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(71) Applicants *(for all designated States except US)*: THE GOVERNMENT OF THE UNITED STATES OF AMERICA represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Office of Technology Transfer, National Institutes of Health, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852 (US). LEIDEN UNIVERSITY [NL/NL]; P.O. Box 9502, NL–2300 RA Leiden (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: TORRENCE, Paul, F. [US/US]; 5245 Forest Drive, Flagstaff, AZ 86004 (US). VAN BOOM, Jacques, H. [NL/NL]; Pieter de Hooglan 14, NL–2343 CR Oegstgeest (NL). VERHEIJEN, Jeroen, C. [NL/NL]; Pieterskerkeloorsteeg 11, NL–2311 TR Leiden (NL). VAN DER MAREL, Gijsbert, A. [NL/NL]; Bronforel 13, NL–2318 MD Leiden (NL).

(74) Agents: FEILER, William, S. et al.; Morgan & Finnegan, L.L.P., 345 Park Avenue, New York, NY 10154 (US).

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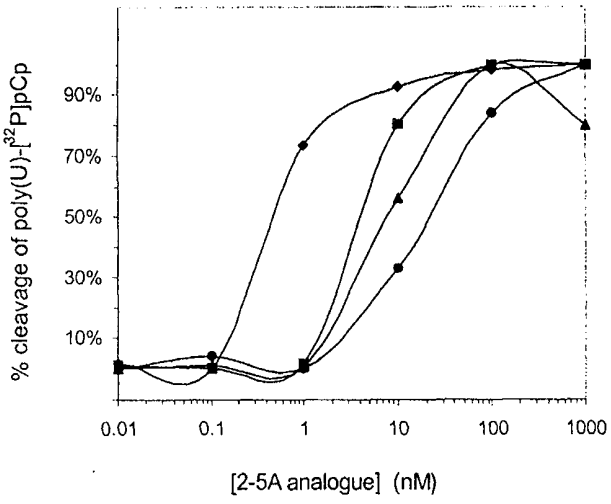

(57) Abstract

Covalent conjugation of a 5'–phosphorylated–2',5'–linked oligoadenylate (2–5A) moiety to an antisense peptide nucleic acid oligomer (PNA) provides a novel chimeric reagent which effects the selective and specific cleavage of a selected target RNA. The 2–5A–antisense PNA chimeras bind the target RNA with high specificity and affinity, and are stable to nucleases. The antisense portion of the chimera recruits a chosen RNA as substrate for cleavage, and the 2–5A portion of the chimera binds and activates RNase L, thus providing a new approach for the targeted ablation of a target mRNA and a reduction in expression of the protein which it specifies. The chimeric molecules are expected to have utility as research tools and as therapeutic agents.

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Peptide Nucleic Acid-Oligoadenylate Chimeric Molecules

FIELD OF INVENTION

The present invention relates to chimeric molecules comprising activators of RNase L covalently linked to peptide nucleic acid oligomers, and to compositions thereof. The invention also relates to methods for sequence-specific cleavage of RNA using these chimeric molecules *in vitro* and *in vivo*.

BACKGROUND OF THE INVENTION

Antisense oligonucleotides hold considerable promise both as research tools for inhibiting gene expression and as agents for the treatment of a myriad of human diseases (Stein and Cheng, Science, 261:1004 (1993); Milligan *et al.*, J. Med. Chem., 36:1923-1937 (1993)). However, the targeted destruction of RNA using antisense oligonucleotides has been difficult to achieve in a versatile, efficient, and reliable manner.

Miller *et al.* were the first to attempt to capitalize on nucleic acid hybridization through the preparation of a series of trinucleotides modified through phosphotriester, 2'-O-methyl or methylphosphonate substitution (Miller, *et al.*, Biochemistry, 13:4887 (1974)). These short, modified DNA sequences, complementary to t-RNA anticodon regions, were found to be able to inhibit protein translation. Zamecnik and Stephenson (Proc. Natl. Acad. Sci. U.S.A., 74:280 (1978)) used a similar strategy to synthesize a 21-deoxyribonucleotide sequence which inhibited the replication of Rous sarcoma virus. Enthusiasm for this approach was generated by the demonstration that human immunodeficiency virus (HIV) could be inhibited through the use of antisense oligonucleotides (Zamecnik, *et al.*, Proc. Natl. Acad. Sci. U.S.A., 83:7706 (1986)). Although the mechanism of action of such antisense reagents is complex and not well understood, it has been demonstrated that hybridization complexes of target messenger RNA and complementary oligo- beta -deoxynucleotides are degraded *in vivo* by the enzyme RNase H, which is present in both eukaryotes and prokaryotes. However, it has been found that many modified antisense oligonucleotides synthesized to improve delivery, cell penetration, or stability will form hybrids with sense strands of RNA but will not act as substrates for RNase H.

Another antisense mechanism which has been found to be operative to some extent is the inhibition of protein synthesis by the passive mechanism of hybridization arrest. By hybridizing an antisense oligonucleotide to a target RNA sequence, the translation of the RNA molecule containing the target sequence can be prevented, thereby inhibiting synthesis of the protein encoded by the RNA molecule. However, because the hybridization of an antisense oligonucleotide to its target ribonucleotide sequence is reversible, this technique cannot totally prevent the translation of the target RNA sequence.

Considerable effort has therefore been directed to the development of oligonucleotides which are able to induce chemical alteration or strand scission of a target RNA molecule. Thus, oligonucleotides have been modified with photoreactive agents such as psoralen or porphyrin (Lee, *et al.*, Nucleic Acids Res., 16:10681 (1988)); oxidative nuclease metal ion complexes such as porphyrin-iron (Doan, *et al.*, Biochemistry, 25:6736 (1986)); phenanthroline-copper (Chen, *et al.*, Proc. Natl. Acad. Sci. USA, 83:7147 (1986)) and ethylene diamine tetraacetic acid-iron (Dreyer, et al, Proc. Natl. Acad. Sci. USA, 82:963 (1985)); nucleases such as staphylococcal nuclease (Corey, *et al.*, J. Am. Chem. Soc., 111:8523 (1989)) and RNase P (Li, *et al.*, Proc. Natl . Acad. Sci. USA, 89:3185 (1992)); and catalytic rRNA sequences (ribozymes) (Rossi, *et al.*, Pharmacol. Ther., 50:245-254 (1991)).

Some of these references discuss methods of digesting RNA molecules having a specific nucleotide sequence. Li *et al.* (Proc. Natl. Acad. Sci. U.S.A., 89:3185-3189 (1992)), for example, showed that RNase P can be used to cleave specific strands of RNA to which antisense oligonucleotides having an ACCA sequence were annealed. Oligonucleotides with an ACCA sequence at one end, referred to as "external guide sequences" (EGS's), were hybridized to a specific sequence on an RNA molecule. The RNA molecule with the bound EGS thereby became a substrate for RNase P and was specifically cleaved by RNase P.

Another method of digesting RNA at a specific location with an antisense oligonucleotide and an RNase was demonstrated by Minshull *et al.* (Nucleic Acids Research, 14:6433-6451 (1986)). Minshull cleaved a specific RNA molecule by first hybridizing an antisense DNA oligonucleotide to the RNA molecule and then treating the hybridized molecule with RNase H. Since RNase H

specifically digests DNA/RNA hybrids, the RNA strand of the hybridized molecule was digested by RNase H.

Corey *et al.* (J. Am. Chem. Soc., 111:8523-8525 (1989)) also discussed a method of targeting a polynucleotide for destruction by a nuclease. Corey fused an antisense oligonucleotide to a nonspecific nuclease. When the oligonucleotide was then hybridized to a polynucleotide with which it could anneal, the nuclease specifically cleaved the targeted polynucleotide strand. This approach has not been applied *in vivo*, however, due to the difficulties involved in passing a molecule as large as a nuclease into an intact living cell.

A number of nonenzymatic strategies for targeting a specific polynucleotide sequence for cleavage have been described. Many of these involve covalently binding a chemical moiety that has polynucleotide cleavage activity to an antisense oligonucleotide. A method disclosed by Chen (Proc. Nat Acad. Sci. U.S.A., 83:7147-7151 (1986)) demonstrated such a strategy. In this method, a 1,10-phenanthroline-copper ion was attached to the 5' end of an oligonucleotide that was complementary to a target polynucleotide sequence. The modified oligonucleotide was hybridized to the complementary target sequence, and cupric ion and 3-mercaptopropionic acid were then added to the reaction mixture. In this environment, the 1,10-phenanthroline-copper ion cleaved the target polynucleotide.

The latent endonuclease RNase L (E.C. 3.1.26.-), formerly known as "2-5A-dependent RNase" cleaves RNA in the presence of the unusual 2',5'-phosphodiester-linked trimeric oligoadenylate ppp5'A2'p5'A2'p5'A (2-5A) (Kerr, *et al.*, Proc. Natl. Acad. Sci. USA, 15:9846 (1978)). The enzyme cleaves RNA on the 3' side of UpNp sequences (where N is A, U, G, or C). RNase L is part of what has been termed the 2-5A system, which is believed to be involved in the regulation of cell growth (Etienne-Smekins, *et al.*, Proc. Natl. Acad. Sci. USA, 80:4609 (1983)) and cell differentiation (Krause *et al.*, Eur. J. Biochem., 146:611 (1985)). RNase L is potently induced by interferon, and appears to mediate certain actions of interferon such as the inhibition of picornavirus replication (Hassel *et al.*, EMBO J., 12:3297-3304 (1993)). Cells and tissues from reptilian, avian, and mammalian species have been found to contain basal levels RNase L. For reviews, see Williams *et al.*, 'The 2-5-A System: Molecular and Clinical Aspects of the Interferon-Related

Pathway" (Alan R. Liss, Inc., New York, 1985) and Player and Torrence, Pharmacol. Ther., 78:55-113 (1998).

The attachment of a 2-5A oligomer to an antisense oligonucleotide results in a chimeric oligonucleotide which activates RNase L, and which directs the activity of RNase L against the complementary target RNA. This approach to antisense therapeutics has been described in several publications: Lesiak *et al.*, Bioconjugate Chem., 4:467-472 (1993); Torrence *et al.*, Carbohydrate Modifications in Antisense Research, Y. Sanghvi and P. Cook, eds, (American Chemical Society, Washington DC), 118-132 (1994); Cirino *et al.*, Proc. Natl. Acad. Sci. U.S.A., 94:1937-1942 (1997); Torrence *et al.*, Antisense Nucleic Acid Drug Dev., 7:203-206 (1997); Xiao *et al.*, J. Med. Chem., 41:1531-1539 (1998); Player *et al.*, Proc. Natl. Acad. Sci. U.S.A. 95:8874-8879 (1998); Kondo *et al.*, Oncogene 16:3323-3330 (1998).

