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(54) Title: ANTISENSE OLIGONUCLEOTIDES THAT INHIBIT EXPRESSION OF HIF-1

(57) Abstract: New antisense oligonucleotide compounds, RX-0047 and RX-0149, inhibit expression of HIF-1 and also induce cytotoxicity in several cancer cell lines.

## Antisense Oligonucleotides that Inhibit Expression of HIF-1

### Field of the Invention

This invention relates to new antisense oligonucleotide compounds, RX-0047 and RX-0149, that inhibit expression of a human protein, HIF-1, and also induce cytotoxicity in several cancer cell lines.

### Background of the Invention

Tumors cannot grow without blood vessels that supply cancer cells with oxygen and nutrients (Blagosklonny, International J. Oncol., 2001 19: 257-262). Control of the hypoxic response in mammalian cells by the transcription factor, HIF-1 is one of the major regulators of cancer cell growth. HIF renders cells capable of surviving hypoxia and stimulating endothelial growth and it is upregulated in a broad range of cancers (Zhong et al., Cancer Res. 1999 59: 5830-5835). One of the most striking examples of the role of HIF in angiogenesis and tumor progression is the loss of function of the protein, VHL, which is a tumor suppressor gene that is mutated in most sporadic clear-cell renal carcinoma and in VHL disease. Therefore disruption of the HIF-1 pathway inhibits tumor growth, indicating HIF-1 as a potential anticancer target. Inhibition of HIF-1 is a mechanism-based anti-angiogenic strategy because it is the HIF-mediated response that drives tumor angiogenesis. United States Patent No., 6,222,018 issued to Semenza, April 24, 2001, relates to the nucleotide sequences encoding HIF-1. The specific oligonucleotides disclosed and claimed in the present invention were not disclosed in that patent.

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### Brief Description of the Drawings

Fig. 1. RX-0047 and RX-0149 inhibit HIF-1 mRNA expression in various cancer cells

Fig. 2. Western blot analysis of inhibition of HIF-1 protein expression by RX-0047 and RX-0149.

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### Summary of the Invention

The present invention is directed to antisense oligonucleotides, which are targeted to a nucleic acid encoding HIF-1 (hypoxia-inducible factor 1), and which modulate the expression of HIF-1. Also provided is a method of inhibiting expression of HIF-1 in cells comprising contacting the cells with the oligonucleotide compounds and compositions of the invention. An advantage of the presently described oligonucleotides is that, in addition to inhibiting expression of HIF-1, they have a cytotoxic effect on several different cancer cell lines. The advantages of the present invention can be obtained by contacting cells of various cancer cell lines with an antisense compound that is specifically hybridizable to a site on the HIF-1 gene having the following sequence: 5' ttggacactgggtggctcatt 3' at site 2,772 of HIF-1 gene (Genebank # NM001530) (Seq. Id. No. 1). Particularly preferred is RX-0047, comprising 5' aatgagccaccagtgtccaa 3' (Seq. Id. No. 2). Similar advantages can be obtained with a compound that is antisense to the sequence 5' gacttggagatgtagctcc 3' at site 1,936 of HIF-1 gene (Genebank # NM001530) (Seq. Id. No. 3). Particularly preferred is RX-0149, comprising, 5' ggagctaacatctccaagtc 3' (Seq. Id. No. 4). The contact occurs under conditions that allow the oligonucleotide to hybridize with the gene encoding HIF-1. After hybridization, the ability of the cells to produce HIF-1 is inhibited, and cancer cell viability is reduced.

### Detailed Description of the Invention

HIF (hypoxia-inducible factor) is a heterodimeric key transcription factor and activated by hypoxia. It not only mediates homeostatic responses including erythropoiesis, angiogenesis and glycolysis to hypoxia in mammals, but also facilitates tumor neovascularization and growth. It is composed of 2 different subunits, HIF-1 $\alpha$  and HIF-1 $\beta$  which is also known as ARNT (Aryl Receptor Nuclear Translocator). These 2 subunits belong to the bHLH (basic helix-loop-helix) and PAS (Per, ARNT, Sim) family. The bHLH domain of these factors is responsible for dimerization through the two helices and for DNA binding through their basic domain. Two separate domains within HIF-1 $\alpha$  and HIF-2 $\alpha$  respond to hypoxia signaling pathways. The first is the oxygen-dependent degradation (ODD), which, at normoxia, is subject to post-translational modification by an

oxygen-dependent prolyl hydroxylase (Jaakkola et al., Science, 2001, 292: 468-472). The hydroxylated proline promotes interaction of HIF with the VHL (von Hippel-Lindau) ubiquitin ligase complex, initiating rapid ubiquitination and subsequent HIF protein destruction via proteasome. Recent report indicated that asparagine hydroxylation of the HIF COOH-terminal transactivation domain (CAD) also plays an important role in proteasome-mediated HIF degradation by inhibiting interaction with the p300/CBP coactivator and reduces the transcriptional activity of HIF-1 during normoxia (Lando et al., Science, 2002, 295: 858-861). Therefore in conjunction with prolyl hydroxylase, asparagine hydroxylase works as a hypoxic switch regulated by O<sub>2</sub> level in the cell. During hypoxia, p42/p44 MAPK activity induces post-translational phosphorylation of HIF-1 and promotes the transcriptional activity of HIF-1. A recent report showed that along with the hydroxylation, ubiquitination and phosphorylation, acetylation plays an important role in regulation of HIF-1 stability (Jeong et al., Cell, 2002, 111: 709-720).

Under hypoxia, HIF-1 $\alpha$  is not hydroxylated because the hydroxylase, which requires dioxygen for activity, is inactive and thus HIF-1 $\alpha$  is not recognized by pVHL and accumulates in the cell. HIF-1 $\alpha$  then translocates to the nucleus and dimerizes with the constitutively present HIF-1 $\beta$  subunit (Semenza, Genes & Development, 1985, 14: 1983-1991). The dimer then binds to the hypoxia responsive element (HRE) in target genes, resulting in their transactivation of genes such as erythropoitin, VEGF (Forsythe et al., Mol. Cell. Biol., 1996, 16: 4604-4613), platelet-derived growth factor- $\beta$  (PDGF- $\beta$ ), glucose transporter (GLUT1) and nitrous oxide synthetase (Neckers, J. Natl. Cancer Ins., 1999, 91: 106-107). Certain hormones and growth factors also lead to increased levels of HIF-1 $\alpha$  as well as mutations in certain oncogenes and tumor-suppressor genes, VHL for example, result in an increase in HIF-1 $\alpha$  level (Ivan and Kaelin, Current Opinion in Genetics & Development, 2001, 11: 27-34). It will be interesting to determine whether hydroxylation or alternative mechanisms are involved in this hypoxia-independent HIF activation.

Several current strategies for cancer therapies that exploit the hypoxic microenvironment and response are as follows; 1) hypoxia-dependent drugs or gene-therapy vectors, 2) inhibition of HIF stability, 3) inhibition of transactivation by HIF, and 4) VEGF inhibitors.

