below using the first of the nine sequences shown above as an example: 5'-UUCGUGAAGACGGUGGGCCGA-3' (SEQ ID NO: 139)

As a fully complementary duplex, the above strand would be predicted to have a loading bias in its favor relative to the passenger strand. With the addition of exemplary overhangs, the final candidate HCV VIROMIR could have the following formula:

5'-UUCGUGAAGACGGUGGGCCGA-3' (SEQ ID NO: 139)
3'-dTdTAAGCACUUCUGCCACCCGG-5' (SEQ ID NO: 140)

It will be appreciated by one skilled in the art that multitargeting interfering RNA molecules will comprise the sequence corresponding to the complement of the seed. In this example, this complementary sequence is CGUGAAGAC (SEQ ID NO: 98).

Example 14

Chemically-Modified Codemirs

Because dsRNA has limited stability in vivo, it is well understood by one skilled in the art that it may be desirable to chemically modify the multitargeting interfering RNA in order to improve stability and activity. Any chemical modification to the multitargeting interfering RNA is within the scope of the invention. As a non-limiting example, we considered the use of 2'-F modified nucleotides within the context of CODEMIR-1 (also known as AM001). To investigate the stability of the modified CODEMIRs, either the Oligreen or Sybr Green I fluorescent dye was used to assess CODEMIR degradation in 10% human serum. These, in particular Sybr Green I, bind avidly to dsRNA to produce enhanced fluorescence. Thus, monitoring of fluorescence during the incubation of dsRNA in 10% human serum, or other biological solution, produces a facile method for monitoring stability. To further clarify the presence of the products of degradation produced, CODEMIR-1 and chemically-modified analogs (with one or both strands 2'-F modified at every C and U position) were incubated in 10% serum for 30 minutes and separated by PAGE. Unmodified duplex RNA exhibited substantial degradation, and was presumably completely inactive. By contrast, duplex RNA in which both strands were 2'-Fluoro modified, exhibited no degradation at all, which corresponds well to the results observed in the fluorescence assay (data not shown). The two duplexes in which only a single strand was modified appeared to

be incompletely degraded to a duplex presumably a hybrid between the full-length modified strand and a partially degraded unmodified strand. Because this degradation product would be likely to have greatly decreased activity, it seems reasonable to infer that only constructs which demonstrate no degradation in the fluorescence assay would retain maximal activity.

However, replacement of all pyrimidine nucleotides with their corresponding 2'-F analogs was somewhat deleterious to activity when assessed by the suppression of VEGF secretion by ARPE-19 cells. In contrast, similar modification of the guide strand did not significantly alter activity. Because efficient loading of the guide strand into RISC requires (in some cases) the cleavage of the passenger strand, we hypothesized that 2'-F modification of the passenger strand inhibited strand loading. To test this, variants of CODEMIR-1 with 2'-F modification at each C and U position in the guide and/or passenger strand, were designed (Table 14-1). In particular, the influence of modification of position 9 (pos9) of the passenger strand (the position critical for passenger strand cleavage during RISC loading) was examined. When tested for their ability to suppress VEGF expression in ARPE-19 cells, a modest difference was observed between the pos9-Fluoro and pos9-ribo variants in which only the passenger strand was modified (ie CODEMIR-144 and 87) although this was not significant. By contrast, the pos9-ribo variant in which both strands were otherwise modified (CODEMIR-145) was significantly ($p<0.01$) more active than the comparable pos9-Fluoro variant (CODEMIR-33). As compared to unmodified CODEMIR-1, the activity of the pos9-Fluoro variant in which only the passenger strand was modified (CODEMIR-87), but not the pos9-ribo variant (CODEMIR-144), was significantly decreased ($p<0.01$).

The stability of the pos9-ribo and pos9-F variants of CODEMIR-1 was assessed in 10% human serum using Sybr Green I. No difference between the pos9-ribo and pos9-Fluoro variants was observed. However, as discussed above, the end product of degradation in the case where only a single strand is modified is unlikely to possess activity. The RNase activity of serum is due to RNase-A like endonuclease(s)(Haupenthal, J. (2006) *Biochem Pharmacol* **71**, 702-710). These RNases are single-strand specific, only cleave 3' of C and U bases and are blocked by 2'-F modification (Kelemen, B.R. (2000) *Biochemistry* **39**, 14487-14494). Thus, a likely model of short RNA duplex degradation in serum is that the "breathing" ends of the duplex are the most vulnerable to RNase degradation whereas the central section of the duplex is protected by its duplex nature. To assess this model, variants of CODEMIR-1 in which only the ends of each strand were 2'-F modified were designed (Table 14-1). Modification of 3 bases from the end of the duplex region (CODEMIR-165)

produced a modest improvement in stability, but this was abolished if only the 3' termini were modified (CODEMIR-167). Interestingly, modification of 5 bases from the end of the duplex region (CODEMIR-166) also produced an increase in stability. However, when tested for VEGF suppressive activity, all of the terminus-modified CODEMIRs displayed significantly impaired activity relative to CODEMIR-1; comparable in activity to the other 2'-F modified CODEMIRs containing a passenger pos9-ribo base (Figure 13).

Overall, therefore, 2'Fluoro modification represents a viable strategy for the chemical modification of the multitargeting interfering RNA of the invention, although extensive modification, particularly that of the pos-9 nucleotide of the passenger strand may reduce activity in cell-based assays. Although the trade-off for stability may in some cases not appear worthwhile, the use of transfection reagents (in this case Lipofectamine) may mask some of the benefits of chemical modification because lipid-based complexation protects nucleic acid from degradation by nucleases. Thus while CODEMIR-1 was amongst the most potent in the cell screen assay (with lipofection), it had minimal stability in serum. Ultimately, whereas the differential activity of multitargeting interfering RNA can be tested in a predictive manner in cell-based assays, the impact of chemical modification would need testing in the context intended for the therapeutic molecule.

Table 14-1. 2'-Fluoro-modified variants of CODEMIR-1 (2'-F-modified nucleotides are bolded and underlined).

CODEMIR	Duplex Sequence (pass/guide)	
CODEMIR-1	5'AGACUCACCCACCCACAUAUU-3'	(SEQ ID NO: 101)
	3'AAUCUGAGUGGGUGGGUGUAU -5'	(SEQ ID NO: 100)
CODEMIR-92	AGACUCACCCACCCACAUAUU	(SEQ ID NO: 101)
	AAUCUGAGUGGGUGGGUGUAU	(SEQ ID NO: 423)
CODEMIR-144	AGACUCACCCACCCACAUAUU	(SEQ ID NO: 424)
	AAUCUGAGUGGGUGGGUGUAU	(SEQ ID NO: 100)
CODEMIR-87	AGACUCACCCACCCACAUAUU	(SEQ ID NO: 425)
	AAUCUGAGUGGGUGGGUGUAU	(SEQ ID NO: 100)
CODEMIR-145	AGACUCACCCACCCACAUAUU	(SEQ ID NO: 424)
	AAUCUGAGUGGGUCGGUGUAU	(SEQ ID NO: 423)
CODEMIR-33	AGACUCACCCACCCACAUAUU	(SEQ ID NO: 425)
	AAUCUGAGUGGGUCGGUGUAU	(SEQ ID NO: 423)
CODEMIR-165	AGACUCACCCACCCACAUAUU	(SEQ ID NO: 426)
	AAUCUGAGUGGGUCGGUGUAU	(SEQ ID NO: 541)

CODEMIR	Duplex Sequence (pass/guide)	
CODEMIR-166	AGACUCACCCACCCACAUAUU	(SEQ ID NO: 427)
	AAUCUGAGUGGGUGGGUGUAU	(SEQ ID NO: 423)
CODEMIR-167	AGACUCACCCACCCACAUAUU	(SEQ ID NO: 426)
	AAUCUGAGUGGGUGGGUGUAU	(SEQ ID NO: 428)

It is well recognized by those skilled in the art that the terminal conjugation of nucleic acid therapeutics to various dyes, pharmacophores, ligands, peptides, linkers, conjugates, polymers, lipids, peptides and other molecules can be used to improve or monitor the uptake, distribution, tissue targeting, stability or biological potency of the said nucleic acid. In most cases, the required conjugation reactions are performed through the formation of a phosphoester linkage by means of an aliphatic chain. To investigate the compatibility of such a strategy in relation to the multitargeting interfering RNA of the invention, we investigated the biological activity of analogs of CODEMIR-1 in which the active guide strand was linked at either the 5' or 3' oligonucleotide terminal. These include phosphate linked aliphatic chains with hydroxyl or amino moieties, polyethylene glycols and abasic sugars (CODEMIRs 146-156; Figure 14). All of these CODEMIRs demonstrated high VEGF suppressive activity when transfected into ARPE-19 cells at 10nM (Figure 15), indicating the compatibility of these modifications with biological activity of the molecules of the invention. The stability (Figure 17) and further RNAi activity (Figure 18) of the chemically modified variants of CODEMIR-1 were also analyzed. It is possible and indeed likely that cell phosphoesterases caused the release of the linker once delivered within the cell and that this would account for the high and uniform activity of these analogs. This indicates that at least some of the linkers could be used in a prodrug approach in which the targeting or protective ligand is shed once the multitargeting interfering RNA has penetrated into cells.

Example 15

Use of ARPE-19 Cells for the Evaluation of Anti-angiogenic CODEMIRs

CODEMIR-1 has been the prototype sequence in which the influence of chemical and sequence modifications have been tested. This CODEMIR may be particularly useful for the treatment of the wet forms of AMD as well as macular edema and diabetic retinopathy. This is because secreted VEGF-A plays a major role in all of these diseases (Witmer et al (2003)

Prog Retin Eye Res, 22, 1-29), although ICAM-1 overexpression may be an early initiating event, particularly for diabetic retinopathy and macular edema (Funatsu et al., (2005) Ophthalmology, 112, 806-16.; Joussen et al. (2002) *Am J Pathol*, 160, 501-9 ; Lu et al. (1999) *Invest Ophthalmol Vis Sci*, 40, 1808-12). We have shown that CODEMIR-1 has demonstrated the ability to suppress both VEGF-A and ICAM-1 production by human retinal epithelium cells (ARPE-19 cell line). Retinal pigmented epithelial cells are a major contributor to the production of these proteins in these ocular angiogenic diseases (Matsuoka et al., (2004) *Br J Ophthalmol*, 88, 809-15, Yeh et al. (2004), *Invest Ophthalmol Vis Sci*, 45, 2368-73). RPE cells are also the primary site of uptake of foreign nucleic acids in the eye and, for these two reasons, are the appropriate cell model for evaluation of anti-angiogenic CODEMIRs. The *in vivo* activities of two oligonucleotide drugs correlated with their activity against RPE cells in culture (Garrett et al. (2001) *J Gene Med*, 3, 373-83; Rakoczy et al. (1996), *Antisense Nucleic Acid Drug Dev*, 6, 207-13) demonstrating the value of this cell culture model. An advantage of this cell line is that it forms polarized monolayers that mimic the RPE layer of the eye (Dunn et al., (1996), *Exp Eye Res*, 62, 155-69), and which can be studied for protracted periods of time. This property was used to evaluate VEGF secretion by repeated sampling of the supernatant of ARPE-19 monolayers (ICAM-1 cannot be studied in this same way because it requires harvesting of the cells). VEGF secretion was suppressed for at least 9 days following a single dose of CODEMIR-1 (Figure 16). This indicates that CODEMIR-1 is expected to produce a durable inhibition of VEGF production in the eye.

Example 16

Mismatches in the Seed Region Impair CODEMIR-1 Activity

In order to validate that seed binding was essential for CODEMIR activity, a number of RNA duplexes based upon CODEMIR-1, but with mismatches to the targets in the seed region, were designed and tested. These were aligned with the human transcriptome using BLAST and three sequences with the lowest predicted off-target siRNA activity were chosen (CODEMIRs 122-124). These feature mismatches at positions 4, 4+6 and 4+6+8, respectively (Table 16-1). Each of these CODEMIRs was assessed for activity against VEGF and ICAM in ARPE-19 cells (Figure 19). A mismatch at position 4 exhibited slightly impaired activity against both VEGF and ICAM-1, whereas mismatches at 4 and 6 or at 4+6+8 greatly reduced VEGF suppression and abolished ICAM-1 suppression, demonstrating that seed binding is important for CODEMIR activity.

Table 16-1 – Variants of CODEMIR-1 with seed mismatches (mismatches are bold).

	Duplex	mRNA binding (RNA hybrid)	
CODEMIR-1	Passenger AGACUCACCCACCCACAUAUU (SEQ ID NO: 101) AAUCUGAGUGGGUGGGUGUAU (SEQ ID NO: 100) Guide	VEGF 5' G A C 3' UAGAC CACCCACCCACAUA (SEQ ID NO: 181) AUCUG GUGGGUGGGUGUAU 5' (SEQ ID NO: 100) 3' A A CCAC C 3' ICAM 5' G CCAC UUAG CUC CCCACCCACAUA (SEQ ID NO: 183) AAUC GAG GGGUGGGUGUAU 5' (SEQ ID NO: 100) 3' U U C C 3'	
CODEMIR-22	Passenger AGACUCACCCACCCAGACAUU (SEQ ID NO: 141) AAUCUGAGUGGGUGGGUCUAU (SEQ ID NO: 142) Guide	VEGF 5' G A C C 3' UAGAC CACCCACCCA AUA (SEQ ID NO: 181) AUCUG GUGGGUGGGU UAU 3' A A C C 5' (SEQ ID NO: 142) ICAM 5' G CCAC C C 3' UUAG CUC CCCACCCA AUA (SEQ ID NO: 183) AAUC GAG GGGUGGGU UAU 3' U U C C 5' (SEQ ID NO: 142)	
CODEMIR-23	Passenger AGACUCACCCACCGAGAUAUU (SEQ ID NO: 429) AAUCUGAGUGGGUGGCUCUAU (SEQ ID NO: 430) Guide	VEGF 5' G A C 3' UAGAC CACCCACC (SEQ ID NO: 181) AUCUG GUGGGUGG 3' A A CUCUAU 5' (SEQ ID NO: 430) ICAM 5' G CCAC CACAUA 3' UUAG CUC CCCACC (SEQ ID NO: 183) AAUC GAG GGGUGG 3' U U CUCUAU 5' (SEQ ID NO: 430)	
CODEMIR-24	Passenger AGACUCACCCAGCGAGAUAUU (SEQ ID NO: 431) AAUCUGAGUGGGUCGCUCUAU (SEQ ID NO: 432) Guide	VEGF 5' G A C 3' UAGAC CACCCA (SEQ ID NO: 433) AUCUG GUGGGU 3' A A CGCUCUAU 5' (SEQ ID NO: 432) ICAM 5' G CCAC CCCACAUA 3' UUAG CUC CCCA (SEQ ID NO: 183) AAUC GAG GGGU 3' U U CGCUCUAU 5' (SEQ ID NO: 432)	

The results demonstrated the more microRNA-like, less siRNA-like, qualities of CODEMIRs with respect to mismatches in the seed.

Example 17

Screening of 32 Variants of CODEMIR-1 for VEGF and ICAM-1 RNAi Activity

The RNAi efficacy of an additional 32 variants of CODEMIR-1, differing in the composition of the 3' tail of the guide strand was analyzed. A consensus sequence of the *VEGF* and *ICAM-1* target sites was generated (allowing wobble base pairing between guide strand and the target site – i.e. allowing G to be equivalent to A and U to be equivalent to C; Figure 20). CODEMIRs representing the 32 (2^5) possible 3' tails targeting both transcripts were designed (Table 17-1). ARPE-19 cells treated with 40 nM of each of these CODEMIRs demonstrated *VEGF* suppression ranging from ~50% to ~90% (Figure 21). CODEMIRs with complementarity to the *VEGF* mRNA at position 13 of the guide strand (i.e. having 14 contiguous bases of complementarity to the target were substantially more effective that those with a mismatch at position 13 (12 bases of contiguous complementarity to the target); presumably because a mismatch at position 13 impairs RISC mediated cleavage of the *VEGF* mRNA.

In keeping with the effects of central mismatches on the activity of siRNAs targeting the CODEMIR-1 binding site (above), no CODEMIR demonstrated less than 40% suppression of *VEGF*. Indeed, transfection of ARPE-19 cells with a synthetic microRNA duplex (hsa-mir-299 herein named CODEMIR-84), which shares part of the CODEMIR-1 seed binding region but has little complementarity to the *VEGF* mRNA in the 3' tail (Table 17-1), also inhibited *VEGF* expression by ~40%. It appears that this level of *VEGF* suppression represents the translational repression induced by nearly any short RNA that binds with perfect complementarity to the CODEMIR-1 seed site of the *VEGF* mRNA. Of those CODEMIRs that displayed greater than 40% VEGF suppression, there was a strong correlation between activity and both the degree of complementarity (an inverse correlation with the number of mismatches) and the length of the complementary seed region (Figure 22). CODEMIRs with a 12 base complementary seed produced only ~70% suppression, even with only a single mismatch (at position 13). By contrast, CODEMIRs with a 14 base complementary seed produced strong suppression (~85%), but activity of these CODEMIRs was impaired by mismatches in the remainder of the 3' tail. All of the CODEMIRs with 17 base complementary seed regions were highly active, with mismatches in the 3' tail having

little effect upon activity. The CODEMIRs with a complementary seed longer than 17 bases had activity that appeared to more closely correlate with strand loading than with any other factor.

