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54 INTERFERING RNA DUPLEX HAVING BLUNT-ENDS AND 3'-MODIFICATIONS

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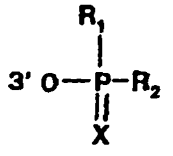
(54) **Title:** INTERFERING RNA DUPLEX HAVING BLUNT-ENDS AND 3'-MODIFICATIONS



(I)

$$3'\,O-\underset{\underset{X}{\|}}{\overset{\overset{R_1}{|}}{P}}-R_2$$

(57) **Abstract:** The present invention relates to double-stranded RNA compounds with at least one blunt end comprising at least one 3'-end of Formula (I): wherein X is O or S R_1 and R_2 are independently OH, NH_2, SH, alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms and functional groups, preferably a heteroatom selected from the group of N, O, or S or a functional group selected from the group OH, NH_2, SH, carboxylic acid or ester; or R_1 and R_2 may be of formula Y-Z where Y is O, N, S and Z is H, alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms, preferably a heteroatom selected from the group of N, O, or S; and wherein said double-stranded RNA mediates RNA interference. Preferred 3'end modifications are: phosphate, phosphorothioate, abasic ribonucleoside, hydroxyphopyl phosphodiester.

INTERFERING RNA DUPLEX HAVING BLUNT-ENDS AND 3'-MODIFICATIONS

FIELD OF THE INVENTION

The invention relates to selective inhibition of target genes using double-stranded RNA and provides compounds useful for this purpose.

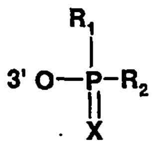
BACKGROUND OF THE INVENTION

Short RNA duplexes have been shown to be the effective guides that mediate RNA interference in many in vitro and in vivo models (Hamilton et al. 1999, Zamore et al., 2000, Caplen et al., 2001, Elbashir et al., 2001, Yang et al., 2000).
Most commonly, synthetic siRNA duplexes are designed such as a stretch of 19 contiguous ribonucleotide base-pairs is flanked with 2-3 unpaired nucleotides at the 3'-end of each strand ("overhangs"). This 21-nt siRNA species has been found to be generated during DICER-mediated cleavage of long ds-RNA in mammalian and non-mammalian systems (Bernstein et al., 2001, Ketting et al., 2001). This particular 21-mer siRNA format has been firstly selected from a drosophila melanogaster model and then highlighted regarding its efficiency (Elbashir et al. 2001). Consequently, the major part of today's studies applying synthetic siRNAs as gene inhibitors is relying on this "wildtype" 21-mer siRNA derivative. For the overhangs usually 2'-deoxynucleotides are used, notably for cost reasons but also with regard to a potential protection against intracellular nuclease activity.

The present invention now provides a new and inventive format for double-stranded RNA ("dsRNA") mediating RNAi. The blunt-ended siRNAs in accordance with the present invention overcome disadvantages of the synthetic siRNAs with 3'-overhangs which are currently used in the art.

SUMMARY OF THE INVENTION

The present invention provides double-stranded RNA with at least one blunt end comprising at least one 3'-end of formula:



$$3'\,\mathrm{O}-\overset{\displaystyle R_1}{\underset{\displaystyle \overset{\|}{X}}{\mathrm{P}}}-R_2$$

formula I

wherein

X is O or S

R_1 and R_2 are independently OH, NH_2, SH, alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms and functional groups, preferably a heteroatom selected from the group of N, O, or S or a functional group selected from the group OH, NH_2, SH, carboxylic acid or ester;

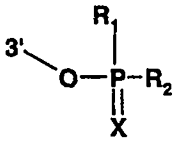
Also, R_1 and R_2 may be of formula Y-Z where Y is O, N, S and Z is H, alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms, preferably a heteroatom selected from the group of N, O, or S;

and wherein said double-stranded RNA mediates RNA interference.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the surprising finding that synthetic double-stranded RNA (dsRNA) molecules with at least one blunt end comprising a certain type of chemical modification efficiently mediate RNA interference. The dsRNAs according to the present invention are particularly useful for high-throughput approaches using siRNAs due to their simplified synthetic procedure, such as for instance the use of a universal solid support.

In one aspect, the present invention relates to double-stranded RNA with at least one blunt end comprising at least one 3'-end of the formula:



$$3'\,\mathrm{O}-\overset{\displaystyle R_1}{\underset{\displaystyle \overset{\|}{X}}{\mathrm{P}}}-R_2$$

Formula I

wherein

X is O or S

R_1 and R_2 are independently OH, NH_2, SH, alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms and functional groups, preferably a heteroatom selected from the group of N, O, or S or a functional group selected from the group OH, NH_2, SH, carboxylic acid or ester;

Also, R_1 and R_2 may be of formula Y-Z where Y is O, N, S and Z is H, alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms, preferably a heteroatom selected from the group of N, O, or S;

and wherein said double-stranded RNA mediates RNA interference.

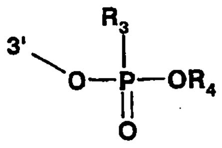
R_1 and R_2 may also form a cyclic structure, e.g. a carbocyclic or heterocyclic ring, the ring structure preferably having from 3 to 7 members.

In a preferred embodiment, Z is one or more abasic nucleoside, preferable ribonucleoside, moieties. The nucleoside moieties may be linked for instance by a phosphodiester or a phosphorothioate group.

In another preferred embodiment, R_1 is OH. In another preferred embodiment, R_1 and R_2 together comprise from 1 to 24 C-atoms more preferably from 1 to 12, or from 2 to 10 and most preferably from 1 to 8 or from 2 to 6. In another preferred embodiment, R_1 and R_2 are independently OH, lower alkyl, lower aryl, lower alkyl-aryl, lower aryl-alkyl, where lower alkyl, lower aryl, lower alkyl-aryl, lower aryl-alkyl can be substituted by additional heteroatoms and functional groups as defined above. In another preferred embodiment, R_1 and R_2 are not both OH.