Given the pivotal role of the HIF transcription factor in the development of cancers, it would be desirable to inhibit its operation during oncogenesis. However, it would also be desirable, to the extent possible, to avoid interrupting the family's roles in other aspects of cellular metabolism. One approach might be to identify the gene that encodes a likely transcription factor that expressed highly during hypoxia, and devise an antisense oligonucleotide that can be used to inhibit that gene's activity in the right context. The inventors have found that two antisense oligonucleotides both exhibit an enhanced ability to inhibit the production of protein by the HIF-1 gene, and further, induce cytotoxicity in a variety of cancer cell lines.

An antisense compound is a tool that can be used to introduce modifications into the nucleic acids found in living cells. The term "antisense" refers to the notion that nucleic acids "encode" proteins. That is, the sequence of nucleotides found in a given nucleic acid determines, among other things, what protein will be produced. A "sense" sequence for a full gene will yield a normal protein in the usual amount, in response to a given stimulus. A "sense" oligonucleotide will hybridize with a normal gene sequence, and will not affect the amount of, or properties of, the protein. A "nonsense" sequence will not yield a product, or may yield a non-functional product. For example, if a "nonsense" codon or oligomer is inserted into a gene, a truncated, non-functional protein may result. An "antisense" oligonucleotide will hybridize with a normal gene, but will yield a protein altered with respect to its structure, or amount. It has been found that antisense oligomers, that is antisense compounds that are relatively short, can be easily inserted into cells, where they alter gene function.

Antisense compounds are commonly used as research reagents for the exploration of gene function because they are able to alter gene expression with exquisite specificity, and may be used to elucidate the function of particular genes. Antisense compounds can be used, for example, to distinguish between functions of various members of a biological pathway.

Antisense oligonucleotides can be used to selectively block disease-causing genes, thereby inhibiting production of disease-associated proteins. Some antisense oligonucleotides have been safely and effectively administered to humans, and numerous clinical trials are presently underway. It is thus possible that oligonucleotides can be used to treat cells, tissues, and animals, especially

humans. In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for a nucleic acid target and increased stability in the presence of nucleobases. The present invention employs oligomeric nucleotide compounds, particularly antisense oligonucleotides, which are targeted to a portion of a nucleic acid encoding HIF-1, and which modulate the expression of HIF-1. The oligonucleotide compounds are designed to specifically hybridize with one or more nucleic acids encoding HIF-1. One oligonucleotide, RX-0047, is targeted to a site on the HIF-1 gene having the following sequence: 5' ttggacactgggtggctcatt 3' at site 2,772 of HIF-1 gene (Genebank # NM001530 ) (Seq. Id. No. 1). The sequence for the backbone of RX-0047 is complementary to this site. The other oligonucleotide, RX-0149, is targeted to a site in the coding region of the HIF-1 gene having the following sequence: 5' gacttgagatgtagctcc 3' at site 1,936 of HIF-1 gene (Genebank # NM001530) (Seq. Id. No. 3). The sequence for the backbone of RX-0149 is complementary to this site. The inventors have found that oligomers comprising either 5 or 10 nucleotides upstream and downstream from the sequence where the 20-mer of RX-0047 and RX-0149 were derived showed a measurable inhibition of HIF-1 mRNA expression. However, the inventors have found that, this oligonucleotide is more sensitive to variability, and that while 18-mer of RX-0149 showed some inhibition of HIF-1 mRNA expression, further truncation from either end resulted in a substantial loss of inhibition of HIF-1 mRNA expression. The oligomers comprising either 5 or 10 nucleotides upstream and downstream from the sequence where the 20-mer of RX-0047 and RX-0149 were derived demonstrated an inhibition of proliferation of cancer cells. The truncated versions of RX-0047 and RX-0149 which showed some inhibition of HIF-1 mRNA expression also showed an inhibition of cancer cell proliferation.

To target an antisense compound to a particular gene means to identify the nucleic acid sequence of interest, and select one or more sites within the nucleic

acid sequence to be modified. Once the target site has been identified, an oligonucleotide is chosen which is sufficiently complementary to the target site so that it will hybridize specifically to the site, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

5 As used herein, the phrase "nucleic acid encoding HIF-1" encompasses DNA encoding HIF-1, RNA (including pre-mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an antisense oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. The functions of DNA to be interfered with include  
10 replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid  
15 function is modulation of the expression, or production of, a protein. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. For the present purposes, the gene encoding HIF-1 is modulated so that expression of HIF-1 is inhibited.

In the context of this invention, "to hybridize" means to hydrogen bond,  
20 which may be via Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide  
25 at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions  
30 in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense

compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there  
5 is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in vitro assays, under conditions in which the assays are performed.

10 While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 10 to about 30 nucleobases. Particularly preferred  
15 are antisense oligonucleotides comprising about 20 nucleobases (i.e. about 20 linked nucleosides). As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group  
20 covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure  
25 can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

30 Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes

of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example,  
5 phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates,  
10 thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Preferred modified oligonucleotide backbones that do not include a  
15 phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide,  
20 sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH<sub>2</sub> component parts.

25 In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization  
30 properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular  $-\text{CH}_2\text{-NH-O-CH}_2-$ ,  $-\text{CH}_2\text{-N(CH}_3\text{)-O-CH}_2-$  [known as a methylene (methylimino) or MMI backbone],  $-\text{CH}_2\text{-O-N(CH}_3\text{)-CH}_2-$ ,  $-\text{CH}_2\text{-N(CH}_3\text{)-N(CH}_3\text{)-CH}_2-$  and  $-\text{O-N(CH}_3\text{)-CH}_2\text{-CH}_2-$  [wherein the native phosphodiester backbone is represented as  $-\text{O-P-O-CH}_2-$ ]. Also preferred are oligonucleotides having morpholino backbone structures.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted  $\text{C}_1$  to  $\text{C}_{10}$  alkyl or  $\text{C}_2$  to  $\text{C}_{10}$  alkenyl and alkynyl. Particularly preferred are  $\text{O}[(\text{CH}_2)_n\text{O}]_m\text{CH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{OCH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{NH}_2$ ,  $\text{O}(\text{CH}_2)_n\text{CH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{ONH}_2$ , and  $\text{O}(\text{CH}_2)_n\text{ON}[(\text{CH}_2)_n\text{CH}_3]_2$ , where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position:  $\text{C}_1$  to  $\text{C}_{10}$  lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH,  $\text{SCH}_3$ , OCN, Cl, Br, CN,  $\text{CF}_3$ ,  $\text{OCF}_3$ ,  $\text{SOCH}_3$ ,  $\text{SO}_2\text{CH}_3$ ,  $\text{ONO}_2$ ,  $\text{NO}_2$ ,  $\text{N}_3$ ,  $\text{NH}_2$ , heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy ( $2'\text{-O-CH}_2\text{CH}_2\text{OCH}_3$ , also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoethoxy, i.e., a  $\text{O}(\text{CH}_2)_2\text{ON}(\text{CH}_3)_2$  group, also known as 2'-DMAOE, as described in examples hereinbelow. Other preferred modifications include 2'-methoxy ( $2'\text{-O-CH}_3$ ), 2'-aminopropoxy ( $2'\text{-OCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$ ) and 2'-fluoro ( $2'\text{-F}$ ). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used

herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-Me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Certain nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2° C. and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety, cholic acid, a thioether, e.g., hexyl-S-tritylthiol, a thiocholesterol, an aliphatic chain, e.g., dodecandiol or undecyl residues, a phospholipid, e.g., dihexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate, a polyamine or a polyethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyloxycholesterol moiety.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which

are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound.