In contrast to the VEGF suppression data, when the 32 CODEMIR-1 variants were assayed for ICAM-1 suppressive activity, no clear trend was discernable with respect to the length of 5' complementarity or the number of mismatches to the target (Figure 23). In part, this may have been because those CODEMIRs with a high degree of complementarity to the ICAM-1 mRNA also contained long sequences of contiguous guanosines (CODEMIR-52, 56, 60, 64, 68, 72, 76 and 80), which may be detrimental to activity. To test this, variants of CODEMIR-56 and 76 were designed in which the guanosine at position 14 of the guide strand (which forms a G:U pairing with the ICAM-1 mRNA) was replaced with an A (to generate an A:U pair with the ICAM-1 mRNA) in order to break the contiguous Gs. These variants (CODEMIRs 120 and 121 – Table 17-2) demonstrated a marked increase in both VEGF and ICAM-1 suppressive activity compared to their respective analogues containing a guanosine at position 14 (Figure 24). The increase in ICAM-1 suppressive activity is potentially attributable to the introduction of an A:U pair in place of a G:U pair in the guide/target interaction (rather than being a direct effect of removing the 7 G motif). However, the fact that activity of these CODEMIRs against VEGF was also improved (despite the fact that the predicted binding of these CODEMIRs to the VEGF mRNA is unchanged compared with CODEMIRs 56 and 76 – Table 17-2) shows that the 7 G motif was detrimental to the activity of CODEMIRs 56 and 76.

One possible strategy to increase the activity of CODEMIRs that have substantial complementarity to the target mRNA is to include inosine bases at crucial points (sites that cannot be matched to all transcripts and are near to the RISC cleavage site). To test the tolerance for inosine bases by the RNAi machinery, three variants of CODEMIR-1 were designed which included inosine bases at positions 13, 15 or 13 and 15 of the guide strand (CODEMIR-100, 101 and 102, respectively; Table 17-3). These CODEMIRs showed comparable ICAM-1 suppressive activity to CODEMIR-1 (which contains a mismatch at position 13), but reduced VEGF suppression relative to CODEMIR-1 in the case of CODEMIR-100 and 102 (Figure 25). The comparable activity against ICAM-1 may result from translational repression that is largely dependent upon the seed binding alone, and so is not affected by alterations in the 3' tail. The VEGF suppressive activity of inosine containing variants of CODEMIR-1 was also compared to similar variants of CODEMIR-1 with mismatches to the VEGF mRNA at positions 13, 15 or 13 and 15 (CODEMIRs 68-71). For

this assay, ARPE-19 cells were transfected with 10nM RNA duplex in order to increase the dynamic range of the assay. None of the inosine containing variants demonstrated substantially improved activity compared to its respective mismatched variant, although, the variant with an inosine at position 13 was slightly more active than the mismatched variant (compare CODEMIR-70 with CODEMIR-100 in Figure 26). Nevertheless, these experiments indicate that inosine substitution can be considered in the design of CODEMIRs.

An alternative to inosine containing CODEMIRs as a strategy for increasing the length of the 5' complementary region is to introduce a single base pair loop (either on the target or the guide strand) so as to increase the length of complementarity on either the target or the guide strand. Two such CODEMIRs (one with a predicted target loop (CODEMIR 105) and one with a predicted guide strand loop (CODEMIR 106) after position 14 of the guide strand – Table 17-3) were tested for the ability to suppress VEGF and ICAM-1 expression (Figure 27). Interestingly, each of these displayed substantially reduced activity compared to CODEMIR-1 (which has an A:A mismatch at position 15). This suggests that symmetrical mismatches (loops) may be tolerated better than asymmetrical mismatches, and is a potentially useful design principle.

Another strategy for increasing the activity of a CODEMIR against multiple targets is to increase the hybridisation strength of the 3' tail by inclusion of chemically modified bases that have increased hybridization potential in RNA:RNA duplexes. Such modified bases include LNA (locked nucleic acid), ENA (ethylene bridge nucleic acid), 2'Fluoro, 2'O-methyl and 2'O-alkyl-ribose among others. Strengthening of hybridisation in the seed region could also be envisaged, however, this may impact negatively on strand-loading bias. Modification of the G at position 16 of the guide strand of CODEMIR-1 was chosen in an effort to increase hybridization and therefore stability of the binding of the tail of the guide strand of CODEMIR-1 to both targets. This modified CODEMIR (CODEMIR-99) has the ribo-base at position 16 replaced with a LNA base. When tested for the ability to suppress VEGF and ICAM-1 expression in ARPE-19 cells, this CODEMIR exhibited comparable activity to CODEMIR-1 (Figure 25). As with the inosine containing variants of CODEMIR-1 (and most variants of CODEMIR-1 generally), the comparable ICAM-1 suppression may reflect a near maximal translational suppression that cannot be improved upon without induction of RISC mediated cleavage. Again, however, this experiment indicates that such chemical modifications are tolerated and can be considered in the design of CODEMIRs.

Modifications such as the LNA modified base could be used in the 3'tail of a candidate multitargeting interfering RNA so as to strengthen the interaction of this portion of the guide strand with the corresponding part of the passenger strand, thereby improving loading bias. Therefore, there are a number of uses for modified bases that are envisaged in this invention.

The data primarily relates to principles of CODEMIR design and in particular supports the approach of obtaining maximum hybridisation of the active RNA to each of its targets. However, these data also support the fact that complete complementarity is not necessary for maximal activity and that significant activity can be obtained with the guide strand even when the guide strand has complete complementarity to just the seed region.

Table 17-1. Sequence listing of 32 systematically designed variants of CODEMIR-1

	Guide strand (5' to 3')	VEGF binding (upper = VEGF mRNA)	ICAM-1 binding (upper = ICAM-1 mRNA)
CODEMIR-1	UAUGUGGGUGGGUGAGUCUAA (SEQ ID NO: 100)	5' G A C 3' UAGAC CACCCACCCACAUA AUCUG GUGGGUGGGUGUAU 3' A A 5' Top strand (SEQ ID NO: 181) Bottom strand (SEQ ID NO: 100)	5' G CCAC C 3' UUAG CUC CCCACCCACAUA AAUC GAG GGGUGGGUGUAU 3' U U 5' Top strand (SEQ ID NO: 183) Bottom strand (SEQ ID NO: 100)
CODEMIR-52	UAUGUGGGUGGGGGGGGUCUCU (SEQ ID NO: 434)	5' U UGUAGACACA C 3' GGGAUUCC CCCACCCACAUA CUCUGGGG GGGUGGGUGUAU 3' U 5' Top strand (SEQ ID NO: 435) Bottom strand (SEQ ID NO: 434)	5' U CC C 3' AG ACCUCCCCACCCACAUA UC UGGGGGGGUGGGUGUAU 3' UC 5' Top strand (SEQ ID NO: 436) Bottom strand (SEQ ID NO: 434)
CODEMIR-53	UAUGUGGGUGGGUGGGGUCUCU (SEQ ID NO: 437)	5' U A C 3' AGAC CACCCACCCACAUA UCUG GUGGGUGGGUGUAU 3' UC G 5' Top strand (SEQ ID NO: 438) Bottom strand (SEQ ID NO: 437)	5' U CC C C 3' AG ACCU CCCACCCACAUA UC UGGG GGGUGGGUGUAU 3' UC U 5' Top strand (SEQ ID NO: 436) Bottom strand (SEQ ID NO: 437)
CODEMIR-54	UAUGUGGGUGGGGGUGUCUCU (SEQ ID NO: 439)	5' U A C 3' AGACAC CCCACCCACAUA UCUGUG GGGUGGGUGUAU 3' UC G 5' Top strand (SEQ ID NO: 438) Bottom strand (SEQ ID NO: 439)	5' U CUUUGUUAGCCACCU C 3' GACAC CCCCACCCACAUA CUGUG GGGGUGGGUGUAU 3' UCU 5' Top strand (SEQ ID NO: 440) Bottom strand (SEQ ID NO: 439)
CODEMIR-55	UAUGUGGGUGGGUGUGUCUCU (SEQ ID NO: 441)	5' U C 3' AGACACACCCACCCACAUA UCUGUGUGGGUGGGUGUAU 3' UC 5' Top strand (SEQ ID NO: 438) Bottom strand (SEQ ID NO: 441)	5' U C CUC C 3' AG CAC CCCACCCACAUA UC GUG GGGUGGGUGUAU 3' UC U U 5' Top strand (SEQ ID NO: 436) Bottom strand (SEQ ID NO: 441)

CODEMIR-56	UAJGUGGGUGGGGGGGUGUCU (SEQ ID NO: 442)	5' U A C 3' AGACAC CCCACCCACAUA UCUGUG GGGUGGGUGCAU 3' GGG 5' Top strand (SEQ ID NO: 438) Bottom strand (SEQ ID NO: 442)	5' U C C 3' AG CACCUCCCCACCCACAUA UC GUGGGGGGGUGGGUGUAU 3' U 5' Top strand (SEQ ID NO: 436) Bottom strand (SEQ ID NO: 442)
CODEMIR-57	UAUGUGGGUGGGGGGGUGUCU (SEQ ID NO: 443)	5' U C 3' AGACAC ACCCACCCACAUA UCUGUG UGGGUGGGUGUAU 3' GG 5' Top strand (SEQ ID NO: 438) Bottom strand (SEQ ID NO: 443)	5' U C C C 3' AG CACCU CCCACCCACAUA UC GUGGG GGGUGGGUGUAU 3' U U 5' Top strand (SEQ ID NO: 436) Bottom strand (SEQ ID NO: 443)
CODEMIR-58	JAUGUGGGUGGGGGGGUGUCU (SEQ ID NO: 444)	5' U C 3' AGACACA CCCACCCACAUA UCUGUGU GGGUGGGUGUAU 3' GG 5' Top strand (SEQ ID NO: 438) Bottom strand (SEQ ID NO: 444)	5' U C C C 3' AG CAC CCCCACCCACAUA UC GUG GGGGUGGGUGUAU 3' U U 5' Top strand (SEQ ID NO: 436) Bottom strand (SEQ ID NO: 444)
CODEMIR-59	JAUGUGGGUGGGGGGUGUCU (SEQ ID NO: 445)	5' G JCCUGUAG C 3' GGAU ACACACCCACCCACAUA UCUG JGUGUGGGUGGGUGUAU 3' 5' Top strand (SEQ ID NO: 446) Bottom strand (SEQ ID NO: 445)	5' C CUUUGCUAGCC CUC C 3' GACAC AC CCCACCCACAUA CUGUG UC GGGUGGGUGUAU 5' 3' U U Top strand (SEQ ID NO: 440) Bottom strand (SEQ ID NO: 445)
CODEMIR-60	JAUGUGGGUGGGGGGGUCCGCU (SEQ ID NO: 447)	5' J G UGUAGACACA C 3' G GAUCCC CCCACCCACAUA C CUGGGG GGGUGGGUGUAU 3' U G 5' Top strand (SEQ ID NO: 433) Bottom strand (SEQ ID NO: 447)	5' C C C 3' AGC ACCUCCCCACCCACAUA UCG UGGGGGGGUGGGUGUAU 3' C 5' Top strand (SEQ ID NO: 436) Bottom strand (SEQ ID NO: 447)
CODEMIR-61	JAUGUGGGUGGGGGGUCCGCU (SEQ ID NO: 448)	5' A A C 3' GAC CACCCACCCACAUA CUG GUGGGUGGGUGUAU 3' UCG G 5' Top strand (SEQ ID NO: 449) Bottom strand (SEQ ID NO: 448)	5' C C C C 3' AGC ACCU CCCACCCACAUA UCG UGGG GGGUGGGUGUAU 3' C U 5' Top strand (SEQ ID NO: 436) Bottom strand (SEQ ID NO: 448)

CODEMIR-62	UAUGUGGGUGGGGCUGUCGCU (SEQ ID NO: 450)	5' A A C 3' GACAC CCCACCCACAUA CUGUG GGGUGGGUGUAU 3' UCG G 5' Top strand (SEQ ID NO: 449) Bottom strand (SEQ ID NO: 450)	5' U C C C 3' AGC AC UCCCCACCCACAUA UCG UG GGGGGUGGGUGUAU 3' C U 5' Top strand (SEQ ID NO: 436) Bottom strand (SEQ ID NO: 450)
CODEMIR-63	UAUGUGGGUGGGGUGUGUCGCU (SEQ ID NO: 451)	5' A C 3' GACACACCCACCCACAUA CUGUGUGGGUGGGUGUAU 3' UCG 5' Top strand (SEQ ID NO: 449) Bottom strand (SEQ ID NO: 452)	5' U C UC C 3' AGC AC C CCCACCCACAUA UCG UG G GGGUGGGUGUAU 3' C U U 5' Top strand (SEQ ID NO: 436) Bottom strand (SEQ ID NO: 451)
CODEMIR-64	UAUGUGGGUGGGGGGGUGGCU (SEQ ID NO: 452)	5' U A A C 3' AG CAC CCCACCCACAUA UC GUG GGGUGGGUGUAU 3' G GGG 5' Top strand (SEQ ID NO: 438) Bottom strand (SEQ ID NO: 452)	5' U C C 3' AGCCACCU CCCACCCACAUA UCGGUGGG GGGUGGGUGUAU 3' U 5' Top strand (SEQ ID NO: 436) Bottom strand (SEQ ID NO: 452)
CODEMIR-65	UAUGUGGGUGGGGUGGGUGGCU (SEQ ID NO: 453)	5' U UG AGACA C 3' CC U CACCCACCCACAUA GG G GUGGGUGGGUGUAU 5' 3' UC UG Top strand (SEQ ID NO: 454) Bottom strand (SEQ ID NO: 453)	5' U C C 3' AGCCACCU CCCACCCACAUA UCGGUGGG GGGUGGGUGUAU 3' U 5' Top strand (SEQ ID NO: 436) Bottom strand (SEQ ID NO: 453)
CODEMIR-66	UAUGUGGGUGGGGGGUGUGGCU (SEQ ID NO: 454)	5' U AG A C 3' GU ACAC CCCACCCACAUA CG UGUG GGGUGGGUGUAU 3' U G G 5' Top strand (SEQ ID NO: 545) Bottom strand (SEQ ID NO: 454)	5' U C C 3' AGCCAC UCCCCACCCACAUA UCGGUG GGGGGUGGGUGUAU 3' U 5' Top strand (SEQ ID NO: 436) Bottom strand (SEQ ID NO: 454)
CODEMIR-67	UAUGUGGGUGGGGUGUGUGGCU (SEQ ID NO: 455)	5' U AG C 3' GU ACACACCCACCCACAUA CG UGUGUGGGUGGGUGUAU 3' U G 5' Top strand (SEQ ID NO: 545) Bottom strand (SEQ ID NO: 455)	5' U UC C 3' AGCCAC C CCCACCCACAUA UCGGUG G GGGUGGGUGUAU 3' U U 5' Top strand (SEQ ID NO: 436) Bottom strand (SEQ ID NO: 455)

CODEMIR-68	UAUGUGGGUGGGGGGGUCUAU (SEQ ID NO: 456)	5' U ACA C 3' GUAGAC CCCACCCACAUA UAUCUG GGGUGGGUGUAU 3' GGG 5' Top strand (SEQ ID NO: 545) Bottom strand (SEQ ID NO: 456)	5' U CC C 3' UAG ACCUCCCCACCCACAUA AUC UGGGGGGGUGGGUGUAU 3' U 5' Top strand (SEQ ID NO: 457) Bottom strand (SEQ ID NO: 456)
CODEMIR-69	UAUGUGGGUGGGUGGGUCUAU (SEQ ID NO: 458)	5' U A C 3' GUAGAC CACCCACCCACAUA UAUCUG GUGGGUGGGUGUAU 3' G 5' Top strand (SEQ ID NO: 545) Bottom strand (SEQ ID NO: 458)	5' U CC C C 3' UAG ACCU CCCACCCCACAUA AUC UGGG GGGUGGGGUGUAU 3' U U 5' Top strand (SEQ ID NO: 457) Bottom strand (SEQ ID NO: 458)
CODEMIR-70	UAUGUGGGUGGGGGUGUCCUAU (SEQ ID NO: 459)	5' U A C 3' GUAGACAC CCCACCCACAUA UAUCUGUG GGGUGGGUGUAU 3' G 5' Top strand (SEQ ID NO: 545) Bottom strand (SEQ ID NO: 459)	5' U C CU C 3' UAG CAC CCCCACCCACAUA AUC GUG GGGGUGGGUGUAU 3' U U 5' Top strand (SEQ ID NO: 457) Bottom strand (SEQ ID NO: 459)
CODEMIR-71	UAUGUGGGUGGGGUGUCCUAU (SEQ ID NO: 460)	5' U C 3' GUAGACACCCACCCACAUA UAUCUGUGGGUGGGUGUAU 3' 5' Top strand (SEQ ID NO: 545) Bottom strand (SEQ ID NO: 460)	5' U C C CUC C 3' UAG CAC CCCACCCACAUA AUC GUG GGGUGGGUGUAU 3' U U U 5' Top strand (SEQ ID NO: 457) Bottom strand (SEQ ID NO: 460)
CODEMIR-72	UAUGUGGUGGGGGGGGUGUAU (SEQ ID NO: 461)	5' G A C 3' ACAC CCCACCCACAUA UGUG GGGUGGGUGUAU 3' UA GGG 5' Top strand (SEQ ID NO: 462) Bottom strand (SEQ ID NO: 461)	5' C C 3' CACCCCCCCACCCCACAUA GUGGGGGGGUGGGGUGUAU 3' UAU 5' Top strand (SEQ ID NO: 453) Bottom strand (SEQ ID NO: 461)
CODEMIR-73	UAUGUGGGUGGGUGGGGUGUAU (SEQ ID NO: 464)	5' U G A C 3' GUA AC CACCCACCCACAUA UAU UG GUGGGUGGGUGUAU 3' G G 5' Top strand (SEQ ID NO: 545) Bottom strand (SEQ ID NO: 464)	5' C C C C 3' CACCU CCCACCCACADA GUGGG GGGUGGGUGUAU 3' UAU U 5' Top strand (SEQ ID NO: 453) Bottom strand (SEQ ID NO: 464)