The term "lower" in connection with organic radicals or compounds means a compound or radical which may be branched or unbranched with up to and including 7 carbon atoms, preferably 1-4 carbon atoms. Lower alkyl represents, for example, methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, tert-butyl, n-pentyl and branched pentyl, n-hexyl and branched hexyl.

In related aspect, the present invention relates to double-stranded RNA with at least one blunt end comprising at least one 3'-end of the formula:



$$3'{-}O-\underset{\underset{O}{\|}}{\overset{\overset{R_3}{|}}{P}}-OR_4$$

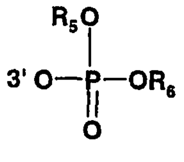
formula II

wherein R_3 is OH, NH_2, SH, alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms, preferably a heteroatom selected from the group of N, O, or S, and R_4 is independently alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms, preferably a heteroatom selected from the group of N, O, or S or a functional group selected from the group OH, NH_2, SH, carboxylic acid or ester, and wherein said double-stranded RNA mediates RNA interference. R_3 and R_4 may also form a cyclic structure, e.g. a carbocyclic or heterocyclic ring, the ring structure preferably having from 3 to 7 members.

R_3 and R_4 may further comprise additional heteroatoms, preferably a heteroatom selected from the group of N, O, or S.

In preferred embodiment, R_3 is OH. In another preferred embodiment, R_3 and R_4, respectively, together comprise from 1 to 24 C-atoms more preferably from 1 to 12, or from 2 to 10 and most preferably from 1 to 8 or from 2 to 6. In another preferred embodiment, R_3 is lower alkyl, lower aryl, lower alkyl-aryl, lower aryl-alkyl, where lower alkyl, lower aryl, lower alkyl-aryl, lower aryl-alkyl can be substituted by additional heteroatoms and functional groups as defined above and R_4 is, independently lower alkyl, lower aryl, lower alkyl-aryl, lower aryl-alkyl, where lower alkyl, lower aryl, lower alkyl-aryl, lower aryl-alkyl can be substituted by additional heteroatoms and functional groups as defined above.

In a related aspect, the present invention relates to double-stranded RNA with at least one blunt end comprising at least one 3'-end of the formula:



$$3'\,O-\underset{\underset{O}{\|}}{\overset{\overset{R_5O}{|}}{P}}-OR_6$$

formula III

wherein R_5 and R_6 are the same or different and are H, alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms, preferably a

heteroatom selected from the group of N, O or S, or a functional group selected from the group OH, NH_2, SH, carboxylic acid or ester wherein said double-stranded RNA mediates RNA interference. R_5 and R_6 may also form a cyclic structure, e.g. a carbocyclic or heterocyclic ring, the ring structure preferably having from 3 to 7 members.
R_5 and R_6 may further comprise additional heteroatoms, preferably a heteroatom selected from the group of N, O or S.

In preferred embodiment, R_5 and R_6, respectively, together comprise from 1 to 24 C-atoms more preferably from 1 to 12 or from 2 to 10 and most preferably from 1 to 6 or from 2 to 6. In another preferred embodiment, R_5 and R_6 are independently lower alkyl, lower aryl, lower alkyl-aryl, lower aryl-alkyl, where lower alkyl, lower aryl, lower alkyl-aryl, lower aryl-alkyl can be substituted by additional heteroatoms and functional groups as defined above.
In another embodiment R_5 and R_6 are not both H.

The RNA molecules of the present invention will have at least at least one strand comprising a 3'-ends of the formula I, II or III. Preferably, both strands of the double-stranded RNA comprise 3'-ends comprising a group of the formula I, II or III. The RNA molecules of the present invention will further have at least one blunt end, preferably two blunt ends. In a particularly preferred embodiment, the RNA molecules of the present invention are blunt ended on both sides and both ends comprise 3'-ends of the formula I, II or III.

The RNA molecules in accordance with the present invention will have at least a partially double-stranded character In a preferred embodiment, they are fully double-stranded. They may be composed of two separate strands, but may also be composed of one strand forming a hairpin loop. In a particularly preferred embodiment, the RNA molecules of the present invention are composed of two separate strands which are fully double-stranded comprising at least one, preferably two, blunt ends.

The RNA molecules according to the present invention mediate RNA interference ("RNAi"). The term "RNAi" is well known in the art and is commonly understood to mean the inhibition of one or more target genes in a cell by dsRNA with a region which is complementary to the target gene. Various assays are known in the art to test dsRNA for its ability to mediate RNAi (see for instance Elbashir et al., Methods 26 (2002), 199-213). The effect of the dsRNA according to the present invention on gene expression will typically result in expression of the target gene being inhibited by at least 10%, 33%, 50%, 90%, 95% or 99% when compared to a cell not treated with the RNA molecules according to the present invention.

The RNA molecules in accordance with the present invention comprise a double-stranded region which is substantially identical to a region of the mRNA of the target gene. Particularly preferred is a region with 100% identity to the corresponding sequence of the target gene. However, the region may also contain one or two mismatches as compared to the corresponding region of the target gene. The present invention includes RNA molecules which target more than one gene. In a preferred embodiment, the RNA molecules of the present invention specifically target one given gene. In order to only target the desired mRNA, the siRNA reagent should have 100% homology to the target mRNA and at least 2 mismatched nucleotides to all other genes present in the cell or organism. Methods to analyze and identify dsRNAs with sufficient sequence identity in order to effectively inhibit expression of a specific target sequence are known in the art. Sequence identity may be optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Another factor affecting the efficiency of the RNAi reagent is the target region of the target gene. The region of a target gene effective for inhibition by the RNAi reagent may be determined by experimentation. Most preferred mRNA target region would be the coding region. Also preferred are untranslated regions, particularly the 3'-UTR, splice junctions. For instance, *transfection assays as described in Elbashir S.M. et al, 2001 EMBO J., 20, 6877-6888* may be performed for this purpose. A number of other suitable assays and methods exist in the art which are well known to a person skilled in the art.

