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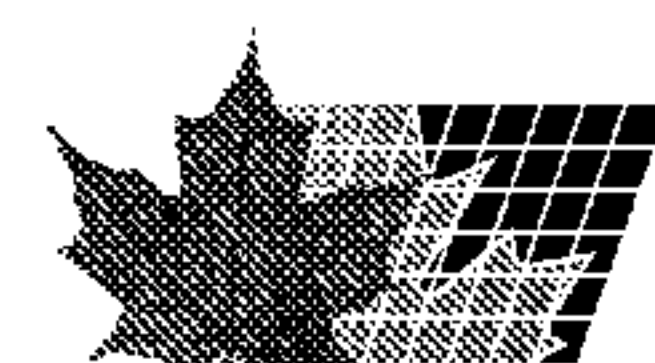
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(54) Title: SYNTHETIC DOUBLE-STRANDED OLIGONUCLEOTIDES FOR SPECIFIC INHIBITION OF GENE
EXPRESSION

(57) **Abrégé/Abstract:**

The invention relates to oligonucleotide derivatives which are at least partially double stranded and which comprise a 2'5' linked oligonucleotide radical on at last one 3'- end. The invention also relates to the use thereof for targeted inhibition of gene expression.



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(54) Title: SYNTHETIC DOUBLE STRANDED OLIGONUCLEOTIDES FOR TARGETED INHIBITION OF GENE EXPRESSION

(54) Bezeichnung: SYNTHETISCHE DOPPELSTRÄNGIGE OLIGONUCLEOTIDE ZUR GEZIELTEN HEMMUNG DER GENEXPRESSION

(57) Abstract: The invention relates to oligonucleotide derivatives which are at least partially double stranded and which comprise a 2'5' linked oligonucleotide radical on at last one 3'-end. The invention also relates to the use thereof for targeted inhibition of gene expression.

(57) Zusammenfassung: Die Erfindung betrifft Oligonucleotid-Derivate, die zumindest teilweise doppelsträngig sind und die an mindestens einem 3'-Ende einen 2'5'-verknüpften Oligonucleotid-Rest aufweisen sowie deren Verwendung zur gezielten Inhibition der Genexpression.



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Description

Synthetic double-stranded oligonucleotides for specific inhibition of gene expression

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The present invention relates to novel oligonucleotide derivatives which are at least partly double-stranded and which have a 2'5'-linked oligonucleotide residue on at least one 3' end and to the use thereof for specific inhibition of gene expression.

10

The inhibition of gene expression with the aid of synthetic nucleic acids is becoming increasingly important. Typical representatives of these synthetic nucleic acids (oligonucleotides) are antisense oligonucleotides, ribozymes, DNA enzymes and external guide sequences (EGS). "Antisense oligonucleotides" are short single-stranded nucleic acid derivatives which bind via Watson-Crick base pairing to a complementary messenger ribonucleic acid (mRNA) whose translation into the corresponding protein is to be inhibited. In most cases antisense oligonucleotides exhibit their action according to a mechanism which is supported by cellular ribonuclease H (RNase H); numerous studies have shown evidence for this. RNase H which is present in all cells recognizes a double strand of DNA and RNA and cuts the mRNA complementary to said oligonucleotide via hydrolysis of one or in most cases more phosphodiester bonds. The way in which the oligonucleotides have to be modified in order for activation of RNase H to take place is known and is described, for example, in Uhlmann (2000) Curr. Opin. Drug Discov. Dev. 3, 203-213. Synthetic ribozymes carry this nuclease activity in their sequence. The most common type of ribozyme is the "hammerhead" ribozyme in which the consensus sequence GAAAC which is derived from naturally occurring ribozymes forms the RNase part and the flanking sequences form the antisense oligonucleotide part. DNA enzymes which, however, are not derived from naturally occurring ribozyme motifs but have been found by in-vitro selection, act in a similar way. EGS are

30

synthetic RNA analogs which activate the cellular RNase P and bind via appropriate flanking sequences to the target mRNA and induce a specific mRNA degradation. All of the abovementioned oligonucleotide derivatives are employed such that the RNA-binding part is single-stranded and that interaction
5 with the target mRNA inhibits the gene expression in a sequence-specific manner.

It is also possible for the gene expression to be inhibited by interaction with particular proteins with the aid of "decoy" oligomers which mimic the binding
10 regions for transcription factors. Treatment with decoy agents makes it possible to intercept particular proteins, in particular transcription factors, in a sequence-specific manner and thereby prevent a transcription activation.

Finally, there are oligonucleotide derivatives which act at the DNA level. These
15 include triplex-forming oligonucleotides (anti-gene oligonucleotides). "Anti-gene oligonucleotides" bind via Hoogsteen base pairing in the large groove of the DNA double helix with formation of a triple helix, thereby causing sequence-specific inhibition of the transcription of the genes. Another group of intracellularly acting oligonucleotide derivatives, the chimeraplasts, are used for
20 specific gene correction.

A common problem of the inhibition of gene expression with the aid of synthetic oligonucleotides is that it is always necessary to assay a relatively large number of oligonucleotides against various regions of the target nucleic acid, in order to
25 identify an efficient sequence. Furthermore, antisense oligonucleotides often inhibit gene expression only inefficiently or incompletely. Moreover, sequence-unspecific side effects were observed, which may be caused by the fact that even relatively short part sequences of about five bases in length activate RNase H. This is shown, for example, by "Woolf et al. (1992). Proc. Natl. Acad.
30 Sci. U.S.A. 89, 7305-7309)". However, there are also side effects which are caused by interaction of the antisense oligonucleotides with proteins.

Recently, the use of double-stranded RNA for inhibiting gene expression has been described. Double-stranded RNA (dsRNA) is a signal for particular cells and organisms to induce a sequence-specific degradation of mRNA according to a process which is known as RNA interference (RNAi). The RNAi
5 phenomenon was observed in a number of different organisms such as, for example, *C. elegans*, flies, fungi, plants and mouse embryos. RNAi is believed to be very similar or identical to post-transcriptional gene silencing (PTGS) found in plants. A simple injection of dsRNA of more than 500 base pairs (bp) in length, whose sense-strand sequence is identical to the target mRNA to be
10 inhibited, can specifically inhibit expression of a target gene having the corresponding DNA sequence. This does not impair the expression of nonhomologous genes and the base sequence of the target gene is not altered. RNAi is a post-transcriptional process in which the dsRNA is first cleaved into relatively small fragments which are then probably used for sequence-specific
15 degradation of the target mRNA. Apart from the double-strand nuclease activity, an ATP-dependent helicase activity is also discussed. However, the detailed mechanism of this process is not known. Studies in plants show that small dsRNA fragments of about 25 nucleotides in length represent the "active species" of the RNAi, which transfer the sequence-specific recognition of the
20 target RNA to a cellular ribonuclease.

The efficiency of inhibiting gene expression with the aid of dsRNA decreases drastically with decreasing fragment length of said dsRNA. Thus it was found that dsRNA of from 400 to 540 bp inhibits the gene expression very effectively,
25 while dsRNA of from 200 to 300 bp does so less efficiently and dsRNA of from 50 to 100 bp has no effect whatsoever. Only recently has it been found that small dsRNA fragments of from 26 to 81 bp in length are after all capable of causing an RNAi-like process. However, the observed inhibition appears to be substantially weaker than in the case of long dsRNA fragments. The inhibition
30 produced by a 717 bp dsRNA was markedly more pronounced than that by a dsRNA of less than 200 bp in length. The 26 bp dsRNA was approx. 250 times

less active than the 81 bp dsRNA. Moreover, the inhibition was sequence-dependent, since a different 27 bp dsRNA was not active at all.

Elbashir et al. Nature (2001) 411, 494 described an inhibition of gene
5 expression in cell culture by a double-stranded RNA comprising 21 nucleotides. The corresponding dsRNA molecules contained on the 3' ends of both strands overhangs of 2 3'5'-linked nucleotides which have either uracil or thymine bases. The authors also note that 2'5'-oligoadenylate-activated ribonuclease
10 processes lead to an intrinsic sequence-unspecific degradation of the target RNA. However, an obvious disadvantage is the fact that a successful inhibition strongly depends on the cell line used.

Previously, the gene expression was efficiently inhibited mainly by using dsRNA of more than 100 bp in length. This relatively long dsRNA is accessible only via
15 in-vitro or in-vivo transcription from the corresponding DNA via suitable transcription systems. Another limitation of RNAi with long dsRNA is the fact that only particular organisms such as *C. elegans*, zebra fish, plants, particular types of fungi, *Drosophila*, oocytes and embryos of mice allow sequence-specific inhibition by dsRNA, while most animal cells when treated with dsRNA
20 cause apoptosis. Long dsRNA still inhibits gene expression when the sequence homology is from 70 to 90%. For this reason, it is possible in the case of gene families with high sequence homology for misinterpretations of the phenotype to occur by simultaneous inhibition of the expression of a plurality of not completely homologous genes.

25

The treatment of cells with dsRNA, for example with dsRNA viruses, generally leads to an apoptotic process or to the sequence-unspecific degradation of the mRNA due to induction of a 2'5'-oligoadenylate-synthase activity. The infected cell synthesizes in response to the viral dsRNA trimeric or tetrameric adenylate
30 (2'5'-A) with the unusual 2'5'-phosphodiester-internucleoside bond. 2'5'-A is phosphorylated by cellular kinases on its 5' end and then activates a nuclease called RNase L. 2'5'-A may also be chemically synthesized and be introduced

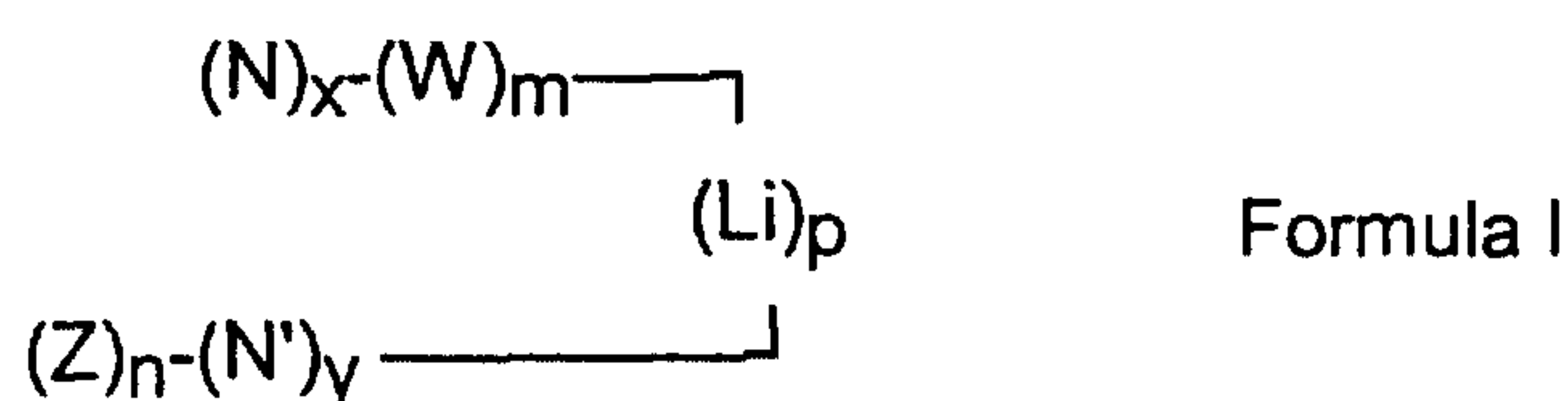
into the cell (Torrence et al. (1994) Curr. Med. Chem 1, 176-191). However, synthetic 2'5'-A activates RNase L only if it has been converted to the 5'-phosphate or 5'-triphosphate form. RNase L activated by 5'-p-2'5'-A (p is phosphate, diphosphate or triphosphate) then degrades the entire RNA of the cell in a sequence-unspecific manner. In addition, it was shown that it is possible to inhibit gene expression sequence-specifically with the aid of antisense oligonucleotide conjugates with a 5'-p-2'5'-A residue. For this purpose, however, it is essential that the 5' end of the 2'5'-A residue is not linked to the oligonucleotide but is present as phosphate or triphosphate (Torrence et al. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1300-4). Furthermore, the target RNA-recognizing oligonucleotide part (antisense part) must be in single-stranded form. For the reasons mentioned above, oligonucleotides having on their 3' ends 2'5'-A residues which consequently have no free 5'-phosphate or triphosphate function have not been described previously as inhibitors of gene expression. The inhibition of gene expression by the single-stranded, 5'-phosphorylated 5'-p-2'5'-A antisense oligonucleotide conjugates is a variation of the antisense principle and is therefore also subject to the limitations of the antisense-oligonucleotide approach.