CODEMIR-74	UAUGUGGGUGGGGGCGUGUAU (SEQ ID NO: 465)	5' U G A C 3' GUA ACAC CCCACCCACAUA UAU UGUG GGGUGGGUGUAU 3' G G 5' Top strand (SEQ ID NO: 545) Bottom strand (SEQ ID NO: 465)	5' C C C 3' CAC JCCCCACCCACAUA GUG GGGGGUGGGUGUAU 3' UAU U 5' Top strand (SEQ ID NO: 463) Bottom strand (SEQ ID NO: 465)
CODEMIR-75	UAUGUGGGUGGGGUGUGUGUAU (SEQ ID NO: 466)	5' U G C 3' GUA ACACACCCACCCACAUA UAU UGUGUGGGUGGGGUGUAU 3' G 5' Top strand (SEQ ID NO: 545) Bottom strand (SEQ ID NO: 466)	5' A CUC C 3' GC CAC CCCACCCACAUA UG GUG GGGUGGGUGUAU 5' 3' UA U U 5' Top strand (SEQ ID NO: 467) Bottom strand (SEQ ID NO: 466)
CODEMIR-76	UAUGUGGGUGGGGGUCGAU (SEQ ID NO: 468)	5' A ACA C 3' GAC CCCACCCACAUA CUG GGGUGGGUGUAU 3' UAG GGG 5' Top strand (SEQ ID NO: 449) Bottom strand (SEQ ID NO: 468)	5' C C 3' ACCUCCCCCACCCACAUA UGGGGGGGUGGGUGUAU 3' UAGC 5 Top strand (SEQ ID NO: 469) Bottom strand (SEQ ID NO: 468)
CODEMIR-77	UAUGUGGGUGGGGGUCGAU (SEQ ID NO: 470)	5' A A C 3' GAC CACCCACCCACAUA CUG GUGGGUGGGUGUAU 3' UAG G 5' Top strand (SEQ ID NO: 449) Bottom strand (SEQ ID NO: 470)	5' C C C 3' ACCU CCCACCCACACA UGGG GGGUGGGUGUAC 3' UAGC U 5' Top strand (SEQ ID NO: 469) Bottom strand (SEQ ID NO: 470)
CODEMIR-78	UAUGUGGGUGGGGGGGUCGAU (SEQ ID NO: 471)	5' A A C 3' GACAC CCCACCCACAUA CUGUG GGGUGGGUGUAU 3' UAG G 5' Top strand (SEQ ID NO: 449) Bottom strand (SEQ ID NO: 471)	5' C CUUGUUAGCCACCU C 3' UGACAC CCCCACCCACAUA GCUGUG GGGGUGGGUGUAU 3' UA 5' Top strand (SEQ ID NO: 472) Bottom strand (SEQ ID NO: 472)
CODEMIR-79	UAUGUGGGUGGGGUGUGUCGAU (SEQ ID NO: 473)	5' A C 3' GACACACCCACCCACAUA CUGUGUGGGUGGGUGUAU 3' UAG 5' Top strand (SEQ ID NO: 449) Bottom strand (SEQ ID NO: 473)	5' A C CUC C 3' G CAC CCCACCCACAUA C GUG GGGUGGGUGUAU 5' 3' UAG U C 5' Top strand (SEQ ID NO: 467) Bottom strand (SEQ ID NO: 473)

CODEMIR-80	UAUGUGGGUGGGGGGGUGGAU (SEQ ID NO: 474)	5' U JG AGACA A C 3' UCC U C CCCACCCACAUA AGG G G GGGUGGGGUGUAU 3' U UG G 5' Top strand (SEQ ID NO: 475) Bottom strand (SEQ ID NO: 474)	5' G C 3' CCACCUCCCCACCCACAUA GGUGGGGGGGUGGGUGUAU 3' UA 5' Top strand (SEQ ID NO: 476) Bottom strand (SEQ ID NO: 474)
CODEMIR-81	UAUGUGGGUGGGGUGGGGUGGAU (SEQ ID NO: 477)	5' U UG AGACA C 3' UCC U CACCCACCCACAUA AGG G GUGGGUGGGGUGUAU 3' U UG 5' Top strand (SEQ ID NO: 475) Bottom strand (SEQ ID NO: 477)	5' G C C 3' CCACCU CCCACCCACAUA GGUGGG GGGUGGGGUGUAU 3' UA U 5' Top strand (SEQ ID NO: 476) Bottom strand (SEQ ID NO: 474)
CODEMIR-82	UAUGUGGGUGGGGGGUGUGGAU (SEQ ID NO: 479)	5' U UGUAG A C 3' UCC ACAC CCCACCCACAUA AGG UGUG GGGUGGGGUGUAU 3' U G 5' Top strand (SEQ ID NO: 475) Bottom strand (SEQ ID NO: 479)	5' G C C 3' CCAC UCCCCACCCACAUA GGUG GGGGGUGGGGUGUAU 3' UA U 5' Top strand (SEQ ID NO: 476) Bottom strand (SEQ ID NO: 479)
CODEMIR-83	UAUGUGGGUGGGGUGUGUGGAU (SEQ ID NO: 479)	5' U UGUAG C 3' UCC ACACACCCACCCACAUA AGG UGUGUGGGUGGGGUGUAU 3' U 5' Top strand (SEQ ID NO: 475) Bottom strand (SEQ ID NO: 479)	5' G UC C 3' CCAC C CCCACCCACAUA GGUG G GGGUGGGGUGUAU 3' UA U U 5' Top strand (SEQ ID NO: 476) Bottom strand (SEQ ID NO: 479)
CODEMIR-84 (hsa-mir-299)	UAUGUGGGAUGGUAAACCGCUU (SEQ ID NO: 193)	5' C C 3' CCA CCCACAUA GGU GGGUGCAU 3' UUCGCCAAAU A 5' Top strand (SEQ ID NO: 480) Bottom strand (SEQ ID NO: 193)	5' U CACCUCC C 3' AGC CCA CCCACAUA UCG GGU GGGUGCAU 3' U CCAAAU A 5' Top strand (SEQ ID NO: 436) Bottom strand (SEQ ID NO: 193)

Table 17-2. Variants of CODEMIR-56 and 76 without 7 G motifs.

	Duplex	mRNA binding (RNA hybrid)
CODEMIR-56	Passenger ACACCCCCCCCACCCACACAUAUU (SEQ ID NO: 481) UCUGUGGGGGGGGGUGGGUGUAU (SEQ ID NO: 482) Guide	VEGF 5' U A C 3' AGACAC CCCACCCACAUA (SEQ ID NO: 438) UCUGUG GGGUGGGUGUAU 3' GGG 5' (SEQ ID NO: 482) ICAM-1 5' U C C 3' AG CACCUCCCCACCCACACA (SEQ ID NO: 436) UC GUGGGGGGGGGGGGGUAU 3' U 5' (SEQ ID NO: 482)
CODEMIR-76	Passenger CGACCCCCCCCACCCACACAUAUU (SEQ ID NO: 483) UAGCUGGGGGGGGGUGGGUGUAU (SEQ ID NO: 484) Guide	VEGF 5' A ACA C 3' GAC CCCACCCACAUA (SEQ ID NO: 449) CUG GGGUGGGUGUAU 3' UAG GGG 5' (SEQ ID NO: 484) ICAM-1 5' C C 3' ACCUCCCCACCCACACA (SEQ ID NO: 485) UGGGGGGGGGGGGGUAU 3' CAGC 5' (SEQ ID NO: 484)
CODEMIR-120	Passenger ACACCUCCCCACCCACACAUAUU (SEQ ID NO: 486) UCUGUGGAGGGGUGGGUGUAU (SEQ ID NO: 487) Guide	VEGF 5' U A C 3' AGACAC CCCACCCACAUA (SEQ ID NO: 438) UCUGUG GGGUGGGUGUAU 3' GAG 5' (SEQ ID NO: 487) ICAM-1 5' U C C 3' AG CACCUCCCCACCCACACA (SEQ ID NO: 436) UC GUGGAGGGGUGGGUGUAU 3' U 5' (SEQ ID NO: 487)
CODEMIR-121	Passenger CGACCUCCCCACCCACACAUAUU (SEQ ID NO: 488) UAGCUGGAGGGGUGGGUGUAU (SEQ ID NO: 489) Guide	VEGF 5' A ACA C 3' GAC CCCACCCACAUA (SEQ ID NO: 449) CUG GGGUGGGUGUAU 3' UAG GAG 5' (SEQ ID NO: 489) ICAM-1 5' C C 3' ACCUCCCCACCCACACAUA (SEQ ID NO: 469) UGGAGGGGUGGGUGUAU 3' UAGC 5' (SEQ ID NO: 489)

Table 17-3. Variants of CODEMIR-1 incorporating LNA or inosine bases, or asymmetric loops.

	Duplex	mRNA binding (RNA hybrid)
CODEMIR-1	Passenger AGACUCACCCACCCACAUAUU (SEQ ID NO: 101) AAUCUGAGUGGGUGGGUGUAU (SEQ ID NO: 100) Guide	VEGF 5' G A C 3' UAGAC CACCCACCCACAUA (SEQ ID NO: 181) AUCUG GUGGGUGGGUGUAU 3' A A 5' (SEQ ID NO: 100)
		ICAM 5' G CCAC C 3' UUAG CUC CCCACCCACAUA (SEQ ID NO: 183) AAUC GAG GGGUGGGUGUAU 3' U U 5' (SEQ ID NO: 100)
CODEMIR-99	Passenger AGACUCACCCACCCACAUAUU (SEQ ID NO: 101) AAUCUGAGUGGGUGGGUGUAU (SEQ ID NO: 490) Guide	VEGF 5' G A C 3' UAGAC CACCCACCCACAUA (SEQ ID NO: 181) AUCUG GUGGGUGGGUGUAU 3' A A 5' (SEQ ID NO: 490)
		ICAM 5' G CCAC C 3' UUAG CUC CCCACCCACAUA (SEQ ID NO: 183) AAUC GAG GGGUGGGUGUAU 3' U U 5' (SEQ ID NO: 490)
CODEMIR-100	Passenger AGACUCACCCACCCACAUAUU (SEQ ID NO: 101) AAUCUGAGIGGGUGGGUGUAU (SEQ ID NO: 491) Guide	VEGF 5' G A C 3' UAGAC CACCCACCCACAUA (SEQ ID NO: 181) AUCUG GIGGGUGGGUGUAU 3' A A 5' (SEQ ID NO: 491)
		ICAM 5' G CC C C 3' UUAG AC UCCCCACCCACAUA (SEQ ID NO: 183) AAUC UG GIGGGUGGGUGUAU 3' A 5' (SEQ ID NO: 491)
CODEMIR-101	Passenger AGACUCACCCACCCACAUAUU (SEQ ID NO: 101) AAUCUGIGUGGGUGGGUGUAU (SEQ ID NO: 492) Guide	VEGF 5' G C 3' UAGACACACCCACCCACAUA (SEQ ID NO: 181) AUCUGIGUGGGUGGGUGUAU 3' A 5' (SEQ ID NO: 492)
		ICAM 5' G CC C C 3' UUAG ACCU CCCACCCACAUA (SEQ ID NO: 183) AAUC UGIG GGGUGGGUGUAU 3' U 5' (SEQ ID NO: 492)

CODEMIR-102	Passenger AGACUCACCCACCCACAUAUU (SEQ ID NO: 101) AAUCUGIGIGGGUGGGUGUAU (SEQ ID NO: 493) Guide	VEGF 5' G C 3' UAGACACACCCACCCACAUA (SEQ ID NO: 181) AUCUGIGIGGGUGGGUGUAU 3' A 5' (SEQ ID NO: 493) ICAM 5' G CC C 3' UUAG ACCUCCCCACCCACAUA (SEQ ID NO: 182) AAUC UGIGIGGGUGGGUGUAU 3' 5' (SEQ ID NO: 493)
CODEMIR-104	Passenger AGACUCACCCACCCACAUAUU (SEQ ID NO: 101) CAUCUGAGUGGGUGGGUGUAU (SEQ ID NO: 494) Guide	VEGF 5' CU A C 3' GUAGAC CACCCACCCACAUA (SEQ ID NO: 495) CAUCUG GUGGGUGGGUGUAU 3' A 5' (SEQ ID NO: 494)
CODEMIR-105	Passenger UAGACCACCCACCCACAUAUU (SEQ ID NO: 496) ACAUCUGGUGGGUGGGUGUAU (SEQ ID NO: 497) Guide	VEGF 5' C A C 3' UGUAGAC CACCCACCCACAUA (SEQ ID NO: 495) ACAUCUG GUGGGUGGGUGUAU 3' 5' (SEQ ID NO: 497)
CODEMIR-106	Passenger GACAUCACCCACCCACAUAUU (SEQ ID NO: 498) AUCUGUAGUGGGUGGGUGUAU (SEQ ID NO: 499) Guide	VEGF 5' UG C 3' UAGACA CACCCACCCACAUA (SEQ ID NO: 545) AUCUGU GUGGGUGGGUGUAU 3' A 5' (SEQ ID NO: 499)
CODEMIR-107	Passenger AGACACACCCACCCACAUAUU (SEQ ID NO: 500) CAUCUGUGUGGGUGGGUGUAU (SEQ ID NO: 501) Guide	VEGF 5' CU C 3' GUAGACACACCCACCCACAUA (SEQ ID NO: 495) CAUCUGUGUGGGUGGGUGUAU 3' 5' (SEQ ID NO: 501)

Bold face indicates LNA modified nucleotide; I indicates inosine base

Example 18

Screening multiple seeds for VEGF activity

The data acquired from the systematic analysis of the VEGF suppressive activity of 32 variants of CODEMIR-1 (above) demonstrated that the length of 5' complementarity to the target as well as the total complementarity to the target are critical determinants of CODEMIR activity, particularly when hybridization of the guide strand supports RISC-mediated cleavage. These factors, as well as those factors related to loading of the active strand in RISC, are the most important CODEMIR design criteria elucidated to date. However, these factors do not allow discrimination between active and inactive seed sites, which is critical for in silico identification of active CODEMIRs. Some of the factors that may be expected to influence the activity of CODEMIR activity at a given seed site are: i) secondary structure at the target site of the mRNA, ii) an adenosine at the final position of the target site (i.e. a uracil at the first position of the guide strand – naturally occurring microRNAs have a preference for this pairing) and iii) the number of seed sites in the target mRNA (i.e. the number of guide strand binding sites in the target). To assess these factors, 12 CODEMIRs were designed (Table 18-1) with seed sites that had the characteristics: i) free target secondary structure, no initial A and only a single site (CODEMIRs 108-110), ii) free target secondary structure, an initial A and only a single site (CODEMIRs 111-113), iii) not free target secondary structure, an initial A and only a single site (CODEMIRs 114-116), and iv) not free target secondary structure, an initial A and two sites (CODEMIRs 117-119). To exclude complications arising from differing 3' tails on the guide strands of these CODEMIRs a uniform 3' tail was incorporated, using the sequence from CODEMIR-84 (miR-299), which has favourable strand loading characteristics and supports translational suppression of VEGF (see above and Figure 4). Each of these CODEMIRs was assayed for VEGF suppressive activity in ARPE-19 cells (Figure 28). However, no discernable trend was observed with respect to the seed characteristics under investigation, despite the fact that some of these CODEMIRs had significant activity relative to the irrelevant control.

Table 18-1 – CODEMIRs targeting different seed sites in the VEGF 3'UTR.