The length of the complementary region of the RNA molecules in accordance with the present invention is preferably from 10 to 100 nucleotides, more preferably 15 to 50 nucleotides, even more preferably 17 to 30 nucleotides and most preferably 19 to 25 nucleotides. In a particularly preferred embodiment the RNA molecules in accordance with the present invention consist of short dsRNA molecules having a length from 15 to 50 nucleotides, more preferably 17 to 30 nucleotides and most preferably 19 to 25 nucleotides.

The 3' end of the dsRNA according to the present invention confer a high in vivo stability in serum or in growth medium for cell cultures. Thus, the dsRNA according to the present invention does not require additional stabilization against nuclease degradation as is common in the art, for instance, by adding a 3'-overhang of 2 or 3 deoxynucleotides. However, the dsRNA according to the present invention may also contain at least one modified or non-natural ribonucleotide. Preferred modifications are include, but are not limited to modifications at the 2' position of the sugar moiety, such as for instance 2'-O--(2-methoxyethyl) or 2'-MOE) (Martin et al., Helv. Chim. Acta, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. Other preferred modifications include backbone modifications including, but not limited to, replacing the phoshoester group connecting adjacent ribonuclotides with for instance phosphorothioates, chiral phosphorothioates or phosphorodithioates. Methods for the synthesis of modified or non-natural ribonucleotide are well known and readily available to those of skill in the art.

The dsRNA molecules may be prepared by a method comprising the steps:
(i) synthesizing the two RNA strands each using, for instance, TOM chemistry as illustrated in Example 1. Other methods to synthesis RNA strands are readily apparent to a person of skill in the art. The reaction may be carried out in solution or, preferably, on solid phase or by using polymer supported reagents.
(ii) combining the synthesized RNA strands under conditions, wherein a dsRNA molecule is formed, which is capable of mediating RNA.

In another aspect, the present invention provides methods for the inhibition of a target gene comprising introducing into a cell dsRNA according to the present invention, which is capable of inhibiting at least one target gene by RNAi. Also, more than one species of dsRNA, which are each specific for another target region, may be introduce into a cell at the same time or sequentially. The dsRNA according to the present invention can be introduced into a cell by various standard methods in genetic engineering, including physical methods, for example, simple diffusion, by injection of a solution containing the nucleic acid, bombardment by particles covered by the nucleic acid, soaking the cell or organism in a solution of the nucleic acid, lipofection or electroporation of cell membranes in the presence of the nucleic acid. A particularly preferred method for delivering nucleic acids is the use of lipofection. The cells are subsequently maintained under conditions under which RNAi occurs. It will be readily apparent to the skilled artisan under what conditions a given cell line is maintained in order that RNAi occurs. This method for the inhibition of a target gene can be used therapeutically (e.g. for knocking down a gene overexpressed in a particular disease) or for research (e.g. examine function of a gene or validate targets for drug discovery). In a preferred embodiment, the gene function is completely eliminated by this method, i.e. knock-out cells for a particular gene can be generated by this method.

The cell may be a plant or an animal cell. In a preferred embodiment, the cell is a mammalian cell, more preferably a human cell. The type and source of the cell is not critical for the invention, thus the invention includes for instance cells from the inner cell mass, extraembryonic ectoderm or embryonic stem cells, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include without limitation adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, dendritic cells, neurons, glia, mast cells, blood cells and leukocytes (e.g., erythrocytes, megakaryotes, lymphocytes, such as B, T and natural killer cells, macrophages, neutrophils, eosinophils, basophils, platelets, granulocytes), epithelial cells, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands, as well as sensory cells.

In another aspect, dsRNA according to the present invention is used for the identification of gene function in an organism wherein the activity of a target gene of previously unknown function is inhibited. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics would envision determining the function of uncharacterized genes by employing the dsRNA according to the present invention to reduce the amount and/or alter the timing of target gene activity. The dsRNA according to the present invention could be used in determining potential targets for pharmaceuticals, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, including the human genome, can be coupled with the invention to determine gene function in mammalian systems, in particular in human cell culture systems.

The ease with which RNA can be introduced into an intact mammalian cell containing the target gene allows the method for the inhibition of a target gene according to the present invention to be used in high throughput screening (HTS). For example, solutions containing RNA molecules according to the present invention that are capable of inhibiting a given target gene can be placed into individual wells positioned on a microtiter plate as an ordered array. Subsequently, intact cells in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity or by proteomic, genomics and standard molecular biology techniques. Thus, the function of the target gene can be assayed from the effects it has on the cell when gene activity is inhibited.

The present invention is not limited to any type of target gene or nucleotide sequence. For example, the target gene can be a cellular gene, an endogenous gene, a pathogen-associated gene, a viral gene or an oncogene. The following classes of possible target genes are listed for illustrative purposes only and are not to be interpreted as limiting: transcription factors and developmental genes (e.g., adhesion molecules, cyclin kinase inhibitors, Wnt family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors); oncogenes (e.g., ABLI, BCLI, BCL2, BCL6, CBFA2, CBL, CSFIR, ERBA, ERBB, ERBB2, ETSI, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCLI, MYCN, NRAS, PIMI, PML, RET, SKP2, SRC, TALI, TCL3, and YES); tumor suppressor genes (e.g., APC, BRAI, BRCA2, CTMP, MADH4, MCC, NFI, NF2, RBI, TP53, and WTI); and enzymes (e.g., ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases, amyloglucosidases, catalases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucose oxidases, GTPases, helicases, integrases, insulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, peroxidases, phosphatases, phospholipases, phosphorylases, proteinases and peptidases, recombinases, reverse transcriptases, telomerase, including RNA and/or protein components, and topoisomerases).