20 Recently, oligonucleotides have been used increasingly as tools for studying the function of new genes (functional genomics). The use of antisense oligonucleotides and ribozymes for sequence-specific inhibition of gene expression of new genes coding for proteins with unknown function is made more difficult by the fact that generally a large variety of oligonucleotides of different sequences have to be assayed, and this is a disadvantage in particular for a high-throughput process.

It is therefore an object of the present invention to provide novel chemically modified oligonucleotides with significantly improved inhibition of gene expression, which circumvent the abovementioned limitations of the conventional methods and agents.

According to the invention, this object is achieved by novel oligonucleotide derivatives which are at least partly double-stranded and which have a 2'5'-linked oligonucleotide residue on at least one 3' end. The sequence of the novel oligonucleotide derivatives is complementary in one strand to the RNA sequence whose translation is to be inhibited and corresponds in the other strand to the RNA to be inhibited. The RNA double strand thus corresponds to the base sequence of the gene whose expression may be inhibited, with the deoxyribonucleotides being replaced by the corresponding ribonucleotides and thymidine being replaced by uridine.

The invention accordingly provides double-stranded nucleic acid derivatives of the formula I,



where

N and N' are naturally or not naturally occurring nucleotides which are at least partly complementary to one another and where at least one nucleotide strand (N)_x or (N')_y is complementary or partially complementary to a target gene or to the RNA corresponding thereto,

x and y independently of one another are 10 to 100, preferably 15 to 45 and particularly preferably 16 to 25,

n is 0 to 20, preferably 2 to 10, particularly preferably 3 to 6,

m is 0 to 20, preferably 2 to 10, particularly preferably 3 to 6,

p is 0 to 20, preferably 0 to 5,

W and Z are naturally or not naturally occurring nucleotides which are linked via a 3'5' or 2'5' internucleoside bond,

Li is a linker which covalently links the two nucleotide strands,

wherein at least two residues Z or W are linked via a 2'5' internucleoside bond and are present in single-stranded form and m and n are not simultaneously zero.

Preference is given to oligonucleotides of the formula I whose homologous target RNA has the following sequence patterns:

10 5'-(U)_v-(N)_z-(U)_w

5'-(U)_v-(N)_z-UX

5'-UX-(N)_z-UX and

5'-(U)_v-(N)_z

15 where v and w independently of one another are 2 to 20, preferably 2 to 10, particularly preferably 2 to 6 and
z is 15 to 25, preferably 16 to 23 and particularly preferably 19 to 21 and
U is uridine, N is A, G, C or U, and X is A, G or C, preferably A.

20 If the gene whose expression is to be inhibited contains, for example, the following DNA sequence

5'-TTTTGAAGCGAAGGTTGTGGATCTG (Seq ID No. 1)

or the following RNA sequence

5'-UUUUGAAGCGAAGGUUGUGGAUCUG (Seq ID No. 2)

25 then the target RNA has the following sequence pattern

5'-(U)_v-(N)_z-UX, where v is 4, z is 19 and X is G.

Furthermore, preference is given to oligonucleotides of the formula I in which one or more phosphodiester bonds have been replaced, for example by phosphorothioate bonds or N3',P5'-phosphoramidate bonds. Particular
5 preference is given to oligonucleotides of the formula I in which one or more phosphodiester bonds have been replaced by phosphorothioate residues. The phosphorothioate residues are preferably introduced on the 3' ends, the 5' ends and on the internal pyrimidine nucleotides C and U, in particular if several pyrimidine nucleotides succeed one another in the sequence. The
10 phosphorothioate residues may be introduced in the upper or lower strand, preferably in both strands.

A particular embodiment of the invention comprises the use of a mixture of two or more oligonucleotide derivatives in accordance with formula 1 for inhibiting
15 gene expression. The oligonucleotide derivatives in this case may be directed against different regions of an RNA or against the RNA of different genes.

Surprisingly, it was found that partially double-stranded nucleic acids which have a 2'5'-linked oligoadenylate residue on at least one end inhibit the gene
20 expression much more strongly than double-stranded RNA comprising only 3'5'-linked nucleotides. The double-stranded RNA fragments having the 2'5'-linked oligoadenylate residue were also more active than the corresponding single strand having a 2'5'-linked oligoadenylate residue. This is surprising in that normally antisense oligonucleotides and sense oligonucleotides cancel out each
25 other's action. Another surprise is that the 2'5'-linked oligoadenylate residue need not have a free end with a 5'-phosphate or 5'-triphosphate residue, in order to be able to exhibit its activity. It also came as a complete surprise that the 2'5'-linked oligoadenylate residue can be bound to the 3'5'-linked RNA directly via the 5' function. Surprisingly, a 2'5'-A residue in the coding strand had
30 only a very small positive effect on the activity of the double strand as long as a 2'5'-A residue was present in the noncoding strand. Surprisingly, double-stranded RNA having on the lower strand overhanging ends of four to six bases

is much more active than one having only two overhanging bases. In contrast to the antisense oligonucleotides of which generally a plurality of sequences (for example 10 to 100) have to be assayed in order to obtain an active sequence, surprisingly all of the assayed double-stranded oligonucleotides of the formula I
5 were inhibition-active if they were homologous to the corresponding gene sequences. Surprisingly, no intrinsic unspecificity with the 2'5'-linked oligonucleotides was observed either. Previously, a 2'5'-active inhibition via double-stranded RNA had always been associated with an unspecific, i.e. sequence-independent, effect (Bass, Nature (2001) 411, 428).

10

A mixture of two 2'5'-(A)₄- oligonucleotides which do not form base pairs is less effective than the double-stranded molecules. Furthermore, compounds of the formula I are less effective or ineffective if their sequence is not perfectly homologous to the target RNA.

15

Surprisingly, the oligonucleotides of the invention also had an inhibitory sequence-specific effect on human primary cells. As far as we know, the inhibition of gene expression by double-stranded oligonucleotides in human primary cells has not been observed previously. It was likewise unexpected that
20 for this only one strand of the double-stranded RNA had to have an overhanging end.

The inventive oligonucleotides of the formula I may also be used for inhibiting gene expression in cells which express only a small amount of, a defective or
25 no 2'5'-oligoadenylate synthase. The described dsRNA molecules comprising 21 nucleotides (Elbashir et al. Nature (2001) 411, 494) do not have these properties.

It is furthermore also possible to use the oligonucleotides of the formula I for
30 treating patients having a deficiency or defect in 2'5'-oligoadenylate synthase. Patients with CFS (chronic fatigue syndrome), for example, may also be treated.

The sequences of the double-stranded nucleic acids which are used for inhibiting the gene expression of particular targets are selected on the basis of the corresponding gene sequences. The sequences of said genes are obtained by sequencing or from gene databases. An example which may be illustrated here is the inhibition of luciferase (firefly) by double-stranded nucleic acids. The accession number for this gene is U47298. The coding region of firefly luciferase comprises 1 653 nucleotides. The following four regions may be selected, inter alia, as target sequences for the inhibition by double-stranded nucleic acids.

10

```

      Gcttttacagatgcacatatcgaggtggacatcacttacg
121  -----+-----+-----+-----+ 160
      cgaaaatgtctacgtgtatagctccacctgtagtgaatgc
15  (Seq ID No. 3)

```

20

```

      ccgcgaacgacattttataatgaacgtgaattgctcaacag
311  -----+-----+-----+-----+ 350
      ggcgcttgctgtaaatattacttgacttaacgagttgtc
      (Seq ID No. 4)

```

25

```

      gcggtcggtaaagttgttccattttttgaagcgaagggttg
1081 -----+-----+-----+-----+ 1120
      cgccagccatttcaacaaggtaaaaaacttcgcttccaac
      (Seq ID No. 5)

```

30

```

      attttttgaagcgaagggttgatctggataccgggaaa
1101 -----+-----+-----+-----+ 1140
      taaaaaacttcgcttccaacacctagacctatggcccttt
      (Seq ID No. 6)

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35

The corresponding double-stranded RNA for these regions then has the following sequence.

40