CODEMIR	Features	Duplex	VEGF binding	
108	Free 2° No A 1 site	Passenger GCGGUUUACCCCCUGAAAUGUU (SEQ ID NO: 502) UUCGCCAAAUGGGACUUUAC (SEQ ID NO: 503) Guide	5' A A 3' CCUGAAACG (SEQ ID NO: 504) GGACUUUAC 5' (SEQ ID NO: 503)	
109		Passenger GCGGUUUACCAGAAGAGACUU (SEQ ID NO: 505) UUCGCCAAAUGGUCUUCUCG (SEQ ID NO: 506) Guide	5' C AAG A 3' GGCG AGAAGAGAC (SEQ ID NO: 507) UCGC UCUUCUCUG 5' (SEQ ID NO: 506)	
110		Passenger GCGGUUUACCUUUAUAUGUUU (SEQ ID NO: 508) UUCGCCAAAUGGAAACAUACA (SEQ ID NO: 509) Guide	5' U AAC UA A 3' GGC UUG UUUGUGUGU (SEQ ID NO: 510) UCG AAU AAAUAUACA 5' (SEQ ID NO: 509)	
111	Free 2° A 1 site	Passenger GCGGUUUACCAUAUUAACACC (SEQ ID NO: 511) UUCGCCAAAUGGCAUAAUUGU (SEQ ID NO: 512) Guide	5' U GGAAAAG U 3' GUGG ACACAACA (SEQ ID NO: 513) CGCC UAUAAAUUGU 5' (SEQ ID NO: 512)	
112		Passenger GCGGUUUACCGUAUAUAAACC (SEQ ID NO: 514) UUCGCCAAAUGGCAUAUAUUU (SEQ ID NO: 515) Guide	5' C A 3' CGUUUU UUGUACAAA (SEQ ID NO: 516) GCCAAA GGCAUAUAUUU 3' UUC U 5' (SEQ ID NO: 515)	
113		Passenger GCGGUUUACCAAUGUGCAAUU (SEQ ID NO: 517) UUCGCCAAAUGGUUACACGUU (SEQ ID NO: 518) Guide	5' G CAG CCCCCCCCC CAGG G 3' GGGC GGU UGCC AACUGUGCAA UUCG CCA AUGG UUACACGUU 3' A 5' Top strand (SEQ ID NO: 519) Bottom strand (SEQ ID NO: 518)	
114	Not Free 2° A 1 site	Passenger GCGGUUUACCACUGGCAGAUU (SEQ ID NO: 520) UUCGCCAAAUGGUGACCGUCU (SEQ ID NO: 521) Guide	5' A AG U 3' GG GCCACUGGCAGA (SEQ ID NO: 522) CC UGGUGACCGUCU 3' UUCG AAA 5' (SEQ ID NO: 521)	

115		Passenger GCGGUUUACCAGAAAUUAAU (SEQ ID NO: 523) UUCGCCAAAUGGUCUUUAAUU (SEQ ID NO: 524) Guide	5' A A 3' AUUAGAAAUUAA (SEQ ID NO: 525) UGGUCUUUAAUU 3' UUCGCCAAA 5' (SEQ ID NO: 524)	
116		Passenger GCGGUUUACCCGGCGAAGAUU (SEQ ID NO: 526) UUCGCCAAAUGGGCCGCUUCU (SEQ ID NO: 527) Guide	5' U A AUGC G 3' GGC G CCCGGCGAAGA (SEQ ID NO: 528) UCG C GGGCCGCUUCU 3' U C AAAU 5' (SEQ ID NO: 527)	
117	Not Free 2' A 2 sites	Passenger GCGGUUUACCGAUACAGAAUU (SEQ ID NO: 529) UUCGCCAAAUGGCUAUGUCUU (SEQ ID NO: 530) Guide	5' A AAAG C 3' GG ACUGAUACAGAA (SEQ ID NO: 531) CC UGGCUAUGUCUU 3' UUCG AAA 5' (SEQ ID NO: 530)	
118		Passenger GCGGUUUACCGGAAGAGGAUU (SEQ ID NO: 532) UUCGCCAAAUGGCCUUCUCCU (SEQ ID NO: 533) Guide	5' U ACUUGA UU A 3' GUGG G GGGAGGGGA (SEQ ID NO: 534) CGCC U CCUUCUCCU 3' UU AAA GG 5'(SEQ ID NO: 533)	
119		Passenger GCGGUUUACCUGCCCAGGAUU (SEQ ID NO: 535) UUCGCCAAAUGGACGGGUCCU (SEQ ID NO: 536) Guide	5' G CAG CCUCUCC A 3' GGGC GGU CCUGCCCAGGA UUCG CCA GGACGGGUCCU 3' AAU 5' Top strand (SEQ ID NO: 537) Bottom strand (SEQ ID NO: 536)	

Example 19

Expression of CODEMIRs as shRNAs

To further confirm, as shown in the HIV VIROMIR example, that CODEMIRs may be expressed as short hairpin RNAs (shRNA), a shRNA CODEMIR was designed based on CODEMIR-1. This hairpin was designed to include the 21 nucleotide core of CODEMIR-1 at the free (non-loop) terminus of the hairpin (Figure 29). The hairpin was cloned into a plasmid vector and expressed from an H1 promoter. ARPE-19 cells were seeded into 96-well plated (4000 cells/well) and transfected with 200 ng plasmid DNA 24 hours later. VEGF and ICAM-1 was evaluated 48 hours later by ELISA and FACS, respectively. The hairpin demonstrated VEGF and ICAM-1 suppressive activity relative to a length matched hairpin control (Figure 30); demonstrating the applicability of expressed short-hairpin CODEMIRs

Results from the study demonstrated that CODEMIRs can be expressed as hairpins. Expression of interfering RNAs as hairpins is well documented and these findings demonstrate that the concept of CODEMIRs is applicable to hairpins as well as to synthetic duplexes.

One skilled in the art will appreciate that the construction of a suitable expression system for shRNA requires consideration of many factors which, for example, influence the amount of RNA produced (promoter, length and stability of transcript etc). Hence, it is evident that further optimisation of an expression system for a CODEMIR-1 shRNA precursor could be considered and would likely lead to higher suppressive capacity.

Example 20

Blunt-ended CODEMIRs

Experiments using cell lysates from Drosophila have identified that 2 nucleotide 3' overhangs at the extremities of the duplex are optimal for RNA silencing. However, in some experiments with mammalian cells, "blunt-ended" duplexes were also found to be considerably active and had increased stability in culture medium containing FBS (Czauderna et al., (2003), *Nucleic Acids Res*, 31, 2705-16).

To test the effect of blunt ends on CODEMIR activity, variants of CODEMIR-1 were designed with either 1 or 0 base overhangs (Table 20-1). When transfected in ARPE-19 cells at 40 nM, these CODEMIRs demonstrated decreased efficacy against VEGF and ICAM-1 as

compared to CODEMIR-1 (Figure 31). In contrast to the results of Czauderna *et al*, it was found that blunt-ended duplexes were not appreciably more stable (data not shown). Furthermore, data have been generated indicating that the presence of a blunt end reduces the loading of the shorter strand (data not shown), thereby explaining the negative result observed in the above experiment. Therefore, whilst blunt-ended duplexes may not be of utility in enhancing the stability of the molecule, this strategy can be used to enhance the loading of an active strand by designing the passenger strand to be shorter than the guide strand and having its 5' end located at the blunt end of the duplex.

Table 20-1 – Variants of CODEMIR-1 with truncated 3' termini

	CODEMIR duplex	Predicted binding (RNA Hybrid)
CODEMIR-1	Passenger AGACUCACCCACCCCACAUAUU (SEQ ID NO: 101) AAUCUGAGUGGGGUGGGGUGUAU (SEQ ID NO: 100) Guide	VEGF 5' G A C 3' UAGAC CACCCACCCCACATA (SEQ ID NO: 181) AUCUG GUGGGUGGGGUGUAU CODEMIR-1 3' A A 5' (SEQ ID NO: 100) ICAM 5' G CCAC C 3' CUAG CUC CCCACCCCACAUA (SEQ ID NO: 183) AAUC GAG GGGUGGGGUGUAU CODEMIR-1 3' U U 5' (SEQ ID NO: 100)
CODEMIR-24	Passenger AGACUCACCCACCCCACAUAU (SEQ ID NO: 129) AUCUGAGUGGGGUGGGGUGUAU (SEQ ID NO: 538) Guide	VEGF 5' G A C 3' UAGAC CACCCACCCCACATA (SEQ ID NO: 181) AUCUG GUGGGUGGGGUGUAU AM024 3' A 5' (SEQ ID NO: 538) ICAM 5' G CCAC C 3' UAG CUC CCCACCCCACAUA (SEQ ID NO: 457) AUC GAG GGGUGGGGUGUAU AM024 3' U U 5' (SEQ ID NO: 338)
CODEMIR-25	Passenger AGACUCACCCACCCCACAUA (SEQ ID NO: 189) UCUGAGUGGGGUGGGGUGUAU (SEQ ID NO: 539) Guide	VEGF 5' U A C 3' AGAC CACCCACCCCACATA (SEQ ID NO: 438) UCUG GUGGGUGGGGUGUAU AM025 3' A 5' (SEQ ID NO: 539) ICAM 5' C C 3' CUC CCCACCCCACAUA (SEQ ID NO: 540) GAG GGGUGGGGUGUAU AM025 3' UCU U 5' (SEQ ID NO: 539)

Example 21

In vivo studies with CODEMIR-1

The activity of CODEMIR-1 and other multitargeting RNA of the invention could be tested in various preclinical models known to those skilled in the art. As a non-limiting example, CODEMIR-1 could be tested in a retinopathy of prematurity model. This model is well known to those working in the field of ocular angiogenesis and is used extensively as one of several models for the development of drugs active against the diseases of interest (AMD, diabetic retinopathy etc). The study could comprise of a suitable number of mouse or rat neonate pups equally divided into treatment groups. The treatment groups could include negative controls such as vehicle, irrelevant or scrambled sequence controls plus a number of multitargeting RNA including CODEMIR-1. One could also consider including siRNA to VEGF or other validated angiogenic targets as known comparators.

In this model, beginning on Day 1 of life, litters are exposed to cycles of hyperoxia followed by several days of room air. The injections could be performed on the last day of cycling, prior to the 4 day normoxia period. Several days later, animals could be injected with FITC-dextran and sacrificed. Fluorescence images of the retinal flat mounts could used to estimate the extent of neoangiogenesis in each animal. In addition, measurement of the production of the target RNA molecules or their encoded proteins (in this case, VEGF and ICAM) could be measured by analysis of homogenized samples or, alternatively, with in situ hybridization.

As a further non-limiting example, CODEMIR-1 could alternatively be evaluated *in vivo* for inhibition of disease-related angiogenesis using the laser-induced Choroidal Neovascularization (CNV) model in rats or primates. In this model, animals under general anaesthesia have their pupils dilated and retina photographed. Choroidal neovascularisation (CNV) is induced by krypton laser photocoagulation. This is performed using laser irradiation to either the left or alternatively, the right eye of each animal from all treatment groups through a slit lamp. A total of 6-11 laser burns are generally applied to each eye surrounding the optic nerve at the posterior pole.

At a suitable time following laser injury, the multitargeting RNA are injected into the affected eyes. The suitable time can be the day following laser induction, or for an assessment against established CNV, the injections can be performed several days or weeks following injury. Intravitreal injections of the oligonucleotides are performed by inserting a 30- or 32-gauge needle into the vitreous. Insertion and infusion can be performed and directly viewed through an operating microscope. Care is taken not to injure the lens or the retina. Ideally, the test compounds are placed in the superior and peripheral vitreous cavity. Periodically after treatment, the neoangiogenesis is evaluated by either imaging and/or direct sampling (eg histology, immunohistochemistry). In all cases, the assessment of CNV is best performed by a skilled operator blinded to the actual treatment to ensure a lack of bias in the recording of the information.

An example of a direct imaging method is Colour Fundus Photography (CFP). Again, under anaesthesia as described above, the pupils are dilated. The fundus is then photographed with a camera using the appropriate film.

Alternatively, or preferably in addition to CFP, fluorescein angiography is used to image the vessels and areas of vascular leakage in the retina. This is performed on all of the animals following the intraperitoneal or intravenous injection of sodium fluorescein. The retinal vasculature is then photographed using the same camera as used for CFP but with a barrier filter for fluorescein angiography added. Single photographs can be taken at 0.5-1 minute intervals immediately after the administration of sodium fluorescein. The extent of fluorescein leakage is scored by a trained operator. The mean severity scores from each of the time points are compared by a suitable statistical analysis and differences considered significant at $p < 0.05$. In addition, the frequency of each lesion score is counted, tabulated and represented graphically.

Alternatively, or in addition, rats can be euthanased at selected time points following treatment (for example 7, 14 and 28 days post injection) and eyes examined by conventional histopathology. A reduction in the number and severity of lesions is expected to be seen with samples treated by active oligonucleotides of the invention.

Other non-limiting examples including testing the multitargeting RNA of the invention in other preclinical models such as those that are well known to those skilled in the art. A non-

exhaustive list includes pulmonary fibrosis (bleomycin induced), paw inflammation (carrageen), joint arthritis, diabetes, viral infection, tumour xenografts etc.

While the foregoing specification teaches the principles of the present invention, with examples provided for the purpose of illustration, it will be understood that the practice of the invention encompasses all of the usual variations, adaptations and/or modifications as come within the scope of the following claims and their equivalents. All references are hereby incorporated into this application in their entirety.

What is Claimed is:

1. A multitargeting interfering RNA molecule comprising a guide strand of the Formula (I):

5'-p-XSY-3'

wherein p consists of a terminal phosphate group that is independently present or absent;

wherein S consists of a first nucleotide sequence of a length of about 5 to about 20 nucleotides that is at least partially complementary to a first portion of each of at least two binding sequences present in distinct genetic contexts in one or more pre-selected target RNA molecules;

wherein X is absent or consists of a second nucleotide sequence;

wherein Y is absent or consists of a third nucleotide sequence, provided that X and Y are not absent simultaneously;

wherein XSY is at least partially complementary to each of said binding sequences to allow a stable interaction therewith.

2. The multitargeting interfering RNA molecule of claim 1, wherein S is completely complementary to the first portion of each of at least two binding sequences.

3. The multitargeting interfering RNA molecule of claim 1 or claim 2, wherein the first portion of each of at least two binding sequences is a seed sequence.

4. The multitargeting interfering RNA molecule of any one of claims 1 to 3, wherein X consists of one or two nucleotides.

5. The multitargeting interfering RNA molecule of any one of claims 1 to 4, wherein Y is at least partially complementary to a second portion of each of the binding sequences, said second portion is adjacent to and connected with the 5'-end of said first portion of the binding sequences.

6. The multitargeting interfering RNA molecule of any one of claims 1 to 5, wherein S is of a length of about 8 to about 15 nucleotides.

7. The multitargeting interfering RNA molecule of any one of claims 1 to 6, wherein XSY is of a length of about 17 to about 25 nucleotides.

8. The multitargeting interfering RNA molecule of any one of claims 1 to 7, further comprising a passenger strand that is at least partially complementary to the guide strand to allow formation of a stable duplex between the passenger strand and the guide strand.

9. The multitargeting interfering RNA molecule of claim 8, wherein the passenger strand and the guide strand are completely complementary to each other.

10. The multitargeting interfering RNA molecule of any one of claims 1 to 9 comprising one or more terminal overhangs.

11. The multitargeting interfering RNA molecule of claim 10, wherein the overhang consists of 1 to 5 nucleotides.

12. The multitargeting interfering RNA molecule of any one of claims 1 to 11, wherein the binding sequences are present in distinct genetic contexts in one pre-selected target RNA molecule.

13. The multitargeting interfering RNA molecule of any one of claims 1 to 11, wherein the binding sequences are present in distinct genetic contexts in at least two pre-selected target RNA molecules.

14. The multitargeting interfering RNA molecule of any one of claims 1 to 13, wherein at least one of the pre-selected target RNA molecules is a non-coding RNA molecule.

15. The multitargeting interfering RNA molecule of any one of claims 1 to 14, wherein at least one of the pre-selected target RNA molecules is a messenger RNA molecule.

16. The multitargeting interfering RNA molecule of any one of claims 1 to 15, wherein at least one of the binding sequences is present in the 3'-untranslated region (3'UTR) of a messenger RNA molecule.

17. The multitargeting interfering RNA molecule of any one of claims 1 to 16, wherein one or more of the pre-selected target RNA molecules are involved in a disease or disorder.

18. The multitargeting interfering RNA molecule of claim 17, wherein one or more of the pre-selected target RNA molecules are involved in a disease or disorder of an animal or a plant.

19. The multitargeting interfering RNA molecule of claim 18, wherein the animal is selected from the group consisting of a rat, a mouse, a dog, a cat, a pig, a monkey, and a human.

20. The multitargeting interfering RNA molecule of any one of claims 1 to 19, wherein one or more of the pre-selected target RNA molecules encode a protein of a class selected from the group consisting of receptors, cytokines, transcription factors, regulatory proteins, signaling proteins, cytoskeletal proteins, transporters, enzymes, hormones, and antigens.

21. The multitargeting interfering RNA molecule of any one of claims 1 to 20, wherein one or more of the pre-selected target RNA molecules encode a protein selected from the group consisting of ICAM-1, VEGF-A, MCP-1, IL-8, VEGF-B, IGF-1, Gluc6p, Inppl1, bFGF, PlGF, VEGF-C, VEGF-D, β-catenin, κ-ras-B, κ-ras-A, EGFR,Bcl-2, presenilin-1, BACE-1, MALAT-1, BIC, TGFβ, and TNF alpha.

22. The multitargeting interfering RNA molecule of any one of claims 1 to 21 that decreases expression of any combination of VEGF-A, κ-ras and Bcl-2 in an expression system.

23. The multitargeting interfering RNA molecule of any one of claims 1 to 21 that decreases expression of both MALAT-1 and BIC in an expression system.

24. The multitargeting interfering RNA molecule of any one of claims 1 to 21 that decreases expression of both ICAM-1 and VEGF-A in an expression system.

25. The multitargeting interfering RNA molecule of any one of claims 1 to 21 that decreases expression of both TGFβ and IL-8 in an expression system.

26. The multitargeting interfering RNA molecule of any one of claims 1 to 21 that decreases expression of both IL-8 and MCP-1 in an expression system.

27. The multitargeting interfering RNA molecule of any one of claims 1 to 21, wherein one or more of the pre-selected target RNA molecules is viral RNA.

28. The multitargeting interfering RNA molecule of claim 27, wherein the virus is selected from the group consisting of a human immunodeficiency virus (HIV), a hepatitis C virus (HCV), an influenza virus, a rhinovirus, and a severe acute respiratory syndrome (SARS) virus.

29. The multitargeting interfering RNA molecule of claim 28, wherein one or more of the pre-selected target RNA molecules encode an essential protein for HIV selected from the group consisting of GAG, POL, VIF, VPR, TAT, NEF, REV, VPU and ENV.

30. The multitargeting interfering RNA molecule of claim 27 wherein one or more of the preselected RNA molecules comprises Hepatitis C Virus (HCV) and one or more of the preselected RNA molecules encodes TNFα.

31. The multitargeting interfering RNA molecule of any one of claims 1 to 30 comprising at least one modified ribonucleotide, universal base, acyclic nucleotide, abasic nucleotide, non-ribonucleotide or combinations thereof.