5 These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving  
10 RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter  
15 oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as  
20 composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase  
25 synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

30 The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the in vivo synthesis of antisense molecules. The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of

compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other  
5 compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

10 The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives.

15 The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

For oligonucleotides, preferred examples of pharmaceutically acceptable  
20 salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid,  
25 oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and  
30 iodine.

The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense

compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding HIF-1, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding HIF-1 can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of HIF-1 in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable

Compositions and formulations for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain

buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

10

### Examples

The following examples illustrate the practice of various aspects of the present inventions. They do not limit the inventions, or the claims, which follow them.

#### **Example 1- Growth of cancer cell lines**

15 Cancer cells used to determine the effect of oligonucleotide compounds were obtained from the following sources: Human OVCAR-3 (ovary), MCF-7 (breast, hormone-dependent), HeLa (cervix), PC3 (prostate), HepG2 (liver), and A549 (lung), HT-29 (colon), PANC-1 (pancreas), Caki-1 (kidney) from the American Type Culture Collection (ATCC) (Manassas, VA); U251 (brain), from  
20 Riken Cell Bank (Japan); MKN-45 (stomach) from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Germany); UMRC2 (kidney) and Lox IMVI (melanoma) from the United States National Cancer Institute (Bethesda, MD). All cell lines except UMRC2, Caki-1 and PANC-1 were grown in RPMI1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine serum  
25 ("FBS"), 1 mM sodium pyruvate, 10 mM HEPES and 100 U/ml penicillin and 100 µg/ml streptomycin ("P/S"). UMRC2, Caki-1 and PANC-1 cells were maintained in Dulbecco's modified Eagle's medium ("DMEM", Invitrogen) supplemented with 10 % FBS, P/S, 10 mM HEPES and 2 mM L-glutamine. All cells were incubated at 37 °C under humidified 5 % CO<sub>2</sub>.

#### **30 Example 2 – Synthesis of Oligonucleotides**

Various nucleotide sequences found in the human HIF-1 $\alpha$  gene coding region known as the open reading frame ("ORF") and 3' untranslated region ("3' UTR") were selected as targets, and the corresponding complementary

oligonucleotides synthesized. The backbone of each oligonucleotide was modified during synthesis to introduce phosphorothioate linkages between nucleotides, except at the 3' and 5' ends, so that an antisense oligonucleotide resulted.

5 Oligonucleotides located in the coding region of HIF-1 $\alpha$  were synthesized using 8909 Expedite DNA synthesizer from Applied Biosystems, Foster City, CA ("ABI"). The synthesis of phosphorothioates was conducted the same manner as for the corresponding phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M 3H-1,2-benzodithiole-3-one 1,1-dioxide in  
10 acetonitrile for the stepwise thiation of the phosphite linkages. After cleavage from the controlled pore glass column and deblocking in concentrated ammonium hydroxide, the oligonucleotide compound was heated in the presence of ammonium hydroxide at 55 °C overnight. The supernatant was transferred to a new tube and ammonium hydroxide was evaporated by Speedvac plus and  
15 UVS400 Universal Vacuum System (Thermo Savant, Holbrook, NY). The oligonucleotide was precipitated with 75 mM NaOAc, pH 7.2 and 2.5 volumes of ethyl alcohol and washed once with ethyl alcohol. The oligonucleotide was dissolved in water and the oligonucleotide concentration was measured by UV spectrophotometer.

### 20 **Example 3 - Transfection**

The day before transfection, cells were trypsinized, counted and plated. For 6 well-plate each well  $2.5 \times 10^5$  cells were plated so that they reach 50-90% confluency at the day of transfection. All the reagents and media used for transfection experiment were obtained from Invitrogen (Carlsbad, CA). The  
25 following solutions were prepared in sterile tubes: Solution A: for each transfection, a mixture of 2  $\mu$ l (0.5  $\mu$ g) DNA, 100  $\mu$ l of serum free medium ("Opti-MEM") and 3  $\mu$ l PLUS reagent was incubated at room temperature for 15 minutes. Solution B: for each transfection, a mixture of 2.5  $\mu$ l of Lipofectamine Reagent and 100  $\mu$ l of serum free medium (Opti-MEM). Solutions A and B were combined and  
30 incubated at room temperature for 15 minutes. For transfection, cells were washed once with 2 ml of serum free medium or PBS and 800  $\mu$ l of serum free medium (Opti-MEM) were added to each well. The combined solution A and B was added to each well and mixed gently. Subsequently cells were incubated for

3 hours at 37 °C, the medium was replaced with regular medium and incubated for the indicated time. For the standard LIPOFECTAMINE 2000 protocol, which was employed for RT-PCR and cytotoxicity experiments, cells were plated in 96-well plates the day before transfection in 100 µl of growth medium. For each well, an oligonucleotide sample was diluted in 25 µl OPTI-MEM reduced serum medium. Separately LIPOFECTAMINE 2000 reagent was diluted (1:50) in 25 µl OPTI-MEM for 5 minutes at room temperature. The oligonucleotide and reagent were mixed and incubated at room temperature for 20 minutes to allow complex formation, and then the complex was added directly to the cells in their growth medium and gently mixed. Subsequently cells were incubated for 4 hours at 37°C, and then the medium was replaced with regular medium and incubated for the indicated time.

**Example 4 - Inhibition of HIF-1 $\alpha$  mRNA expression by antisense oligonucleotides**

The antisense oligonucleotides were then tested for their ability to down-regulate, or inhibit, the expression of mRNA encoding HIF-1 $\alpha$ . The level of expression of HIF-1 $\alpha$  mRNA in cells transfected with the antisense oligonucleotides was measured by RT-PCR analysis. Samples were taken at 2 hours after transfection (change of media), RNA was isolated and subjected to RT-PCR analysis.