A gene derived from any pathogen may be targeted for inhibition. For example, the gene could cause immunosuppression of the host directly or be essential for replication of the pathogen, transmission of the pathogen, or maintenance of the infection. Cells at risk for infection by a pathogen or already infected cells, such as cells infected by human immunodeficiency virus (HIV) infections, influenza infections, malaria, hepatitis, plasmodium, cytomegalovirus, herpes simplex virus, and foot and mouth disease virus may be targeted for treatment by introduction of RNA according to the invention. The target gene might be a pathogen or host gene responsible for entry of a pathogen into its host, drug metabolism by the pathogen or host, replication or integration of the pathogen's genome, establishment or spread of an infection in the host, or assembly of the next generation of pathogen. Methods of prophylaxis (i.e., prevention or decreased risk of infection), as well as reduction in the frequency or severity of symptoms associated with infection, can be envisioned.

In another aspect, the invention further provides a method for identifying and/or characterizing pharmacological agents acting on at least one target protein comprising: contacting a eukaryotic cell, preferably a mammalian cell, more preferably a human cell capable of expressing at least one endogenous gene coding for the protein(s) of interest with (a) at least one dsRNA molecule according to the present invention, which is capable of inhibiting the expression of the gene(s) encoding the protein(s) of interest and (b) a test substance or a collection of test substances wherein pharmacological properties of said test substance or said collection are to be identified and/or characterized. The cells might be concomitantly or sequentially contacted with the dsRNA and the compound(s) to be tested, the order in which the cells are contacted with the dsRNA and the compound(s) is of not crucial. In a preferred embodiment, the cells further comprise at least one exogeneous nucleic acid coding for variant or mutated form of the protein(s) of interest, wherein the expression of said exogenous nucleic acid is less inhibited by said dsRNA.

In another aspect, the invention also provides a kit comprising reagents for inhibiting expression of a target gene in a cell, wherein said kit comprises dsRNA according to the present invention. The kit comprises at least one of the reagents necessary to carry out the in vitro or in vivo introduction of the dsRNA according to the present invention to test samples or subjects. In a preferred embodiment, such kits also comprise instructions detailing the procedures by which the kit components are to be used.

Another aspect of the present invention provides pharmaceutical compositions and formulations which include dsRNA according to the present invention which is capable of inhibiting at least one target gene by RNAi. The pharmaceutical compositions may also contain more than one species of dsRNA which are each specific for another target region. 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.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers. The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation may be a lyophilized powder which may contain any or all of the following: 1-50 mM histidine, 0. 1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

The determination of an effective dose is well within the capability of those skilled in the art. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions that exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration. Normal dosage amounts may vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

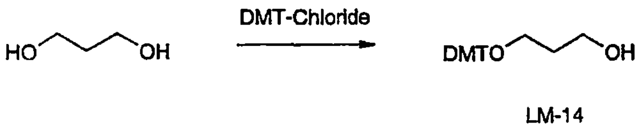
The following examples are intended to illustrate, but not further limit, the invention.

EXAMPLES

Example 1:

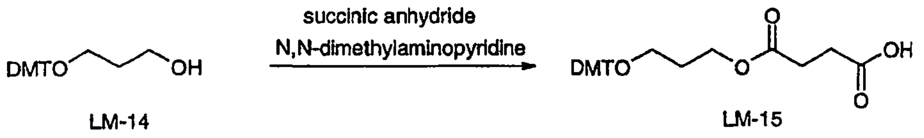
Procedures for synthesis of solid support LM-17

3-[Bis-(4-methoxy-phenyl)-phenyl-methoxy]-propan-1-ol (LM-14)



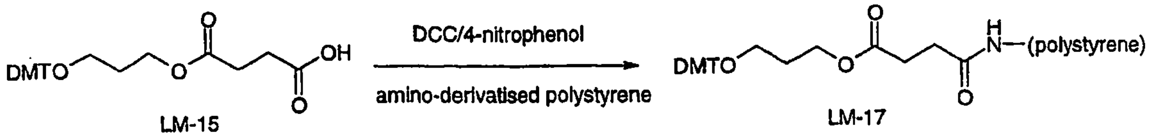
To a solution of 1,3-propanediol (20 ml, 268 mmol) in absolute pyridine (20 ml) under an argon atmosphere was added 4,4'-dimethoxytriphenylchloromethane (1.87 g, 5.4 mmol). After stirring at room temperature for 10 minutes, the mixture was poured onto an ice/water mixture and extracted with ethyl acetate (twice). The combined organic phases were washed with water (twice) and brine, dried over sodium sulfate, filtered and concentrated. The obtained brown liquid was purified by column chromatography (silica gel, eluent: ethyl acetate/hexane = 1:2) yielding 1,56 g (77%) of **LM-14** as a slightly yellow oil.

Succinic acid mono-{3-[bis-(4-methoxy-phenyl)-phenyl-methoxy]-propyl} ester (LM-15)



To a solution of **LM-14** (250 mg, 0.69 mmol) in absolute pyridine (5 ml) under an argon atmosphere was added N,N-dimethylaminopyridine (45 mg, 0.37 mmol). After addition of succinic anhydride (57 mg, 0.57 mmol) the mixture was stirred at room temperature overnight. The reaction mixture was diluted with ethyl acetate and washed with brine (3 times). The organic phase was dried over sodium sulfate, filtered, concentrated to yield crude **LM-15** (536 mg) which was used without further purification in the subsequent step.