32. The multitargeting interfering RNA molecule of any one of claims 1 to 13, wherein S consists essentially of a nucleotide sequence selected from the group consisting of:

UAUGUGGGUGGG (SEQ ID NO: 1), UGUUUUG (SEQ ID NO: 2), ACCCCGUCUCU (SEQ ID NO: 5), AGCUGCA (SEQ ID NO: 7), AAACAAUGGAAUG (SEQ ID NO: 8), GGUAGGUGGGUGGG (SEQ ID NO: 10), CUGCUUGAU (SEQ ID NO: 12), UCCUUUCCA (SEQ ID NO: 13), UUUUUCUUU (SEQ ID NO: 14), UUCUGAUGUUU (SEQ ID NO: 15), UCUUCCUCUAU (SEQ ID NO: 16), UGGUAGCUGAA (SEQ ID NO: 17), CUUUGGUUCCU (SEQ ID NO: 18), CUACUAAUGCU (SEQ ID NO: 19), UCCUGCUUGAU (SEQ ID NO: 20), AUUCUUUAGUU (SEQ ID NO: 21), CCAUCUUCCUG (SEQ ID NO: 22), CCUCCAAUUCC (SEQ ID NO: 23), CUAAUACUGUA (SEQ ID NO: 24), UUCUGUUAGUG (SEQ ID NO: 25),

GCUGCUUGAUG (SEQ ID NO: 26), ACAUUGUACUG (SEQ ID NO: 27), UGAUAUUUCUC (SEQ ID NO: 28), AACAGCAGUUG (SEQ ID NO: 29), GUGCUGAUAUU (SEQ ID NO: 30), CCCAUCUCCAC (SEQ ID NO: 31), UAUUGGUAUUA (SEQ ID NO: 32), CAAAUUGUUCU (SEQ ID NO: 33), UACUAUUAAAC (SEQ ID NO: 34), GCCUAUCAUAU (SEQ ID NO:58), UGGUGCCUGCU (SEQ ID NO: 59), AAUUAAUAUGGC (SEQ ID NO: 60), CCCUCUGGGCU (SEQ ID NO: 61), UUCUUCCUCAU (SEQ ID NO: 62), UAUUUAUACAGA (SEQ ID NO: 63), CACCAAAAUUC (SEQ ID NO: 64), UGAGUNNGAACAUU (SEQ ID NO: 72) where N is any base, CUCCAGG (SEQ ID NO: 74), UCAGUGGG (SEQ ID NO: 76), UCCUCACAGGG (SEQ ID NO: 78), GUGCUCAUGGUG (SEQ ID NO: 79), CCUGGAGCCCUG (SEQ ID NO: 80), UCUCAGCUCCAC (SEQ ID NO: 81), ACCCUCCGCACC (SEQ ID NO: 86), GUGUUGAAG (SEQ ID NO: 88), UUCCACAAC (SEQ ID NO: 90), UCCACUGUC (SEQ ID NO: 92), CAGAAUAG (SEQ ID NO: 93), AACUCUCUA (SEQ ID NO: 94) and CGUGAAGAC (SEQ ID NO: 98).

33. The multitargeting interfering RNA molecule of any one of claims 1 to 13, wherein S consists essentially of a nucleotide sequence selected from the group consisting of: UAUGUGGGUGGG (SEQ ID NO: 1), UCCUCACAGGG (SEQ ID NO: 78), GUGUUGAAG (SEQ ID NO: 88), UUCCACAAC (SEQ ID NO: 90), AACUCUCUA (SEQ ID NO: 94) and CGUGAAGAC (SEQ ID NO: 98).

34. The multitargeting interfering RNA molecule of any one of claims 1 to 13, wherein S consists essentially of a nucleotide sequence of 6 or more contiguous bases contained within any of the sequences selected from the group consisting of: UAUGUGGGUGGG (SEQ ID NO: 1), UCCUCACAGGG (SEQ ID NO: 78), GUGUUGAAG (SEQ ID NO: 88), UUCCACAAC (SEQ ID NO: 90), AACUCUCUA (SEQ ID NO: 94) and CGUGAAGAC (SEQ ID NO: 98).

35. The multitargeting interfering RNA molecule of claim 8, consisting essentially of:

5' UUCCUCACAGGGCAGUGAUUC 3' (SEQ ID NO: 122)
3'UUAAAGAGUGUCCCGUCACUA 5', (SEQ ID NO: 124)

5' UACAAAUCUACUUCAACAUUU 3' (SEQ ID NO: 131)
3'GUAUGUUUAGAUGAAGUUGUG 5', (SEQ ID NO: 132)

5' AACAUAUGUUCUUCAACAUUU 3' (SEQ ID NO: 133)

3'GUUUGUAUACAAGAAGUUGUC 5', (SEQ ID NO: 134)

36. The multitargeting interfering RNA molecule of claim 9, consisting essentially of:

5' UAUGUGGGUGGGUGAGUCUAA 3' (SEQ ID NO: 100)
3'UUAUACACCCACCCACUCAGA 5', (SEQ ID NO: 101)

5' UGUUUUGUUGUUACAUAUGAC 3' (SEQ ID NO: 102)
3'UUACAAAACAACAAUGUAUAC 5', (SEQ ID NO: 103)

5' UAUGUGGGUGGGGUGUCUCUA 3' (SEQ ID NO: 104)
3'UUAUACACCCACCCCACAGAG 5', (SEQ ID NO: 105)

5' UAUGUGGGUGGGGUGGUCUAA 3' (SEQ ID NO: 106)
3'UUAUACACCCACCCCACCAGA 5', (SEQ ID NO: 107)

5' UAUGUGGGUGGGGUGGUGUCU 3' (SEQ ID NO: 108)
3'UUAUACACCCACCCCACCACA 5', (SEQ ID NO: 109)

5' UAUGUGGGUGGGUGAGUGUCU 3' (SEQ ID NO: 110)
3'UUAUACACCCACCCACUCACA 5', (SEQ ID NO: 111)

5' CUCACCCACCCACAUACAUUU 3' (SEQ ID NO: 112)
3'CUGAGUGGGUGGGUGUAUGUA 5', (SEQ ID NO: 113)

5' UCACCCACCCACAUACAUAUU 3' (SEQ ID NO: 114)
3'UGAGUGGGUGGGUGUAUGUAU 5', (SEQ ID NO: 115)

5' UCACCCACCCACAUACAUUUU 3' (SEQ ID NO: 116)
3'UGAGUGGGUGGGUGUAUGUAA 5', (SEQ ID NO: 117)

5' UAUGUGGGUGGGUGAGUCUA 3' (SEQ ID NO: 118)
3'UAUACACCCACCCACUCAGA 5', (SEQ ID NO: 119)

5' GGGUUUACCAGGAACAUCGUU 3' (SEQ ID NO: 120)
3'UACCCAAAUGGUCCUUCUACC 5', (SEQ ID NO: 121)

5' UUCCUCACAGGGCAGUGAUUC 3' (SEQ ID NO: 122)
3'UUAAGGAGUGUCCCGUCACUA 5', (SEQ ID NO: 123)

5' UUCCUCACAGGGCAGUGGUUC 3' (SEQ ID NO: 125)
3'UUAAGGAGUGUCCCGUCACCA 5', (SEQ ID NO: 126)

5' CCCGGACCCUUAGAGAGUUUU 3' (SEQ ID NO: 127)
3'ACGGGCCUGGGAAUCUCUCAA 5', (SEQ ID NO: 128)

5' UACCCUCGCACCGAUCUCCCAA 3' (SEQ ID NO: 129)
3'UUAUGGGAGCGUGGCUAGAGGG 5', (SEQ ID NO: 130)

5′ UUCCACAACACAAGCUGUGUU 3′ (SEQ ID NO: 135)
3′UUAAGGUGUUGUGUUCGACAC 5′, (SEQ ID NO: 136)

5′ GGACCCUUAGAGAGUUUCAUU 3′ (SEQ ID NO: 137)
3′CCCCUGGGAAUCUCUCAAAGU 5′, (SEQ ID NO: 138)

5′ UUCGUGAAGACGGUGGGCCGA 3′ (SEQ ID NO: 139)
3′dTdTAAGCACUUCUGCCACCCGG 5′, (SEQ ID NO: 140)

or

5′ AGACUCACCCACCCAGAUAUU 3′ (SEQ ID NO: 141)
3′AAUCUGAGUGGGUGGGUCUAU 5′ (SEQ ID NO: 142)

37. The multitargeting interfering RNA molecule of claim 9, consisting essentially of:

5′ UAUGUGGGUGGGUGAGUCUAA 3′ (SEQ ID NO: 100)
3′ UUAUACACCCACCCACUCAGA 5′, (SEQ ID NO: 101)

5′ GGACCCUUAGAGAGUUUCAUU 3′ (SEQ ID NO: 137)
3′ GGCCUGGGAAUCUCUCAAAGU 5′, (SEQ ID NO: 138)

or

5′ UUCGUGAAGACGGUGGGCCGA 3′ (SEQ ID NO: 139)
3′ dTdTAAGCACUUCUGCCACCCGG 5′, (SEQ ID NO: 140)

38. The multitargeting interfering RNA molecule of claim 37, comprising a least one modified ribonucleotide, universal base, acyclic nucleotide, abasic nucleotide and non-ribonucleotide, overhang variation or a combination thereof.

39. A biological system comprising a multitargeting interfering RNA molecule of any one of claims 1 to 37.

40. The biological system of claim 39 being a virus, a microbe, a cell, a plant, or an animal.

41. A vector comprising a nucleotide sequence that encodes the multitargeting interfering RNA molecule of any one of claims 1 to 37.

42. The vector of claim 41 being a viral vector.

43. The vector of claim 42 that is derived from a virus selected from the group consisting of an adeno-associated virus, a retrovirus, an adenovirus, a lentivirus, and an alphavirus.

44. A cell comprising the vector of claim 41 or claim 42.

45. The multitargeting interfering RNA molecule of any one of claims 1 to 37 produced from a short hairpin RNA molecule.

46. A vector for the short hairpin RNA molecule of claim 45.

47. A cell comprising the vector of claim 46.

48. A pharmaceutical composition comprising a multitargeting interfering RNA molecule of any one of claims 1 to 37 and an acceptable carrier.

49. A pharmaceutical composition comprising an expression vector of claim 41 and an acceptable carrier.

50. A pharmaceutical composition comprising an expression vector of claim 46 and an acceptable carrier.

51. A method of inducing RNA interference in a biological system, comprising the step of introducing a multitargeting interfering RNA molecule of any one of claims 1 to 37 into the biological system.

52. A method of inducing RNA interference in a biological system, comprising the steps of:

(a) selecting one or more target RNA molecules;

(b) designing a multitargeting interfering RNA molecule comprising a guide strand that can form stable interactions with at least two binding sequences present in distinct genetic contexts in the set of one or more target RNA molecules;

(c) producing the multitargeting interfering RNA molecule; and

(d) administering the multitargeting interfering RNA molecule into the biological system, whereby the guide strand of the multitargeting interfering RNA molecule forms stable interactions with the binding sequences present in

distinct genetic contexts in the target RNA molecules, and thus induces RNA interference of the target RNA molecules.

53. The method of claim 52, wherein the biological system is a virus, a microbe, a cell, a plant, or an animal.

54. The method of claim 52, wherein the biological system is an animal selected from the group consisting of a rat, a mouse, a dog, a cat, a pig, a monkey, and a human.

55. The method of any one of claims 52 to 54, wherein the one or more target RNA molecules comprises RNA molecules that are involved in a disease or disorder of the biological system.

56. The method of any one of claims 52 to 55, wherein one or more of the target RNA molecules comprises one or more RNA molecules selected from the biological system.

57. The method of any one of claims 52 to 56, wherein one or more of the target RNA molecules comprises one or more RNA molecules selected from a second biological system that is infectious to the biological system.

58. The method of any one of claims 52 to 57, wherein the target RNA molecules comprise one or more RNA molecules selected from the biological system and one or more target RNA molecules selected from a second biological system that is infectious to the biological system.

59. The method of any one of claims 52 to 58, wherein the target RNA molecules comprise one or more RNA molecules selected from an animal or a plant and one or more RNA molecules selected from a microbe or a virus that is infectious to the animal or the plant.

60. The method of any one of claims 52 to 59, wherein the target RNA molecules comprise one or more RNA molecules selected from a human and one or more RNA molecules selected from a virus selected from the group consisting of a human immunodeficiency virus (HIV), a hepatitis C virus (HCV), an influenza virus, a rhinovirus, and a severe acute respiratory syndrome (SARS) virus.

61. The method of any one of claims 52 to 60, wherein the one or more target RNA molecules comprise a RNA molecule encoding a protein of a class selected from the group consisting of receptors, cytokines, transcription factors, regulatory proteins, signaling proteins, cytoskeletal proteins, transporters, enzymes, hormones, and antigens.

62. The method of any one of claims 52 to 61, wherein the one or more target RNA molecules comprise a RNA molecule encoding a protein selected from the group consisting of ICAM-1, VEGF-A, MCP-1, IL-8, VEGF-B, IGF-1, Gluc6p, Inppl1, bFGF, PlGF, VEGF-C, VEGF-D, β-catenin, κ-ras-B, κ-ras-A, EGFR, Bcl-2, presenilin-1, BACE-1, MALAT-1, BIC, TGFβ, and TNF alpha.

63. The method of claim 62, wherein the one or more target RNA molecules encode ICAM-1 and VEGF-A.

64. The method of any one of claims 52 to 63, wherein the one or more target RNA molecules comprises a viral RNA molecule.

65. The method of any one of claims 52 to 64, wherein the one or more target RNA molecules comprises a virus selected from the group consisting of a human immunodeficiency virus (HIV), a hepatitis C virus (HCV), an influenza virus, a rhinovirus, and a severe acute respiratory syndrome (SARS) virus.

66. The method of claim 65, wherein the one or more target RNA molecules comprise a RNA molecule encoding an essential protein for HIV selected from the group consisting of GAG, POL, VIF, VPR, TAT, NEF, REV, VPU and ENV.

67. The method of claim 59, wherein the target RNA molecules comprises a RNA molecule encoding a human protein TNFalpha, LEDGF(p75), BAF, CCR5, CXCR4, furin, NFkB, STAT1.

68. The method of claim 59, wherein one or more of the preselected RNA molecules comprises Hepatitis C Virus (HCV) and one or more of the preselected RNA molecules encodes TNFα.

69. A method of treating a disease or condition in a subject, the method comprising the steps of:

(a) selecting one or more target RNA molecules, wherein the modulation in expression of the target RNA molecules is potentially therapeutic for the treatment of the disease or condition;

(b) designing a multitargeting interfering RNA molecule comprising a guide strand that can form stable interactions with at least two binding sequences present in distinct genetic contexts in the one or more target RNA molecules;

(c) producing the multitargeting interfering RNA molecule;

(d) administering the multitargeting interfering RNA molecule into the subject, whereby the guide strand of the multitargeting interfering RNA molecule forms stable interactions with the binding sequences present in distinct genetic contexts in the one or more target RNA molecules, and thus induces RNA interference and modulation of expression of the target RNA molecules.

70. A method for designing a multitargeting interfering RNA molecule, comprising the steps of:

a) selecting one or more target RNA molecules, wherein the modulation in expression of the target RNA molecules is desired;

b) obtaining at least one nucleotide sequence for each of the target RNA molecules;

c) selecting a seed sequence of 6 nucleotides or more, said seed sequence occurs in at least two distinct genetic contexts in nucleotide sequences obtained in b) for the target RNA molecules;

d) selecting at least two binding sequences, wherein each of binding sequences comprises the seed sequence, and the binding sequences are present in distinct genetic contexts in the target molecules; and

e) designing a multitargeting interfering RNA molecule having a guide strand that shares a substantial degree of complementarity with each of the at least two binding sequences to allow stable interaction therewith.

71. The method of claim 70 comprising designing a passenger strand that is at least partially complementary to the guide strand to allow formation of a stable duplex between the passenger strand and the guide strand.

72. A method for designing a multitargeting interfering RNA molecule, comprising the steps of:

a) selecting one or more target RNA molecules, wherein the modulation in expression of the target RNA molecules is desired;

b) obtaining at least one nucleotide sequence for each of the target RNA molecules;

c) selecting a length, n, in nucleotides, for a seed sequence; wherein n = about 6 or more;

d) generating a collection of candidate seed sequences of the length n from each nucleotide sequences obtained in step b), wherein each candidate seed sequence occurs at least once in nucleotide sequences obtained in step b);

e) determining the genetic context of each of the candidate seed sequences in each nucleotide sequence obtained in step b), by collecting, for each occurrence of the candidate seed sequence, a desired amount of the 5' and 3' flanking sequence;

f) selecting a seed sequence of the length n from the candidate seed sequences, wherein the seed sequence occurs at least in two distinct genetic contexts in nucleotide sequences obtained in step b);

g) selecting a consensus target sequence, wherein said consensus target sequence comprises the seed sequence and a desired consensus sequence for the sequence flanking either one or both of the 5' and 3' ends of the seed; and

h) designing a multitargeting interfering RNA molecule comprising a guide strand that shares a substantial degree of complementarity with the consensus target sequence to allow stable interaction therewith.

73. The method of claim 72 wherein the step of generating a collection of candidate seed sequences comprises the steps of beginning at a terminus, sequentially observing the nucleotide sequence using a window size of n and stepping along the nucleotide sequence with a step size of 1.

74. The method of claim 72 or claim 73, wherein the step of selecting seed sequences comprises the step of discarding any sequence of the length *n* that:

i) is composed of a consecutive string of 5 or more identical single nucleotides;

ii) is composed of only adenosine and uracil;

iii) is predicted to occur with an unacceptably high frequency in the non-target transcriptome of interest;

iv) is predicted to have a propensity to undesirably modulate the expression or activity of one or more cellular component; or

v) is any combination of i) to iv).

75. The method of any one of claims 72 to 74, wherein the step of selecting a consensus target sequence comprises the step of discarding any sequence that is composed of only a single base, is composed only of A and U, has a consecutive string of 5 or more bases which are C, is G/C rich at the 3' end, is predicted to occur with unacceptable frequency in the non-target transcriptome of interest; or any combination thereof.

76. The method of any one of claims 72 to 75 comprising designing a passenger strand that is at least partially complementary to the guide strand to allow formation of a stable duplex between the passenger strand and the guide strand.

77. The method of any one of claims 72 to 76, further comprising the step of modifying the multitargeting interfering RNA molecule,

i) to improve the incorporation of the guide strand of the multitargeting interfering RNA molecule into the RNA induced silencing complex (RISC);

ii) to increase or decrease the modulation of the expression of at least one target RNA molecule;

iii) to decrease stress or inflammatory response when the multitargeting interfering RNA molecule is administered into a subject;

iv) to alter half life in an expression system; or

v) any combination of i) to iv).