Through this activator-antisense approach, RNase L is converted from a non-specific nuclease to a highly specific endoribonuclease that selectively cleaves mRNA targets. This has been demonstrated in a cell-free system from Daudi cells, a human lymphoblastoid cell line, in which a modified HIV-1 vif mRNA was targeted for cleavage by an activator-antisense DNA complex (Torrence *et al.*, Proc. Natl. Acad. Sci. U.S.A. 90:1300-1304 (1993)). Subsequently, purified RNase L has been directed by an activator-antisense DNA complex to cleave selectively an mRNA target encoding the protein kinase PKR in the presence of a non-targeted mRNA (Maran *et al.*, Science, 265:789-792 (1994)). In HeLa cells, the use of activator-antisense DNA complexes, which were directed to a sequence in PKR mRNA, resulted in the ablation of PKR mRNA and enzyme activity such that the double-stranded RNA-mediated activation of transcription factor NF-κB was ablated. Recently, it was shown that the activation of RNase L by an activator-antisense DNA chimera results in the catalytic degradation of PKR mRNA (K_{cat} of about 7 sec^{-1}) (Maitra *et al.*, J. Biol. Chem., 270:15071-15075 (1995)).

More recently, it has been shown that a 19-mer antisense oligonucleotide linked to an RNase L activator, and directed against the RNA component of human telomerase, is effective in inhibiting tumor growth. (Telomerase is found in 70-80% of all malignant tumors but is undetectable in most

normal cells, which makes it a strong candidate for the targeted antisense approach.) Both *in vitro* and *in vivo* experiments involving malignant human gliomas demonstrated that, by treating malignant glioma cells in culture with 2-5A antisense against telomerase RNA, the vast majority of cells can be killed within 14 days. When the antisense molecules were injected into human tumors implanted in nude mice, the tumor mass was significantly reduced over a 14-day period (Kondo *et al.*, Oncogene 16:3323-3330 (1998).

The recruitment of RNase L for antisense applications has also been described by Torrence *et al.* in US patent 5,583,032 (incorporated herein by reference, in its entirety) and in US patent applications 08/962,690 filed November 3, 1997 and 60/044,507, filed April 21, 1997 (both of which are incorporated herein by reference, in their entirety).

Antisense oligonucleotides have great potential as therapeutic agents, due to their ability to inhibit gene expression in a sequence-specific manner. However, the therapeutic use of antisense oligonucleotides is hampered by their susceptibility to ubiquitous endonucleases and exonucleases, which rapidly degrade exogenous oligonucleotides (de Mesmaeker *et al.*, Acc. Chem. Res., 28:366-374 (1995)). Much effort has gone into the design of oligonucleotide analogs which are resistant to nucleases. Among the more promising analogs are peptide nucleic acids (PNAs), in which the sugar-phosphate backbone of the oligonucleotide is replaced with a polyamide or "pseudopeptide" backbone. These PNA oligomers, which lack phosphodiester bonds, are entirely resistant to nucleases. See, for example, Nielsen *et al.*, patent application PCT/US97/12811, publication No. WO 98/03542.; Hyrup and Nielsen, Bioorg. Med. Chem. 4:5-23 (1996); and Nielsen *et al.*, Science 254:1497-1500 (1991).

A PNA antisense agent will hybridize with high affinity and selectivity to the complementary mRNA strand (Demidov et al;., Biochem. Pharmacol. 48:1310-1313 (1994); Knudsen and Nielsen, Nucleic Acids Res. 24:494-500 (1996)). Unfortunately, the resulting hybrid RNA-PNA duplex is resistant to RNase H, the enzyme specific for DNA/RNA hybrids that plays a major role in the standard mechanism of action of antisense oligonucleotides. Prior to the present invention, the effect of an antisense PNA has therefore been limited to steric

inhibition of translation, because of the absence of catalytic RNase H degradation of PNA-RNA hybrids (Hanvey *et al.*, Science 258:1481-1485 (1992)). Because hybridization of the sense and antisense strands is reversible, a relatively high concentration of antisense PNA must be attained in order to bind a high proportion of the complementary mRNA at equilibrium, if significant inhibition of translation is to be observed.

There remains a need for antisense reagents and therapeutics which exhibit the sequence specificity, affinity, and nuclease stability of PNA oligomers, while retaining the advantage of catalytic RNase degradation of the target RNA.

BRIEF DESCRIPTION OF THE INVENTION

Definitions.

The term "2-5A" refers to any oligonucleotide activator of RNase L.

The term "oligonucleotide" refers to any number of nucleotides linked by phosphodiester, phosphorothiodiester, methylphosphonodiester, or methylphosphonothiodiester moieties.

The terms "activator of RNase L", "RNase L activator", and "RNase L activating moiety" refer to any compound or chemical moiety that is capable of activating RNase L.

The terms "polyamide nucleic acid" and "PNA" refer to an oligomer of amino acid monomers, wherein each monomer comprises a terminal amino group and a terminal carboxyl group, and the monomers are linked through amide bonds between the terminal amino group of one monomer and the terminal carboxyl group of the next monomer. Each monomer further comprises a covalently attached purine or pyrimidine base, and the bases are oriented along the oligomer chain so as to be capable of forming Watson-Crick base pairs with a single-stranded oligonucleic acid.

The term "peptide nucleic acid" as used herein refers to a PNA which is an oligomer of N-(2-aminoethyl)glycine, wherein purine and pyrimidine bases are covalently attached through an acetyl linker to the glycine alpha nitrogens.

The term "nucleobase" refers to a purine or pyrimidine base that is capable of forming Watson-Crick base pairs to one or more of the bases adenine,

thymine, guanosine, cytosine, and uracil. The term is intended to include analogues of purines and pyrimidines as well, such as deazapurines and deazapyimidines, that are likewise capable of forming Watson-Crick base pairs with to one or more of the bases adenine, thymine, guanosine, cytosine, and uracil.

Nucleobase sequences preceded by "(pna)" refer to polyamide nucleic acids having that sequence of nucleobases, and do not refer to any particular polarity of the polyamide chain, i.e. the amino and carboxy termini of the polyamide backbone could be at either end of the given nucleobase sequence.

Nucleobase sequences preceded by "pna" refer to oligo[N-(2-aminoethyl)-N-(1-(purinyl or pyrimidinyl)acetyl)glycine] nucleic acids having that sequence of nucleobases. Sequences not preceded by "(pna)" or "pna" refer to ordinary oligonucleotides having the given sequence.

Description of the invention

One object of the invention is to provide 2-5A-polyamide nucleic acid chimeric molecules, which comprise a polyamide nucleic acid (PNA) moiety covalently attached to an RNase L activating moiety. These chimeric molecules are capable of binding to and activating RNase L, and can direct the nuclease activity of RNase L to a target RNA molecule having a sequence complementary to that of the polyamide nucleic acid moiety of the chimeric molecule. The peptide nucleic acid moiety is preferably an oligo[N-(2-aminoethyl)-N-(1-(purinyl or pyrimidinyl)acetyl)glycine], and the activator moiety preferably comprises a 2',5'-oligoadenylate oligonucleotide. The activator moiety is preferably attached through its 2' end to the peptide nucleic acid, and preferably to the amino terminus of the peptide nucleic acid. The activator moiety may optionally comprise one or more 5' thiophosphate groups. Another object of the invention is to provide compositions comprising the chimeric molecules of this invention.

Another object of the invention is to provide methods for cleaving a specifically selected strand of RNA (the "target RNA"), using the chimeric molecules of this invention. Yet another object of the invention is to provide methods of degrading and/or reducing the concentration of a target RNA molecule, by contacting the target RNA with RNase L in the presence of a 2,5-A-peptide nucleic acid chimeric molecule of this invention having a polyamide nucleic acid

sequence complementary to that of the target RNA.

Another object of this invention is to provide pharmaceutical compositions comprising the chimeric molecules of the invention, and methods of treating mammals with these compositions.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Structure of 5'-O-phosphoryladenylyl(2'→5')adenylyl-(2'→5')adenylyl(2'→5')adenosine, a 2',5'-oligoadenylate example of a 2-5A activator of RNase L.

Figure 2. Retrosynthetic analysis of target 2-5A-PNAs **2-4**. (DMTr, dimethoxytrityl; MMTr, monomethoxytrityl; TBDMS, t-butyldimethylsilyl; CNE, cyanoethyl; HMBA, p-hydroxymethylbenzoate.)

Figure 3. Synthesis of 2-5A-PNA adducts **2-4**. (Abbreviations as defined in Figure 2.)

Figure 4. The effect of 2-5A and 2-5A-PNA chimeras on the ability of RNase L to cleave a radiolabeled poly(U) substrate, plotted as a function of concentration. (◆), 2-5A; (■), 2-5A-pnaA$_4$; (▲), 2-5A-pnaA$_8$; (●), 2-5A-pnaA$_{12}$.

Figure 5. A representative autoradiograph showing the effect of 2-5A and 2-5A-PNA chimeras on the ability of RNase L to cleave the synthetic oligonucleotide r([^{32}P]pC$_{11}$U$_2$C$_7$) [r=ribo]. Lane 1, no 2-5A; lanes 2-6, 10^{-7}-10^{-11}M 2-5A; lanes 7-11, 10^{-5}-10^{-9}M 2-5A-pnaA$_4$; lanes 12-16, 10^{-5}-10^{-9}M 2-5A-pnaA$_8$; lanes 17-21, 10^{-5}-10^{-9}M 2-5A-pnaA$_{12}$.

Figure 6. The displacement of (2-5A-[^{32}P]pCp) from binding sites in CEM cell lysate by 2-5A analogues, plotted as a function of concentration. (◆), 2-5A; (■), 2-5A-pnaA$_4$; (▲), 2-5A-pnaA$_8$; (●), 2-5A-pnaA$_{12}$.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides chimeric molecules which comprise an antisense polyamide nucleic acid moiety that binds or anneals to the target strand of RNA, and an activator of RNase L attached to the antisense polyamide nucleic acid moiety. The antisense peptide nucleic acid moiety may comprise any polyamide nucleic acid oligomer that is capable of forming a double helix with single-stranded nucleic acids having a complementary sequence of base pairs. The polyamide nucleic acid backbone may be based on any amino acids known to the art, such as for example N-(2-aminoethyl)glycine (Nielsen *et al.*, patent application PCT/US97/12811, publication No. WO 98/03542) or N-(1-aminoacyl)proline (Lowe, patent application PCT/GB97/02820, publication No. WO 98/16550). Other PNA backbone polymers are also known to the art: Dueholm *et al.*, Bioorg. Med. Chem. Lett., 4:1077 (1994); Hyrup *et al.*, J. Chem. Soc. Chem. Commun., 518 (1993); Hyrup *et al.*, J. Am. Chem. Soc., 116:7964 (1994), Krotz *et al.*, Tetrahedron Lett., 6:6937 (1995). The polyamide nucleic acid backbone is preferably based on N-(2-aminoethyl)glycine, and is more preferably an oligo[N-(2-aminoethyl)-N-(1-(purinyl or pyrimidinyl)acetyl)glycine]. Whatever the nature of the polyamide backbone, the antisense polyamide nucleic acid moiety has a nucleobase sequence complementary to the sequence of the selected target RNA.