Solid support **LM-17**



To a solution of the crude **LM-15** (0.69 mmol) in a mixture of absolute DMF (5 ml) was added pyridine (0.13 ml), 4-nitrophenol (143 mg, 1.0 mmol) and N,N-dicyclohexylcarbodiimide (DCC, 156 mg, 0.76 mmol) under an argon atmosphere. The yellow solution was stirred at room temperature for two days, after which a yellow suspension had formed. After filtration over celite, amino-derivatised polystyrene (457 mg) was added to the filtrate. Triethylamine (0.046ml) was added and the mixture was shaken using a mechanical bottle shaker for 24 hours. The mixture was filtered and the solid support was washed twice each with DMF (5 ml), methanol (5 ml) and diethyl ether (5 ml) to give **LM-17** (453 mg). Quantitation (absorption at 498 nm) of trityl groups released upon treatment with 0.1 ml of a solution of p-toluenesulfonic acid (1.9 g) in acetonitrile (100ml) revealed a loading of 14 µmol/g.

Example 2

1. Synthesis of Oligoribonucleotides (siRNA's)

Modified synthetic oligoribonucleotides described in this invention can be prepared using standard TOM-phosphoramidite chemistry on ABI394 or Expedite/Moss Synthesizers (Applied Biosystems). Phosphoramidites are dissolved in acetonitrile at 0.05 M concentration (0.2M on OligoPilot II), coupling is made by activation of phosphoramidites by a 0.2 M solution of benzimidazolium triflate in acetonitrile. Coupling times are usually comprised between 3-6 minutes. A first capping is made using standard capping reagents.. Oxidation is made by a 0.1 M iodine solution in THF/Pyridine/Water (77:20:3) or 0.5M t-butylhydroxyperoxide (Fluka) in dichloromethane for two minutes. A second capping is performed after oxidation. Oligonucleotide growing chains are detritylated for the next coupling by 2% dichloroacetic acid in dichloromethane or dichloroethane. After completion of the sequences the support-bound compounds are cleaved and deprotected as "Trityl-on" by a Methylamine solution (41% aqueous methylamine/33% ethanolic methylamine 1:1 v/v) at 35°C for 6 h. Resulting suspensions are lyophilised to dryness. 2'-O-silyl groups are removed upon treatment with 1M tetrabutylammonium fluoride 10min at 50°C and 6h at

35°C. The obtained crude solutions are directly purified by RP-HPLC. The purified detritylated compounds are analysed by Electrospray Mass spectrometry and Capillary Gel Electrophoresis and quantified by UV according to their extinction coefficient at 260 nM. Oligonucleotide sequences are listed in Table 1:

NickName	Target	Antisense strand	Sense strand
siRNA 8646	p2x3	ACU CCA UCC AGC CGA GUG Aasg	UCA CUC GGC UGG AUG GAG Utst
C3-8646	p2x3	ACU CCA UCC AGC CGA GUG A-C3	UCA CUC GGC UGG AUG GAG U-C3
phosphate-8646	p2x3	ACU CCA UCC AGC CGA GUG A-p	UCA CUC GGC UGG AUG GAG U-p
abasic-8646	p2x3	ACU CCA UCC AGC CGA GUG A-ab-ab	UCA CUC GGC UGG AUG GAG Uab-ab
siRNA 8549	luc	UCG AAG UAC UCA GCG UAA GdTdT	CUU ACG CUG AGU ACU UCG AdTdT
siRNA-10557	GAPDH	GGC CAU CCA CAG UCU UCU Ggg	CAG AAG ACU GUG GAU GGC CUU
MM siRNA-10559	GAPDH	GGC CAG CCA CAU UCG UCU Ugg	AAG ACG AAU GUG GCU GGC CUU
C3-10569	GAPDH	GGC CAU CCA CAG UCU UCU G-C3	CAG AAG ACU GUG GAU GGC C-C3
MM C3-10571	GAPDH	GGC CAG CCA CAU UCG UCU U-C3	AAG ACG AAU GUG GCU GGC C-C3
siRNA2	hGAPDH	CAU GUA GUU GAG GUC AAU Gaa	CAU UGA CCU CAA CUA CAU GUU
siRNA2 MM	hGAPDH	CAU GUA GAU GAU GUC GAU Gaa	CAU CGA CAU CAU CUA CAU GUU
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C3-siRNA2 MM	hGAPDH	CAU GUA GAU GAU GUC GAU G-C3	CAU CGA CAU CAU CUA CAU G-C3
OH-siRNA	hGAPDH	CAU GUA GAU GAU GUC GAU G	CAU UGA CCU CAA CUA CAU G
P2-siRNA	hGAPDH	CAU GUA GAU GAU GUC GAU G-p	CAU UGA CCU CAA CUA CAU G-p

N: RNA, n: 2'-methoxyethyl ribonucleoside, dN: deoxyribonucleoside, ab: abasic ribonucleoside, p: phosphate, s: phosphorothioate, C3: hydroxypropylphosphodiester

Example 3

3.1 Materials and Methods

Materials: Oligofectamine and other cell culture reagents are obtained from Life Technologies, GibcoBRL, now Invitrogen, Gaithersburg, MD). JetPEI is purchased from Polyplus-Transfection (Illkirch, France).

Cell lines: Stably transfected Chinese hamster ovary cells (CHO-K1) (ATCC CCL61, American Type Culture Collection, Rockville, MD) expressing recombinant rat P2X$_3$ are generated as described (Hemmings et al., NAR 31 (2003), 2117-2126). CHO cells are cultured in minimal essential medium (MEM-*α*) supplemented with 10% (v/v) FBS, 2 mM glutamine in a 5% CO$_2$-humidifed chamber. HeLa cells are cultured in Dubelco's modified essential medium (DMEM 41965) supplemented with 10% (v/v) FBS, 2 mM glutamine in a 5% CO$_2$-humidifed chamber.

Oligonucleotide synthesis: Oligoribonucleotides are purchased at Qiagen or synthesized using TOM-phosphoramidite chemistry, as described by the manufacturer (Qiagen) and purified by RP-HPLC. 3'-hydroxypropylphosphate oligoribonucleotides are synthesized on solid support LM-17 (loading: 14 μmol/g). Oligonucleotide chain elongation, cleavage from the support, deprotection and purification are identical to that 21-mer oligoribonucleotides having two deoxynucleotides as 3'-overhangs.