78. The method of claim 72, further comprising repeating the steps c) to g) of claim 72 with a new value of *n*.

79. The method of claim 72, further comprising the steps of making the designed multitargeting interfering RNA molecule and testing it in a suitable expression system.

80. A method of designing a full length multitargeting interfering RNA from a seed sequence, comprising the steps of:

a. deducing the sequence of the complete complement of the seed sequence;
b. generating permutations for the extension of the complete complement of the seed sequence to the desired length *n*;
c. creating a collection of putative guide strand sequences, each of which comprises the sequence of the complete complement of the seed sequence and one of the permutations generated in step b);
d. using RNAhybrid to determine the binding pattern and the minimum free energy (mfe) of the putative guide strand sequences created in step c) against all the target sequences comprising the seed sequence;
e. discarding putative guide strand sequences where
    i. there is a contiguous run of 5 or more G residues; and
    ii. the Load Bias is < 1.2; and
f. selecting a guide strand sequence of the length *n* for a multitargeting interfering RNA sequence from the list of the remaining putative guide strand sequences based on their Relative Activity Score.

81. The method of claim 80, wherein the desired length *n* is 21 bases.

82. The method of claim 80 or claim 81 further comprising the steps of producing the multitargeting interfering RNA comprising the guide strand sequence and testing the multitargeting interfering RNA in an expression system.

83. A method of making a multitargeting interfering RNA molecule, comprising the steps of:

i) designing a multitargeting interfering RNA molecule having a guide strand that can form stable interactions with at least two binding sequences present in distinct genetic contexts in a set of pre-selected target RNA molecules; and

ii) producing the multitargeting interfering RNA molecule.

84. A method for making a pharmaceutical composition comprising the step of mixing a multitargeting interfering RNA molecule of any one of claims 1 to 37and a pharmaceutically acceptable carrier.

85. A multitargeting interfering RNA molecule comprising a guide strand that forms stable interactions with at least two binding sequences present in distinct genetic contexts in one or more pre-selected target RNA molecules.

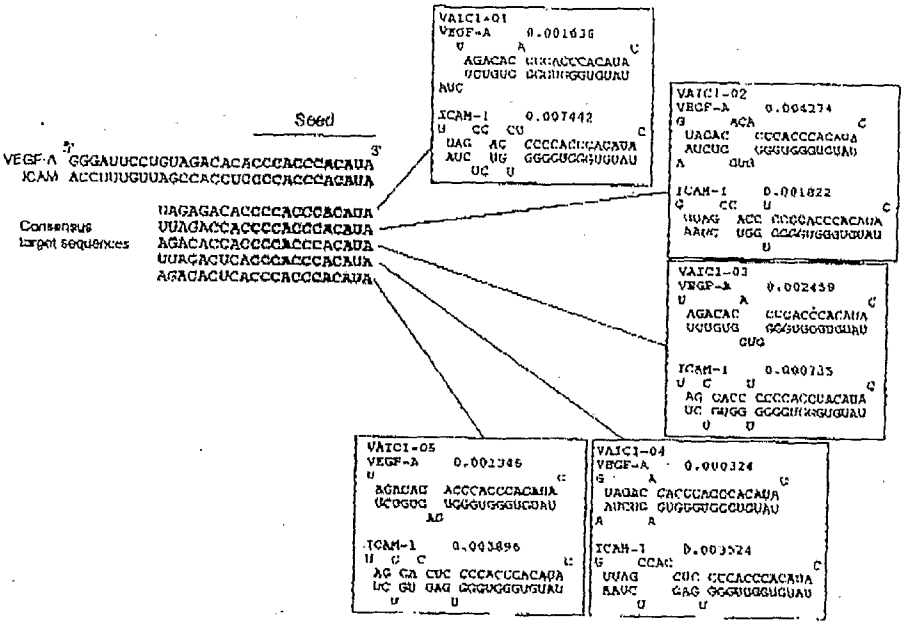
Seed
5'
3'
VEGF-A GGGAUUCCUGUAGACACACCCACCCACAUA
ICAM ACCUUUGUUAGCCACCUGCCCACCCAGAUA
Consensus
target sequences
UAGAGACACCCCACCCACAUA
UUAGACCACCCCACCCACAUA
AGACACCACCCCACCCACAUA
UUAGACUCACCCACCCACAUA
AGACACUCACCCACCCACAUA
VAIC1-01
VEGF-A 0.001636
ICAM-1 0.007442
VAIC1-02
VEGF-A 0.004274
ICAM-1 0.001822
VAIC1-03
VEGF-A 0.002439
ICAM-1 0.000735
VAIC1-05
VEGF-A 0.001346
ICAM-1 0.003896
VAIC1-04
VEGF-A 0.000324
ICAM-1 0.000524

Figure 1

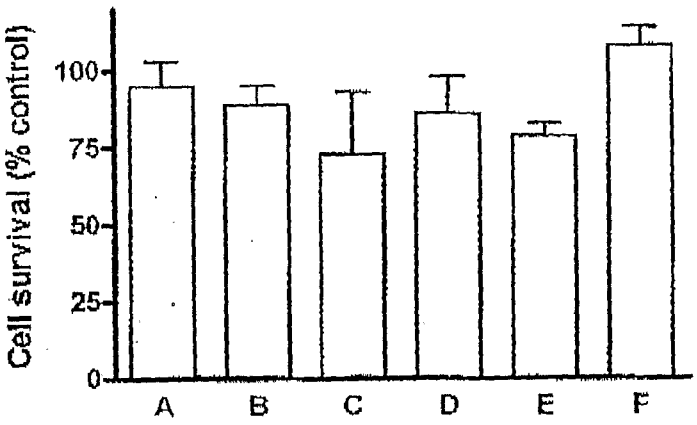

Figure 2

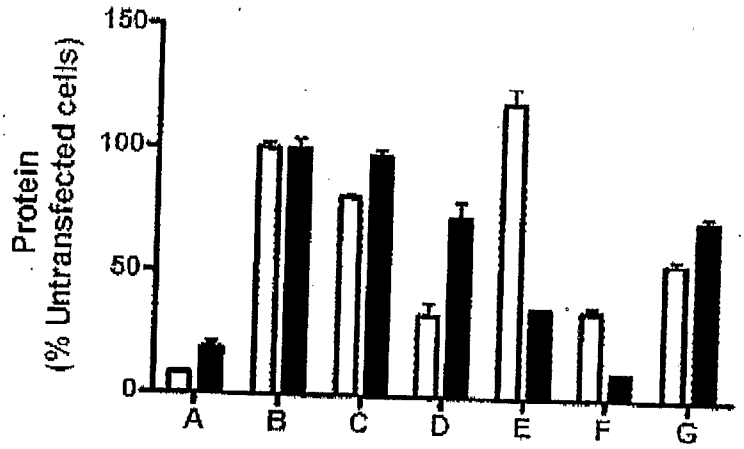

Figure 3

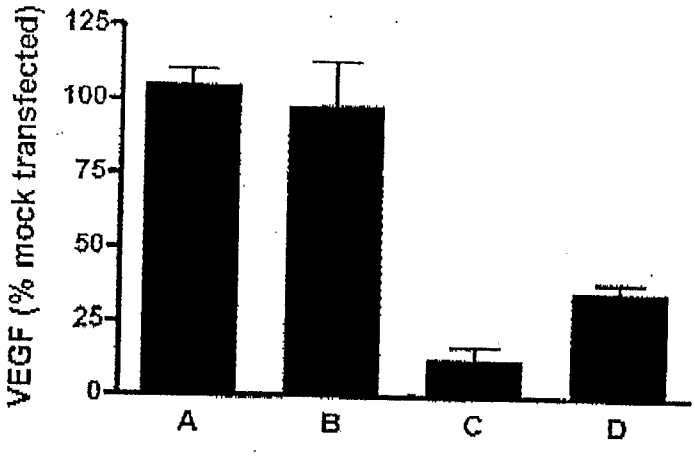

Figure 4

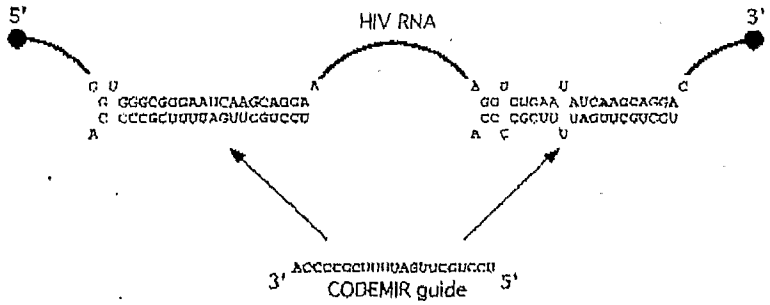

Figure 5

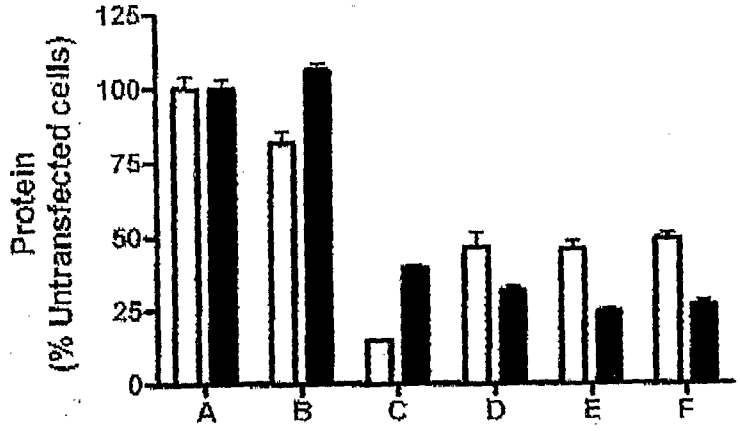

Figure 6

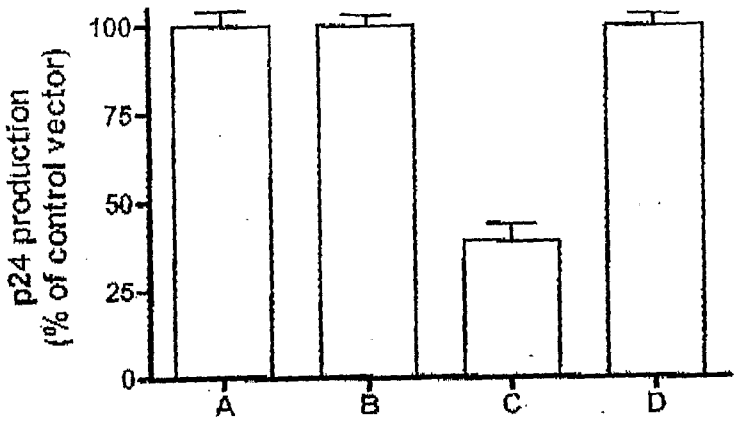

Figure 7

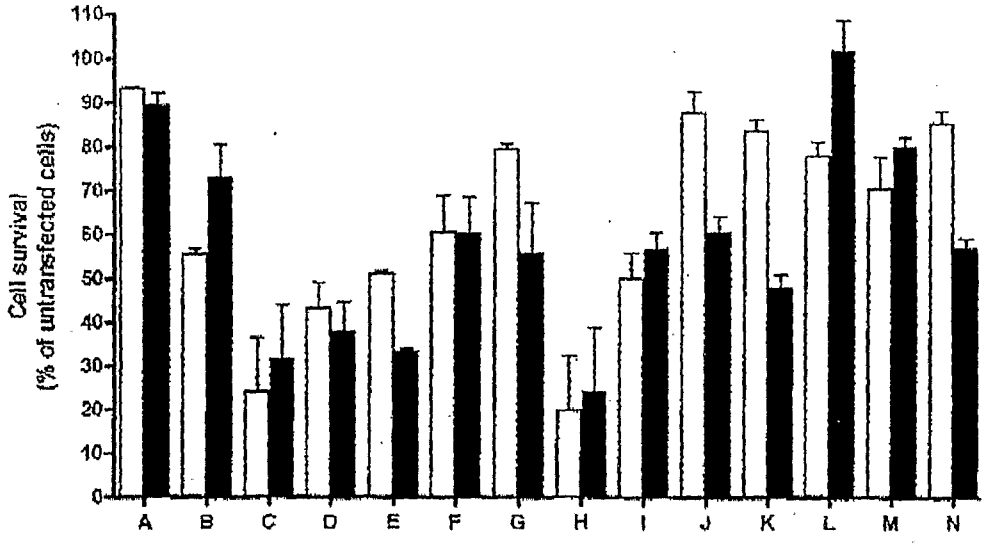
Cell survival
(% of untransfected cells)
110
100
90
80
70
60
50
40
30
20
10
0
A
B
C
D
E
F
G
H
I
J
K
L
M
N

Figure 8

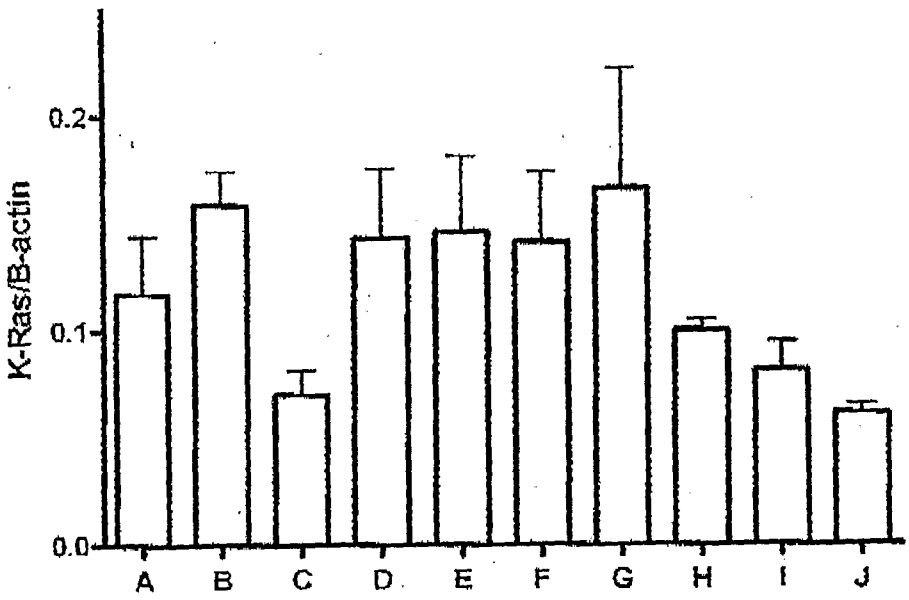

Figure 9

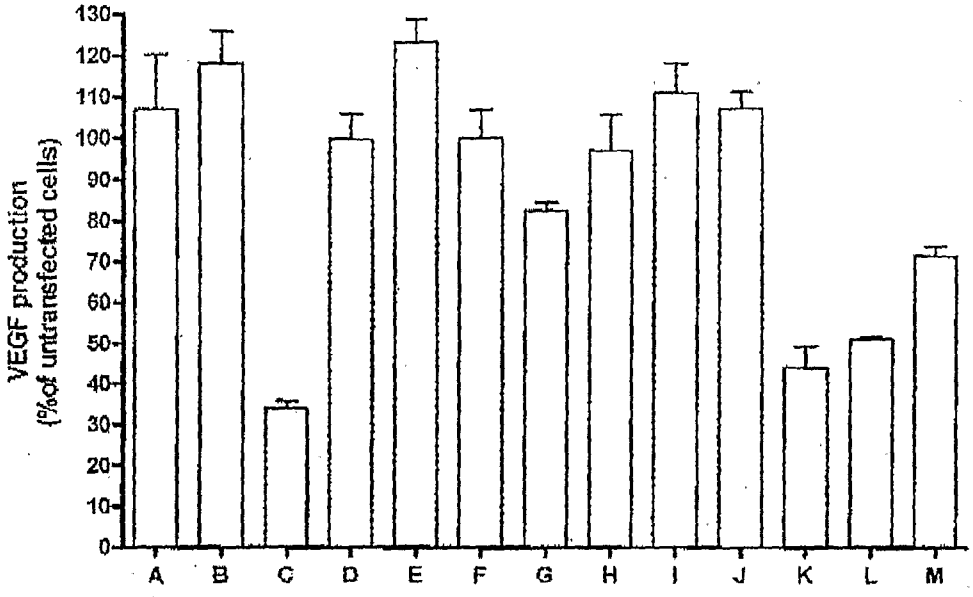
VEGF production
(%of untransfected cells)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
A
B
C
D
E
F
G
H
I
J
K
L
M