The activator of RNase L may be any RNase L activator. Several such activators are known in the art. For example, a 3'-deoxyadenosine may be substituted for an adenosine within the 2-5A moiety (Torrence *et al.*, J. Biol. Chem., 263:131-1139 (1988)). 3'-O-methylation at the 2' terminal of the 2-5A moiety also generates an activator (Baglioni *et al.*, J. Biol. Chem., 256:3253-3257 (1981)). Other modifications of the 2-5A moiety that produce RNase L activators are substitution of thiophosphate and phosphorothioate for the phosphate groups (Charubala *et al.*, Nucleosides and Nucleotides, 10:383-388 (1991)), β,γ-difluoromethylene phosphonate analogues (Bisbal *et al.*, Biochemistry, 26:5172-5178 (1987)), and bromination of an adenosine, for example in the activator p5'A2'p5'A2'p5'(Br^8A)2'p5'(Br^8A) (Torrence *et al.*, Antiviral Res., 18:275-289 (1993)). One or more of these modifications may optionally be employed in carrying out the methods of this invention, particularly where enhanced stability of

the chimera to phosphodiesterase is desirable.

The RNase L activator moiety preferably comprises a 2',5'-oligoadenylate oligonucleotide having three or more adenosine residues, more preferably three or four adenosine residues. In a preferred embodiment of the invention, the 2',5'-oligoadenylate oligonucleotide is p5'A2'p5'A2'p5'A2'p5'A. Preferably, a mono-, di-, or tri-phosphate group, or one of the thio analogues thereof, is present at the 5' end of the 2-5A moiety. In another preferred embodiment, the RNase activator moiety comprises one or more 5' thiophosphate groups.

The RNase L activator moiety is coupled at its 2' end, preferably through the 2' position, to the PNA moiety, and is preferably coupled through a flexible linker. The linker may be any bifunctional linker that provides sufficient flexibility and spacing between the activator moiety and the PNA moiety to allow RNase L-mediated cleavage of the target RNA strand. Linkers suitable for attaching oligonucleotides to peptide nucleic acids are known to the art, such as, for example, N-(2-hydroxyethyl)-N-[1-(purinyl or pyrimidinyl)acetyl]glycine (Petersen *et al.*, Bioorg. Med. Chem. Lett., 5:1119-1124 (1995); Breipohl *et al.*, Tetrahedron 53:14671-14686 (1997); Efimov *et al.*, Nucl. Acids Res., 26:566-575 (1998); Peyman *et al.*, Angew. Chem. Int. Ed. Engl., 36:2809-2812 (1998); Uhlmann *et al.*, Nucleosides and Nucleotides 16:603-608 (1997)). Aminonucleosides may also be used as linkers (Finn *et al.*, Nucl. Acids Res., 24:3357-3363 (1996); Dueholm *et al.*, J. Org. Chem., 59:5767-5773 (1994)). In a preferred embodiment, the linker is an oligo[butanediol monophosphate] linker as exemplified herein.

The polarity of the antisense nucleobase sequence with respect to the RNase L activator moiety is not critical to antisense activity (Xiao *et al.*, J. Med. Chem. 41:1531-1539 (1998)). The RNase L activator therefore may be linked to either end of the antisense PNA moiety. The RNase L activator moiety is preferably linked to the amino terminus of the PNA moiety as exemplified herein. The polarity of the polyamide backbone with respect to the antisense nucleobase sequence is also not critical, (Lagriffoul *et al.*, Bioorg. Med. Chem Lett., 4:1081 (1994)), and a given nucleobase sequence may begin at either the amino or carboxyl terminus of the polyamide backbone. The antisense polyamide nucleic acid moiety is preferably

between about 6 and about 50 nucleobases in length, more preferably between about 10 and about 30 nucleobases in length, and most preferably between about 12 and about 25 nucleobases in length.

Preferably, the antisense moiety hybridizes, binds, anneals to, or otherwise forms a complex with, a single stranded region of the target strand of RNA contained in the cell. In certain preferred embodiments the target RNA is the RNA component of telomerase, the mRNA encoded by a mutant gene associated with a dominant autosomal disease, or a transcript of an oncogene or a proto-oncogene, or is the transcript of a viral gene or the genome or antigenome of an RNA virus.

In one embodiment, the present invention relates to methods of inhibiting the action of teleomerase with chimeric molecules comprising an activator of RNase L covalently attached to an antisense PNA that is capable of binding to the RNA component of telomerase. By way of example, a polyamide nucleic acid having a nucleobase sequence

(pna)GCGCGGGGAGCAAAAGCAC,

covalently linked to an activator of RNase L, will be useful for inhibiting telomerase. The activator of RNase L is preferably a 2',5'-oligoadenylate oligonucleotide having three or more adenosine residues, more preferably three or four adenosine residues. Such chimeras, and pharmaceutical compositions comprising them, would be useful for inhibiting the growth of tumors in mammals.

An embodiment of this invention suitable for ablating the bcr/abl mRNA, the chimeras and methods of which may also be useful for treating chronic myelogenous leukemias, includes a polyamide nucleic acid having the following nucleobase sequence:

(pna)GAA GGG CTT TTG AAC TCT

(pna)GAA GGG CTT CTT CCT TAT TGA

(pna)GCC CAC CGG GTC CAC CA

covalently linked to an activator of RNase L. (Maran, *et al.*, Blood, in press (1998)).

An embodiment of this invention suitable for ablating the ICAM-1 mRNA, the chimeras and methods of which may also be useful for treating Crohns Disease and other inflammatory conditions, includes a polyamide nucleic acid

having the following nucleobase sequence:

(pna)GCC CAA GCT GGC ATC CGT CA

covalently linked to an activator of RNase L. This embodiment of the invention is directed to the mRNA encoding the intracellular adhesion protein ICAM-1. (Bennet *et al.*, J. Immunol., 152:3530-3540 (1994)).

An embodiment of this invention invention suitable for ablating the *ha-ras* mRNA, the chimeras and methods of which may also be useful for treating cancer, includes a polyamide nucleic acid having the following nucleobase sequence:

(pna)TCC GTC ATC GCT CCT CAG GG

covalently linked to an activator of RNase L. This embodiment of the invention is directed to the mRNA transcribed from the ha-ras gene. (Monia et al., J. Biol. Chem., 271:14533-14540 (1996)).

An embodiment of this invention suitable for ablating the HIV *gag* gene or *gag* mRNA, the chimeras and methods of which may also be useful for treating HIV infection, includes a polyamide nucleic acid having the following nucleobase sequence:

(pna)TCTTCCTCTCTCTACCCACGCTCTC

covalently linked to an activator of RNase L. This embodiment of the invention is directed to the HIV *gag*-1 RNA (Agrawal and Tang, Antisense Res. Dev., 2:261-266 (1992)).

An embodiment of this invention suitable for ablating the CMV immediate-early RNA, the chimeras and methods of which may also be useful for treating CMV retinitis, includes a polyamide nucleic acid having the following nucleobase sequence:

(pna)TTT GCT CTT CTT CTT GCG

covalently linked to an activator of RNase L. This embodiment of the invention is directed to the mRNA of cytomegalovirus. A phosphorothioate antisense oligonucleotide having this sequence (Vitravene^{TM}, fomivirsen) has been approved for treatment of CMV retinitis. (Anderson *et al.*,. Antimicrob. Agents Chemother., 40:2004-2011 (1996); Azad *et al.*, Antiviral Res., 28:101-111 (1995); Azad *et al.*,

Antimicrob. Agents Chemother., 37:1945-1954 (1993); Leeds *et al.*, Drug. Metab. Dispos., 25:921-926 (1997).

The present invention also relates to covalently-linked chimeras of an activator of RNase L and a polyamide nucleic acid (PNA) that is capable of binding to the genomic RNA strand of an RNA virus and/or binding to the antigenomic or mRNA of a negative strand RNA virus. In accordance with the present invention, the methods and chimeras of the invention may be applied to target any negative strand RNA virus, including, but not limited to, parainfluenza virus, mumps virus, rabies, influenza virus, and arenaviruses such as LCMV (lymphocytic chorio-meningitis virus), Lassa virus, Machupo virus, Sabia virus and Junin virus. The invention in one embodiment relates to a covalently linked chimera of a PNA that is capable of binding to the genomic or antigenomic template RNA strand of a negative strand RNA virus and/or binding to an mRNA of a viral protein (an "antisense PNA") coupled to an activator of RNase L

Negative strand RNA viruses have multiple genes, *i.e.*, the virion contains the complement of the coding strand. On entry into a host cell the genome is transcribed to produce the various mRNA encoding the viral proteins and also to produce an entire complementary RNA, *i.e.*, the antigenome, from which the genomic strands of the progeny virus are transcribed. According to the invention, the sequence of the antisense PNA is selected so that the activator-antisense chimera binds to and thereby causes the catalytic destruction of the RNA virus genomic or antigenomic strand.

The present invention also relates to methods of inhibiting infection by RNA viruses with chimeric molecules comprising an activator of RNase L covalently attached to an antisense PNA that is capable of binding to the genome, antigenome, or mRNA of an RNA virus. In the presence of RNase L, the chimeric molecules specifically lead to cleavage of the viral genomic or antigenomic RNA or mRNA.

In one embodiment of the present invention, the antisense PNA moiety of the chimera is complementary to a region of the virus RNA antigenome which is characterized by an absence of self-hybridizing secondary structure. According to the invention, the non-self-hybridizing portion of the antigenome to be

targeted by the antisense PNA moiety can be determined from the sequence of the RNA antigenome by using a secondary-structure-determining algorithm such as MFOLD™. A suitable portion of the antigenome is one that is normally in a single stranded conformation, *i.e.*, forms a loop in the stem and loop secondary structure of the RNA. A similar selection of non-self-hybridizing portions of the viral mRNA may be carried out. In those embodiments of the present invention where the antisense activator chimeras are designed to target antigenomic RNA, they are also complementary to the mRNA that directs translation of the viral proteins.

In a preferred embodiment the antisense PNA moiety is complementary to a portion of the RSV genome or antigenome that is normally single stranded. The activator is attached through a linker to either the amino or the carboxy terminus of the antisense PNA moiety by a linker.

The RNase L activator-antisense PNA chimeras of the present invention can be designed to be complementary to either the genomic or antigenome of any negative strand RNA virus. This embodiment of the present invention is illustrated by the following description of chimeras directed to RSV strain A2, but the invention can be practiced with any other RNA virus having a known genomic sequence. The antigenomic sequence of negative strand RNA viruses can be derived therefrom by routine techniques.