Purity is assessed by capillary gel electrophoresis. Quantification is carried out by UV according to the extinction coefficient at 260 nM. Annealing of double-stranded RNA (dsRNA) is performed as described elsewhere (Elbashir et al., Methods 26 (2002), 199-213). Oligonucleotide sequences that are used in this report are listed in Table 1 and are characterized by electrospray mass spectroscopy.

Cell transfection: Cationic lipid-oligonucleotide (Oligofectamine) and polymer-oligonucleotide (jetPEI) mixtures are prepared immediately prior to transfection as previously described. Eighteen hours before transfection, 4 x 10^4 cells are plated into 24-well plates in a volume of 0.5 ml MEM-α for CHO cells or 0.5 ml DMEM for HeLa cells (both supplemented with 10% (v/v) FBS, 2 mM glutamine) per well. Prior to the transfection, growth medium is removed from the cells and replaced with 500 μl of OptiMEM and 100 μl of the transfecting reagent/oligonucleotide mixture. Plates are incubated at 37º in a humidified 5% CO2 incubator. After 4 hrs, 60 μl of FBS are added to each well, and the incubation was prolonged for 20h.

RNA harvesting and Real-Time quantitative PCR mRNA analysis: Total RNA is isolated 24 h after oligonucleotide transfection with the RNeasy 96 kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. The RNA samples are mixed with reagents from the Reverse Transcriptase Q-PCR mastermix kit (Eurogentec) and run according to the included protocol.

3.2 Blunt ended siRNA activity

A CHO cell-line expressing the rat P2X$_3$ purino-receptor is used to compare the relative activity of different mRNA inhibitors such as first and second generation oligonucleotides (ASO) as well as short interfering RNAs. Use of linear PEI as "universal" transfection reagent allows to perform head-to-head comparisons between these various gene expression inhibitors. The siRNA sequence was selected from an optimal ASO sequence previously characterized (Dorn et al., Antisense & Nucleic Acid Drug Development 11 (2001): 165–174), is designed to target the P2X3 coding sequence and has been shown to efficiently and selectively down-regulate P2X3 expression at the molecular level, as demonstrated by Q-RT-PCR, immunodetection and functional assay. As positive control, we use the siRNA sequence having two target-complementary 2'-MOE modified overhangs, linked together via a phosphorothioate internucleosidic bond. We then synthesize the corresponding 19-mer blunt-ended siRNA so that after ammonia mediated cleavage from the solid support, the released compound sustains a hydroxypropylphosphate (hpp) group on the 3'-position of its last ribonucleotide.

As an intermediate between the 21-mer siRNA (two complementary overhangs) and the blunt-ended 19-mer, a sequence homologous 21-nt siRNA with two abasic nucleoside overhangs is also synthesized. Such non-nucleosidic overhangs allows us to assess whether one of the steps involved in the RNAi pathway, and more particularly during the unwinding of the duplex, would absolutely require a 3'-elongation on siRNA.

The siRNAs are transfected into CHO cells using JetPEI at a N/P ratio of 5. mRNA levels are assessed by RT-Q-PCR 24 hours after transfection. As shown in Fig. 2, a decrease in the GAPDH mRNA level is detected in CHO cells with comparable level of down-regulation for all three compounds. The blunt-ended 19bp siRNA having a 3'-hydroxypropylphosphate ribonucleotide shows the same efficacy in inhibiting P2X$_3$ mRNA as the "wild-type" 21-mer siRNA.

We now apply a similar format modification to siRNA targeting an endogenous gene and design a siRNA sequence targeting the Chinese hamster GAPDH mRNA in a way that it is also specific and fully homolog to the human GAPDH gene. After transfection into CHO cells of the 21-mer siRNA (2 deoxyribonucleotidic overhangs) and the blunt-ended 3'-hydroxypropylphosphate siRNA (19-mer) at concentrations ranging from 25 to 200 nM, RT-Q-PCR analysis of the chinese hamster GAPDH mRNA level shows an equivalent mRNA down-regulation for the two compounds, although down-regulation is slightly more pronounced in the case of a "wild-type" siRNA at high concentrations. However, when transfected at the same concentrations with the cationic lipid Oligofectamine, both compounds showed exactly the same mRNA inhibition.

Taken together, these data shows that modification or absence of oligonucleotidic overhangs on the 3'-end of siRNAs are in CHO cells not critical regarding their interfering activity.

3.3 Blunt-ended siRNA activity in human cells

To validate this first result in human cells, siRNA sequence targeting the human GAPDH mRNA open reading frame are designed. Both blunt-ended and wild-type siRNAs silence the human GAPDH in HeLa cells with the same level of target down-regulation (Figure. 3).

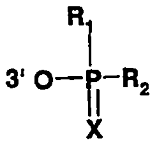
3.4 3'-end functional requirements of blunt-ended siRNAs

Having shown in two different mammalian cell-lines and against different targets that depletion of the overhangs does not impair the silencing activity of the resulting blunt-ended siRNAs, we also evaluate whether the 3'-ribonucleotidic position on the first 3'-ribonucleotide of a blunt-ended siRNA would require a specific chemical moiety to be optimal in the silencing process.

As shown in Figure 4, both blunt-ended 3'-hydroxy and 3'-phosphate siRNAs lead to a similar level of targeted GAPDH mRNA down-regulation in HeLa cells as the wild-type 21-mer siRNA, with a high selectivity as compared to the three nt mismatch controls.