UMRC2 cells ( $2.5 \times 10^5$  cells per well) on a 6-well plate were transfected with the experimental oligonucleotides and the transfected cells were used to isolate total RNA. Total RNA was isolated by using RNA-STAT kit (TEL-TEST, Inc., Friendswood, TX), according to the supplier's manual [See also Chomczynski, P. and Sacchi, N in *Anal. Biochem.* 162: 156-159 (1987)]. Briefly, media were removed from the two 6-well plates and total 0.5 ml RNA-STAT solution was added and mixed by pipetting several times, and transferred to an eppendorf tube. 0.1 ml of chloroform was added to the tube, and the tube was shaken vigorously for 15 seconds, and then incubated for 3 minutes at room temperature before centrifugation at 14,000 rpm for 15 minutes at 4°C. The top layer was transferred to a new tube and 0.3 ml of isopropanol was added and incubated for 10 minutes at room temperature. Subsequently the RNA precipitate was centrifuged at 14,000 rpm for 10 minutes. The resulting pellet was washed

with 70 % ethanol, dried briefly and reconstituted with 20  $\mu$ l water. RNA concentration was determined by spectrophotometer. RT reaction was carried out using M-MLV enzyme kit (Invitrogen). 5  $\mu$ g of total RNA was used to synthesize cDNA in 20  $\mu$ l RT reaction. First-strand cDNA was synthesized by incubating total RNA, oligo dT (0.5 mg) and dNTP (0.5 mM) mixture at 65 °C for 5 minutes and by quick-chilling on ice. First-strand buffer, 7.4 mM DTT and 1  $\mu$ L M-MLV Reverse Transcriptase (200 units) was added to the above reaction mixture and incubated at 37 °C for 50 minutes and the enzyme inactivation was followed at 70 °C for 15 minutes. HIF-1 cDNA synthesized by RT reaction was measured by PCR using Sapphire RCR mix (SuperBio Inc., Seoul, Korea) with appropriate primers. For HIF-1 $\alpha$  mRNA detection, primers, 5' GCACAGGCCACATTCACG 3' (Seq. Id No. 5) and 5' TGAAGATTCAACCGGTTTAAGGA 3' (Seq. Id No. 6). Beta-actin was used as an internal PCR control. Primers for beta-actin were 5' CCCATGCCATCCTGCGTCTG 3' (Seq. Id. No. 7) and 5' ACGGAGTACTTGCGCTCAG 3' (Seq. Id. No. 8). PCR products were analyzed on 1.5 % agarose gel by electrophoresis.

A total of 124 oligonucleotides were initially screened, and the results from the preferred four are shown in Table 1 below. Each oligonucleotide was retested to confirm the down-regulation of mRNA expression level. Each reaction was performed in duplicate.

**TABLE 1. Expression of HIF-1 $\alpha$  mRNA Inhibited**

Rexahn#	Region	Target Site*	5'--Sequence--3'	Seq. Id. No.	% Inhibition
RX-0047	3' UTR	2772	aatgagccaccagtgtccaa	2	54
RX-0073	3' UTR	3195	attgtgcaattgtggctacc	9	28
RX-0149	Coding	1936	ggagctaacatctccaagtc	4	57
RX-0158	Coding	2217	gtgatgatgtggcactagta	10	40

\*Genebank # NIM001530

RX-0047, RX-0073, RX-0149 and RX-0158 are new sequences designed by the inventors. These were chosen as representatives from two regions found in the reference, the 3' UTR and ORF regions of the HIF-1a gene, both exhibiting the

highest % inhibition for that region according to the test used in that reference. All of the new sequences exhibited enhanced % inhibition over the reference, using the test described herein. However, it was found that % inhibition did not correlate with cytotoxicity, as discussed in Example 6. Subsequently, two oligonucleotides that exhibited both high % inhibition of HIF-1 mRNA expression and cytotoxicity in UMRC2 cells were selected for testing in 12 other cancer cell lines. A list of the nucleotides tested is shown in Table 2, below.

TABLE 2. List of HIF-1 S-Oligonucleotides

ID	5'-Sequence-3'	start	length	Sequence Id. #
RX-0001	ctccatggtgaatcgggtccc	251	20	11
RX-0002	gccggcgccctccatggtga	260	20	12
RX-0028	ctcaggtggctgtcagggc	48	20	13
RX-0029	ctcgtgagactagagagaag	111	20	14
RX-0030	aagtcagaggtgggggtgc	145	20	15
RX-0031	gagatctggctgcatctc	331	18	16
RX-0032	gaactcacattatgtggaag	398	20	17
RX-0033	cacagaggcctatcaagat	422	20	18
RX-0034	tagctgatgtaagcctcat	442	20	19
RX-0035	ccagcatccagaagttcct	472	20	20
RX-0036	catctcaatatccaaatca	492	20	21
RX-0037	atcatctgtgcttcatgt	512	20	22
RX-0038	catgaccatcatctgtga	572	20	23
RX-0039	gtattgttcacattatcag	602	20	24
RX-0040	cactgtgtccagttagtca	639	20	25
RX-0041	catggtcacatggatgagta	669	20	26
RX-0042	taagcatttctcatttcc	690	20	27
RX-0043	ttcacaaggcatttctgtg	712	20	28
RX-0044	ttagggtaacattcattctg	771	20	29
RX-0045	tatgttcatagttcttctc	797	20	30
RX-0046	atacctccatgttcagac	819	20	31
RX-0047	aatgagccaccagtgccaa	2772	20	2
RX-0048	ataaatagactgcttaggt	2782	20	32
RX-0049	cagtattgtagccaggcttc	2832	20	33
RX-0050	ttgaactaaccaagttgtg	2852	20	34
RX-0051	gctgtctgtgatccagcatt	2928	20	35
RX-0052	atgctactgcaatgcaatgg	2976	20	36
RX-0053	aaaggttactgccttcttac	3091	20	37
RX-0054	tcaactgcctatgatcatga	3113	20	38
RX-0055	gtactgctggcaaagcatta	3173	20	39
RX-0056	attccatgagtaactgctgg	3233	20	40
RX-0057	gattaacaatgtcatgttcc	3320	20	41
RX-0058	ataataaacatacagcatt	3358	20	42