The present invention also relates to a chimera of an activator of RNase L, coupled to a PNA which is complementary to a region of the virus RNA genomic strand characterized by repeated, conserved, or consensus sequences.

In a preferred embodiment of the present invention, the antisense PNA moiety of the chimera is complementary to a region of the viral genomic RNA strand characterized by repeated or consensus sequences. In a particular embodiment, the antisense PNA moiety has a sequence of approximately 17 nucleobases that is complementary to a number of repeated or consensus sequences that occur within the critical gene-end, intragenic, and gene-start signals of the RSV virus RNA genome. Each gene of the RSV genomic RNA begins with a conserved nine-nucleotide gene-start signal, 3'CCCCGUUUA, with the exception of the L gene, which has the signal 3'CCCUGUUUUA. Transcription begins at the first nucleotide of the gene start signal. Each RSV gene terminates with a semi-

conserved 12- to 13- nucleotide gene-end signal, 3' UCAAUUNAUAUAUUUU, which directs transcriptional termination and polyadenylation.

In a preferred embodiment, the following sequence is used as the antisense PNA moiety of the chimera:

(pna)AAA AAT GGG GCA AAT AA

This 17-mer targets a number of sequences that occur within the critical gene-end-intragenic-gene-start signals of the RSV genomic RNA.

In another embodiment of the present invention, the genomic strand of RSV may be targeted with a chimera having an antisense PNA moiety which comprises any of the following sequences:

(pna)GAA GAT GGG GCA AAT AC

(pna)AAG GGA GGG GCA AAT AT

(pna)ACA CAT GGG GCA AAT AA

(pna)AAC ACA GGG GCA AAT AT

(pna)AAA ACT GGG GCA AAT AT

(pna)AGT TGT GGG ACA AAA TG

Critical sequences abstracted from the RSV genome are illustrated in Table 1. Here it is clear that the above preferred 17mer antisense PNA moiety is a perfect hybridization match for three vital RSV genomic RNA signal sequences. Also clear is the fact that this consensus PNA antisense sequence may additionally target other critical regions with lowered but significant efficiency. For instance, the nucleotide sequence signal at the F/intragenic M2 gene start signal has only two mismatches to the consensus antisense sequence. Moreover, one of these is a terminal mismatch which would have a relatively small effect on hybrid duplex stability. Likewise, the signal at the NS2-intragenic-NS2 gene-start has three mismatches, but only one is of the more significant internal variety. Following this logic, the expected order of hybridization efficiency of the consensus antisense PNA 17-mer with the different listed targets would be: 1=2=4>8>3>6,7>5>>9.

TABLE 1

2-5A-Antisense Polyamide Nucleic Acids Targeting RSV Genomic RNA

	antisense PNA	(pna)AAA AAT GGG GCA AAT AA						mismatches	
								terminal	internal
1	3'-leader/NS1 start	3'UUU	UUA	CCC	CGU	UUA	UU5'	0	0
2	NS1/NS2 gene start	3'UUU	UUA	CCC	CGU	UUA	UU5'	0	0
3	NS2/N gene start	3'CUU	CUA	CCC	CGU	UUA	UG5'	2	1
4	N/P gene start	3'UUU	UUA	CCC	CGU	UUA	UU5'	0	0
5	P/M gene start	3'UUC	CCA	CCC	CGU	UUA	UA5'	1	3
6	M/SH gene start	3'UGU	GUA	CCC	CGU	UUA	UU5'	0	2
7	G/F gene start	3'UUG	UGA	CCC	CGU	UUA	UA5'	1	2
8	F/M2 gene start	3'UUU	UGA	CCC	CGU	UUA	UA5'	1	1
9	L gene start	3'UCA	ACA	CCC	UGU	UUU	AC5'	1	7

The result of this design is that a single 2-5A-PNA antisense chimera would be targeted, with varying degrees of efficiency, to a large number of nucleotide sequence signals that are critical for transcription of the RSV genome to yield RSV mRNAs. Such a strategy should lead to a number of disruptions in the parent RSV genomic RNA, any one of which would, according to the model of RSV transcription and replication, be sufficient to shut down virus replication.

In accordance with the present invention, the activator-antisense chimeras can also be designed to target repeated, conserved, or consensus sequences of the genomic strand of other negative strand RNA viruses. For example, Sendai, vesicular stomatitis and influenza viral genes are transcribed from 3' to 5' from a single promoter at the 3' terminus. The 3' and 5' termini also contain sequences required for viral replication and viral packaging. In one embodiment of the invention, these sequences are targeted by the antisense PNA chimeras of the present invention to specifically cleave the genomic strand of these negative strand RNA genomes.

The examples herein demonstrate that 2',5'-oligoadenylate-peptide nucleic acid conjugates are capable of binding to and activating the 2-5A-dependent RNase L. Although the adducts evaluated (**2-4**) were 1 - 2 orders of magnitude less effective than parent 2-5A (1) in activation of RNase L, their potency is comparable to previous 2',5'-oligoadenylate-3',5'-oligodeoxynucleotide conjugates. For instance, Maitra *et al.* (J. Biol. Chem., 270:15071-15075 (1995)) found that 2-5A-antiPKR was 2-fold less effective than 2-5A alone at inducing the degradation of PKR RNA by pure human RNase L, but that the corresponding 2-5A-sensePKR

construct or a chimera against an unrelated RNA sequence (2-5A-antiHIV) were 10-100 fold less potent than the parent 2-5A tetramer. Similarly, 2-5A-antiBCR and 2-5A-iso-antiBCR (a "tail-to-tail" chimera) were 10-fold less potent at inducing degradation of the non-targeted oligonucleotide $rC_{11}U2C7$ (Xiao *et al.*, J. Med. Chem., 41:1531-1539 (1998)), and the 2-5A-antisense anti-RSV chimera that inhibits RSV replication (Player *et al.*, Proc. Natl. Acad. Sci. U.S.A. 95:8874-8879 (1998)), as well as the 2-5A-anti-gag(HIV) chimera that causes targeted degradation of a *gag* RNA (Player *et al.*, Antiviral Chem. Chemother. 9:225-231 (1998)), were an order of magnitude less effective than 2-5A itself. The mechanism underlying this difference has not yet been investigated, but may involve steric interference by the antisense moiety in the interaction of 2-5A and the enzyme, or in the process of enzyme dimerization (Carroll *et al.*, J. Biol. Chem., 272:19193-19198 (1997)).

Insofar as the PNA chimeras of this invention possess an RNase L activation potency similar to that of 2-5A-antisense chimeras with an all-nucleic-acid backbone, they represent an important advance in the application of both the 2-5A-antisense and PNA strategies to the control of gene expression. Most importantly, the 2-5A-dependent RNase L augments the PNA-antisense agents with a catalytic mode of targeted RNA destruction. The *in vivo* lifetime of these chimeras, relative to the corresponding 2-5A-oligonucleotide antisense conjugates, will be improved since the PNA backbone is highly resistant to attack by the nucleases present in cells and sera (Demidov et al;., Biochem. Pharmacol. 48:1310-1313 (1994)); and this longevity is gained without compromise to the affinity or selectivity of target hybridization (Knudsen and Nielsen, Nucleic Acids Res. 24:494-500 (1996)).

Another advantage of the PNA antisense oligomer is the synthetic flexibility of the pseudopeptide linkage which facilitates further modifications, for example those directed toward improved cellular uptake of the chimera [de Mesmaeker *et al.*, Acc. Chem. Res., 28:366-374 (1995); Hyrup and Nielsen, Bioorg. Med. Chem. 4:5-23 (1996); Simmons *et al.*, Bioorg. Med. Chem. Lett. 7:3001-3006 (1997)], or those directed toward improving solubility or affinity for the target RNA, for example by appending a polyamine moiety (Gangamani *et al.*, Biochem. Biophys. Res. Commun., 240:778-782 (1997). Such modifications of the chimeric

molecules are contemplated to be within the scope of this invention.

An unexpected advantage of the chimeras of the present invention is their ability to broaden the substrate specificity of RNase L, as illustrated by the examples herein. This enzyme is normally specific for single-stranded RNA, but surprisingly, cleavage induced by the chimeras of this invention may occur at sites where the target RNA is presumably hybridized to the antisense PNA moiety. Also surprising is that the chimeras induce RNase L to cleave the targe RNA at sites other than at the uridine residues normally preferred by RNase L.

The invention also provides a method of cleaving a specifically selected (target) strand of RNA, which comprises the steps of: (a) hybridizing the strand of RNA with a chimeric molecule of this invention to form a complex of the RNA strand and the chimeric molecule, and (b) allowing the resulting complex to react with RNase L; thereby specifically cleaving the target strand of RNA. The chimeric molecules of the invention comprise (a) an antisense peptide nucleic acid moiety that binds or anneals to the target strand of RNA and (b) an activator of RNase L attached to the antisense peptide nucleic acid moiety.

Another aspect of this invention is a method of cleaving a specifically selected target strand of RNA contained in a cell, wherein the cell contains RNase L, comprising the steps of: (a)contacting the cell with a chimeric molecule of the invention, (b) entry of the chimeric molecule into the cell; (c) allowing the chimeric molecule to form a complex with the target strand of RNA; and (d) allowing the complex to react with RNase L; whereby the target strand of RNA is cleaved. It will be appreciated that the cell may be an isolated cell in culture, or may be a cell in its natural environment as part of a multicellular animal. The animal is preferably a mammal, and most preferably a human.

Preferably, the chimeric molecule is contacted with said cell at a concentration of between 0.1 μM and 100 μM, more preferably at a concentration of between about 1.0 μM and 5.0 μM. PNA-DNA chimeras are known to be capable of passing into cells at concentrations within this range (Uhlmann *et al.*, Angew. Chem. Int. ed. Engl. 35:2632-2635 (1996)). Optionally, methods of enhancing the passing of oligonucleotides into cells may be employed, for example by electroporation and by complexation with ionic surfactants, as is known to those

skilled in the art. It is within the ability of those skilled of the art to vary the dosage, if necessary or desired, and depending upon the mode of delivery and the cellular milieu, so as to obtain the desired concentration of chimeric molecules in a multicellular animal.

The invention also provides compositions comprising the chimeric molecules of this invention. Pharmaceutical compositions comprising the chimeric molecules of this invention will comprise an effective amount of the chimera, and may additionally comprise pharmaceutically acceptable carriers, solvents, diluents, and additives known to the art. Examples of additional pharmaceutically acceptable components of these pharmaceutical compositions include water, saline, buffers, surfactants, dispersants, and preservatives. The pharmaceutical compositions are preferably sterilized by ultrafiltration or other methods known to the art. The compositions may take the form of sterile solutions or lyophilized powders, or may be in solid dosage forms for implantation or oral or rectal administration.

The chimeric molecules and compositions of this invention are expected to be useful for reducing the concentration of RNA species *in vitro* and *in vivo*, in any application where antisense molecules are of use. In the presence of RNase L, the chimeric molecules of the present invention are also expected to be useful wherever ribozymes are of use. Among the research applications of the chimeric molecules of this invention is the sequence-specific inhibition of a targeted mRNA in order to effectively "knock out" the associated gene, as an aid to elucidating the function of the encoded protein.

Among the therapeutic uses for the chimeras and compositions of this invention are the treatment of diseases or infections, particularly viral diseases and infections, by ablation of viral mRNA, genomic, or antigenomic RNA, by the reduction of expression of proteins associated with pathophysiological effects (such as TNF and auto-antibodies), and by the reduction of transcription of oncogenes or tumor-promoting gene products (such as angiogenic growth factors). Another therapeutic use for the chimeras of the invention is the inhibition of tumor growth in mammals by chimeras targeted to the RNA component of telomerase. In addition, chimeric molecules targeted to the mRNA encoded by mutated genes responsible for autosomal dominant inherited diseases, such as retinitis pigmentosa, myotonic

dystrophy, and the like, are expected to be useful for ameliorating the symptoms of such diseases, whenever the DNA sequence of the mutant gene is known. The invention provides a method for treating such diseases by administration of a chimeric molecule of the invention, wherein the antisense polyamide nucleic acid moiety of the chimera has a nucleobase sequence complementary to the mRNA encoded by said gene, in an amount sufficient to reduce the transcription rate of the mutant gene.

The activator-antisense chimeras of the invention may also be used to inhibit infection by a negative strand RNA virus to which the activator-antisense chimera is targeted, for example RSV infection. The activator-antisense chimeras of the invention can be administered to a subject having an RSV or other respiratory viral infection by any route effective to deliver the activator-antisense chimeras to the epithelium of the bronchi, bronchioles and alveoli of the subject. In one embodiment the activator-antisense chimeras are delivered by use of an inhaled aerosol, according to the techniques well known in the art for the delivery of ribavirin. In a further embodiment of the invention a mixture of ribavirin and a chimera of the invention can be administered in a common pharmaceutical carrier.

In an alternative embodiment the chimera can be administered parentally, *e.g.*, by intravenous infusion. When delivered by intravenous administration, the dose of the chimera can be determined by routine methods well known to pharmacologists so that the serum concentration approximates the concentration at which RNA cleavage activity is seen in the *in vitro* examples described herein. When delivered by aerosol administration the dose should be selected so that the tissue concentration in the lung approximates the concentration at which RNA cleavage activity is seen in the *in vitro* examples.

Another aspect of the invention relates to methods of preparing the chimeras of the invention. In one embodiment, the synthesis process comprises the steps of

(a) preparing a polyamide nucleic acid moiety on a solid-phase resin by N-terminal extension,

(b) attaching to the N-terminal of said polyamide nucleic acid moiety an N-(2-hydroxyethyl)-N-[1-(purinly or pyrimidinyl)acetyl]glycyl moiety,

(c) attaching to the terminal hydroxyl group introduced in step (b) an O-(4-hydroxybutyl)phosphoryl moiety,

(d) attaching to the terminal hydroxyl group introduced in step (c) an additional O-(4-hydroxybutyl)phosphoryl moiety,

(e) attaching to the terminal hydroxyl group introduced in step (d) an O-(2'-adenosyl)phosphoryl moiety,

(f) attaching to the 5' hydroxyl group of the terminal adenosyl moiety another O-(2'-adenosyl)phosphoryl moiety,

(g) repeating step (f) between one and three times,

(h) attaching to to the 5' hydroxyl group of the terminal adenosyl moiety a monophosphate, diphosphate, or triphosphate moiety,

(i) cleaving the chimeric molecule from the resin, optionally with the simultaneous removal of protecting groups, and

(j) removing any remaining protecting groups from the chimeric molecule.

While the examples presented below describe a number of embodiments of this invention, it is apparent to those skilled in the relevant arts that the compounds, compositions, and methods of this invention can be altered to provide other embodiments which nonetheless remain within the scope of this invention. Therefore, it will be appreciated that the scope of this invention is not defined by the specific embodiments which are presented merely by way of example.

All references cited in this disclosure are incorporated by reference herein, in their entirety.

EXAMPLES

Synthesis of chimeric molecules.

Retrosynthetic analysis revealed that the solid-phase assembly of target 2-5A-PNA adducts **2-4** could be attained starting from known building blocks **5-9** following a protocol similar to that described for the synthesis of DNA-PNA chimeras (Uhlmann *et al.*, Nucleosides and Nucleotides 16:603-608 (1997), van der Laan *et al.*, Tetrahedron Lett. 38:2249-2252 (1997)).

Thus, highly cross-linked polystyrene beads were functionalized with a glycine unit via a *p*-hydroxymethylbenzoic acid linker, resulting in immobilized **10** (Figure 2). The PNA part of the chimera was constructed by sequential elongation of the amino-terminus using monomethoxytrityl protected PNA adeninyl monomer **5** (Will *et al.*, Tetrahedron 51:12069-12082 (1995)) and HATU as the coupling reagent. The synthesis of the PNA sequence was completed by introduction of the 3'-linker by reaction of **6** (Petersen *et al.*, Bioorg. Med. Chem. Lett., 5:1119-1124 (1995)) with the amino group of immobilized PNA **11** to give **12**. At this stage, elongation of the solid-phase bound oligomer was feasible using standard phosphoramidite chemistry. The butyl spacers were introduced by the o-nitrophenyltetrazole mediated reaction (D. Filipov, Ph.D. Thesis, Leiden University (1988)) of (2-cyanoethyl)-N,N-diisopropyl-4-O-(4,4'-dimethoxytrityl)butylphosphoramidite **7** (Lesiak *et al.*, Bioconjugate Chem., 4:467-472 (1993)) with the free hydroxyl group in **12**. The 2',5'-oligoadenylate part of the chimera was appended to the growing oligomer by elongation of **13** with the protected adenosine 2'-phosphoramidite 8 in four consecutive coupling cycles. The 5'-terminal phosphate triester was introduced by reaction of the free hydroxyl in resulting **14** with 2-[[2-(4,4'-dimethoxytrityloxy)ethyl]sulfonyl]ethyl-(2-cyanoethyl)-N,N-diisopropylphosphoramidite **9**, to afford fully protected 2-5A-PNA adduct **15**. In the final stage of the synthesis, the chimeric oligonucleotide **15** was released from the resin with concomitant deprotection using methanolic ammonia and desilylation with tetraethylammonium fluoride. The crude mixture was desalted by gel filtration and purified by reverse-phase HPLC. This procedure furnished 2-5A-PNAs **2-4**, of the general formula 2-5A-pnaA$_n$, where n = 4, 8, or 12. The homogeneity and identity of the products were confirmed by HPLC analysis and mass spectrometry.

Synthesis procedures

All solvents (Biosolve™, DNA synthesis grade) were used as received. Solid phase syntheses were performed on a Pharmacia Gene Assembler™ using highly cross-linked polystyrene beads as the solid support (loading: 26-28 μmol/g) on a 1 μmol scale. The support was functionalized with a glycine moiety via a p-hydroxymethylbenzoic acid linker. Assembly of the PNA part was

established using solutions of 0.3 M of monomers 5 and 6, 0.3 M DiPEA and 0.3 M HATU in acetonitrile/ dimethylformamide (1/1, v/v). Prior to coupling, the monomers were pre-activated for 1 min by mixing equal amounts of the PNA monomer (15 equiv per μmol support), HATU and DiPEA solutions. The protocol for one PNA chain extension cycle consisted of (1) wash: acetonitrile/dimethylformamide (1/1, v/v), 2.5 ml; (2) coupling: PNA + HATU + DiPEA in acetonitrile/dimethylformamide (1/1, v/v), 15 min; (3) wash: acetonitrile/dimethylformamide (1/1, v/v), 2.5 ml, acetonitrile, 2.5 ml; (4) capping: Ac_2O/lutidine/N-methylimidazole/tetrahydrofuran (1/1/1/7, v/v/v/v), 2.0 ml; (5) wash: acetonitrile, 2.5 ml, dichloromethane, 3.5 ml; (6) detritylation: 2% trichloroacetic acid in dichloromethane, 3 min; (7) wash: dichloromethane, 2.5 ml, acetonitrile, 5 ml. The introduction of the phosphate bonds was carried out with 15 equiv of cyanoethyl phosphoramidites **7-9** using 5-(o-nitrophenyl)tetrazole (8 equiv) as the activator. Standard DNA capping, washing, oxidation and detritylation cycles were used. Coupling yields were gauged spectrophotometrically (254 nm) by the absorption of the released trityl cation after each deprotection step. After the last elongation step, the oligomers were cleaved from the support with concomitant deprotection of the phosphate groups and exocyclic amino groups by treatment with methanolic ammonia (1.5 ml) at 50°C for 16 h. The samples were filtered and the silyl protective groups were removed by treatment with tetraethylammonium fluoride (0.5 M in dry acetonitrile) at ambient temperature for 16 h. Desalting was established using a G-25 column with a 0.15 M solution of ammonium bicarbonate as the eluting agent. RP-HPLC purification and analysis were carried out with a LiChrospher™ 100 RP-18 endcapped column (10.0 x 250 mm and 4.0 x 250 mm, respectively). Gradient elution was performed at 40°C by building up a gradient starting with buffer A (50 mM triethylammonium acetate in water) and applying buffer B (50 mM triethylammonium acetate in acetonitrile/water, 1/1, v/v) with a flow rate of 1.0 ml/min or 5.0 ml/min for analysis and purification, respectively. The identity of the oligomers was confirmed using electrospray mass spectrometry. For 2-5A-pnaA4 (**2**), calculated for $C_{105}H_{138}N_{53}O_{49}P_7$: 3143; found 3142.7. For 2-5A-pnaA8 (**3**), calculated for $C_{149}H_{190}N_{81}O_{57}P_7$: 4244; found 4244.5.

Chimeric molecules having other nucleobase sequences may be prepared by the method described above, by altering the order in which the PNA monomers are introduced to the reaction vessel and coupled to the growing oligomer.