Claims:

1. Double-stranded RNA with at least one blunt end comprising at least one 3'-end of formula:



$$3'\,O-\overset{\displaystyle R_1}{\underset{\displaystyle \overset{\|}{X}}{P}}-R_2$$

formula I

wherein

X is O or S

R_1 and R_2 are independently OH, NH_2, SH, alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms and functional groups, preferably a heteroatom selected from the group of N, O, or S or a functional group selected from the group OH, NH_2, SH, carboxylic acid or ester;

or R_1 and R_2 may be of formula Y-Z where Y is O, N, S and Z is H, alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be substituted by additional heteroatoms, preferably a heteroatom selected from the group of N, O, or S;

and wherein said double-stranded RNA mediates RNA interference.

2. Double-stranded RNA according to claim 1, wherein R_1 is OH.

3. Double-stranded RNA according to claim 1 or 2 comprising a region of 15 to 30 nucleotides complementary to a target gene.

4. Double-stranded RNA according to claim 1, 2 or 3 wherein R_1 and R_2 are independently OH, NH_2, SH, lower alkyl, lower aryl, lower alkyl-aryl, lower aryl-alkyl, where lower alkyl, lower aryl, lower alkyl-aryl, lower aryl-alkyl can be substituted by additional heteroatoms and functional groups, preferably a heteroatom selected from the group of N, O or S or a functional group selected from the group OH, NH_2, SH, carboxylic acid or ester; or, R_1 and R_2 may be of formula Y-Z where Y is O, N, S, B and Z is alkyl, aryl, alkyl-aryl, aryl-alkyl, where alkyl, aryl, alkyl-aryl, aryl-alkyl can be

substituted by additional heteroatoms, preferably a heteroatom selected from the group of N, O, or S, provided that not both R_1 and R_2 are OH.

5. Double-stranded RNA according to claim 1, 2, 3 or 4 wherein R_1 is OH and R_2 comprises from 1 to 24 C-atoms.

6. Double-stranded RNA according to any of claims1, 2, 3, 4 or 5 wherein said RNA comprises two blunt ends.

7. Double-stranded RNA according to claim 1 to 5~~6~~ wherein Z is one or more abasic nucleosides.

8. A method for the inhibition of a target gene comprising introducing into a cell a dsRNA according to any of claims 1 to 6~~7~~.

9. The method of claim 7~~8~~ wherein the target gene is a pathogen-associated gene, a viral gene or an oncogene.

10. A kit comprising reagents for inhibiting expression of a target gene in a cell, wherein said kit comprises dsRNA according to any of claims 1 to 6~~7~~ into a cell and a means for introduction of a dsRNA into a cell in an amount sufficient to inhibit expression of the target gene.

11. A pharmaceutical composition comprising an effective amount of at least one dsRNA according to any of claims 1 to 6~~7~~ for the inhibition of a target gene.

12. A method for identifying and/or characterizing pharmacological agents acting on at least one target protein comprising: contacting a eukaryotic cell capable of expressing at least one endogenous gene coding for the protein(s) of interest with

    (a) at least one dsRNA molecule according to the present invention, which is capable of inhibiting the expression of the gene(s) encoding the protein(s) of interest and

(b) a test substance or a collection of test substances wherein pharmacological properties of said test substance or said collection are to be identified and/or characterized.

13. Use of a double stranded RNA according to claims 1 to 6~~7~~ for the inhibition of a target gene.

FIGURE 1

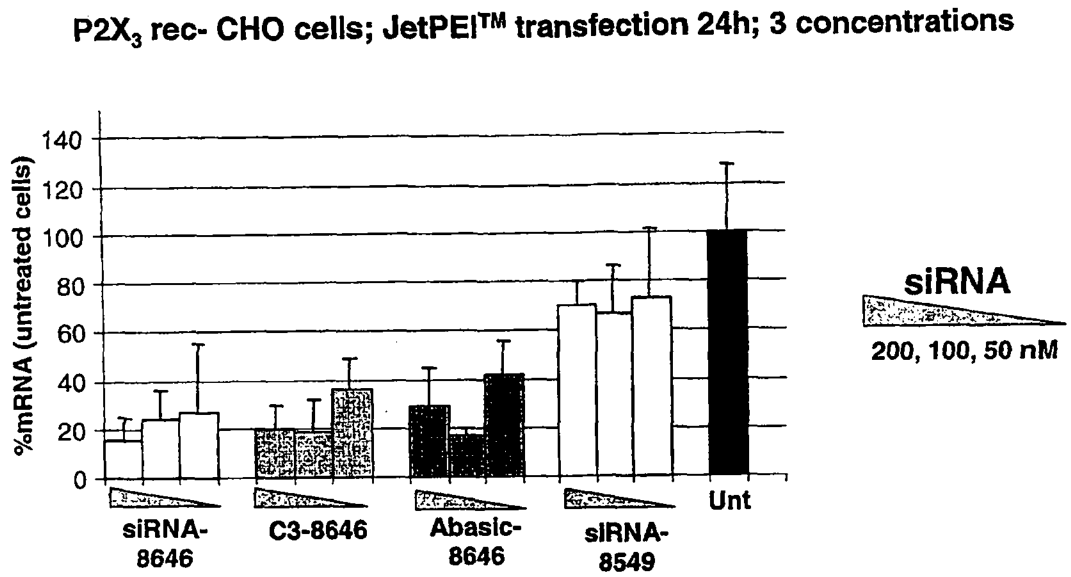
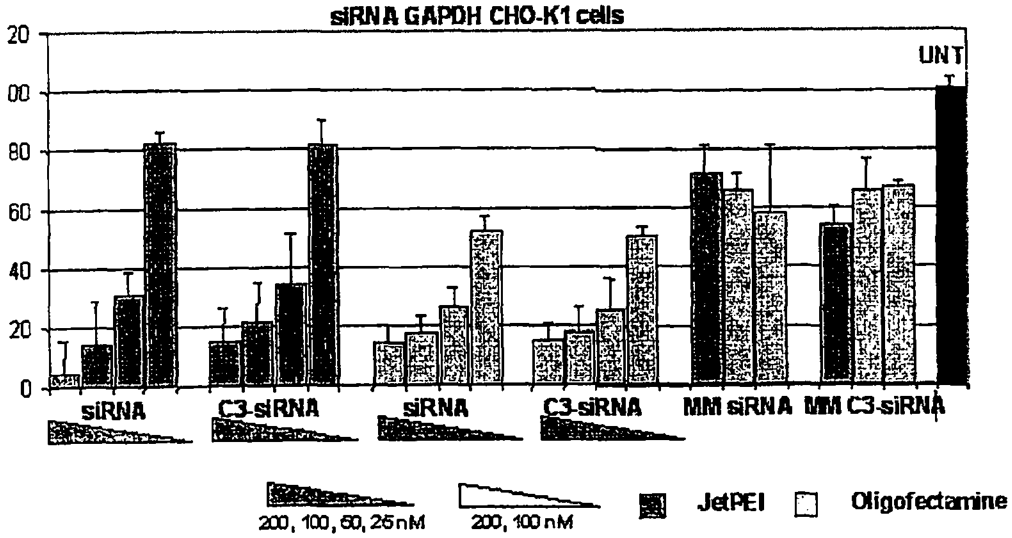