ID	5'-Sequence-3'	start	length	Sequence Id. #
RX-0059	tattatgtaaattggctttac	3386	20	43
RX-0060	ttctagatatatgcatatct	3411	20	44
RX-0061	atcagatgatttctctgaat	3463	20	45
RX-0062	aactccacaactacatagg	3523	20	46
RX-0063	gtttaatatcagttacacaa	3543	20	47
RX-0064	tataccaacagggtaggcag	3582	20	48
RX-0065	ctgcctgtataggagcatt	2671	20	49
RX-0066	caccctgcagtaggttctg	2691	20	50
RX-0067	taactgatccaaagctctg	2721	20	51
RX-0068	aaaattagatgtagaaaata	2811	20	52
RX-0069	agtagaaaggggatcaaaaa	2872	20	53
RX-0070	aaagagcattaatgtaaatt	2893	20	54
RX-0071	caaaaaactgagaaaatga	2948	20	55
RX-0072	aaattatattggcatctct	3069	20	56
RX-0073	atfgtgcaattgtggctacc	3195	20	9
RX-0074	atttcttctaaaaactagt	3271	20	57
RX-0075	ggtttaacaattcataggc	3299	20	58
RX-0076	ccaaataaatgccacatacc	3431	20	59
RX-0077	tttgagctggcaaagtgact	3491	20	60
RX-0078	tctgtttacagtctgctca	3611	20	61
RX-0079	atgcttctaaaactactcaa	3751	20	62
RX-0080	aacaagatattactgtgac	3791	20	63
RX-0081	cagttagtgtagatccaacc	3857	20	64
RX-0082	ataaaaagggtgcattttta	3001	20	65
RX-0083	ccctagccaaaaataaataa	3021	20	66
RX-0084	attcgaaaaaggataaact	3041	20	67
RX-0085	aaaagggtgtaaaaattttc	3131	20	68
RX-0086	atttatgtaaaatgtgaaaa	3151	20	69
RX-0087	aattttgctaagaatgcatg	3641	20	70
RX-0088	agcaaattaacatactaggc	3661	20	71
RX-0089	aaatcaaacattgtattttg	3681	20	72
RX-0090	taatagcg acaaagtcata	3701	20	73
RX-0091	ctacatgaaaaaaaggatgt	3721	20	74
RX-0092	aaclataatctctaaaata	3771	20	75
RX-0117	tgaatgtggcctgtgcagtg	841	20	76
RX-0118	tgaggtgggtactgttgggt	871	20	77
RX-0119	cagcaccaagcagggtcatag	911	20	78
RX-0120	tcttctggctcatatcccat	1051	20	79
RX-0121	ttggtcagatgatcagagtc	1111	20	80
RX-0122	tgtcctgtgggtgactgtcc	1156	20	81
RX-0123	accagacatatccacctct	1195	20	82
RX-0124	tggttgagaattcttgggt	1241	20	83
RX-0125	ttcacacatacaatgcactg	1261	20	84
RX-0126	tgtgtaataataaccactcac	1288	20	85
RX-0127	aggacacattctgtttgtg	1327	20	86
RX-0128	ttcatatctgaagattcaac	1354	20	87
RX-0129	tcaactttgggtgaatagctg	1381	20	88
RX-0130	ggctactgtatctctgat	1401	20	89
RX-0131	catcaggttccttctaagt	1431	20	90

ID	5'-Sequence-3'	start	length	Sequence Id. #
RX-0132	gctggggccagcaaagtaa	1453	20	91
RX-0133	agatatgattgtctccag	1475	20	92
RX-0134	ctggatcatcagttctgtgt	1514	20	93
RX-0135	aatggfacttcccaagttg	1534	20	94
RX-0136	atttatattctgtaatttt	1586	20	95
RX-0137	ggtaatggagacattgcca	1606	20	96
RX-0138	gtgcagggtcagcactactt	1653	20	97
RX-0139	taatgcaactcttgattga	1673	20	98
RX-0140	ctctggatttggtctaatt	1694	20	99
RX-0141	gtaaaagaaagttccagtga	1714	20	100
RX-0142	tgtctgatcctgaatctggg	1739	20	101
RX-0143	acttgtctagtgcctccat	1772	20	102
RX-0144	ggactattaggctcagggtga	1792	20	103
RX-0145	gaccatatacactatccacat	1829	20	104
RX-0146	ccaattccaactgaattca	1851	20	105
RX-0147	gttcttgcctctgtgtctt	1889	20	106
RX-0148	taaactctgtgtcctgagtag	1916	20	107
RX-0149	ggagctaacaatctccaagtc	1936	20	4
RX-0150	tgcttctaattggtgacaac	2001	20	108
RX-0151	aactgtgcttggaggacttg	2042	20	109
RX-0152	tgagtctgctggaataactgt	2062	20	110
RX-0153	ttagcagtaggttcttgat	2083	20	111
RX-0154	ttaattcatcagtggtggca	2118	20	112
RX-0155	atatttaatgtcttccata	2157	20	113
RX-0156	aggagatggagatgcaatca	2177	20	114
RX-0157	gtttcttatgtatgtgggt	2197	20	115
RX-0158	gtgatgatgtggcactagta	2217	20	10
RX-0159	acttgagtatctctatag	2237	20	116
RX-0160	ctctgittggtgaggctgtc	2258	20	117
RX-0161	ctgtctgttctatgactcct	2286	20	118
RX-0162	tagggtctctggatgagat	2310	20	119
RX-0163	ttcctcaggaactgtagttc	2357	20	120
RX-0164	attctgcaaagctagtatct	2390	20	121
RX-0165	agtgaaccatcatgttccat	2428	20	122
RX-0166	tccaattcctactgcttgaa	2440	20	123
RX-0167	tcggctgctgtaataatgt	2470	20	124
RX-0168	tgatgtagtagctgcatgat	2492	20	125
RX-0169	tagattgcatccttttaca	2526	20	126
RX-0170	cagtctacatgctaaatcag	2591	20	127
RX-0171	tcattccattgattgccccag	2611	20	128
RX-0172	tcagctgtggaatccactt	2631	20	129
RX-0173	aacttcacaatcataactgg	2651	20	130

Fig. 1 shows down-regulation of HIF-1 mRNA level in 13 cancer cell lines (UMRC2, OVCAR-3, MKN-45, A549, PC3, U251, Lox IMVI, HeLa, HepG2, HT-29, Caki-1, PANC-1 and MCF-7) after transfection with 0.3  $\mu$ M RX-0047 and RX-

0149. High level down-regulation of HIF-1 was observed in UMRC2, PANC-1, OVCAR-3, MCF-7, Lox IMVI, A549 and PC3 cell lines, moderate level down-regulation was observed in HT-29 and Caki-1 cells, and low level down-regulation was observed in HeLa, HepG2, MKN-45, and U251. As shown in Fig. 1, the level of down-regulation of HIF-1 mRNA expression by RX-0047 and RX-0149 was similar except RX-0047-treated cells showed little higher level of down-regulation than RX-0149-treated in a few cell lines.