Figure 10

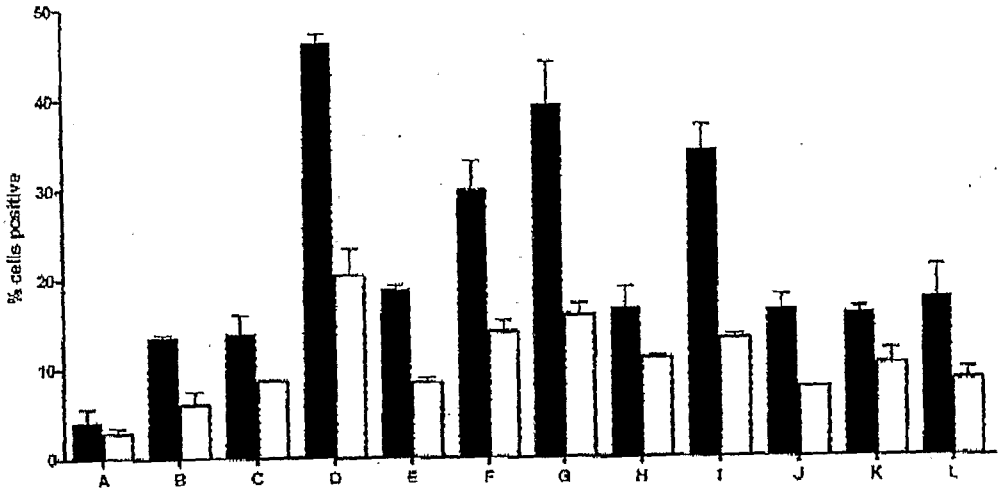

Figure 11

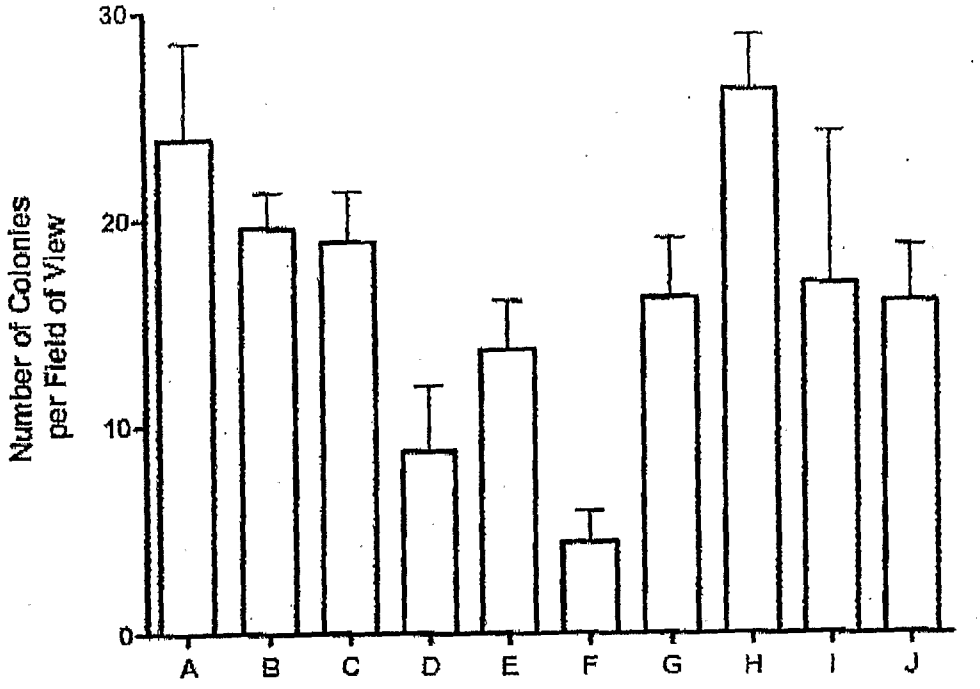

Figure 12

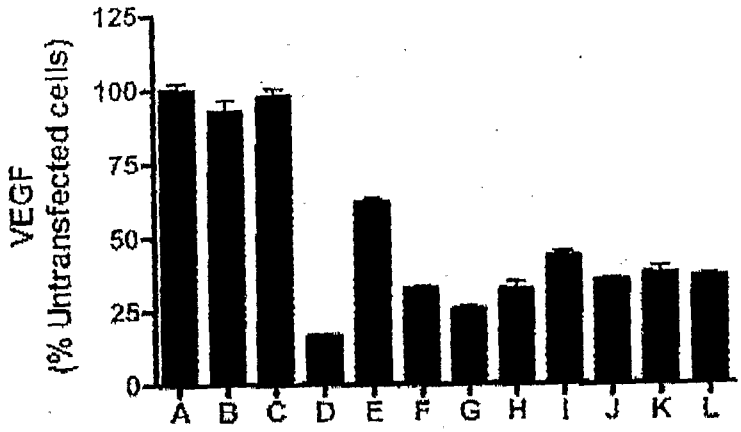

Figure 13

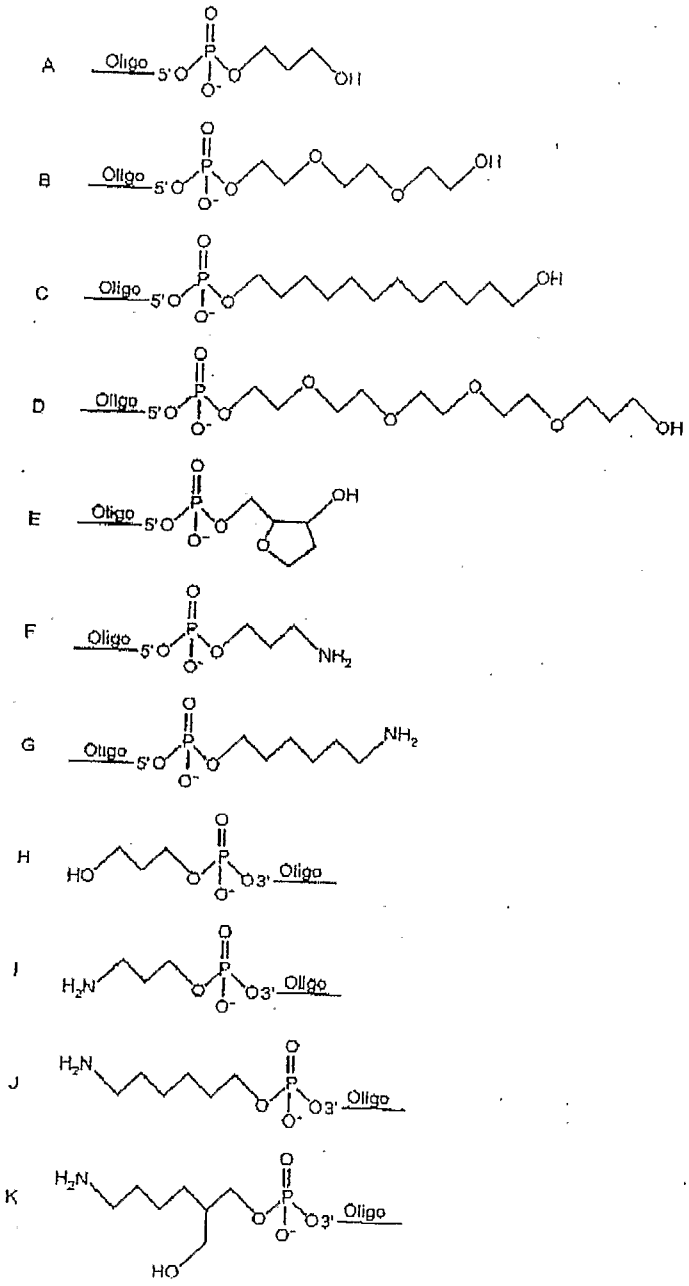
A
Oligo
5'
OH
B
Oligo
5'
OH
C
Oligo
5'
OH
D
Oligo
5'
OH
E
Oligo
5'
OH
F
Oligo
5'
NH2
G
Oligo
5'
NH2
H
HO
3'
Oligo
I
H2N
3'
Oligo
J
H2N
3'
Oligo
K
H2N
3'
Oligo
HO

Figure 14

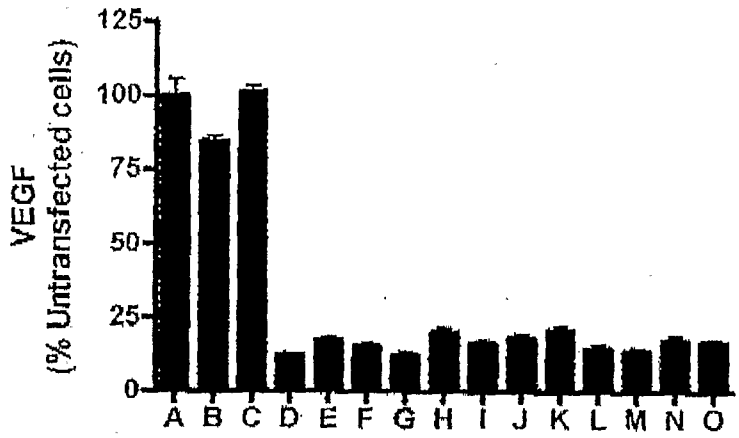
VEGF
(% Untransfected cells)
125
100
75
50
25
0
A B C D E F G H I J K L M N O

Figure 15

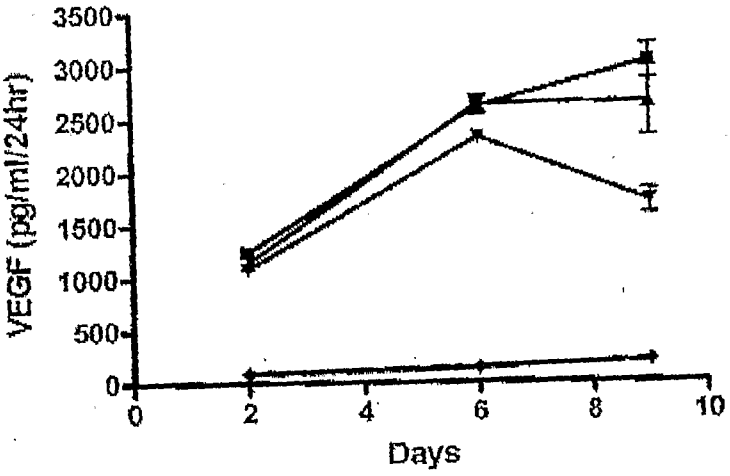
3500
3000
2500
2000
1500
1000
500
0
VEGF (pg/ml/24hr)
0
2
4
6
8
10
Days

Figure 16

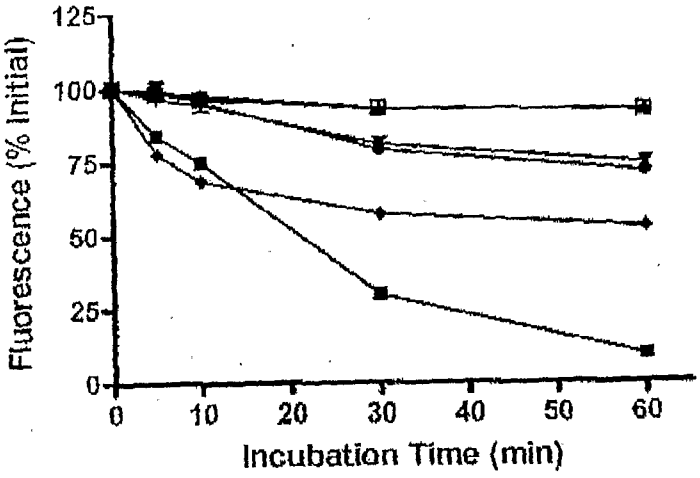
125
100
75
50
25
0
Fluorescence (% Initial)
0
10
20
30
40
50
60
Incubation Time (min)

Figure 17

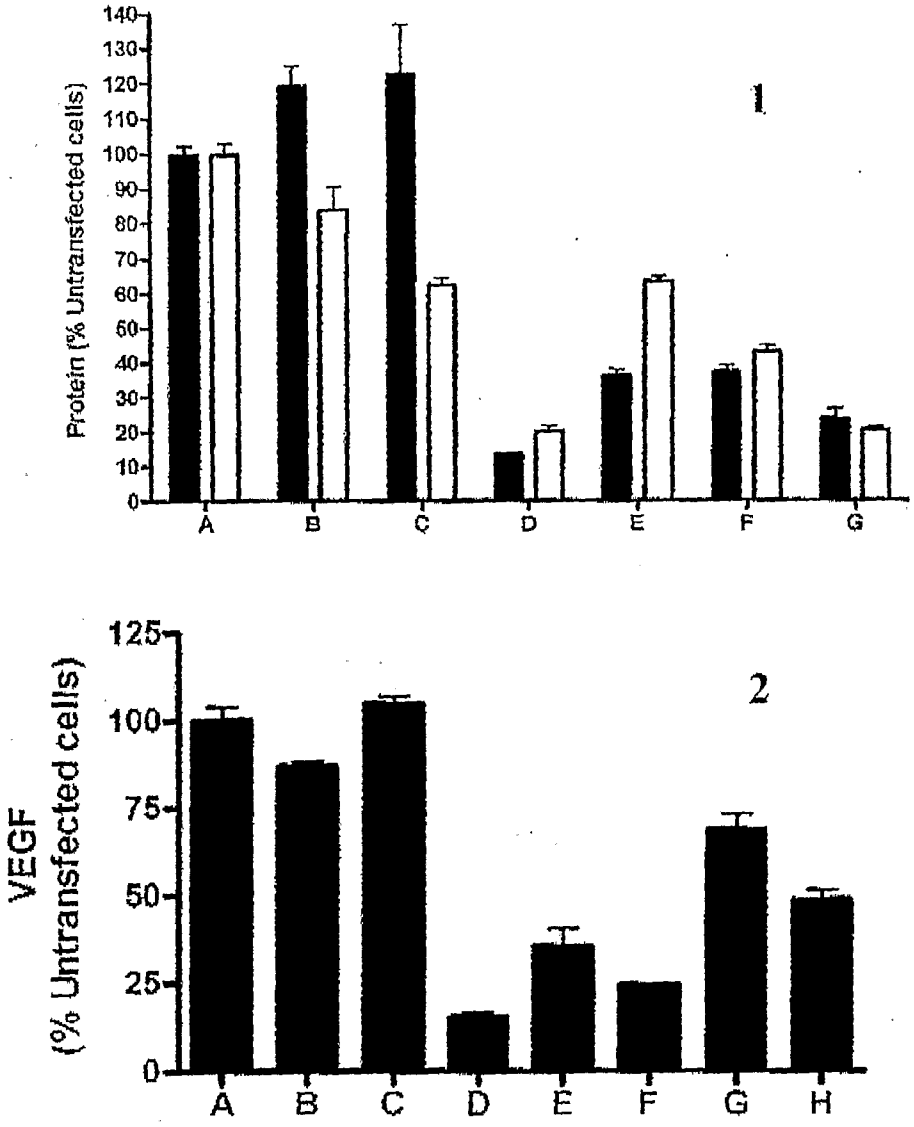
1
Protein (% Untransfected cells)
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
A
B
C
D
E
F
G
2
VEGF
(% Untransfected cells)
125
100
75
50
25
0
A
B
C
D
E
F
G
H

Figure 18

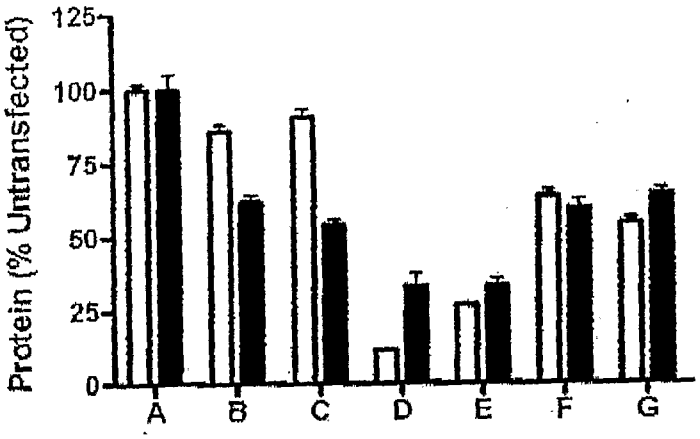

Figure 19

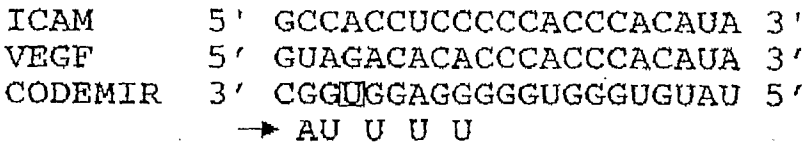
ICAM 5' GCCACCUCCCCCACCCACAUA 3'
VEGF 5' GUAGACACACCCACCCACAUA 3'
CODEMIR 3' CGGUGGAGGGGGUGGGUGUAU 5'
AU U U U

Figure 20

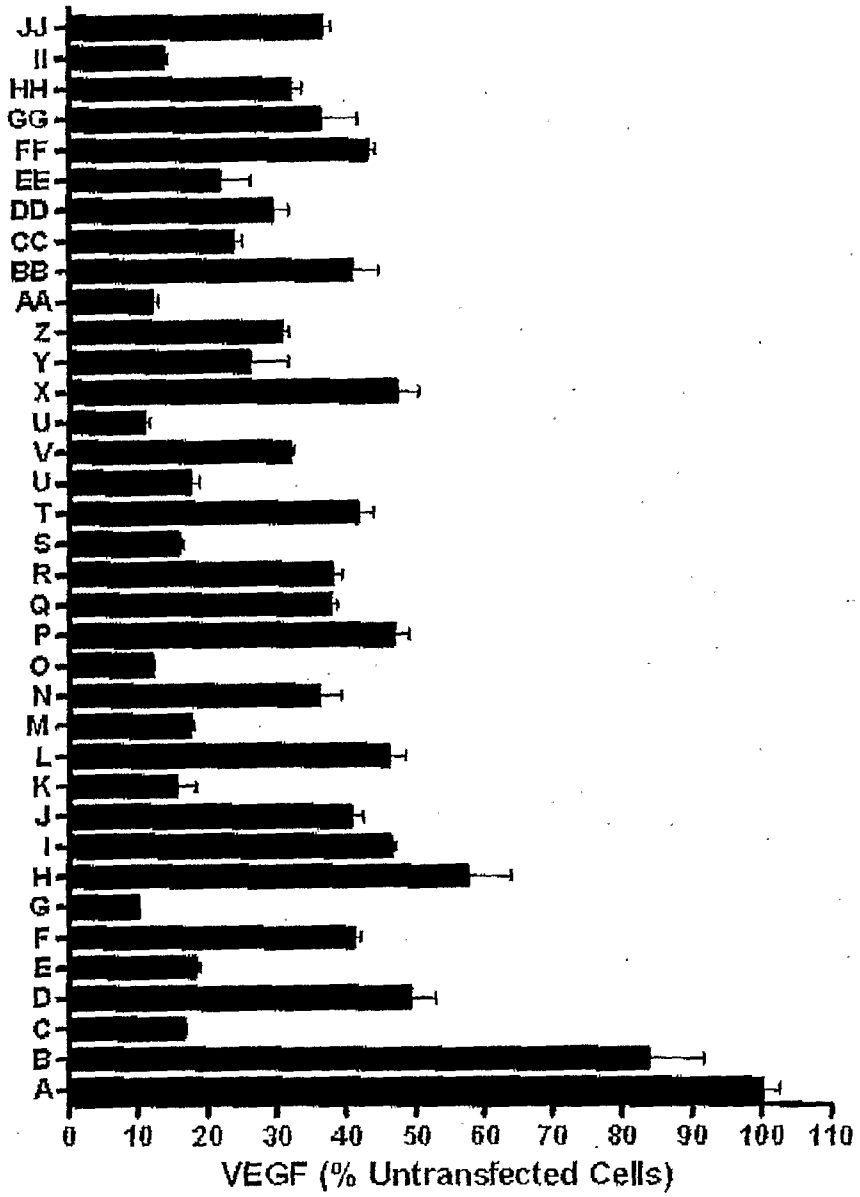
JJ
II
HH
GG
FF
EE
DD
CC
BB
AA
Z
Y
X
U
V
U
T
S
R
Q
P
O
N
M
L
K
J
I
H
G
F
E
D
C
B
A
0 10 20 30 40 50 60 70 80 90 100 110
VEGF (% Untransfected Cells)