FIGURE 2



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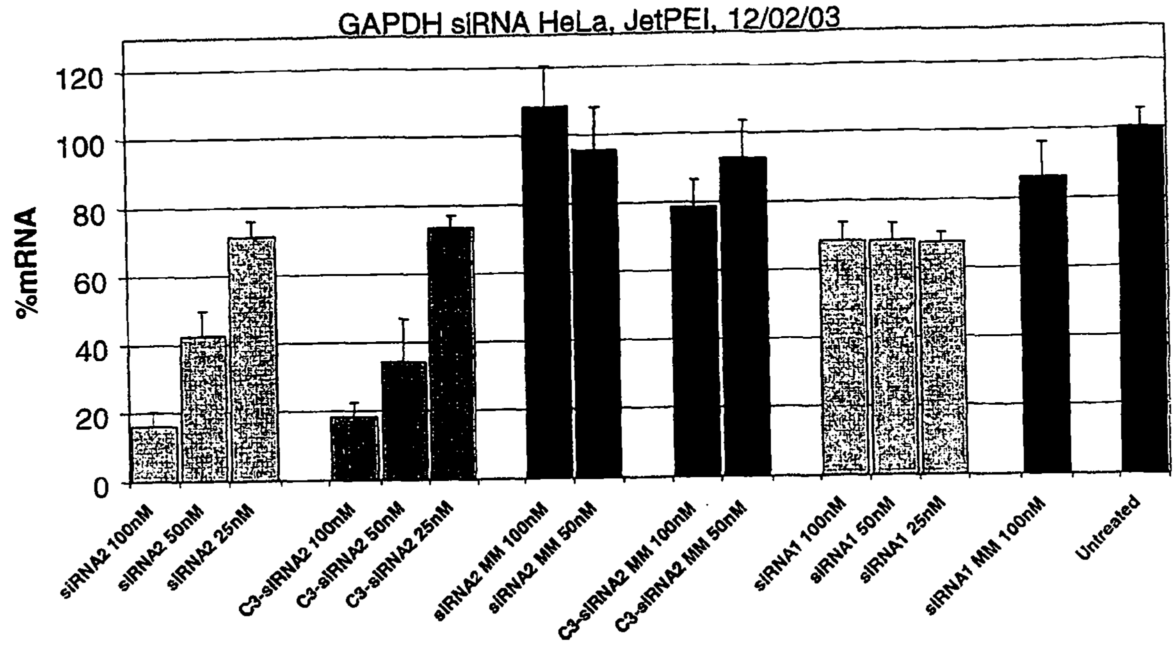

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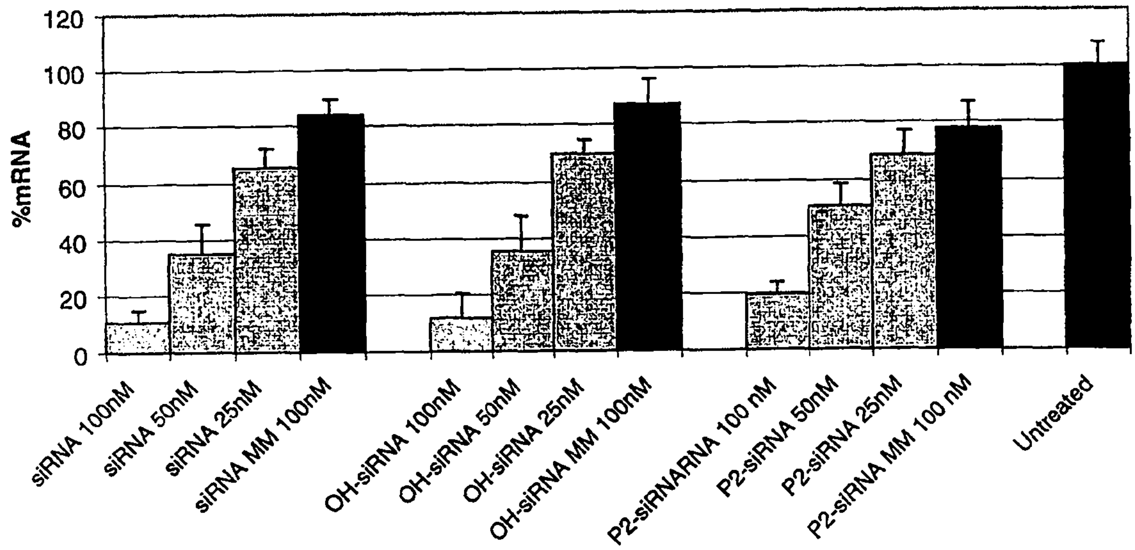

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