RNase L cleavage

To ascertain the ability of the 2-5A-PNA chimeras to activate RNase L, two independent assays were used. The first assay employed radiolabeled poly(U) as the substrate. Under the assay conditions, the 2-5A tetramer **1** activated pure recombinant human RNase L to degrade poly(U)-[^{32}P]pCp, with 50% cleavage occurring at a concentration (EC_{50}) of 0.4 ± 0.04 nM (Figure 4). Each of the three 2-5A-PNA analogues showed a concentration-dependent ability to cause cleavage of the poly(U). The most effective was the tetraadenylate derivative **2**, which possessed an EC_{50} of 4 ± 0.5 nM. Only slightly less effective was the octaadenylate **3**, with an EC_{50} of 7 ± 3 nM. Significantly less effective was the chimera that contained the adenylate 12-mer **4**, with an EC_{50} of 25 ± 4 nM. For comparison the term C_{rel} was used which defines the relative activity of the 2-5A tetramer as unity, and compares (ratio of EC_{50}'s) the relative concentration of other activators needed to effect 50% cleavage. The greater the C_{rel}, the less effective the activator. These data thus indicate the following (decreasing) order of effectiveness: 2-5A (**1**) (Crel=1); 2-5A-pnaA4 (**2**) (Crel=9); 2-5A-pnaA8 (**3**) (Crel=17); 2-5A-pnaA12 (**4**) (C_{rel}=62) (Table 2). Thus, while none of the chimeras was as potent as the 2-5A standard, each brought about 50% RNA substrate cleavage at concentrations within one to two orders of magnitude of 2-5A. In addition, the PNA congeners exhibited decreasing activation potency as the length of the PNA antisense oligomer increased.

In a second independent approach, activation of the 2-5A-dependent RNase L by the various 2-5A-PNA chimeras was ascertained by monitoring the ability of pure recombinant human RNase L to cleave the radiolabeled synthetic ribonucleotide, r([^{32}P]p$C_{11}U_2C_7$). Use of this oligonucleotide was introduced by Carroll and co-workers (J. Biol. Chem. 271:4988-4992 (1996)) who found that RNase L caused scission of the short RNA 3' to both uridine nucleotides, with initial cleavage occurring to yield r(pC_{11}UpUp), and a second cleavage to give r(C_{11}Up) at

higher enzyme concentration or longer incubation times. In the present assays, activity has been represented in terms of the concentration of 2-5A or analogue required to effect 50% cleavage to r([^{32}P]pC$_{11}$UpUp) (EC_{50}). Under these assay conditions, the 2-5A tetramer **1** possessed a mean EC_{50} of 0.3 ± 0.01 nM (Figure 5). The 2-5A-PNA chimera tetramer **2** showed a mean EC_{50} of 3 ± 1 nM , a 10-fold reduction in RNase L activation ability. The octaadenylate congener **3** gave an average EC_{50} of 5 ± 1 nM, and compound **4** (the dodecaadenylate chimera) had a mean EC_{50} of 32 ± 1 nM, a 100-fold reduction in activation compared to the tetrameric 2-5A standard. Based on the above data, the 2-5A-peptide nucleic acids could be ranked in the following order of decreasing ability to activate RNase L, using a C_{rel} value as defined for the poly(U) cleavage assay: 2-5A (**1**) (C_{rel}=1) > 2-5A-pnaA4 (**2**) (C_{rel}=11) ~ 2-5A-pnaA8 (**3**) (C_{rel}=14) > 2-5A-pnaA12 (**4**) (C_{rel}=99) (Table 2).

Table 2.

Relative activities (C_{rel})[a] of 2-5A-PNA chimeras and the parent 2-5A compound in cleavage activity and radiobinding assays.

Compound	Poly(U) Cleavage	$pC_{11}U_2C_7$ Cleavage	Radiobinding
2-5A	1	1	1
2-5A-pnaA$_4$	9 ± 1 (5)	11 ± 2 (3)	3 ± 1 (3)
2-5A-pnaA$_8$	17 ± 8 (4)	14 ± 3 (3)	8 ± 7 (4)
2-5A-pnaA$_{12}$	62 ± 10 (5)	99 ± 2 (3)	43 ± 26 (6)

[a]C_{rel} is the EC_{50} (or IC_{50}) of the 2-5A-PNA chimera divided by the EC_{50} (or IC_{50}) of the 2-5A standard, with 2-5A arbitrarily assigned a C_{rel} value of 1. These values are given as the mean ± standard deviation, with the number of experiments (n) given in brackets. The experimental EC_{50} values for 2-5A were 0.4 ± 0.04 nM (n=5; poly(U) assay) and 0.3 ± 0.01 nM (n=3; $pC_{11}U_2C_7$ assay). Similarly, the experimental IC_{50} for probe displacement by 2-5A in the radiobinding assay was 3.3 ± 1.5 nM (n=6).

RNase L activation and cleavage of a poly-uridine (Poly(U)) substrate.

Pure recombinant human RNase L was prepared by a modification of a previously described procedure (Player *et al.*, in *Methods: A companion to Methods in Enzymology*, **15** (1998)). Poly(U) was obtained commercially as a mixture of high molecular weight uridine polymers. Using T4 RNA ligase, the poly(U) was 3'-labeled with 5'-[^{32}P]pCp and then HPLC purified. This procedure and the assay have been described previously [Silverman and Krause, *Lymphokines and Interferons: A practical approach*, Clemens *et al.*, eds, 149-193 (1987); Player et al, *supra*]. 2 µL of a 10x cleavage buffer (100 mM HEPES, pH 7.5, 1.0 M KCl,

50 mM $Mg(OAc)_2$, 10 mM ATP, and 143 mM 2-mercaptoethanol) and 12-16 µL of RNase-free water were used in each cleavage reaction. To this, 2 µL of a 10x solution of 2-5A analogue (final concentrations 10-5 to 10-10 M) and recombinant RNase L enzyme (final concentration of 130 nM) were added, and lastly 2µL of poly(U)-[^{32}P]pCp substrate (final concentration 10uM in UMP equivalents) to make a final volume of 20 µL. After a 15 min incubation at 30 °C, 4 volumes of 5mg/ml carrier (yeast) RNA was added, and then 10 M ammonium acetate to a final concentration of 2-2.5 M. After mixing with 2 volumes of cold ethanol, the reaction mixtures were left on ice for 30 min, and the precipitated RNA pelleted with a brief spin at 4 °C (12 000 x g for 2 min.). The presence of cleaved fragments of poly(U)-[^{32}P]pCp was assessed by counting aliquots of the supernatant in scintillation fluid.

RNase L activation and cleavage of a synthetic oligonucleotide substrate.

This assay relied upon the RNase L-induced cleavage of the synthetic oligonucleotide, $rC_{11}U_2C_7$ [Carroll *et al.*, J. Biol. Chem., 272:19193-19198 (1997)]. Oligoribonucleotide $rC_{11}U_2C_7$ was prepared by Midland Certified Reagent Co. (Midland, TX) and 5'-labeled with polynucleotide kinase and γ-^{32}P-ATP (DuPont-NEN, Wilmington, DE). For cleavage assays, the cleavage buffer was 25 mM Tris HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 100 µM ATP, and 10 mM DTT. For each assay, components were added in the following order: 14 - 18 µL cleavage buffer, 2 µL 2-5A-antisense chimera at 10x the desired final concentration, and 2 µL of an RNase L solution to give a final concentration of 130 nM RNase L. Cleavage reaction mixtures were held for 10 min on ice after addition of RNase L. The substrate [^{32}P]$pC_{11}U_2C_7$ (2 µL of a solution to give a final substrate concentration of 10 nM) was added last and the mixture incubated at 37 °C for 15 min. 20 µL of RNase-free loading buffer was then added, and the samples applied to a 1 mm 20% PAGE/8M urea gel for electrophoresis at 350 V for 6 h at 1 °C. The gels were exposed to film, and the films developed and scanned to quantify cleavage densitometrically.

For all RNase L-catalyzed RNA degradation experiments, the EC_{50} was defined (after background subtraction) as the effective concentration that brought about 50% degradation of RNA substrate.

Binding to RNase L

The ability of the 2-5A-PNA chimeras to bind to RNase L was evaluated in a nitrocellulose filter assay that depended on the competition of the radioligand, 2-5A-[^{32}P]pCp, with the unlabeled 2-5A analogues, for binding to the RNase L in CEM lysate. The results are presented as IC_{50} values (the molar concentration of 2-5A or analogue required to displace 50 % of the radioactive probe). Also, the data were normalized to the 2-5A standard (defined as unity), and the relative IC_{50} concentrations (C_{rel}) are given in Table 2.

The binding ability of the 2-5A-PNA adducts showed length-dependence (Figure 6). In conditions where the 2-5A tetramer (**1**) gave an IC_{50} of 3 ± 1 nM, the tetraadenylate and octaadenylate congeners (**2** and **3**) were approximately equivalent in binding affinity as judged by their respective IC_{50}'s of 8 ± 3 nM and 21 ± 13 nM. However, the extension of the adenylate sequence to a dodecamer, giving an overall 13-mer as the antisense domain in the 2-5A-PNA chimera (**4**), with an IC_{50} of 123 ± 61 nM, caused a significant decrease in binding. Therefore, the binding data were in accord with the data from the two activation assays since there was a correspondence between a diminished ability to activate RNase L and the ability to bind to RNase L

Radiobinding assays.

Human CEM cell cytoplasmic extracts were prepared according to previously described methodology (Kovacs *et al.*, Bioorg. Chem. 21:192-208 (1993)). The radiobinding assay probe p5'A2'(p5'A2)2 p5'A3'[^{32}P]p5'C3'p, was synthesized by the T4 RNA ligase-catalyzed addition of [^{32}P]5'pCp to the 3' end of 2-5A (1), using a published procedure with subsequent HPLC purification (Player et al, *supra*; Silverman and Krause, *supra*). To assay a given 2-5A analogue, serial dilutions were prepared in water. Each binding assay consisted of 5 µL of 2-5A or 2-5A analogue, 5 µL of CEM cell lysate, and 15 µL of a master mix that consisted of one part of buffer A [20 mM Tris HCl (pH 7.6), 85 mM KCl, 5 mM $Mg(OAc)_2$ 1 mM ATP, 5% (v/v) glycerol] and two parts of buffer B (same as buffer A, but without ATP) and sufficient 2-5A-[^{32}P]pCp probe to give 10^4 to 2 x 10^4 cpm/assay. The order of addition for each assay was 2-5A or analogue, then master mix, then lysate. Assay mixtures were incubated at 4°C for 2 h, after which they were applied to nitrocellulose filters that were subsequently washed (3x) with water. The filters

were placed in scintillant and counted in a liquid scintillation counter.

The present invention is not to be limited in scope by the specific embodiments described above, which are intended only as illustrations of individual aspects of the invention. Modifications which are obvious to those of ordinary skill in the art are intended to be within the scope of the following claims.

CLAIMS

We claim:

1. A method of cleaving a specifically selected strand of RNA, comprising the steps of:
   (a) hybridizing said strand of RNA with a chimeric molecule to form a complex of said strand and said chimeric molecule, said chimeric molecule comprising
      (i) an antisense polyamide nucleic acid moiety that binds or anneals to said strand of RNA and
      (ii) an RNase L activator moiety attached to said antisense polyamide nucleic acid moiety; and
   (b) allowing said complex to react with RNase L; thereby specifically cleaving said strand of RNA.