```

      GCUUUUACAGAUGCACAUUAUCGAGGUGGACAUCACUUACG
121  -----+-----+-----+-----+ 160
      CGAAAAUGUCUACGUGUAUAGCUCCACCUGUAGUGAAUGC
      (Seq ID No. 7, 8)

```

311 CCGCGAACGACAUUUAUAAUGAACGUGAAUUGCUC AACAG 350
 -----+-----+-----+-----+
 GGCGCUUGCUGUAAAUUUACUUGCACUUAACGAGUUGUC
 (Seq ID No. 9, 10)
 5
 1081 GCGGUCGGUAAAGUUGUCCAUUUUUUGAAGCGAAGGUUG 1120
 -----+-----+-----+-----+
 CGCCAGCCAUUUCAACAAGGUAAAAACUUCGCUUCCAAC
 (Seq ID No. 11, 12)
 10
 1101 AUUUUUUGAAGCGAAGGUUGUGGAUCUGGAUACCGGGAAA 1140
 -----+-----+-----+-----+
 UAAAAACUUCGCUUCCAACACCUAGACCUAUGGCCCUUU
 (Seq ID No. 13, 14)
 15

The inventive double-stranded nucleic acids derived therefrom have, for
 example, the sequences listed below and are characterized in that two or more
 20 nucleotides (indicated here by lower-case letters) in at least one strand are
 linked via a 2'5'-internucleoside bond. The overhanging ends may not be
 complementary to the target RNA. The number represents the region on the
 RNA, up means upper (coding) strand and lo means lower (noncoding) strand.
 Preference is given to 2'5'-linked adenylate residues. If in the formula I p equals
 25 zero, the two strands are held together only via hydrogen bonds.

30 5' UACAGAUGCACAUAUCGAGGUGaaaa luc-126_up
 3' aaaaAUGUCUACGUGUAUAGCUCCAC luc-126_lo
 35 5' UAUAAUGAACGUGAAUUGCUCaaaa luc-326_up
 3' aaaaAUUUACUUGCACUUAACGAG luc-326_lo
 40 5' GGUAAAGUUGUCCAUUUUUUaaaa luc-1087_up
 3' aaaaCCAUUUCAACAAGGUAAAAAA luc-1087_lo
 45 5' GAAGCGAAGGUUGUGGAUCUGaaaa luc-1108_up
 3' aaaaCUUCGCUUCCAACACCUAGAC luc-1108_lo

45 luc-126_up: Seq ID No. 15;
 luc-126_lo: Seq ID No. 16;
 luc-326_up: Seq ID No. 17;
 luc-326_lo: Seq ID No. 18;

luc-1087_up: Seq ID No. 19;
 luc-1087_lo: Seq ID No. 20;
 luc-1108_up: Seq ID No. 21;
 luc-1108_lo: Seq ID No. 22;

5

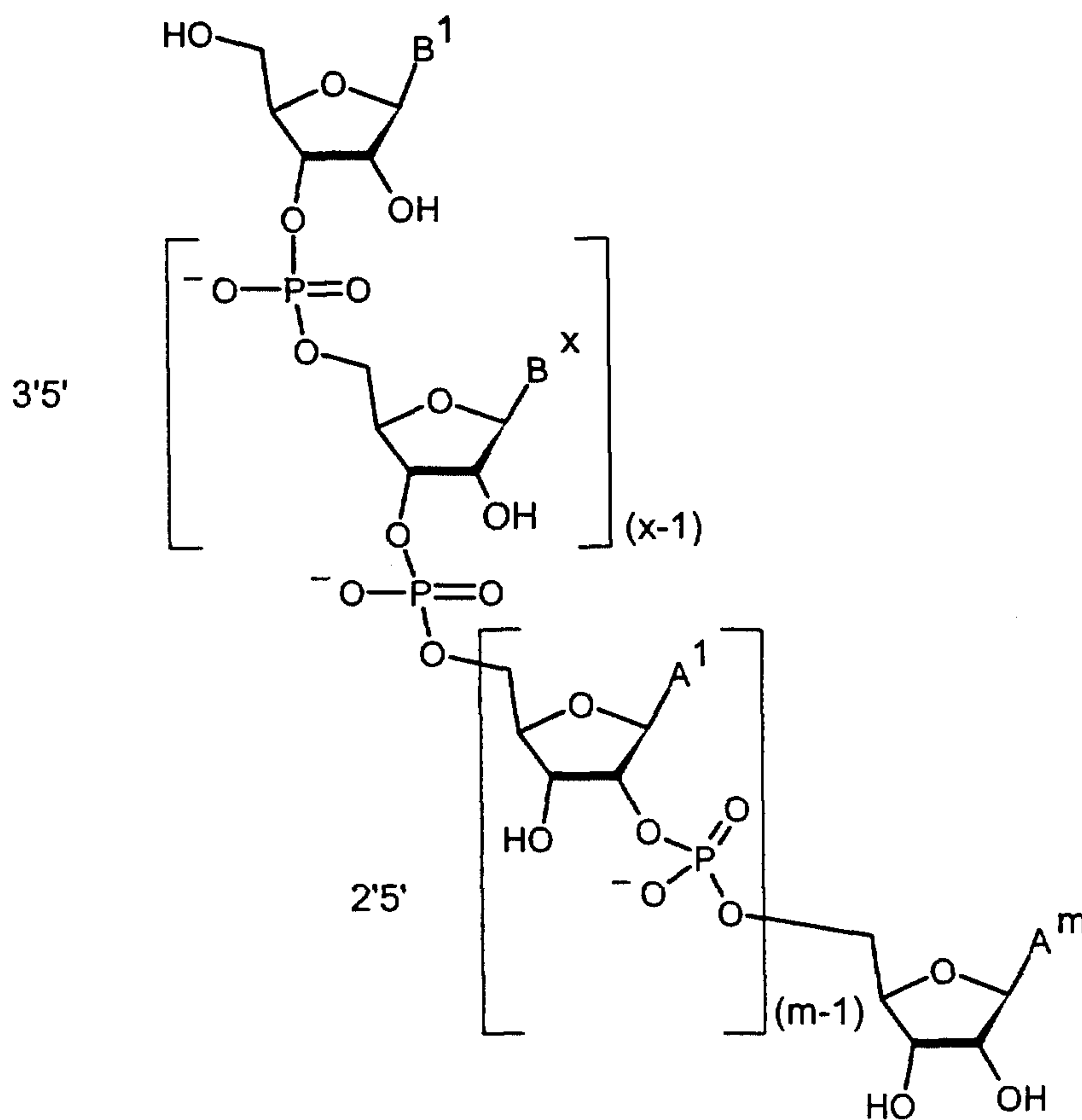
However, the oligonucleotides may also have, for example, 6 overhanging nucleotides in the lower strand, which are partially or completely 2'5'-linked.

10 5' GAAGCGAAGGUUGUGGAUCUG (Seq ID No. 23)
 3' aaaaAACUUCGCUUCCAACACCUAGAC (Seq ID No. 24)

5' GAAGCGAAGGUUGUGGAUCUG
 3' aaaaaaCUUCGCUUCCAACACCUAGAC

15

An example of the structure of a strand of the double-stranded oligonucleotide is given below:



20

In order to test for biological activity, the following oligonucleotides may be prepared, hybridized to a double strand, where appropriate, and tested for inhibition of luciferase activity in assay mixtures.

5	3'	aaaaCCAUUUCAACAAGGUAAAAAA	luc-1087_lo
	5'	GGUAAAGUUGUCCAUUUUUUaaaa	luc-1087_up
10			
	5'	GGUAAAGUUGUCCAUUUUUUaaaa	luc-1087_up
	3'	aaaaCCAUUUCAACAAGGUAAAAAA	luc-1087_lo
15	5'	GGUAAAGUUGUCCAUUUUUU	luc-1087_up
	3'	aaaaCCAUUUCAACAAGGUAAAAAA	luc-1087_lo
20			
	3'	aaaaCUUCGCUUCCAACACCUAGAC	luc-1108_lo
	5'	GAAGCGAAGGUUGUGGAUCUGaaaa	luc-1108_up
25			
	5'	GAAGCGAAGGUUGUGGAUCUGaaaa	luc-1108_up
	3'	aaaaCUUCGCUUCCAACACCUAGAC	luc-1108_lo
30	5'	GAAGCGAAGGUUGUGGAUCUG	luc-1108_up
	3'	aaaaCUUCGCUUCCAACACCUAGAC	luc-1108_lo
35	5'	GAAGCGAAGGUUGUGGAUCUGaaaa	luc-1108_up
	3'	aaaaaaCUUCGCUUCCAACACCUAGAC	luc-1108_lo
40	5'	GAAGCGAAGGUUGUGGAUCUG	luc-1108_up
	3'	aaaaaaCUUCGCUUCCAACACCUAGAC	luc-1108_lo
	5'	GAAGCGAAGGUUGUGGAUCUGaaaa	luc-1108_up
	3'	teg-aaCUUCGCUUCCAACACCUAGAC	luc-1108_lo-teg
45	5'	GGUAAAGUUGUCCAUUUUUUaaaa	luc-1087_up
	3'	aaaaCUUCGCUUCCAACACCUAGAC	luc-1108_lo
	3'	aaaaCCAUUUCAACAAGGUAAAAAA	luc-1087_lo
	3'	aaaaCUUCGCUUCCAACACCUAGAC	luc-1108_lo

In order to show that the oligonucleotides of the invention may also be used for inhibiting gene expression in cells of other species, in particular in human primary cells, a compound of the invention may be directed, for example,
 5 against a human gene or the corresponding RNA thereof and assayed in human cells (HUVEC, human umbilical vein endothelial cells). For this, Edg-1 DNA (accession number M31210) from the gene database, for example, could be transcribed into the corresponding double-stranded RNA and the following two regions (175 and 725) could be selected for synthesizing appropriate
 10 oligonucleotides.

Edg-1 RNA:

"175"

15 GACCUCGGUGGUGUUCAUUCUCAUCUGCUGCUUUAUCAUCCUGGAGAACAUUUUGUCUU
 141 -----+-----+-----+-----+-----+-----+ 200
 CUGGAGCCACCACAAGUAAGAGUAGACGACGAAAUAGUAGGACCUCUUGUAGAAACAGAA
 (Seq ID No. 25, 26)

20 "725"

AUUUCCAAGGCCAGCCGCAGCUCUGAGAAUGUGGCGCUGCUCAAGACCGUAAUUAUCGUC
 721 -----+-----+-----+-----+-----+-----+ 780
 UAAAGGUUCCGGUCGGCGUCGAGACUCUACACCGCGACGAGUUCUGGCAUUAUAGCAG
 (Seq ID No. 27, 28)

25

Examples of the possible structure of the corresponding oligonucleotides are disclosed below:

30

5' AUCAUCCUGGAGAACAUUUU **edg-1-175_up**
 3'-aaaaUAGUAGGACCUCUUGUAGAAA **edg-1-175_lo**

35

5' CCAAGGCCAGCCGCAGCUCUG **edg-1-725_up**
 3'-aaaaGGUUCCGGUCGGCGUCGAGAC **edg-1-725_lo**

Mismatch control

5' CCACGGACAGACGCCGCUGUG **edg-1-mm_up**
 3'-aaaaGGUGCCUGUCUGCGGCGAC **edg-1-mm_lo**

5' AUCAUCCUGGAGACAUCUUU-**FITC** **edg-1-175_up_FITC**
 3'-aaaaUAGUAGGACCUCUUGUAGAAA **edg-1-175_lo**

edg-1-175_up: Seq ID No. 29;
 10 edg-1-175_lo: Seq ID No. 30;
 edg-1-725_up: Seq ID No. 31;
 edg-1-725_lo: Seq ID No. 32;
 edg-1-mm_up: Seq ID No. 33;
 edg-1-mm_lo: Seq ID No. 34;

15

The number represents the region on the edg-1 RNA, up means upper (coding) strand and lo means lower (noncoding) strand. The mismatch control forms a perfectly paired double strand but differs from edg-1 RNA in 5 nucleotides (underlined as mismatch). FITC is a commercially available fluorescent marker.

20

Furthermore, the following oligonucleotides directed against edg-1 were prepared, which have improved nuclease stability and increased inhibitory activity and are derived from the above edg-1 sequences.

25 5' A U*C A U*C*C*U G G A G A A*C A*U C*U*U*U-**FITC**
 5' A U*C A U*C*C*U G G A G A A*C A*U C*U*U*U-**teg**
 3'-a*a*a a U*A G*U A G G A C*C*U C*U*U G*U*A G A A*A

30 5' C*C*A A G G*C*C A G*C*C G*C A G C*U*C*U*G-**teg**
 3'-a*a*a a G G U*U*C*C G G*U*C G G*C G*U*C G A G A*C

5' C*C*A C*G G A C*A G A C*G C*C*G C*U*G*U*G-**teg**
 35 3'-a*a*a a G G U*G C*C*U G*U*C*U G*C G G*C G A*C A*C

Another embodiment of the present invention uses double-stranded oligonucleotides which have a 2'5'-linked overhanging residue only in one strand, preferably the noncoding strand, the two strands being held together via one or more covalent bonds. A possible example of this covalent bond is a linker of the (Li)_p type..

edg-1-175_hairpin

10

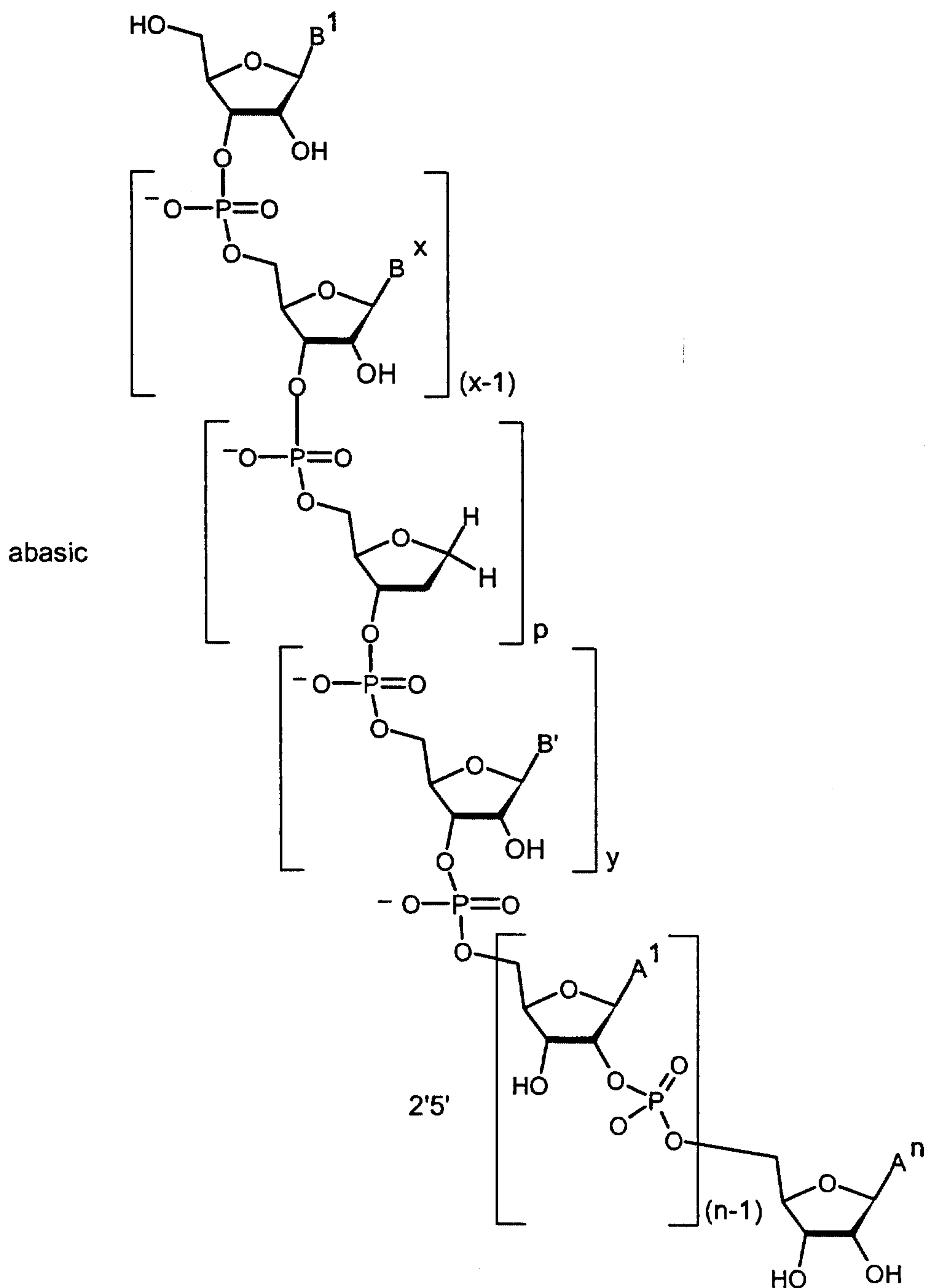
5' AUCAUCCUGGAGAACAUCUUU—
(Li)_p
3'—aaaaUAGUAGGACCUCUUGUAGAAA—

15 For example, the two nucleic acid strands may be held together via a plurality of nucleotide residues, preferably four to five nucleotide residues (Li is N, preferably thymidine, p is 4 to 20, preferably 4 or 5).

5' AUCAUCCUGGAGAACAUUU—
20 (N)_p
3' -aaaaUAGUAGGACCUCUUGUAGAAA—

In the case of the abasic linkers, the molecule has, for example, the following formula:

25



However, the two strands may also be held together via non-nucleotide residues. Examples of suitable non-nucleotide linkers are one or more
 5 oligoethylene glycol phosphate residues, preferably tri- and hexaethylene glycol phosphate residues. Examples of other linkers are alkanediol phosphates, preferably propane-1,3-diol phosphate, butane-1,4-diol phosphate and dodecyl-1,12-diol phosphate. Further examples of linkers are the abasic 1',2'-dideoxyribose units which are generally 3'-O, 5'-O-linked. The linker

reagents required for the synthesis are mostly commercially available (e.g. Spacer 9, Spacer 18, Spacer C3, Spacer C12, dSpacer (abasic) from Glen Research, Sterling, VA). The linker may also incorporate functional groups which, for example, increase the cellular uptake of the double-stranded
 5 oligonucleotide, increase the bioavailability, increase the nuclease stability or increase the biological activity. It is furthermore possible to incorporate groups for labeling, for example fluorescent markers or biotin markers, into the linker.

Other examples of linkers for linking the two strands are disulfide bridges (-S-S-) or pyrothiophosphate bridges $-(O_3)-P-S-S-P(O_3)-$.
 10

Alternatively, it is possible to hold the double strands together noncovalently via lipophilic or ionic interactions or via hydrogen bonds.

15 The specificity of the inhibition of luciferase expression was checked on the basis of double-stranded control oligonucleotides which are not completely homologous to the target RNA and have, for example, 2 or 4 base mismatches. Other control oligonucleotides vary with respect to the overhanging ends.

20 5' GAAGCGAAGGUUGUGGAUCUG
 3' aaaaCUUCGCUUCCAACACCUAGAC

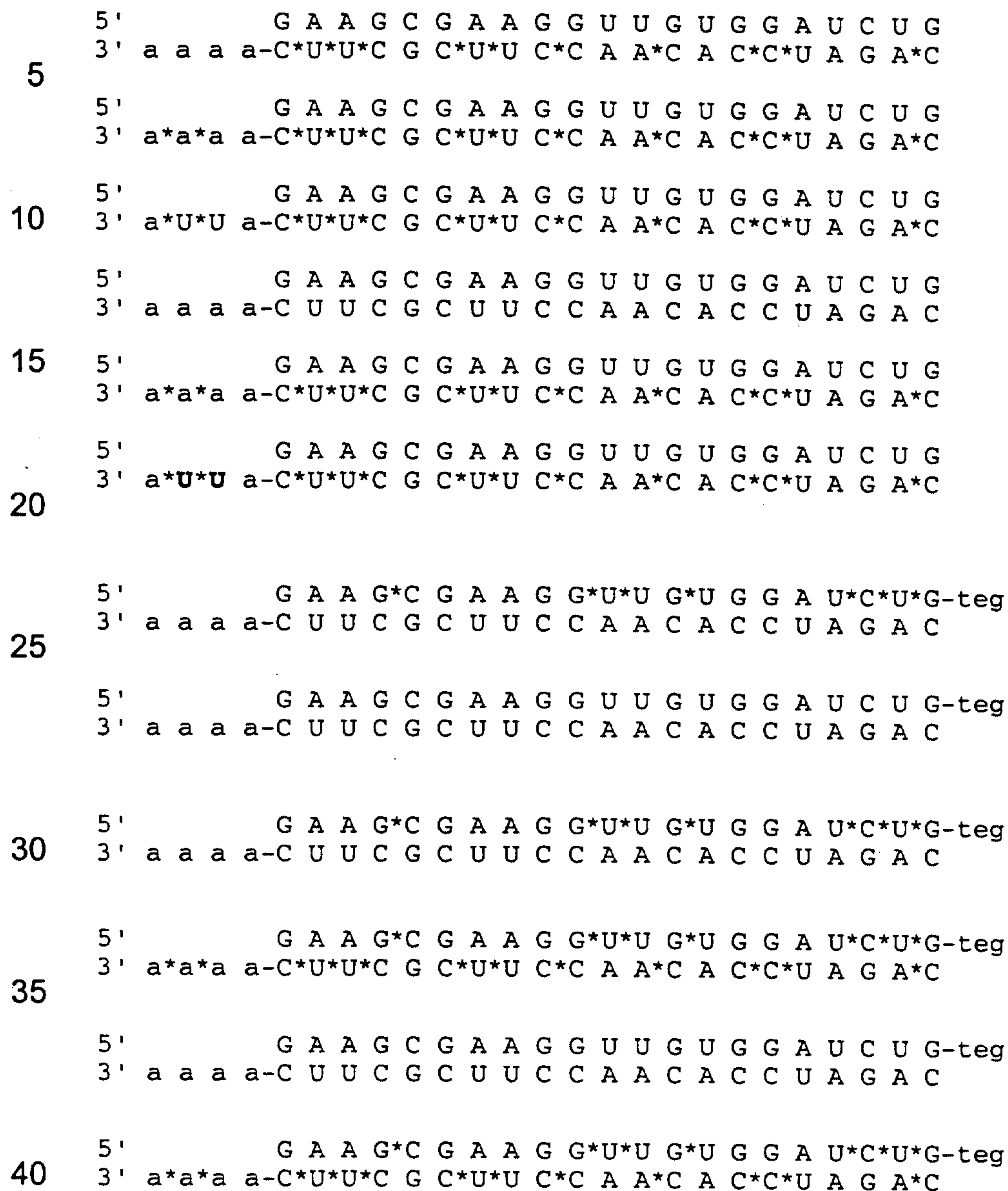
5' GAAGCGAAGUUGGUGGAUCUG 2mm (Seq ID No. 35)
 3' aaaaCUUCGCUUCAACACCUAGAC (Seq ID No. 36)

25 5' GAAGAGAAGUUGGUGGCUCUG 4mm (Seq ID No. 37)
 3' aaaaCUUCUCUUCAACACCGAGAC (Seq ID No. 38)

30 5' GAAGCGAAGGUUGUGGAUCUG
 3' CUUCGCUUCCAACACCUAGAC (Seq ID No. 39)

5' GAAGCGAAGGUUGUGGAUCUGaaaa
 3' CUUCGCUUCCAACACCUAGAC (Seq ID No. 40)

35 The following oligonucleotides which have, for example, either phosphorothioate (asterisks) or 2'O-methylribonucleotide (underlined) modifications in the upper or lower strand or in both strands were prepared.



The inventive nucleic acid derivatives of formula I are synthesized from oligonucleotides. For example, an oligonucleotide may be synthesized completely from the nucleotides adenosine phosphate, guanosine phosphate, inosine phosphate, cytidine phosphate, uridine phosphate and thymidine phosphate. Preference is given to oligonucleotides which are synthesized from ribonucleotides, the "oligoribonucleotides". In other embodiments of the present invention, an oligonucleotide may contain, where appropriate, one or more

modifications, for example chemical modifications. An oligonucleotide may have a plurality of identical and/or different modifications.

Examples of chemical modifications are known to the skilled worker and are described, for example, in E. Uhlmann and A. Peyman, *Chemical Reviews* 90 (1990) 543 and "Protocols for Oligonucleotides and Analogs" *Synthesis and Properties & Synthesis and Analytical Techniques*, S. Agrawal, Ed, Humana Press, Totowa, USA 1993, J. Hunziker and C. Leumann 'Nucleic Acid Analogs: Synthesis and Properties' in *Modern Synthetic Methods* (Ed. Beat Ernst and C. Leumann) Verlag Helvetica Chimica Acta, Basle, p. 331-417, RP Iyer et al. *Curr Opin Mol Therap* (1999) 1:344-358; S. Verma and F. Eckstein, *Annu Rev Biochem* (1998) 67:99-134; JW Engels and E. Uhlmann : Chemistry of oligonucleotides. In: *Pharmaceutical aspects of oligonucleotides*. Couvreur P, Malvy C (Eds), Taylor & Francis, London, (2000): 35-78.