Figure 21

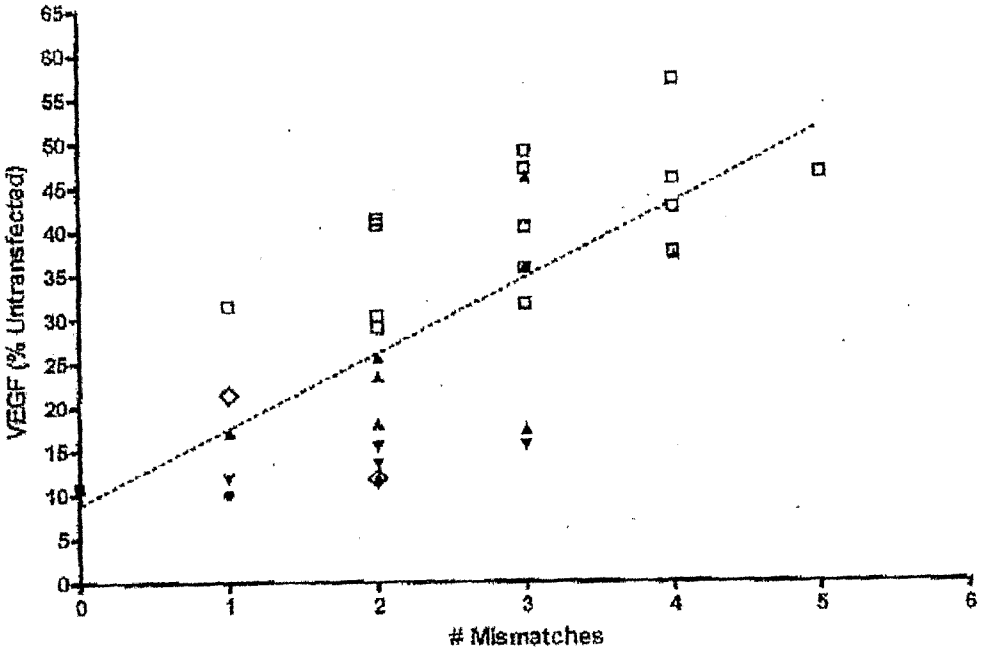
VEGF (% Untransfected)
65
60
55
50
45
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
Mismatches

Figure 22

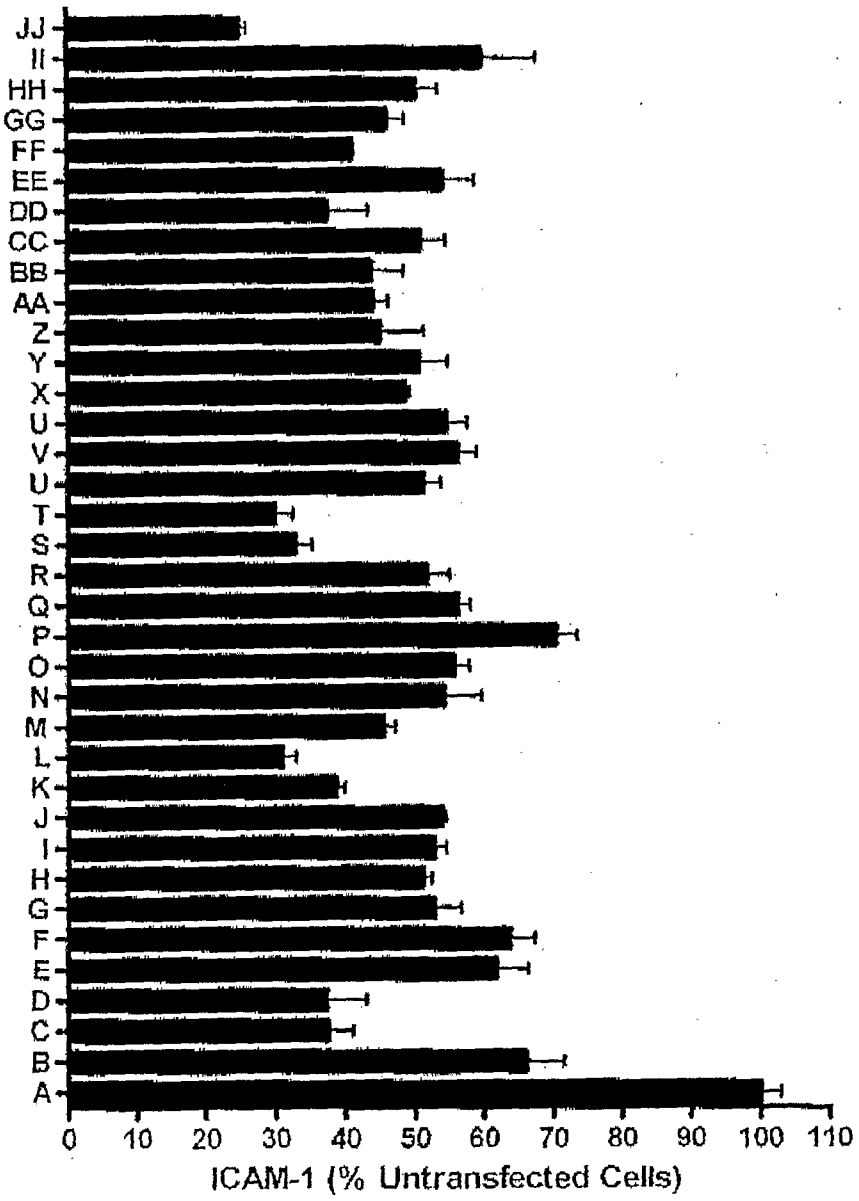
JJ
II
HH
GG
FF
EE
DD
CC
BB
AA
Z
Y
X
U
V
U
T
S
R
Q
P
O
N
M
L
K
J
I
H
G
F
E
D
C
B
A
0
10
20
30
40
50
60
70
80
90
100
110
ICAM-1 (% Untransfected Cells)

Figure 23

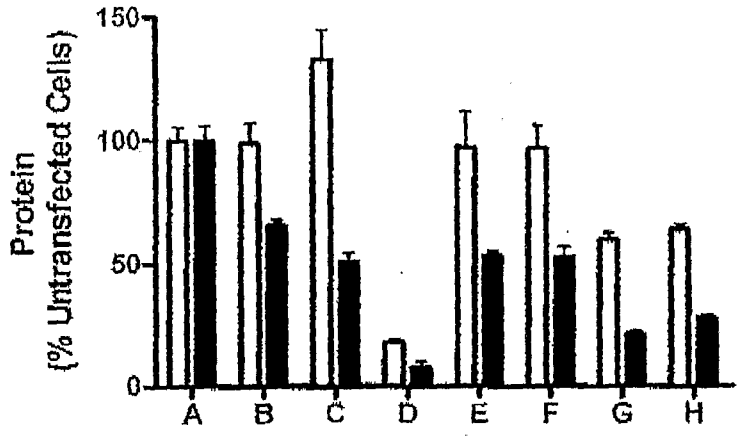

Figure 24

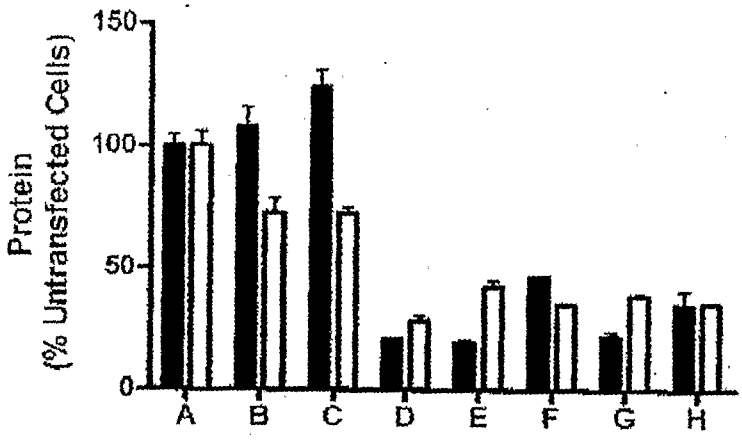

Figure 25

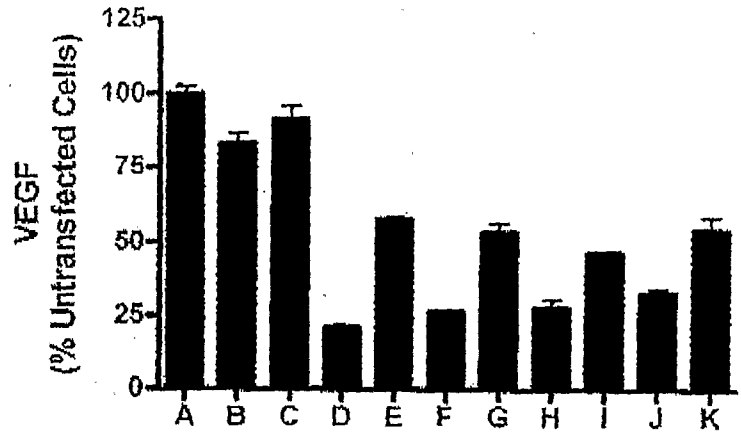

Figure 26

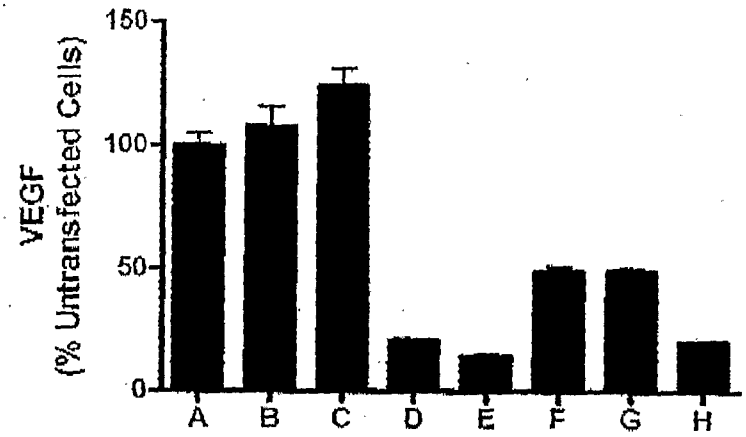

Figure 27

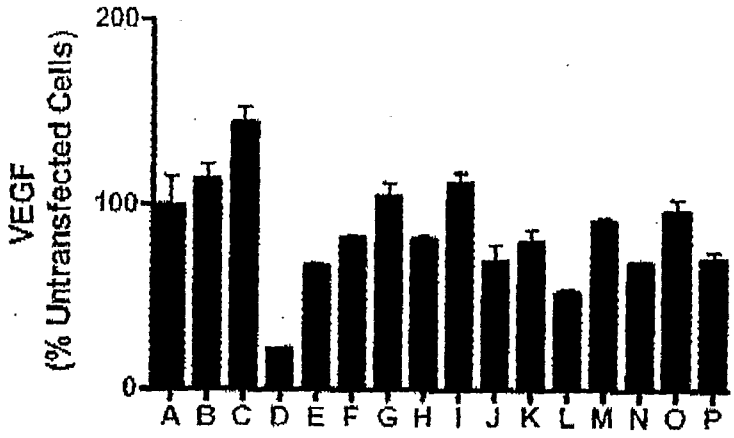

Figure 28

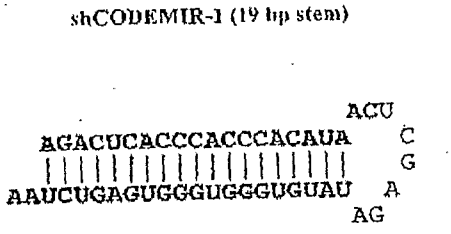
shCODEMIR-1 (19 bp stem)
ACU
AGACUCACCCACCCACAUA
C
G
AAUCUGAGUGGGUGGGUGUAU
A
AG

Figure 29

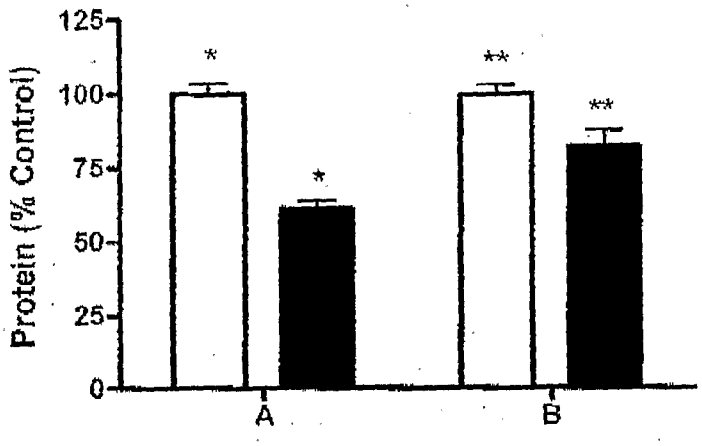

Figure 30

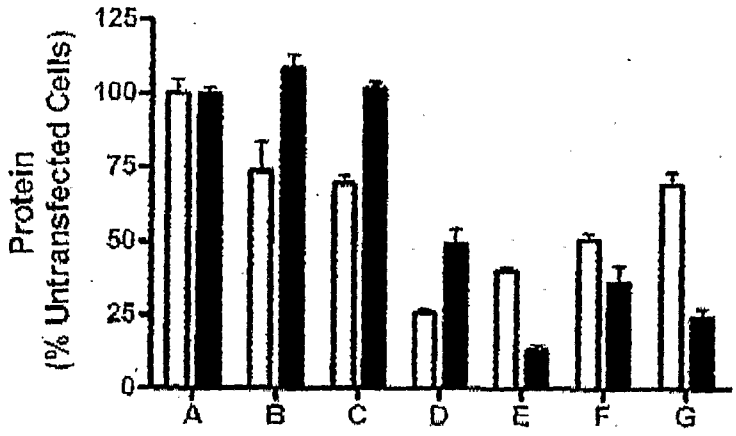

Figure 31

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<170> PatentIn version 3.3

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13

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uccuuucca

9

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uuuuucuuu 9

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cuaauacugu a 11

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11

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<400> 30
gugcugauau u
11

<210> 31
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<400> 31
cccaucucca c
11

<210> 32
<211> 11
<212> RNA
<213> Synthetic

<400> 32
uauugguauu a
11

<210> 33
<211> 11
<212> RNA
<213> Synthetic

<400> 33
caaauuguuc u
11

<210> 34
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11

<210> 35
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<400> 36
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9

<210> 37
<211> 9
<212> RNA
<213> Synthetic

<400> 37
aaagaaaaa 9

<210> 38
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aaacaucaga a 11

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2006/001741

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C07H 21/02 (2006.01) ***C12N 15/00*** (2006.01) A61K 31/7105 A61K 31/7115 A61K 31/712 A61K 31/713

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE CA WPIDS BIOSIS. KEYWORDS: MULTIVALENT, MULTITARGET, BISPECIFIC, KINETIC, SIRNA, INTERFER?, RNA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ANDERSON, J. et al. *Bispecific Short Hairpin siRNA Constructs Targeted to CD4, CXCR4, and CCR5 Confer HIV-1 Resistance.* Oligonucleotides. 2003, vol. 13, pages 303-312. See page 305 Figure 1(B).	1-38, 45, 51, 85
A	LEIRDAL, M. et al. *Gene silencing in mammalian cells by preformed small RNA duplexes.* Biochemical and Biophysical Research Communications. 2002, vol. 295, pages 744-748. See page 745 figure 1.	1-38, 45, 51, 85
A	WO/2003/070912 A2 (Ribozyme Pharmaceuticals, Inc.) 28 August 2003. page 8 lines 26-28, page 11 lines 11-14	1-51

☐ Further documents are listed in the continuation of Box C ☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 February 2007	15 MAR 2007
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer IAN DOWD Telephone No : (02) 6283 2273

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2006/001741

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: **1-85 (all in part)**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
The ISA considers that claims 1-85 encompass such a broad range of subject matter all based on multitargeting interfering RNA molecules, that a complete and meaningful search over the full scope of each claim is not possible on economic grounds. Therefore the search has been based on the purpose/functionality of the RNA molecules, namely, their multitargeting interfering properties.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2006/001741

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member					
WO 03070912	AU	10904/95	AU	10973/95	AU	14571/95
	AU	15665/95	AU	18214/95	AU	26422/95
	AU	36657/99	AU	38111/01	AU	38724/99
	AU	42229/93	AU	42489/93	AU	43454/01
	AU	44120/96	AU	47698/93	AU	48760/99
	AU	52096/98	AU	60255/98	AU	60315/98
	AU	65165/99	AU	67085/98	AU	68041/98
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WO	2006060598	WO	2006078798	WO	2006128141
WO	2007022369				

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX