Title: HIGH SPECIFICITY HAIRPIN ANTISENSE OLIGONUCLEOTIDES

Abstract: This invention relates to hairpin antisense oligonucleotides and their therapeutic use.
HIGH SPECIFICITY HAIRPIN ANTISENSE OLIGONUCLEOTIDES

This invention relates to antisense oligonucleotides and their therapeutic use.

Background of the Invention

Antisense oligonucleotides are therapeutic agents. Conventional antisense oligonucleotides are linear oligonucleotide sequences that are designed to bind to target sequences in messenger RNAs and thereby inhibit the translation of the messenger RNA into its encoded protein, or initiate a chain of events that causes the degradation of the messenger RNA with the effect that its encoded protein cannot be synthesized. The therapeutic use of antisense oligonucleotides may be hampered by poor specificity. For some applications of antisense oligonucleotides, the sequence of each messenger RNA is widely different from other messenger RNAs, and specificity is not a limitation. However, that is not always true. In some diseases the translation of a "wild-type" messenger RNA does not cause a pathogenic condition, but if a particular nucleotide substitution is present in that messenger RNA, then its expression does cause a pathogenic condition. It is desirable to be able to use an antisense oligonucleotide to inhibit the expression of such an RNA. Typically, the mutant messenger RNA is associated with cells that are cancerous. For example, the human ras gene becomes cancer-inducing by the acquisition of a single nucleotide point mutation within its coding sequence (Monia et al., 1992). In this and similar situations, it is important that the antisense oligonucleotide bind to the cancer-causing variant of the ras messenger RNA, but that it not bind to the normal ras messenger RNA that is present in healthy cells.

Relatively long oligonucleotides are used as antisense agents in order that they form strong hybrids and select against unrelated messenger RNAs. These agents do not discriminate in practice against single nucleotide differences. Shortening the length of the antisense oligonucleotide to improve its specificity for the mutant target sequence as compared to the almost identical wild-type sequence is not very useful,
because this weakens the hybrids that it forms, and as a result it is not able to inhibit the expression of mutant messengers RNA.

The present invention markedly improves the specificity of antisense oligonucleotides and antisense therapy.

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Summary of the Invention

This invention includes modified antisense oligonucleotides having increased specificity. The antisense oligonucleotides of this invention, when not bound to target, assume a structured hairpin configuration comprising a single-stranded loop and a double-stranded stem. The loop is complementary to the target. Interaction of the loop to the target results in the formation of a loop-target hybrid, which causes the stem to unwind, or dissociate. Antisense oligonucleotides according to this invention are able to discriminate between an intended target messenger RNA and another messenger RNA differing from the target by a single nucleotide substitution.

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Antisense oligonucleotides according to this invention have an internal sequence flanked by a pair of sequences that are complementary to one another and hybridize to one another under conditions of use in the absence of target messenger RNA. We refer to the three sequences as the 5' arm, the loop, and the 3' arm. Hybridization to the arms to one another creates a double-stranded region, or stem.

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Hybridization of the loop sequence to its perfectly complementary target sequence causes the stem to dissociate, or unwind. This hairpin structure, which is not possessed by conventional linear antisense oligonucleotides, markedly improves the ability to discriminate between two messenger RNAs that differ by a single nucleotide.

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This invention also includes therapeutic use of these hairpin antisense oligonucleotide, which may be administered in the conventional fashion already known for linear antisense oligonucleotides, for example, by encapsulating the agents in liposomes that fuse with all membranes and thereby deliver there contents into cells. Antibodies anchored on liposomes can target the liposomes to cancerous
tumors. Administration is in a therapeutically effective amount by which we mean an amount that is capable of producing a medically desirable result in a treated mammal, for example, a human patient.

**Description of the Preferred Embodiments**

The use of extremely specific hairpin antisense oligonucleotides that selectively inhibit the expression of a pathogenic gene, without inhibiting the expression of a related gene that differs from it by only a single nucleotide substitution, is based on the finding that the presence of the hairpin stem enhances the specificity of the probe sequence located in the loop of the hairpin structure. Hairpin antisense oligonucleotides are simple to design. The arm sequences of hairpin antisense oligonucleotides can be chosen independently of the identity of the target sequence. Only the loop portion of the hairpin antisense oligonucleotide needs to be complementary to the target. The loop sequences in hairpin antisense oligonucleotides are sufficiently long to be specific to a chosen sequence and relatively long as compared to the arms such that hybridization of the loop sequence alone drives the opening of the hairpin stem. The length of the loop sequence in a hairpin antisense deoxyribonucleotide according to this invention ranges from 7 to 30 nucleotides, preferably 10 to 25 nucleotides and more preferably 15-25 nucleotides, whereas the length of the arm sequence ranges from 3 to 8 nucleotides, with the loop sequence always being longer than the stem sequence. The specificity of hairpin antisense oligonucleotides increases as the length (or GC content) of the arm sequences is increased. Whether a particular hairpin antisense oligonucleotide actually exhibits the desired level of specificity can be tested in vitro by labeling the hairpin antisense oligonucleotide with terminal interactive labels according to the methods of Tyagi and Kramer (1996) and then detecting hybridization by observing the increase in fluorescence intensity. A hairpin antisense oligonucleotide will bind to a perfectly complementary target sequence in a messenger RNA, that is, a target perfectly complementary to the loop, but will not bind to a sequence in a messenger RNA that differs from the target sequence by a single nucleotide substitution, and, thus, is not perfectly complementary to the loop.
Another useful aspect of hairpin antisense oligonucleotides is that because they are in the form of a hairpin that possesses a double-stranded stem, they are naturally more resistant to cellular nucleases than conventional linear antisense oligonucleotides.