15

The chemical modification of an oligonucleotide may include, for example,

a) replacing completely or partially the phosphoric diester bridges with, for example, phosphorothioate, phosphorodithioate, $\text{NR}^1\text{R}^{1'}$ phosphoramidate, boranophosphate, $(\text{C}_1\text{-C}_{21})\text{-O-alkyl phosphate}$, $[(\text{C}_6\text{-C}_{12})\text{aryl-(C}_1\text{-C}_{21})\text{-O-alkyl}]$ phosphate, $(\text{C}_1\text{-C}_8)\text{alkyl phosphonate}$ and/or $(\text{C}_6\text{-C}_{12})\text{aryl phosphonate}$ bridges, where

R^1 and $\text{R}^{1'}$ independently of one another are hydrogen, $(\text{C}_1\text{-C}_{18})\text{alkyl}$, $(\text{C}_6\text{-C}_{20})\text{aryl}$, $(\text{C}_6\text{-C}_{14})\text{aryl-(C}_1\text{-C}_8)\text{alkyl}$, preferably hydrogen, $(\text{C}_1\text{-C}_8)\text{alkyl}$ and/or methoxyethyl, particularly preferably hydrogen, $(\text{C}_1\text{-C}_4)\text{alkyl}$ and/or methoxyethyl,

25

or

R^1 and $\text{R}^{1'}$, together with the nitrogen atom to which they are bound, form a 5-6-membered heterocycle which may additionally contain another heteroatom selected from the group consisting of O, S, N;

30

b) replacing completely or partially the 3'- and/or 5'-phosphoric diester bridges with "dephospho" bridges (described, for example, in Uhlmann, E. and

- Peyman, A. in "Methods in Molecular Biology", Vol. 20, "Protocols for Oligonucleotides and Analogs", S. Agrawal, Ed., Humana Press, Totowa 1993, Chapter 16, 355ff), for example with formacetal, 3'-thioformacetal, methylhydroxylamine, oxime, methylenedimethylhydrazo, dimethylenesulfone and/or silyl groups;
- c) replacing partially the sugar phosphate backbone, for example with "morpholino" oligomers (described, for example, in E. P. Stirchak et al., Nucleic Acids Res. 17 (1989) 6129 and in J. Summerton and D. Weller, Antisense and Nucleic Acid Drug Dev. 7 (1997) 187-195) and/or with polyamide nucleic acids ("PNAs") (described, for example, in P. E. Nielsen et al, Bioconj. Chem. 5 (1994) 3) and/or phosphomonoester nucleic acids ("PHONAs") (described, for example, in Peyman et al., Angew. Chem. Int. Ed. Engl. 35 (1996) 2632-2638);
- d) replacing partially the β -D-ribose units with, for example, β -D-2'-deoxyribose, α -D-2'-deoxyribose, L-2'-deoxyribose, 2'-F-2'-deoxyribose, 2'-F-2'-deoxyarabinofuranose, 2'-O-(C₁-C₆)alkylribose, 2'-O-(C₂-C₆)alkenylribose, 2'-[O-(C₁-C₆)alkyl-O-(C₁-C₆)alkyl]ribose, 2'-NH₂-2'-deoxyribose, β -D-xylofuranose, β -D-arabinofuranose, α -arabinofuranose, 2,4-dideoxy- β -D-erythrohexopyranose, conformationally restricted sugar analogs such as LNA (Locked nucleic acids; Singh et al., Chem. Commun. 4 (1998) 455; Singh et al. Chem. Commun. 12 (1998) 1247) and carbocyclic (described, for example, in Froehler, J.Am.Chem.Soc. 114 (1992) 8320) and/or open-chain sugar analogs (described, for example, in Vandendriessche et al., Tetrahedron 49 (1993) 7223) and/or bicyclo sugar analogs (described, for example, in M. Tarkov et al., Helv. Chim. Acta 76 (1993) 481). The 2'-modified oligonucleotide analogs are described in detail in Manoharan, Biochim. Biophys. Acta (1999) 117 and conformationally restricted oligonucleotide analogs in Herdewijn, Biochim. Biophys. Acta (1999) 167;
- e) modifying and, respectively, completely or partially replacing the natural nucleoside bases with, for example, 5-(hydroxymethyl)uracil, 5-aminouracil,

pseudouracil, pseudoisocytosine, dihydrouracil, 5-(C₁-C₆)alkyluracil, 5-(C₂-C₆)-alkenyluracil, 5-(C₂-C₆)alkynyluracil, 5-(C₁-C₆)alkylcytosine, 5-(C₂-C₆)alkenylcytosine, 5-(C₂-C₆)alkynylcytosine, 5-fluorouracil, 5-fluorocytosine, 5-chlorouracil, 5-chlorocytosine, 5-bromouracil, 5-bromocytosine or 7-deaza-7-substituted purines.

Heterocyclic base modifications are described, for example, in Herdewijn, *Antisense & Nucl. Acid Drug Dev.* (2000) 297.

The chemical modification of the oligonucleotide furthermore comprises conjugating an oligonucleotide with one or more molecules which influence advantageously the properties (e.g. nuclease stability, affinity for target sequence, pharmacokinetics) of said oligonucleotide and/or, during hybridization of the modified oligonucleotide to the target sequence, attack said target sequence with binding and/or crosslinking (oligonucleotide conjugates). Examples thereof are conjugates with polylysine, with intercalators such as pyrene, acridine, phenazine, phenanthridine, with fluorescent compounds such as fluorescein, with crosslinkers such as psoralen, azidoproflavin, with lipophilic molecules such as (C₁₂-C₂₀)alkyl, with lipids such as 1,2-dihexadecyl-rac-glycerol, with steroids such as cholesterol or testosterone, with vitamins such as vitamin E, with poly- or oligoethylene glycol, with (C₁₂-C₁₈)alkyl phosphate diesters and/or with -O-CH₂-CH(OH)-O-(C₁₂-C₁₈)alkyl. Such molecules may be conjugated at the 5' and/or 3' end and/or within the sequence, for example at a nucleobase. Examples of oligonucleotide conjugates known to the skilled worker are described in Manoharan (2001) *Conjugated Oligonucleotides in Antisense technology*. In: Crooke (Editor) *Antisense Technology*. Marcel Dekker, New York.

A specific embodiment of the chemical modification relates to conjugation of the oligonucleotide a) with lipophilic molecules, for example (C₁₂-C₂₀)alkyl, b) with steroids such as cholesterol and/or testosterone, c) with poly- and/or oligoethylene glycol, d) with vitamin E, e) with intercalators such as pyrene, f)

with (C₁₄-C₁₈)alkyl phosphate diesters and/or g) with -O-CH₂-CH(OH)-O-(C₁₂-C₁₆)alkyl.

Another specific embodiment of the chemical modification relates to
 5 derivatization of the oligonucleotide, as described in WO 01/08707, as aryl ester conjugate, for example as FDA conjugate, which derivatization benefits the cellular uptake of said oligonucleotides.

Methods for preparing said oligonucleotide derivatives are known to the skilled
 10 worker and described, for example, in Uhlmann, E. & Peyman, A., Chem. Rev. 90 (1990) 543 and/or M. Manoharan in "Antisense Research and Applications", Crooke and Lebleu, Eds., CRC Press, Boca Raton, 1993, chapter 17, p. 303ff. and/or EP-A 0 552 766. The double strand may be prepared, for example, via hybridization of the two single strands by heating and then cooling solutions of
 15 said two single strands in diluted buffer. In principle, the methods described for gene synthesis can be used for preparing the double strand (Chemical and Enzymatic Synthesis of Gene Fragments (Editors: Gassen and Lang)Verlag Chemie, Weinheim (1982)).

20 In further specific embodiments of the present invention, the oligonucleotide may have on its 3' and/or 5' end 3'-3' and/or 5'-5' inversions. This type of chemical modification is known to the skilled worker and described, for example, in M. Koga et al., "Alternating α,β -oligothymidylates with alternating (3'→3') and (5'→5')-internucleotidic phosphodiester linkages as models for antisense
 25 oligodeoxyribonucleotides." J. Org. Chem. 56 (1991) 3757.

The 2'5'-overhanging residue may contain, for example, adenosine, 3'-deoxyadenosine (cordycepin), inosine, 8-bromoadenosine, 8-methyladenosine and other 8-substituted adenosine derivatives. The ribose residue may also be derivatized as 3'-O-methyladenosine. The internucleoside bonds in the 2'5'-
 30 overhanging part are preferably phosphodiester and phosphorothioate bonds. Common derivatives of 2'5'-adenylate, and the synthesis and RNase L activation thereof are described in the literature (Player et al. (1998) Pharmacol. Ther. 78, 55).

The invention further provides methods for preparing the oligonucleotides. The oligonucleotides described may be prepared with the aid of various known chemical methods, as described, for example, in Eckstein, F. (1991)
5 "Oligonucleotides and Analogues, A Practical Approach", IRL Press, Oxford. The oligonucleotides may also be prepared by methods which, where appropriate, contain one or more enzymic steps.

The invention furthermore provides the use of the oligonucleotides for
10 modulating and for completely or partially inhibiting the expression of particular target genes, for example for completely or partially inhibiting translation. The invention furthermore relates to the use of said oligonucleotides for modulating and for completely or partially inhibiting expression in cells which have only a small amount of, a defective or no 2'5'-oligoadenylate synthase.

15

The invention furthermore provides the use of said oligonucleotides as pharmaceuticals or to the use of said oligonucleotides for the production of pharmaceuticals. In particular, it is possible to use said oligonucleotides in pharmaceuticals which are suitable for the prevention and/or treatment of
20 diseases which accompany the expression or overexpression of particular genes.

The invention further provides the use of said oligonucleotides or of pharmaceuticals containing said oligonucleotides for the treatment of diseases
25 in which specific genes are the cause or are involved, due to overexpression.

The pharmaceuticals of the present invention may be used, for example, for the treatment of disorders caused by viruses, for example by CMV, HIV, HSV-1, HSV-2, hepatitis B, hepatitis C viruses, or papillomaviruses. Pharmaceuticals of
30 the present invention are particularly suitable for the treatment of RNA viruses such as, for example, polio viruses, VSV or Influenza virus, in particular also of double-stranded RNA viruses such as reoviruses, for example.

The pharmaceuticals of the present invention are also suitable, for example, for cancer treatment. In this case it is possible, for example, to use oligonucleotide sequences which are directed against targets responsible for the development or growth of cancers. Examples of such targets are:

5

1) nuclear oncoproteins such as, for example, c-myc, N-myc, c-myb, c-fos, c-fos/jun, PCNA, p120,

10

2) cytoplasmic/membrane-associated oncoproteins such as, for example, EJ-ras, c-Ha-ras, N-ras, rrg, bcl-2, cdc-2, c-raf-1, c-mos, c-src, c-abl, c-ets,

15

3) cellular receptors such as, for example, EGF receptor, Her-2, c-erbA, VEGF receptor (KDR-1), retinoid receptors, protein kinase regulatory subunit, c-fms, Tie-2, c-raf-1 kinase, PKC-alpha, protein kinase A (R1 alpha),

4) cytokines, growth factors, extracellular matrix such as, for example, CSF-1, IL-6, IL-1a, IL-1b, IL-2, IL-4, IL-6, IL-8, bFGF, VEGF, myeloblastin, fibronectin,

5) inhibitors of tumor suppressor genes such as, for example, MDM-2.

20

The pharmaceuticals of the present invention are further suitable, for example, for the treatment of disorders which are influenced by integrins or cell-cell adhesion receptors, for example by VLA-4, VLA-2, ICAM, VCAM or ELAM.

25

The pharmaceuticals of the present invention are also suitable, for example, for preventing restenosis. In this connection, it is possible to use, for example, oligonucleotide sequences which are directed against targets responsible for proliferation or migration. Examples of such targets are:

30

1) nuclear transactivator proteins and cyclins such as, for example, c-myc, c-myb, c-fos, c-fos/jun, cyclins and cdc2 kinase,

2) mitogens or growth factors such as, for example, PDGF, bFGF, VEGF, EGF, HB-EGF and TGF- β ,

35

3) cellular receptors such as, for example, bFGF receptor, EGF receptor and PDGF receptor.

The invention further relates to oligonucleotides for the treatment of asthma, with expression of the adenosine-A1 receptor, adenosine-A3 receptor, Bradikinin receptor or of IL-13 being inhibited with the aid of suitable
5 oligonucleotides.

The invention also relates to oligonucleotides, for example, for the treatment of cardiovascular diseases, with, for example, expression of the β 1-adrenergic receptor or of a protein from the EDG family such as, for example, Edg-1 being
10 inhibited.

The invention also relates to oligonucleotides, for example, for the treatment of diabetes, with expression of PTP-1B being inhibited, for example.

15 The pharmaceuticals may be used, for example, in the form of pharmaceutical preparations which may be administered orally, for example in the form of tablets, coated tablets, hard or soft gelatin capsules, solutions, emulsions or suspensions. They may also be administered rectally, for example in the form of suppositories, or parenterally, for example in the form of injection solutions.
20 Pharmaceutical preparations may be produced by processing said compounds in therapeutically inert organic and inorganic carriers. Examples of such carriers for tablets, coated tablets and hard gelatin capsules are lactose, corn starch or derivatives thereof, talc and stearic acid or salts thereof. Carriers suitable for the preparation of solutions are water, polyols, sucrose, invert sugar and glucose.
25 Carriers suitable for injection solutions are water, alcohols, polyols, glycerol and vegetable oils. Carriers suitable for suppositories are vegetable and hardened oils, waxes, fats and semisolid polyols. The pharmaceutical preparations may also contain preservatives, solvents, stabilizers, wetting agents, emulsifiers, sweeteners, colorants, flavorings, salts for modifying the osmotic pressure,
30 buffers, coating agents, antioxidants and, where appropriate, other therapeutically active substances.

Preferred administration forms are topical administrations, local administrations such as, for example, with the aid of a catheter or by inhalation, injections or infusions, and oral administration. For injection, the oligonucleotide derivatives are formulated in a liquid solution, preferably in a physiologically acceptable
5 buffer such as, for example, Hank's solution or Ringer's solution. However, the oligonucleotides may also be formulated in solid form and be dissolved or suspended prior to use. The dosages preferred for systematic administration are from approx. 0.01 mg/kg to approx. 50 mg/kg body weight and day.

10 The invention furthermore relates to pharmaceutical preparations which contain oligonucleotides and/or physiologically tolerated salts thereof in addition to pharmaceutically suitable carriers and/or additives.

The oligonucleotides and/or physiologically tolerated salts thereof may be
15 administered to animals, preferably to mammals, and in particular to humans as pharmaceuticals on their own, in mixtures with one another or in the form of pharmaceutical preparations which permit topical, percutaneous, parenteral or enteral application and which contain as active ingredient an active dose of at least one oligonucleotide in addition to common pharmaceutically suitable
20 carriers and additives. The preparations normally contain about from 0.1 to 90% by weight of the therapeutically active compound. For the treatment of skin disorders such as, for example, psoriasis or vitiligo, a topical application, for example in the form of ointments, lotions or tinctures, emulsions, or suspensions is preferred.

25

The pharmaceutical preparations are produced in a manner known per se (e.g. Remingtons Pharmaceutical Sciences, Mack Publ. Co., Easton, PA.), with pharmaceutically inert inorganic and/or organic carriers being used. For the production of pills, tablets, coated tablets and hard gelatin capsules, lactose,
30 corn starch and/or derivatives thereof, talc, stearic acid and/or salts thereof, etc. may be used, for example. Examples of carriers for soft gelatin capsules and/or suppositories are fats, waxes, semisolid and liquid polyols, natural and/or

hardened oils, etc. Examples of carriers suitable for the preparation of solutions and/or syrups are water, sucrose, invert sugar, glucose, polyols, etc. Carriers suitable for the preparation of injection solutions are water, alcohols, glycerol, polyols, vegetable oils, etc. Carriers suitable for microcapsules, implants and/or rods are mixed polymers of glycolic acid and lactic acid. Liposome formulations which are known to the skilled worker (N. Weiner, Drug Develop Ind Pharm 15 (1989) 1523; "Liposome Dermatics, Springer Verlag 1992), for example HVJ liposomes (Hayashi, Gene Therapy 3 (1996) 878), are also suitable. Dermal administration may also be carried out, for example, with the aid of ionophoretic methods and/or with the aid of electroporation. In addition, it is possible to use lipofectins and other carrier systems, for example those which are used in gene therapy. Particularly suitable systems are those which can be used to introduce oligonucleotides into eukaryotic cells with great efficiency.

In addition to the active substances and the carriers, a pharmaceutical preparation may also contain additives such as, for example, fillers, extenders, disintegrants, binding agents, lubricants, wetting agents, stabilizers, emulsifiers, preservatives, sweeteners, colorants, flavorings or aromatizers, thickening agents, diluents, buffer substances, furthermore solvents and/or solubilizers and/or agents for achieving a depot effect, and also salts for modifying the osmotic pressure, coating agents and/or antioxidants. They may also contain two or more different oligonucleotides and/or their physiologically tolerated salts and furthermore, in addition to at least one oligonucleotide, one or more other therapeutically active substances.

25

The dose may vary within wide limits and, in each individual case, has to be adjusted to the individual circumstances.

30 Examples

1. Synthesis of the oligonucleotides of the formula 1

a) 3' aaaaaUGUCUACGUGUAUAGCUCCAC (The bases indicated by lower-case letters have a 2'5'-internucleoside bond).

5 The syntheses were carried out in an ABI 394 DNA or Expedite synthesizer (Applied Biosystems, Weiterstadt, Germany). The synthesis cycles recommended by the manufacturer were used but for the ribonucleoside-2'-O-phosphoramidites the condensation step was doubled (with a coupling time of in
10 30 s. The solid phase used was a 1000 Å controlled pore glass (CPG) support which had 5'-O-dimethoxytrityl-N-6-benzoyladenine (NSS-6101-10A, Chemgenes, Waltham, MA) bound via the 2' or 3' position of the sugar. After removing the 5'-O-dimethoxytrityl group by cleavage with trichloroacetic acid, the 2'5'-linked oligonucleotide part was synthesized by four condensations with
15 5'-O-dimethoxytrityl-N-6-benzoyl-3'-O-tertbutyldimethylsilyladenine-2'-O-phosphoramidite (ANP-5681, Chemgenes). This was followed by synthesizing the 3'5'-linked oligonucleotide part by repeated condensation with the corresponding 5'-O-dimethoxytrityl-2'-O-tertbutyldimethylsilylnucleoside-3'-O-phosphoramidites (ANP-5671 to ANP-5680, Chemgenes). The CPG support
20 was incubated with 750 µl of conc. ammonia/ethanol (3:1, v:v) with shaking at 30°C for 24 hours in order to remove the oligomer from the support and to deprotect the phosphate and amino protective groups. The supernatant was separated from the support which was then washed twice more with 150 µl of conc. ammonia/ethanol (3:1, v:v). The combined supernatants were
25 concentrated under reduced pressure and the residue was incubated with shaking in 1200 µl of triethylamine x 3HF (very toxic) at 30°C for 24 hours in order to remove the silyl protective groups. This is followed by adding 700 µl of n-butanol, cooling the mixture on dry ice for 30 minutes and centrifugation. The pellet was washed with butanol two more times. In addition, a sodium chloride
30 precipitation was then carried out. 116 OD (260) of the crude product which shows only one main band in gelelectrophoresis were obtained. The product

was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 8256.0, found 8256.8).

b) 3' aaaaAUAUUACUUGCACUUAACGAG

5

The synthesis was carried out analogously to that of example 1a), with the 2'5'-linked oligonucleotide part being synthesized by three condensations with 5'-O-dimethoxytrityl-N-6-benzoyl-3'-O-tertbutyldimethylsilyladenosine-2'-O-phosphoramidite (ANP-5681, Chemgenes). 112 OD (260) of the crude product which shows only one main band in gelelectrophoresis were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 7958.9, found 7958.6).

10

c) 3' aaaaCCAUUUCAACAAGGUAAAAAA

15

The synthesis was carried out analogously to that of example 1b). 117 OD (260) of the crude product which shows only one main band in gelelectrophoresis were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 8012.0, found 8011.8).

20

d) 3' aaaaCUUCGCUUCCAACACCUAGAC

The synthesis was carried out analogously to that of example 1b). 117 OD (260) of the crude product which shows only one main band in gelelectrophoresis were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 7868.8, found 7868.6).

25

e) 3' aaaaaaCUUCGCUUCCAACACCUAGAC

30

The synthesis was carried out analogously to that of example 1a), with the 2'5'-linked oligonucleotide part being synthesized by five condensations with 5'-O-dimethoxytrityl-N-6-benzoyl-3'-O-tertbutyldimethylsilyladenosine-2'-O-

phosphoramidite (ANP-5681, Chemgenes). 112 OD (260) of the crude product which shows only one main band in gelelectrophoresis were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 8527.2, found 8527.5).

5

f) 3' teg-AACUUCGCUUCCAACACCUAGAC (where teg is a triethylene glycol phosphate residue)

The synthesis was carried out analogously to that of example 1a), using a
10 triethylene glycol succinate-derivatized CGP support and preparing the 2'5'-linked oligonucleotide part according to the sequence by condensation with 5'-O-dimethoxytrityl-N-6-benzoyl-3'-O-tertbutyldimethylsilyladenosine-2'-O-phosphoramidite (ANP-5681, Chemgenes). 83 OD (260) of the crude product which contains a triethylene glycol phosphate residue on the 2' position of the
15 3'-terminal adenosine were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 7422.5, found 7422.6).

g) 5' GAAGCGAAGGUUGUGGAUCUGaaaa

20

The synthesis was carried out analogously to that of example 1b). 108 OD (260) of the crude product which shows only one main band in gelelectrophoresis were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 8149.0, found 8148.9).

25

h) 5'-GGUAAAGUUGUCCAUUUUUUaaaa

The synthesis was carried out analogously to that of example 1b). 112 OD (260) of the crude product which shows only one main band in gelelectrophoresis
30 were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 7930.7, found 7930.7).

i) 5'-G A A G C G A A G G U U G U G G A U C U G

The synthesis was carried out analogously to that of example 1a) but only 3'5'-internucleotide bonds were introduced. 82 OD (260) of the crude product which shows only one main band in gelelectrophoresis were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 6832.2, found 6831.8).

j) 3' a a a a-C*U*U*C G C*U*U C*C A A*C A C*C*U A G A*C

10

The synthesis was carried out analogously to that of example 1b). The phosphorothioate residue was introduced by using the Beaucage reagent (RN-1535, Chemgenes, Waltham, MA) rather than the iodine solution in the particular oxidation step. 112 OD (260) of the crude product which shows only one main band in gelelectrophoresis were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 8029.4, found 8031.2).

k) 3' a*a*a a-C*U*U*C G C*U*U C*C A A*C A C*C*U A G A*C

20

The synthesis was carried out analogously to that of example 1j). 128 OD (260) of the crude product which shows only one main band in gelelectrophoresis were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 8061.6, found 8062.8).

25

l) 3' A*U*u a-C*U*U*C G C*U*U C*C A A*C A C*C*U A G A*C (contains only one 2'5'-internucleotide bond between u a)

The synthesis was carried out analogously to that of example 1k). 96 OD (260) of the crude product which shows only one main band in gelelectrophoresis were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 8015.5, found 8017.8).

30

m) 3' a a a a-C U U C G C U U C C A A C A C C U A G A C (The bases indicated by lower-case letters have a 2'5'-internucleoside bond; the underlined nucleotides are 2'-O-methylribonucleotides)

- 5 The synthesis was carried out analogously to that of example 1a). In the case of the underlined nucleotides, 5'-O-dimethoxytrityl-2'-O-methyl-ribonucleoside-3'-O-phosphoramidites (ANP-5751 to ANP-5758, Chemgenes) were condensed. 99 OD (260) of the crude product which shows only one main band in gelelectrophoresis were obtained. The product was further characterized by
10 means of HPLC and electrospray mass spectrometry (negative mode) (calc. 8163.4.6, found 8165.1).

n) 3' a*a*a a-C*U*U*C G C*U*U C*C A A*C A C*C*U A G A*C

- 15 The synthesis was carried out analogously to that of example 1m). 127 OD (260) of the crude product which shows only one main band in gelelectrophoresis were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 8356.1, found 8357.2).

20

o) 3'-aaaaUAGUAGGACCUCUUGUAGAAA (edg-1-175_lo)

- The synthesis was carried out analogously to that of example 1b). 134 OD (260) of the crude product which shows only one main band in gelelectrophoresis
25 were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 8037.9, found 8038.9).

p) 3'-aaaaGGUUCCGGUCGGCGUCGAGAC (edg-1-725_lo)

- 30 The synthesis was carried out analogously to that of example 1b). 134 OD (260) of the crude product which shows only one main band in gelelectrophoresis

were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 8075.9, found 8076.9).

q) 3'-aaaaGGUGCCUGUCUGCGGCGACAC (edg-1-mm_1o)

5

The synthesis was carried out analogously to that of example 1b). 109 OD (260) of the crude product which shows only one main band in gelelectrophoresis were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 8035.9, found 8037.0).

10

r) 5' AUCAUCCUGGAGAACAUCUUU (edg-1-175_up)

The synthesis was carried out analogously to that of example 1a) but only 3'5'-internucleotide bonds were introduced. The solid phase used was a 1000 Å controlled pore glass (CPG) support which held 5'-O-dimethoxytrityluridine (NSS-6104-10U, Chemgenes, Waltham, MA) bound via the 2' or 3' position of the sugar. 110 OD (260) of the crude product which shows only one main band in gelelectrophoresis were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 6618.0, found 6618.5).

20

s) 5' AUCAUCCUGGAGAACAUCUUU-FITC (edg-1-175_up_FITC)

The synthesis was carried out analogously to that of example 1a) but only 3'5'-internucleotide bonds were introduced. The solid phase used was a 500 Å controlled pore glass (CPG) support containing a protected fluorescein derivative (NSS-97505-A1CL, Chemgenes, Waltham, MA). 79 OD (260) of the crude product which shows only one main band in gelelectrophoresis were obtained. The product was further characterized by means of HPLC and electrospray mass spectrometry (negative mode) (calc. 7428.7, found 7432.3).

30

2. Inhibition of luciferase expression in SL-3 cells

In order to test for biological activity, the following oligonucleotides were prepared, where appropriate hybridized to give the double strand and tested for inhibition of luciferase activity in the following assay mixtures.

				Assay mixture	
10	3'	aaaaCCAUUUCAACAAGGUAAAAAA	luc-1087_lo	1	
	5'	GGUAAAGUUGUCCAUUUUUUaaaa	luc-1087_up	2	
15	5'	GGUAAAGUUGUCCAUUUUUUaaaa	luc-1087_up	3	
	3'	aaaaCCAUUUCAACAAGGUAAAAAA	luc-1087_lo		
	5'	GGUAAAGUUGUCCAUUUUUU	luc-1087_up	4	
20	3'	aaaaCCAUUUCAACAAGGUAAAAAA	luc-1087_lo		
	3'	aaaaCUUCGCUCCAACACCUAGAC	luc-1108_lo	5	
25	5'	GAAGCGAAGGUUGUGGAUCUGaaaa	luc-1108_up	6	
	5'	GAAGCGAAGGUUGUGGAUCUGaaaa	luc-1108_up	7	
30	3'	aaaaCUUCGCUCCAACACCUAGAC	luc-1108_lo		
	5'	GAAGCGAAGGUUGUGGAUCUG	luc-1108_up	8	
	3'	aaaaCUUCGCUCCAACACCUAGAC	luc-1108_lo		
35	5'	GAAGCGAAGGUUGUGGAUCUGaaaa	luc-1108_up	9	
	3'	aaaaaaCUUCGCUCCAACACCUAGAC	luc-1108_lo		
40	5'	GAAGCGAAGGUUGUGGAUCUG	luc-1108_up	10	
	3'	aaaaaaCUUCGCUCCAACACCUAGAC	luc-1108_lo		
	5'	GAAGCGAAGGUUGUGGAUCUGaaaa	luc-1108_up	11	
45	3'	teg-aaCUUCGCUCCAACACCUAGAC	luc-1108_lo-teg		
	5'	GGUAAAGUUGUCCAUUUUUUaaaa	luc-1087_up	12	
	3'	aaaaCUUCGCUCCAACACCUAGAC	luc-1108_lo		

3'	aaaaCCAUUUCAACAAGGUAAAAAA	luc-1087_lo	13
3'	aaaaCUUCGCUUCCAACACCUAGAC	luc-1108_lo	

5

The assay mixtures 1, 2, 5, 6, 12 and 13 contain RNA which is not in double-stranded form. In contrast, the oligonucleotides in the assay mixtures 3, 4, 7, 8, 9, 10 and 11 are paired as double strands. The double strands 7, 9 and 11 have in both strands an overhanging 2'5'-linked oligonucleotide residue. The

10 oligoribonucleotide luc-1108_lo contains on its 3' end only two overhanging 2'5'-linked nucleotides and a triethylene glycol phosphate residue (leg). The double strands of assay mixtures 8 and 10 have only one strand with a 2'5'-linked overhang.

15 Transfection: on the day before the experiment, 2×10^6 cells/ml were plated out into 6-well plates. The oligonucleotides were hybridized by heating the two strands in diluted buffer and subsequently cooling them to give the double strand and taken up in 100 μ l of SF 900II SFM (SF-900 serum-free insect medium II; Gibco BRL 10902-096). For transfection, 10 μ l of lipofectin (1 mg/ml; 20 Gibco BRL) were mixed with 100 μ l of SF 900II SFM and incubated at room temperature for 15 min. This was followed by pipetting together the lipofectin mix and the nucleic acid and incubating at room temperature for 15-45 min. In the meantime, the cells were washed with 3 ml of serum-free medium and 800 μ l of SF 900II SFM and the nucleic acid/lipofectin mixture were 25 successively added to the cells, followed by incubation at 25 degrees overnight. On the next day, 1 ml of medium and + serum (Gibco BRL 10122-166; final concentration 2%) is added.

Dual-luciferase reporter (DLR; Promega E1960) assay system:

30

The Promega DLR assay allows the sequential determination of the firefly luciferase and Renilla luciferase activities having different nucleic acid sequences from a single sample. The oligonucleotides according to the formula I, which were to be measured, were directed against firefly luciferase. Thus, only firefly luciferase activity but not Renilla luciferase activity should be inhibited. Thus, apart from the inhibitory action, the specificity may also be tested for.

The passive lysis of the cells in the well plates was carried out by first removing the medium and washing the cells with PBS (phosphate-buffered saline (Gibco BRL 14200-067)). The medium was completely removed by suction and then the PLB (passive lysis buffer, diluted 1:5 with water; 500 μ l of PLB (1x) to be introduced into one well of a 6-well plate) was added thereto. This was followed by a 15-minute incubation with shaking at room temperature.

The luciferase assay reagent II (LAR II) was prepared by resuspending the luciferase assay substrate (LAS) in 10 ml of luciferase assay buffer II (LAB II). The Stop & Glo reagent was prepared by adding 200 μ l of the Stop & Glo substrate (solution) into the bottle containing dry Stop & Glo substrate and mixing the solution for 10 seconds using a vortexer. In order to produce a 1x Stop & Glo solution, 20 μ l of the 50x Stop & Glo substrate and 1 ml of the Stop & Glo buffer are combined. This is sufficient for 10 assays.

DLR-assay: 100 μ l of LAR II were introduced together with 20 μ l of cell lysate into a well and mixed by pipetting up and down for 2-3 seconds. After luminometric measurement of firefly luciferase activity, 100 μ l of Stop & Glo reagent were added, the solution was mixed and then the Renilla-luciferase activity was determined. The luminescence was determined using the Fluoroskan Ascent FL luminometer (Thermo Labsystems, Frankfurt, Germany).

Assay mixture	Type	% Inhibition of firefly luciferase
1	ss	13
2	ss	12
3	ds	44
4	ds	53
5	ss	19
6	ss	17
7	ds	56
8	ds	57
9	ds	42
10	ds	51
11	ds	20
12	ss	7
13	ss	18

ss is single strand, ds is double strand

The double-stranded oligonucleotides (3,4, 7-10) inhibited firefly-luciferase activity to a substantially greater extent than the corresponding single-stranded molecules (1,2, 5,6, 12 and 13), with the exception of the double strand with only 2 overhanging nucleotides in the lower strand (assay mixture 11). An overhanging 2'5'-(A)₄ residue in the upper strand had no effect or only a very small positive effect on the activity of the double strand, as long as there was a 2'5'-(A)₄ residue in the lower strand (cf. 3 vs. 4 and 7 vs. 8). A 2'5'-(A)₄ residue in the lower strand caused a markedly improved action of the double strand compared with a 2'5'-(A)₂ residue (cf. 7 and 8 vs. 11). 5). A mixture of two single-stranded 2'5'-(A)₄ oligonucleotides, which were unable to form a double strand due to noncomplementary bases (12 and 13), was much less effective than double strands with complementary bases and overhanging 2'5'-adenylate residues.

Likewise, the following modified oligonucleotides of the formula I were assayed in the assay mixtures 14 to 18.

		Assay mixture
5	5' G A A G C G A A G G U U G U G G A U C U G 3' a*a*a a-C*U*U*C G C*U*U C*C A A*C A C*C*U A G A*C	14
10	5' G A A G C G A A G G U U G U G G A U C U G 3' a a a a-C U U C G C U U C C A A C A C C U A G A C	15
15	5' G A A G*C G A A G G*U*U G*U G G A U*C*U*G-teg 3' a*a*a a-C*U*U*C G C*U*U C*C A A*C A C*C*U A G A*C	16
	5' G A A G*C G A A G G*U*U G*U G G A U*C*U*G-teg 3' a*a*a a-C*U*U*C G C*U*U C*C A A*C A C*C*U A G A*C	17
20	5' G A A G*C G A A G G*U*U G*U G G A U*C*U*G-teg	18
	* is phosphorothioate, <u>N</u> : 2'-O-methyl-RNA, aaaa: 2'5'-linked adenylate	

Assay mixture	Type	% Inhibition of firefly luciferase
14	ds	67
15	ds	2
16	ds	93
17	ds	0
18	ss	0
1100 bp dsRNA		94
without dsRNA		0

25 ss is single strand, ds is double strand

The introduction of phosphorothioate residues at particular positions in the lower strand (assay mixture 14) or in the lower and upper strands (assay mixture 16) led to a markedly improved action of the oligonucleotides of the invention, while introduction of 2'-O-methyl radicals in the entire lower or upper strand (assay mixtures 15 and 17) led to a strong decrease in activity. Surprisingly, the small oligonucleotide of assay mixture 16 inhibited the

expression of firefly luciferase just as well as the very long (approx. 1100 bp) double-stranded RNA. The single strand (assay mixture 18) was likewise ineffective.

5 3. Inhibition of edg-1 expression in human primary umbilical cells (HUVEC).

In order to show that the oligonucleotides of the invention can also be used for inhibiting gene expression in human primary cells, said oligonucleotides were also directed against a human gene or the corresponding RNA and tested on
10 human cells (HUVEC, human umbilical vein endothelial cells).
The appropriate oligonucleotides were synthesized.

			Assay mixture
15			
	5' AUCAUCCUGGAGAACAUCUUU	edg-1-175_up	14
	3'-aaaaUAGUAGGACCUCUUGUAGAAA	edg-1-175_lo	
	5' CCAAGGCCAGCCGCAGCUCUG	edg-1-725_up	15
20	3'-aaaaGGUUCCGGUCGGCGUCGAGAC	edg-1-725_lo	
	Mismatch control		
	5' CCACGGACAGACGCGCUGUG	edg-1-mm_up	16
25	3'-aaaaGGUGCCUGUCUGCGGCGACAC	edg-1-mm_up	
	5' AUCAUCCUGGAGAACAUCUUU-FITC	edg-1-175_up_FITC	17
	3'-aaaaUAGUAGGACCUCUUGUAGAAA	edg-1-175_lo	

The double-stranded oligonucleotides of assay mixtures 14 to 16 contain only in
30 one strand (noncoding) a 2'5'-linked overhanging residue. This feature is also a particular embodiment of the present invention.

Cells (HUVECs) and detection of cellular uptake.

Transfection: 24h prior to the actual transfection, primary HUVECs (2nd passage, isolated according to Jaffe *et al.*, 1973, J. Clin. Invest 52, pp.2745), were plated out at a density of 2.5×10^5 cells/well in 6-well plates coated with collagen-I from rats (Biocoat™, #354400, Becton Dickinson). Equimolar amounts
5 of strand and counterstrand of the particular oligonucleotides (in each case 1 mM in sterile-filtered PBS, pH 7.4, Gibco BRL # 14200-067) were mixed and hybridized by incubating at 95°C for five minutes, subsequent cooling to room temperature and incubating at 4°C for five minutes. For transfection, 6 µl of lipofectin (1 mg/ml; Gibco BRL, # 18292-011) were mixed with 200 µl of serum-
10 free Opti-MEM 1 medium (Gibco BRL, #31985-047) and incubated at room temperature for 15 minutes. In a parallel reaction, a solution of 10 µM (→ final concentration 0.1 µM) or 100 µM (→ final concentration 1 µM) hybridized oligonucleotide (in PBS, pH 7.4) was diluted in a ratio of 1:10 with serum-free Opti-MEM 1 medium and mixed with the same volume of preincubated lipofectin
15 solution. After incubation at room temperature for 15 minutes, the volume of said mixture was increased to 2 ml with serum-free Opti-MEM 1 medium and the cell lawn was washed once with PBS and then incubated with said mixture at 37°C, 5% CO₂ and 95% humidity for 4 hours. Subsequently, the cell lawn was washed again with PBS and then overlaid with serum-containing EGM
20 medium (CellSystems, # CC-3024 + EGM supplements # CC-3124) and incubated for a further 24 or 48 h. In the case of uptake studies using fluorescently labeled oligonucleotides, the cells were incubated for 4 hours, then fixed with 5% paraformaldehyde (in PBS, pH 7.4) and directly photographed in an inverted fluorescence microscope (Zeiss Axiovert™ 135M) with its 200-fold
25 magnification using a cooled CCD camera (ORCA-1, Bfi optilas) and excitation through an FITC filter (excitation: 490 nm, emission: 510 nm) and processed via AQM2000 software (Kinetic Imaging).

Western blot analysis: the cells were lysed by washing the cell lawn once with PBS and then overlaying it with 200 µl/well 2 x Laemmli buffer (Bio-Rad #161-
30 0737). After incubation at room temperature for five minutes, the cell lysate was collected using a cell scraper (Becton Dickinson, #3085) and, prior to discontinuous 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE,

Laemmli *et al.*, 1970, Bio-Rad-Criterion-System #345-0014), heated at 95°C for 5 minutes and 45 µl of this solution were applied to each slot. The gel was run in 1 x Tris/glycine/SDS buffer (Bio-Rad # 161-0732). For the immunoblot, the gel was transferred with the aid of the Bio-Rad criterion Western blot apparatus (#170-4070) to a nitrocellulose (NC) membrane (Amersham # RPN 2020D) in 1 x Tris/glycine buffer (Bio-Rad #161-0732, + 10% methanol). The NC membrane was then saturated at room temperature for 1 hour using 1 x TBS buffer (Bio-Rad # 170-6435), which contained 5% milk powder ("Blotto", Bio-Rad #170-6404) and 0.1% Tween 20 (Bio-Rad # 170-6531). After washing the membrane three times in Blotto-free TBS-Tween (TBST) buffer, the membrane was incubated with the anti-hEDG-1 primary antibody (polyclonal rabbit serum obtained by immunization with the EDG-1-specific peptide sequence CKAHRSSVSDYVNYD, coupled to KLH and affinity-purified against the abovementioned peptide sequence) in a 1:50 dilution in TBST-Blotto at 4°C overnight. After washing three times with TBST, the secondary antibody (anti-rabbit, alkaline phosphatase-coupled, Dianova # 111-055-045) was incubated in a 1:2000 dilution in TBST-Blotto at room temperature for one hour. After another washing step (see above), the ECF ("enhanced chemifluorescence") detection reaction (Amersham #RPN5785) was carried out, and the NC membrane which was covered with clingfilm was incubated with 1 ml of ECF substrate (Amersham Pharmacia #RPN5785) at room temperature for 5 minutes and then detected using a Fluor-Imager 595 scanner (Amersham Pharmacia). The signal was quantified using the ImageQuant™ software (Amersham Pharmacia) and normalized to the β-tubulin signal which was obtained after destaining (Alpha Diagnostic Kit # 90100) the NC membrane once and incubating the β-tubulin-specific primary antibody (affinity-purified rabbit antibody, Santa Cruz # sc-9104) according to the above-described method.

		EDG-1 Protein (% of control)	
Concentration of ds RNA (μ M)	Assay mixture 14 (Region "175")	Assay mixture 15 (Region "725")	Assay mixture 16 (mismatch)
0.0	100	100	100
0.1	51	121	119
1.0	23	51	118

Treatment of the primary HUVEC cells with the double-stranded oligonucleotides of the invention led to a dose-dependent inhibition of Edg-1 expression. The inhibition proved to be target gene-specific, since, after treatment with the edg-1-specific oligonucleotides, only the edg-1 protein levels and not the tubulin levels were reduced in assay mixtures 14 and 15. The inhibition also proved to be sequence-specific with regard to the oligonucleotides used, since only the edg-1-homologous oligonucleotides of assay mixtures 14 and 15 inhibited edg-1 expression, while the double-stranded nucleic acid of assay mixture 16 which differs from the edg-1 sequence in 5 nucleotides did not inhibit edg-1 expression.

We believe that this is the first experiment to describe sequence-specific inhibition of gene expression in human primary cells by double-stranded RNA.

The cellular uptake in HUVEC cells was checked with the aid of the fluorescently labeled double-stranded oligonucleotide of assay mixture 17. After incubating the assay mixture 17 for 4 hours, good cellular uptake was detected with the aid of fluorescence microscopy. The double-stranded oligonucleotide taken up was mainly located in the cytoplasm, whereas a single-stranded FITC-labeled oligonucleotide was mainly found in the nucleus under the same conditions.

4. Inhibition of edg-1 expression in human primary umbilical cells (HUVEC) with the aid of phosphorothioate-modified oligomers.

5 The oligoribonucleotide analogues which had been modified with phosphorothioate at particular positions were used, as described in example 3, in primary human cells in order to inhibit gene expression of Edg-1 in human cells (HUVEC, human umbilical vein endothelial cells).

Assay mixture 18:

10

5' A U*C A U*C*C*U G G A G A A*C A*U C*U*U*U-teg
3'-a*a*a a U*A G*U A G G A C*C*U C*U*U G*U*A G A A*A

Assay mixture 19:

15

5' C*C*A A G G*C*C A G*C*C G*C A G C*U*C*U*G-teg
3'-a*a*a a G G U*U*C*C G G*U*C G G*C G*U*C G A G A*C

Assay mixture 20:

20

mismatch:

5' C*C*A C*G G A C*A G A C*G C*C*G C*U*G*U*G-teg
3'-a*a*a a G G U*G C*C*U G*U*C*U G*C G G*C G A*C A*C

25 where * is phosphorothioate; (a*a*a a) is a 2'5'-linked adenylate (partially modified with *) and teg is a triethylene glycol phosphate.

30 The double-stranded oligoribonucleotides of assay mixtures 18 to 20 contain only in one strand (noncoding) a 2'5'-linked overhanging residue, with only particular internucleotide bonds being modified with phosphorothioate. This feature is likewise a particular embodiment of the present invention.

		EDG-1 protein (% of control)	
Concentration of ds RNA (μ M)	Assay mixture 18 (region "175")	Assay mixture 19 (region "725")	Assay mixture 20 (mismatch)
0.0	100	10	100
0.1	46	37	112
1.0	27	27	109

This experiment was repeated using a greater variation in the dosage.

		EDG-1 protein (% of control)	
Concentration of ds RNA (μ M)	Assay mixture 18 (region "175")	Assay mixture 19 (region "725")	Assay mixture 20 (mismatch)
0	100.0	100.0	100.0
0.01	107.7	95.6	93.4
0.05	71.9	93.4	115.7
0.1	49.9	61.7	100.8
0.5	43.5	25.8	125.0
1.0	25.1	12.8	113.5

- 5 The treatment of the primary HUVEC cells with the chemically modified double-stranded oligoribonucleotides of the invention led to a dose-dependent inhibition of edg-1 expression. Said inhibition proved to be target gene-specific, since, after treatment with the edg-1-specific oligonucleotides, only the Edg-1 protein levels but not the tubulin levels were reduced in assay mixtures 18 and 19. The
- 10 inhibition also proved to be sequence-specific with regard to the oligonucleotides used, since only the edg-1-homologous oligonucleotides of assay mixtures 18 and 19 inhibited edg-1 expression, while the double-stranded nucleic acid of assay mixture 20 which differs from the edg-1 sequence in 5 nucleotides did not inhibit edg-1 expression.

46

SEQUENCE LISTING

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<120> Synthetic double stranded oligonucleotides for specific inhibition of gene expression

<130> 9982-786

<140> CA 2,453,301

<141> 2002-07-05

<150> DE 101 33 858.9

<151> 2001-07-12

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26

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<210> 26
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<223> Description of Artificial Sequence: Part of human
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53

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<210> 32

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<213> Artificial Sequence

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<210> 33

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21

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<223> Description of Artificial Sequence: Part of human
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<210> 35

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<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Mutation

54

<400> 35
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<210> 36
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<212> RNA
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<220>
<223> Description of Artificial Sequence: Mutation

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<210> 37
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<220>
<223> Description of Artificial Sequence: Mutation

<400> 37
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<210> 38
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<212> RNA
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<220>
<223> Description of Artificial Sequence: Mutation

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<210> 39
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<223> Description of Artificial Sequence: Mutation

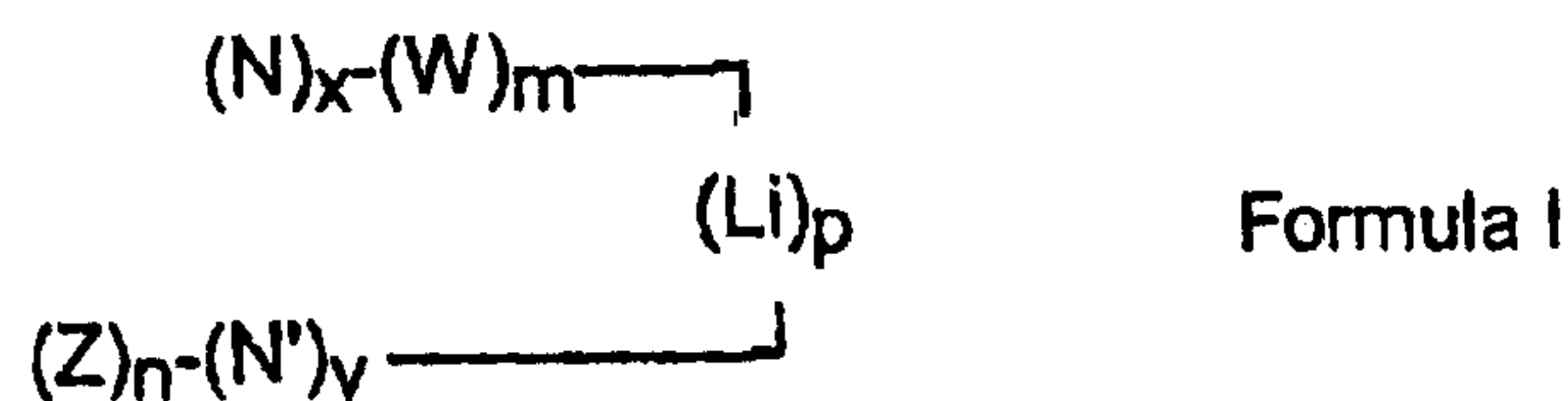
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cagauccaca accuucgcuu c

21

Claims:

1. An oligonucleotide of the formula I



where

N and N' are naturally or not naturally occurring nucleotides which are complementary to one another and where at least one nucleotide strand (N)_x or (N')_y is complementary or partially complementary to a target gene or to the RNA corresponding thereto,

x and y are each 21,

n is independently 4 to 6,

m is independently 0 to 20,

p is independently 0 to 10,

W and Z are naturally or not naturally occurring nucleotides which are linked via a 3'5' or 2'5' internucleoside bond,

Li is a linker which covalently links the two nucleotide strands,

wherein at least four residues Z are linked via a 2'5' internucleoside bond and are present in single-stranded form.

2. The oligonucleotide of the formula I as claimed in claim 1, wherein at least two residues W are linked via a 2'5' internucleoside bond and are present in single-stranded form.

3. The oligonucleotide of the formula I as claimed in claim 1 or 2, wherein m is 0 to 10.

4. The oligonucleotide of the formula I as claimed in claim 3, wherein m is 0 to 6.

5. The oligonucleotide of the formula I as claimed in claim 4, wherein m is 0.

6. The oligonucleotide of the formula I as claimed in any one of claims 1 to 5, wherein p is 0 to 5.

7. The oligonucleotide of the formula I as claimed in claim 6, wherein p is zero.

8. The oligonucleotide of the formula I as claimed in any one of claims 1 to 7, with the proviso that its homologous target RNA has one of the following sequence patterns:

5'-(U)_v-(M)_z-(U)_w

5'-(U)_v-(M)_z-UX

5'-UX-(M)_z-UX and

5'-(U)_v-(M)_z,

where v is independently 2 to 20,

where w is independently 2 to 20,

z is independently 15 to 25,

U is a uridine and M is A, G, C or U and X is A, G or C.

9. The oligonucleotide of the formula I as claimed in claim 8, wherein v is 2 to 10.

10. The oligonucleotide of the formula I as claimed in either of claims 8 or 9, wherein v is 2 to 6.

11. The oligonucleotide of the formula I as claimed in any one of claims 8 to 10, wherein w is 2 to 10.
12. The oligonucleotide of the formula I as claimed in claim 11, wherein w is 2 to 6.
13. The oligonucleotide of the formula I as claimed in any one of claims 8 to 12, wherein z is 16 to 23.
14. The oligonucleotide of the formula I as claimed in claim 13, wherein z is 19 to 21.
15. The oligonucleotide of the formula I as claimed in any one of claims 1 to 14, wherein Z is adenosine or 3'-deoxyadenosine.
16. The oligonucleotide of the formula I as claimed in any one of claims 1 to 15, in which one or more natural phosphodiester bonds have been replaced by unnatural internucleotide bonds which stabilize against nuclease degradation.
17. The oligonucleotide of the formula I as claimed in any one of claims 1 to 16, in which one or more natural phosphodiester bonds have been replaced by phosphorothioate bonds.
18. The oligonucleotide of the formula I as claimed in any one of claims 1 to 17, in which a plurality of natural phosphodiester bonds have been replaced by phosphorothioate bonds, with said modifications being located on the ends and on internal pyrimidine nucleotides.
19. The oligonucleotide of the formula I as claimed in any one of claims 1 to 18, wherein Li is a nucleotide N.

20. The oligonucleotide of the formula I as claimed in any one of claims 1 to 19, wherein Li is a non-nucleotide linker.

21. An in vitro method for inhibiting gene expression of a target gene in a cell wherein an oligonucleotide complementary to an appropriate target gene as claimed in any one of claims 1 to 20 is introduced into a cell, said cell is incubated and inhibition of the gene expression of the target gene is then determined by comparative measurements of the amount of the corresponding mRNA or corresponding gene product in a control cell.

22. The method as claimed in claim 21 for inhibiting gene expression of a target gene in a cell in which 2'5'-oligoadenylate synthase is underexpressed in comparison with a control cell or is defective.

23. A pharmaceutical comprising an oligonucleotide as claimed in any one of claims 1 to 20 and an additive or carrier.

24. The pharmaceutical of claim 23, further comprising an excipient.

25. The use of a pharmaceutical as claimed in claim 23 or 24 for inhibiting gene expression of a target gene in a cell.

26. A method for preparing an oligonucleotide as claimed in any one of claims 1 to 19, wherein the oligonucleotide is first prepared in solution or on the solid phase by successive coupling or coupling in blocks, and after the preparation, isolated and purified.

27. The method of claim 26, further comprising hybridizing the prepared oligonucleotides to give a double stranded oligonucleotide.

28. A method for preparing a pharmaceutical, wherein an oligonucleotide as claimed in any one of claims 1 to 20 is prepared and admixed with further additives or carriers.

29. The method of claim 28, further comprising admixing an excipient.