### Example 5 - Western blot analysis of HIF-1 protein levels

10 Various cancer cell lines were transfected as described above in Example 3 with the preferred RX-0047 and RX-0149 oligonucleotides at a concentration of 0.3  $\mu$ M. Cells were treated with an iron-chelator, deferrioxamine at 100  $\mu$ M final concentration 6 hour prior to 24 hour post-transfection. For nuclear extract preparation, the following method was used. For 10 cm dish, cells were washed  
15 gently with ice-cold PBS containing 0.1 mM  $\text{NaVO}_4$  (2 x 6 ml), resuspended with 1 ml of ice-cold PBS with 0.1 mM  $\text{NaVO}_4$  and centrifuged at 2000 rpm for 5 minutes. The pellet was resuspended in 0.3-0.5 ml of CE buffer, pH 7.6 (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1x protease inhibitor cocktail and 0.1 mM  $\text{NaVO}_4$ ) with 0.5 % NP40 and cells were allowed to swell on  
20 ice for 5 minutes. The preparation was spun at 2000 rpm for 5 minutes. The cytoplasmic extract was removed from the pellet and transferred to a new tube. The nuclei were washed gently with 0.5 ml of CE buffer without NP40. The nuclei were centrifuged as above at 2,000 rpm for 5 minutes. 50  $\mu$ l of NE buffer, pH 8.0 (20 mM Tris-HCl, 420 mM NaCl, 1.5 mM  $\text{MgCl}_2$ , 0.2 mM EDTA, 1 mM PMSF, 25  
25 % glycerol, 0.1 mM  $\text{NaVO}_4$ , and 1x protease inhibitor cocktail) was added to nuclear pellet and vortexed to resuspend the pellet. The extract was incubated on ice for 40 minutes with a periodic vortexing to resuspend the pellet and the CE and NE fractions were spun at maximum speed for 15 minutes to pellet any  
30 nuclei. The supernatant was transferred to a new tube (soluble nuclear fraction) and stored at  $-70^\circ\text{C}$ . BCA protein assay reagent (Pierce Biotechnology, Rockford, IL) was used to measure protein concentration. Crude cell extracts were used to determine HIF-1 protein expression by SDS-PAGE and subsequent Western analysis using an anti-HIF1 antibody (Transduction Labs, Lexington, KY). Anti-beta-actin antibody (Santa Cruz Biotechnology) was used as an internal control.

Results are shown in Fig. 2. Both RX-0047 and RX-0149 demonstrated inhibition of HIF-1 protein expression, to a greater or lesser degree in all cell lines.

### Example 6 – Cell Cytotoxicity Test

5 Human cancer cell lines were used to test cell cytotoxicity of experimental oligonucleotides. Sulforhodamine B ("SRB") method [Skehan et al., *J. National Cancer Institute*, 82: 1107-1112 (1990)] was used to assess the cell survival after RX-oligonucleotide transfection.

10 Cells were plated onto a 96-well plate and transfected with the oligonucleotides the next day. Following a 72-hour incubation period, the surviving cells were stained with sulforhodamine B and measured using a microplate reader. Briefly, 1,000-10,000 cells were plated onto each well in a 96-well plate and transfected with experimental oligomers using Lipofectamine 2000 reagent (Invitrogen). After 4 hour incubation, the transfection agent was removed and the  
15 fresh media were added to each well. After 72 hours incubation, media were removed. Cells were fixed with 10 % trichloroacetic acid ("TCA"), incubated for 1 hour at 4 °C, and washed 4 times with tap water. Subsequently cells were stained with 0.4 % sulforhodamine B in 1 % acetic acid for 30 minutes, washed 4 times with 1 % acetic acid, and air-dried again. After 5 minutes agitation in 10 mM Tris  
20 solution, optical density of the samples was read at 530 nm using Benchmark Plus Microplate reader (Bio-Rad Laboratories, Hercules, CA).

The experimental compounds which showed down-regulation of HIF-1 mRNA, were used to test their effect on UMRC2 cell viability. The following oligo  
25 compounds, RX-0047, RX-0073, RX-0149 and RX-0158 were used for cytotoxicity test. RX-0047 and RX-0149 showed the most potent cell cytotoxic effect compared with the other oligonucleotides tested. Interestingly RX-0118 and RX-0121, new oligonucleotides that had exhibited 74 and 45 % inhibition of mRNA respectively, did not exhibit as much cytotoxicity as RX-0047 and RX-0149. Therefore, the test for mRNA inhibition did not correlate with cytotoxicity.

30 The two best candidates from mRNA inhibition studies, RX-0047 and RX-0149, were screened for cytotoxicity against a variety of cancer cell lines. RX-0047 reduced cell viability in the following human cancer cell lines; PC3 (prostate), U251 (brain), HeLa (cervix), OVCAR-3 (ovary), Lox IMVI (melanoma), HepG2 (liver), MCF-7 (breast), UMRC2 (kidney), MKN-45 (stomach), PANC-1

(pancreas), HT-29 (colon), Caki-1 (kidney) and A549 (lung). The cell cytotoxicity of RX-0047 increased with the concentration of RX-0047 among different cell lines tested. 0.1  $\mu$ M of RX-0047 caused more than 50 % cell death in all 13 cell lines tested. Similar results were obtained for RX-0149. Again, cytotoxicity of RX-0149 was demonstrated in all cell lines, and it increased with concentration to varying degrees among the different cell lines. 0.1  $\mu$ M of RX-0149 in PC3, U251, HeLa, OVCAR-3, Lox IMVI, MCF-7, MKN-45, A549, Caki-1 and UMRC2 caused more than 50 % of cell death. But more than 50 % of cells in PANC-1, HT-29 and HepG2 survived at 0.1  $\mu$ M.

10

### Example 7 - IC<sub>50</sub> measurement of cell cytotoxicity for RX-0047 and RX-0149 oligonucleotides

The experimental oligonucleotides were screened for relative effective dosage. Thirteen different cancer cell lines were transfected with RX-0047 or RX-0149 at concentrations ranging from 0.01  $\mu$ M to 1  $\mu$ M and after 72 hours post-transfection, cells were stained with sulforhodamine B and the number of surviving cells were counted using Bio-Rad Microplate reader (Bio-Rad Laboratories). The IC<sub>50</sub> value, or concentration of drug needed to kill half the cells, was calculated using the KaleidaGraph Software (Synergy Software, Reading, PA) program. The results are reported in Table 3, below.

20

TABLE 3

Cell	IC <sub>50</sub> (nM)												
	PC3	U251	HeLa	OVCAR3-	Lox-IMVI	HepG2	MCF7	UMRC2	MKN-45	A549	Caki-1	HT-29	PANC-1
RX-0047	39	6	15	3	26	31	14	8	10	6	15	82	18
RX-0149	115	12	29	7	83	113	22	17	26	12	23	500	100

For comparison, it is noted that the IC<sub>50</sub> for UMRC2 is 8 nM for RX-0047 and 17 nM for RX-0149. Similar level of cytotoxic effect was observed in U251, OVCAR-3, and A549 cell lines.

25

**Example 8: Sequence Variability**

In order to determine whether the full 20-mer backbone of RX-0047 and RX-0149 are required to down-regulate HIF-1 mRNA expression, 18-, 16-, 14-, and 10-mer oligonucleotides were synthesized and their effects on mRNA expression and cytotoxicity were analyzed. RT-PCR analysis data indicated that the 18-mer version of RX-0047 showed some inhibition of HIF-1 mRNA expression. Whereas the 16- and 14- and 10-mer versions showed less stronger inhibition of HIF-1 mRNA expression. This suggests that the sequence truncation of RX-0047 down to 16-, 14- and 10-mer did have some effect on the desired inhibition of HIF-1 mRNA expression. For RX-0149, the 18- and 16-mer versions of RX-0149 showed similar inhibition of HIF-1 mRNA expression as the 20-mer version of RX-0149. However, when the sequence was truncated to the 14-, and 10-mer versions of RX-0149, the inhibition became insignificant. This indicates that for RX-0047, the 18- and 16-mer versions also worked as efficiently as the 20-mer version. For RX-0149, the 20-mer full-length sequence is required to achieve the maximum inhibition of HIF-1 mRNA expression.

In conjunction with the above data, we observed that oligomers comprising either 5 or 10 nucleotides, both upstream and downstream from the sequence where the 20-mer of RX-0047 was derived, showed a measurable inhibition of HIF-1 mRNA expression.

Cytotoxicity was tested using the same oligomers comprising 5 or 10 nucleotides upstream and downstream from the sequence where 20-mer of RX-0047 was derived in UMRC2 cell line. All 4 modified oligomers demonstrated cytotoxic effects comparable to the 20-mer of RX-0047, consistent with the RT-PCR data. The truncated versions of RX-0047 and RX-0149 described above also showed a similar inhibition pattern of cancer cell proliferation as observed in RT-PCR analysis.

**Example 9: Ex Vivo Xenograft Study**

In order to observe the inhibition of growth of tumors using one of the presently invented compounds, RX-0047, in animal models, an ex vivo xenograft study of nude mice was conducted. The A549 human lung cancer cell line was grown in a 4:1 mixture of Dulbecco's modified Eagle's medium and medium 199

supplemented with 10% cosmic calf serum (HyClone, Logan, UT). Cells were maintained at 37°C under 5% CO<sub>2</sub>.

A marker gene, luciferase, was introduced into tumor cells. The following methods were utilized. Cells were infected with luciferase using a lentiviral vector  
5 containing the luciferase gene and a G418/neo selection marker. Cells were incubated for 24 hours in the presence of viral supernatant. Media was changed following the infection, and the G418 selection was initiated 3-4 days following the infection. Luciferase-positive cells were confirmed using a luminometer.

Immunodeficient mice (Nu/Nu; Harlan Sprague Dawley, Inc., Indianapolis,  
10 IN) were maintained in pathogen-free conditions within the animal resources center (ARC) at University of Texas Southwestern Medical Center and treated according to ARC and IACUC guidelines.

For the A549 lung cancer xenograft model, cells ( $4 \times 10^6$  cells ) were injected subcutaneously into both flanks of each mouse. Test articles were  
15 administered for 14 days via Alzet pump system. Alzet micro-osmotic pumps (Alza, Palo Alto, CA) were implanted subcutaneously (sc) for 10 g mice and intraperitoneally (IP) for 20 g mice. Pumping rate was maintained at 0.25  $\mu$ l/hr ( $\pm$  0.05  $\mu$ l/hr) with continuous infusion for 14 days. Sterile technique was used during filling and handling of the pump and surgical implantation.

For the A549 lung cancer metastasis model, mice were  $\gamma$ -irradiated and  $1 \times 10^6$  cells were introduced intravenously through the tail vein. Animals were  
20 imaged using luciferase-based bioluminescence imaging each week. Mice were terminated either based on negative results (after 3-4 months) or were imaged each week until the tumor burden exceeded 10% of the host animal's normal body  
25 weight (1-2 cm in diameter for an adult mouse) per ARC/IACUC guidelines.

Table 4 shows the measurement of luciferase-base bioluminescence as an indicator of tumor growth in control and RX-0047-treated athymic nude mice sc-implanted with A549 human lung carcinoma xenografts. One week following RX-0047 treatment, 3-fold reduction in tumor size was observed compared to the  
30 control animals. Two weeks following RX-0047 treatment, a 2-fold reduction in tumor size was observed. This indicates that RX-0047 is a potent anti-tumor agent in tumor xenograft model.

TABLE 4

Treatment	rlu* (1 week after treatment)	rlu* (2 weeks after treatment)
Control	$2.0 \times 10^8$	$3.8 \times 10^8$
RX-0047, 30mg/kg/day	$5.8 \times 10^7$	$1.7 \times 10^8$

rlu\* = relative light units

5

In the A549 metastasis model, animals were treated with daily IP doses of RX-0047 (60 mg/kg/day for 9 days followed by 30 mg/kg/day for additional 5 days). The imaging of the metastatic tumor was performed at 2 and 3 weeks following 14-days of RX-0047 IP administration. As shown in Table 5, 2 weeks post treatment with RX-0047, the lung metastasis was not detected while lung metastasis in the control group was observed. Three weeks following RX-0047 treatment, lung metastasis was decreased 60-fold as compared to the control group. This indicates that RX-0047 is potentially a strong inhibitor of lung metastases.

15

TABLE 5

Treatment	rlu* (2 week after treatment)	rlu* (3 weeks after treatment)
Control	$1.9 \times 10^7$	$7.7 \times 10^7$
RX-0047	0	$1.3 \times 10^6$

rlu\* = relative light units

20

25

### Claims

What is claimed is:

1. A compound, RX-0047, having a sequence comprising Seq. Id. No. 2 5'  
5 aatgagccaccagtgtccaa 3', targeted to a nucleic acid molecule encoding human HIF-1, wherein said oligonucleotide compound inhibits the expression of human HIF-1.
2. The compound of claim 1, wherein the compound is an antisense oligonucleotide.
- 10 3. The antisense oligonucleotide of claim 2 having at least one modified internucleoside linkage that is a phosphorothioate linkage.
4. A method of inhibiting the expression HIF-1 in human cells or tissues comprising contacting said cells or tissues with the compound of Claim 1.
5. A method of inducing cytotoxicity in a cancer cell comprising the step of  
15 introducing into the cell an oligonucleotide that hybridizes to a human HIF-1 sequence, comprising, RX-0047, Seq. Id. No. 2, 5' aatgagccaccagtgtccaa 3'.
6. A compound, RX-0149, 5' ggagctaacatctccaagtc 3', Seq. Id. No. 4, targeted to a nucleic acid molecule encoding HIF-1, wherein said compound inhibits the expression of human HIF-1.
- 20 7. The compound of claim 6, wherein the compound is an antisense oligonucleotide.
8. The antisense oligonucleotide of claim 7 having at least one modified internucleoside linkage that is a phosphorothioate linkage.
9. A method of inhibiting the expression of HIF-1 in human cells or tissues  
25 comprising contacting said cells or tissues with the compound of Claim 6.
10. A method of inducing cytotoxicity in a cancer cell comprising the step of introducing into the cell an oligonucleotide that hybridizes to a human HIF-1 sequence, comprising Seq. Id. No. 4 RX-0149, 5' ggagctaacatctccaagtc 3'.

Fig. 1. RX-0047 and RX-0149 inhibit HIF-1 mRNA expression in various cancer cell lines

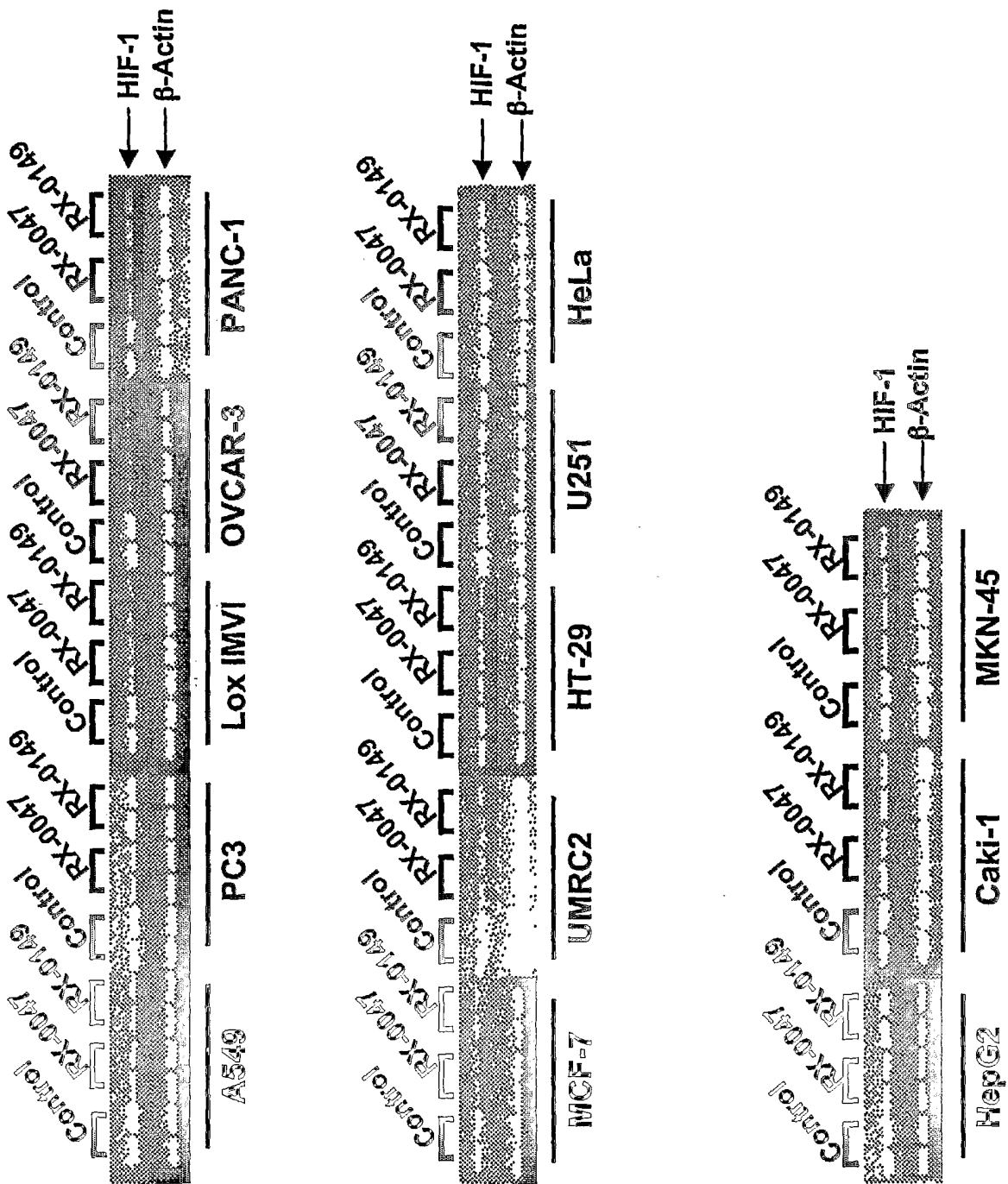


Fig. 2

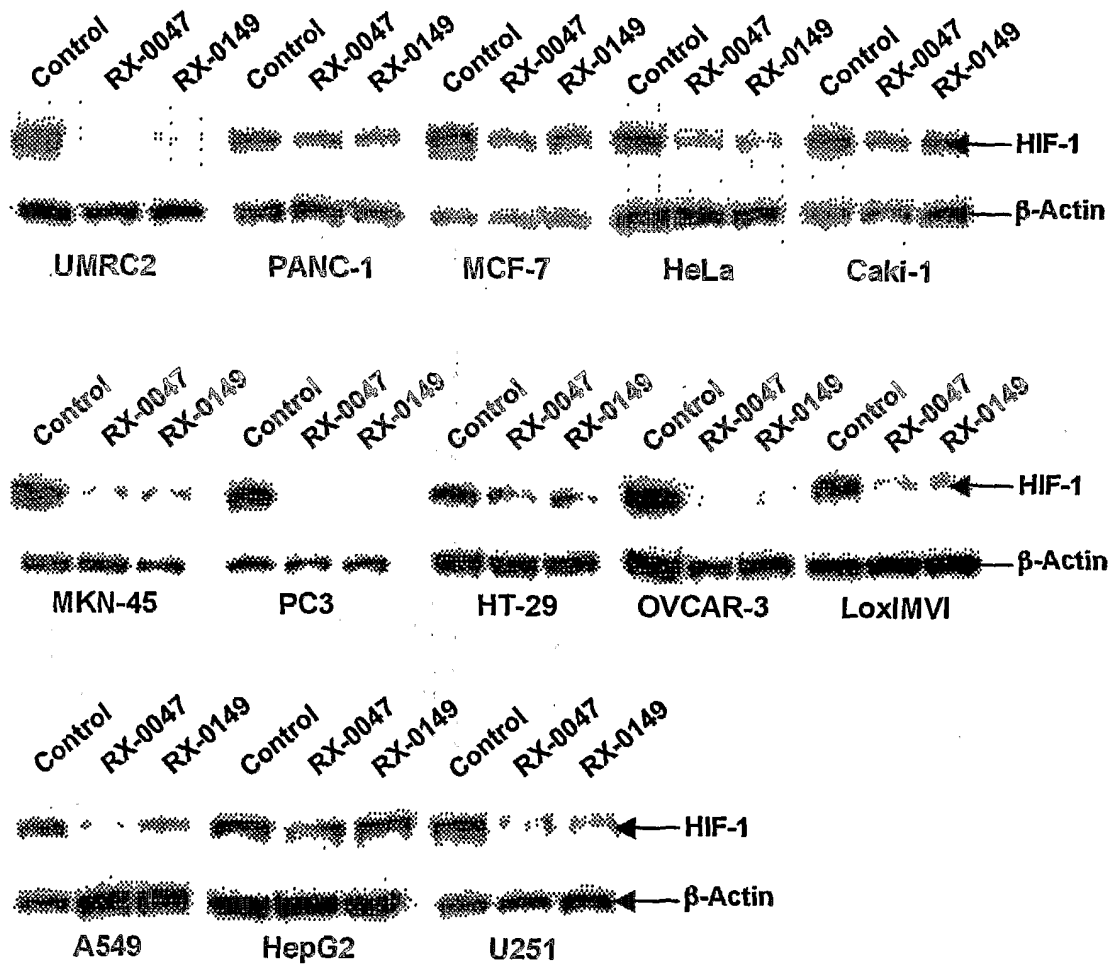


Fig. 2. Western blot analysis of inhibition of HIF-1 protein expression by RX-0047 and RX-0149

## SEQUENCE LISTING

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