Hairpin antisense oligonucleotides of the present invention can contain deoxyribonucleotides, ribonucleotides, peptide nucleic acids (PNA), other modified nucleotides, or combinations of these. Modified nucleotides may include, for example, 2'-O-methylribonucleotides or nitropyrole-based nucleotides. Modified internucleotide linkages may also be included, for example phosphorothioates. The advantage of using such modifications for a particular application will be apparent to persons familiar with the art. In particular, hairpin antisense oligonucleotides constructed from modified nucleotides may form stronger hybrids than if the hairpin primers were constructed from deoxyribonucleotides, thus enabling structured target sequences (such as those that occur in messenger RNAs) to be more easily accessed.

In addition, hairpin antisense oligonucleotides constructed from modified nucleotides, or that contain modified internucleotide linkages, will resist degradation by cellular nucleases, rendering them more effective as therapeutic agents.

Hairpin antisense oligonucleotides according to this invention may have an arm that is at least partially complementary to the intended target. However, it must open when tested against a target consisting of only the perfect complement of the loop.
Example

We have designed a highly specific hairpin antisense oligonucleotide that binds to a mutant carcinogenic form of ras messenger RNA, but does not bind to the wild-type form of ras messenger RNA. These two RNAs differ from one another by a single nucleotide substitution within codon 12 (Monia et al., 1992). This embodiment is made of deoxyribonucleotides. The sequence of the hairpin antisense oligonucleotide is 5'-CGCTGGCCCGCGGAGCGACACAGCCAGC-3', where underlines identify the arm sequences that hybridize to each other, the 5' arm being the sequence CGCTGG. In the absence of target strands that are complementary to the single-stranded loop, the two arms hybridize to one another to form a stem. If the stem sequences had not been added to the loop sequence in this antisense oligonucleotide, it would not have sufficient capacity to discriminate between wild-type ras messenger RNA and mutant ras messenger RNA (Monia et al., 1992). However, the hairpin antisense oligonucleotide is much more specific for its intended target sequence because the sequence in the loop must initiate the binding of the oligonucleotide to the target sequence, and this interaction is much more specific than the binding of a conventional linear antisense oligonucleotide to the same target sequence because the interactive sequence in the loop is embedded within a hairpin stem. By only binding to mutant ras RNA, hairpin antisense oligonucleotides selectively inhibit the growth of cells that express the mutant messenger RNA (cancer cells), and do not inhibit the growth of cells that express the wild-type messenger RNA (healthy cells).
References


We claim:

1. An antisense oligonucleotide consisting of a central loop sequence that is complementary to a selected messenger RNA target sequence and that is flankded by 3' and 5' arm sequences that are complementary to one another, wherein in the absence of said target sequence said oligonucleotide assumes an hairpin structure having a double-stranded stem, wherein interaction of said loop sequence with said target sequence causes dissociation of said stem, and wherein interaction of said loop sequence with a sequence complementary thereto except for a single nucleotide does not cause said stem to dissociate.

2. The antisense oligonucleotide of claim 1 comprising nucleotides selected from the group consisting of deoxyribonucleotides, ribonucleotides, peptide nucleic acids, 2'-O-methylribonucleotides and nitropyrole-based nucleotides.

3. The antisense oligonucleotide of claim 1 comprising modified internucleotide linkages.

4. A therapeutic method comprising administering to a patient an antisense oligonucleotide according to claim 1.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7): C12P 19/34; C07H 21/04; A01N 43/04; A61K 31/70
US CL.: 536/23.1, 24.5; 514/44; 453/91.1, 91.31
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S.: 536/23.1, 24.5; 514/44; 453/91.1, 91.31

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAplus, MEDLINE, BIOSIS, USPAT, EPO, JPO, DERWENT
search terms: (hairpin or stem loop or central loop) and (antisense or oligonucleotide)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tbody>
<tr>
<td>X</td>
<td>US 5,514,546 A (KOOL) 07 May 1996, see figure 1.</td>
<td>1-4</td>
</tr>
<tr>
<td>A</td>
<td>JENDIS et al. Inhibition of Replication of Fresh HIV Type 1 Patient Isolates by a Polypurine Tract-Specific Self-Complementary Oligodeoxynucleotide. AIDS Research and Human Retroviruses. 1996, Vol. 12, No. 12, pages 1161-1168, especially page 1167.</td>
<td>1-4</td>
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☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

Date of the actual completion of the international search: 12 JULY 2000

Date of mailing of the international search report: 18 AUG 2000

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Form PCT/ISA/210 (second sheet) (July 1998)