2. The method of claim 1, wherein said activator moiety comprises a 2',5'-oligonucleotide.

3. The method of claim 2, wherein said activator moiety comprises a 2',5'-oligoadenylate oligonucleotide.

4. The method of claim 3, wherein said 2',5'-oligoadenylate oligonucleotide is p5'A2'p5'A2'p5'A2'p5'A.

5. The method of any one of claims 1-3, wherein said activator moiety comprises one or more 5' thiophosphate groups.

6. A method of cleaving a specifically selected strand of RNA contained in a cell, wherein said cell contains RNase L, comprising the steps of:
   (a) contacting said cell with a chimeric molecule, said chimeric molecule comprising
      (i) an antisense polyamide nucleic acid moiety that binds or anneals to said strand of RNA and

(ii) an RNase L activator moiety attached to said antisense polyamide nucleic acid moiety;

(b) permitting entry of said chimeric molecule into said cell;

(c) allowing said chimeric molecule to form a complex with said strand of RNA; and

(d) allowing said complex to react with RNase L;

whereby said strand of RNA is cleaved.

7. The method of claim 6, wherein said activator moiety comprises a 2',5'-oligoadenylate oligonucleotide.

8. The method of any one of claims 6-7, wherein said activator moiety comprises one or more 5' thiophosphate groups.

9. The method of claim 7, wherein said 2',5'-oligoadenylate oligonucleotide is p5'A2'p5'A2'p5'A2'p5'A.

10. The method of any one of claims 6-9, wherein said chimeric molecule is contacted with said cell at a concentration of between 0.1 μM and 100 μM.

11. The method of any one of claims 6-9, wherein said chimeric molecule is contacted with said cell at a concentration of between about 1.0 μM and 5.0 μM.

12. The method of any one of claims 6-9, wherein said antisense moiety binds or anneals to a single stranded region of said strand of RNA contained in said cell.

13. The method of any one of claims 6-9, wherein said strand of RNA contained in said cell is the RNA transcript of an oncogene or a proto-oncogene.

14. The method of any one of claims 6-9, wherein said strand of RNA contained in said cell is the RNA transcript of a viral protein.

15. The method of any one of claims 6-9, wherein said strand of RNA contained in said cell is the genome of an RNA virus.

16. A chimeric molecule comprising an antisense polyamide nucleic acid moiety attached to an RNase L activator moiety.

17. The chimeric molecule of claim 16, wherein said antisense polyamide nucleic acid moiety is between about 6 and about 50 nucleobases in length.

18. The chimeric molecule of claim 16, wherein said antisense polyamide nucleic acid moiety is between about 10 and about 30 nucleobases in length.

19. The chimeric molecule of claim 16, wherein said antisense polyamide nucleic acid moiety is between about 12 and about 25 nucleobases in length.

20. The chimeric molecule of any one of claims 16 - 19, wherein said antisense polyamide nucleic acid moiety comprises an oligo[N-(2-aminoethyl)-N-(1-(purinyl or pyrimidinyl)acetyl)glycine].

21. The chimeric molecule of any one of claims 16 - 20, wherein said activator moiety comprises a 2',5'-oligoadenylate oligonucleotide.

22. The chimeric molecule of any one of claims 16-21, wherein said activator moiety comprises one or more 5' thiophosphate groups.

23. The chimeric molecule of any one of claims 16-22, wherein said antisense polyamide nucleic acid moiety comprises the sequence (pna)AAAAATGGGGCAATAA.

24. The chimeric molecule of any one of claims 16-22, wherein said antisense polyamide nucleic acid moiety comprises a sequence selected from the group consisting of
(pna)GAAGATGGGGCAAATAC,
(pna)AAGGGAGGGGCAAATAT,
(pna)ACACATGGGGCAAATAA,
(pna)AACACAGGGGCAAATAT,
(pna)AAAACTGGGGCAAATAT, and
(pna)AGTTGTGGGACAAAATG.

25. The chimeric molecule of any one of claims 16-22, wherein said antisense polyamide nucleic acid moiety comprises the sequence (pna)GCGCGGGGAGCAAAAGCAC.

26. A pharmaceutical composition comprising a pharmaceutically effective amount of a chimeric molecule according to any one of claims 16-22, and a pharmaceutically acceptable carrier.

27. A pharmaceutical composition comprising a pharmaceutically effective amount of a chimeric molecule according to any one of claims 23-24, and a pharmaceutically acceptable carrier.

28. A pharmaceutical composition comprising a pharmaceutically effective amount of a chimeric molecule according to claim 25, and a pharmaceutically acceptable carrier.

29. A method of treating a subject having a Respiratory Syncytial Virus infection, comprising administering to said subject an amount of a composition according to claim 27 sufficient to reduce the replication rate of said virus.

30. A method of inhibiting the growth of a tumor in a mammal, comprising administering to said mammal an amount of a composition according to claim 28 sufficient to reduce the growth rate of said tumor.

31. A method of treating a subject having a viral infection, comprising administering to said subject an amount of a composition according to claim 26 sufficient to reduce the replication rate of said virus, wherein the antisense polyamide nucleic acid moiety has a nucleobase sequence complementary to the genome or antigenome of said virus.

32. A method of treating a subject having an autosomal dominant disease caused by a mutant gene, comprising administering to said subject an amount of a composition according to claim 26 sufficient to reduce the expression of said

gene, wherein the antisense polyamide nucleic acid moiety has a nucleobase sequence complementary to the mRNA encoded by said gene.

33. A method of making a chimeric molecule comprising an antisense polyamide nucleic acid moiety attached to an RNase L activator moiety, comprising the steps of

(a) preparing a polyamide nucleic acid moiety on a solid-phase resin by N-terminal extension,

(b) attaching to the N-terminal of said polyamide nucleic acid moiety an N-(2-hydroxyethyl)-N-[1-(purinly or pyrimidinyl)acetyl]glycyl moiety,

(c) attaching to the terminal hydroxyl group introduced in step (b) an O-(4-hydroxybutyl)phosphoryl moiety,

(d) attaching to the terminal hydroxyl group introduced in step (c) an additional O-(4-hydroxybutyl)phosphoryl moiety,

(e) attaching to the terminal hydroxyl group introduced in step (d) an O-(2'-adenosyl)phosphoryl moiety,

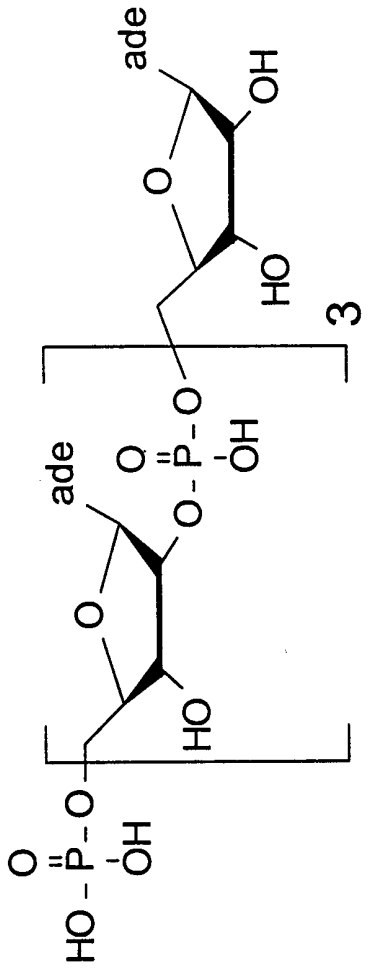
(f) attaching to the 5' hydroxyl group of the terminal adenosyl moiety another O-(2'-adenosyl)phosphoryl moiety,

(g) repeating step (f) between one and three times,

(h) attaching to the 5' hydroxyl group of the terminal adenosyl moiety a monophosphate, diphosphate, or triphosphate moiety,

(i) cleaving the chimeric molecule from the resin, optionally with the simultaneous removal of protecting groups, and

(j) removing any remaining protecting groups from the chimeric molecule.



1

Fig. 1

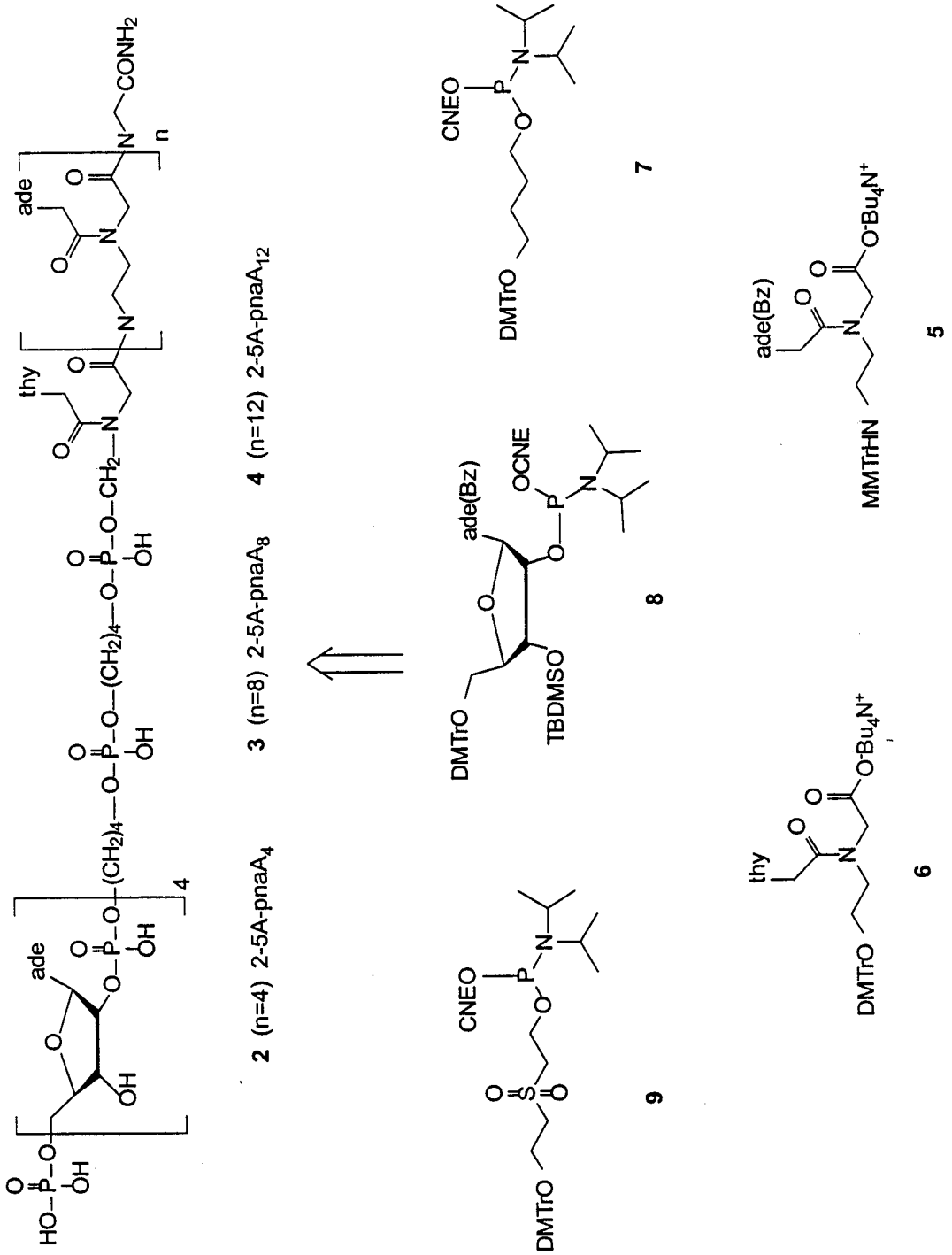


Fig. 2

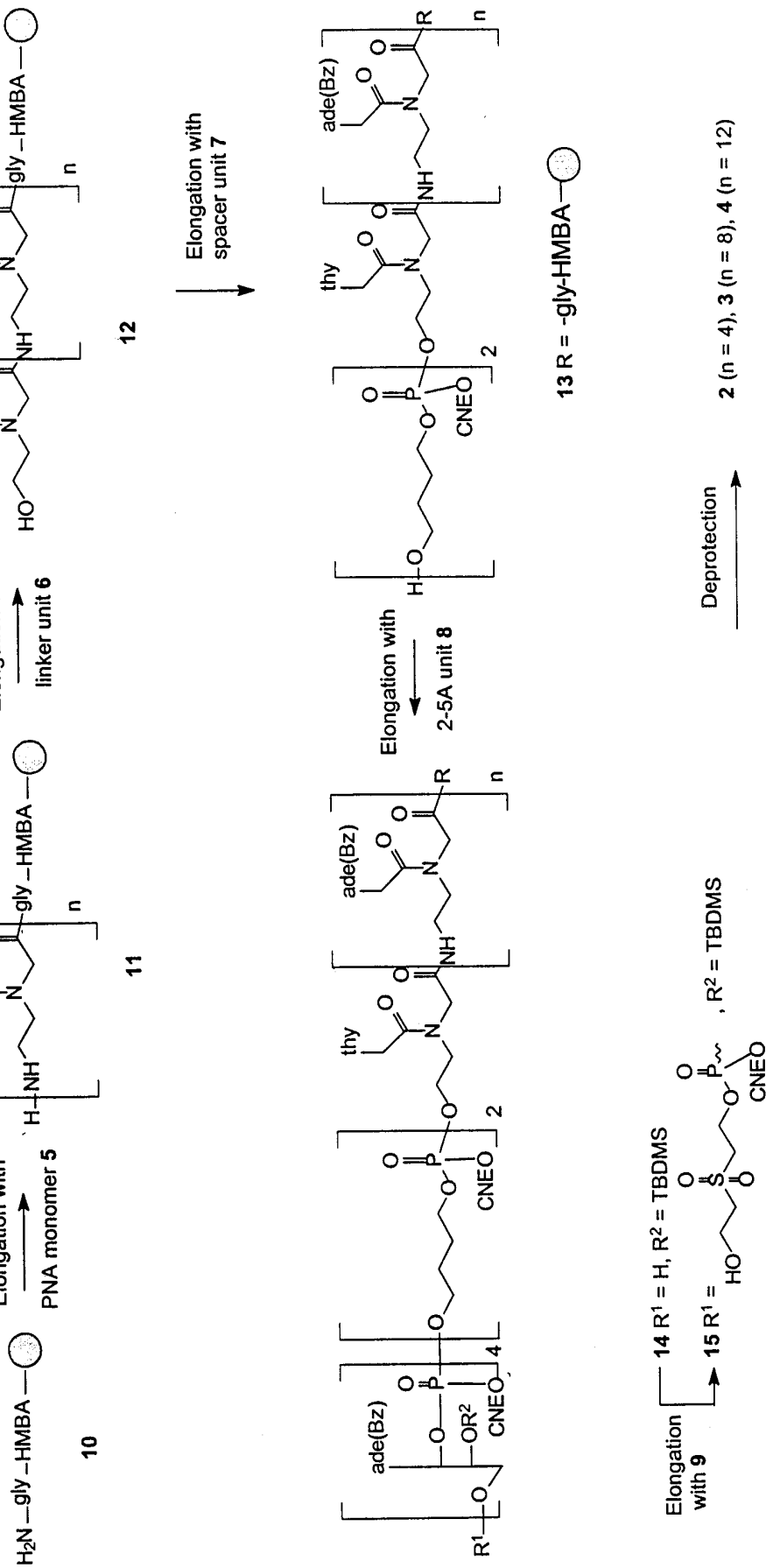

H2N—gly–HMBA—
10
Elongation with
PNA monomer 5
11
Elongation with
linker unit 6
12
Elongation with
spacer unit 7
Elongation with
2-5A unit 8
13 R = -gly-HMBA—
Deprotection
2 (n = 4), 3 (n = 8), 4 (n = 12)
Elongation with 9
14 R1 = H, R2 = TBDMS
15 R1 = , R2 = TBDMS
ade(Bz)
thy
CNEO
HO

Fig. 3

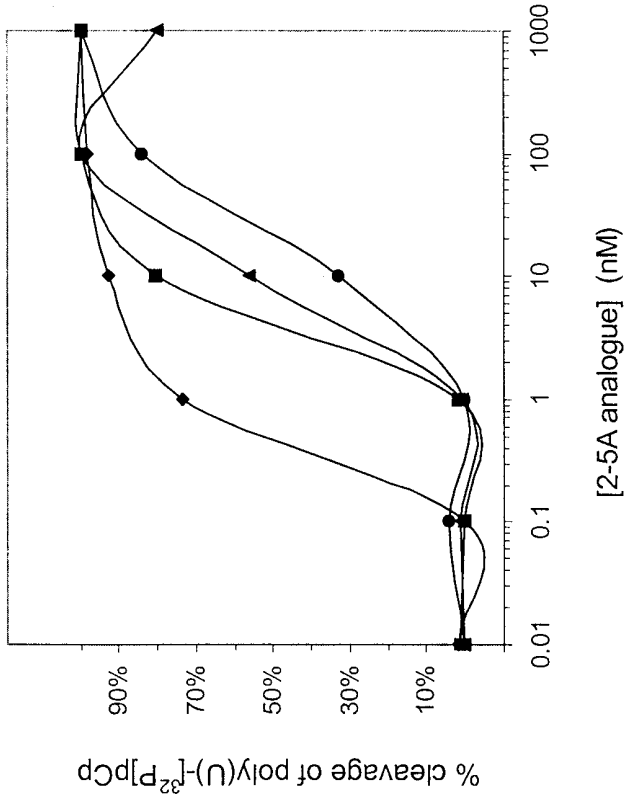

% cleavage of poly(U)-[32P]pCp
90%
70%
50%
30%
10%
0.01
0.1
1
10
100
1000
[2-5A analogue] (nM)

Fig. 4

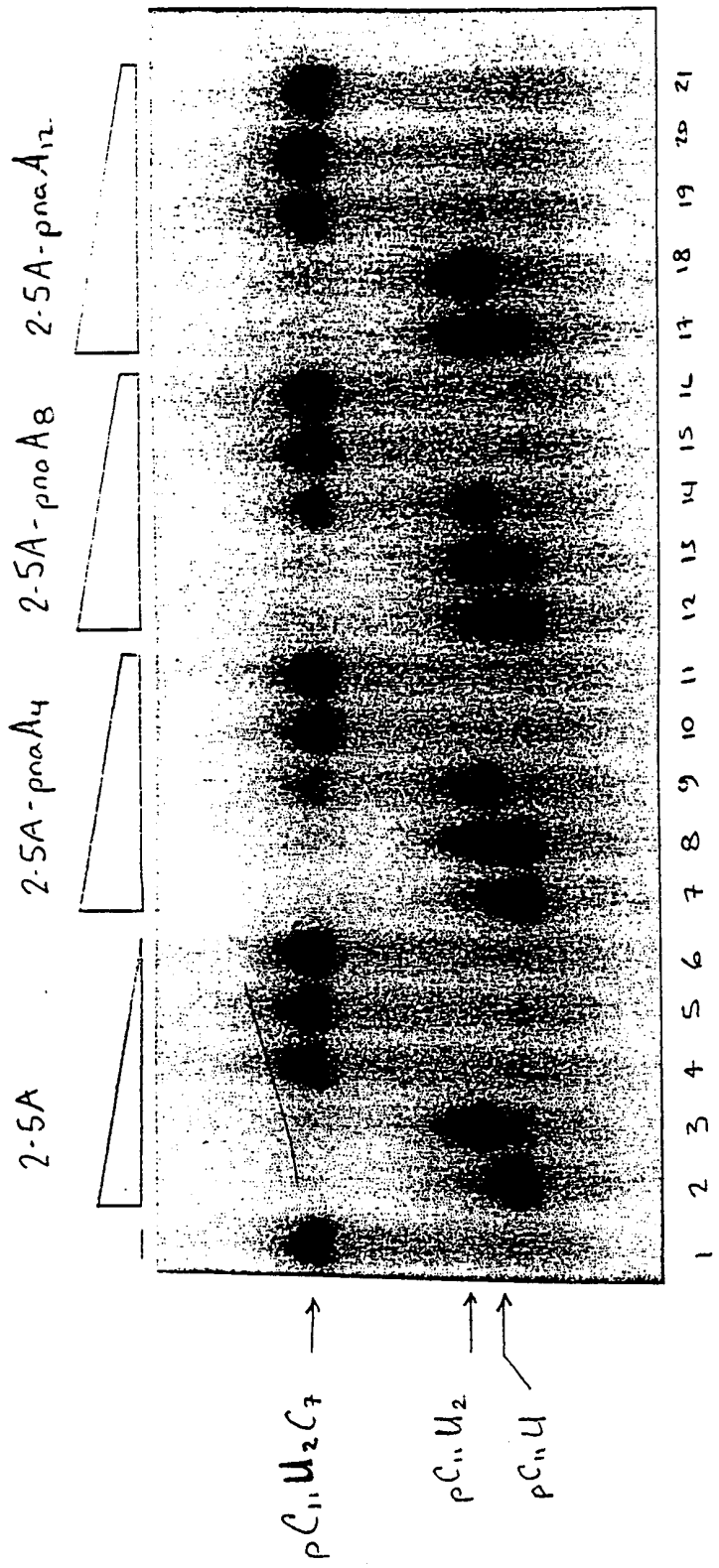

2-5A
2-5A-pnaA4
2-5A-pnaA8
2-5A-pnaA12
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
pC1, U2 C7
pC1, U2
pC1, U

Fig. 5

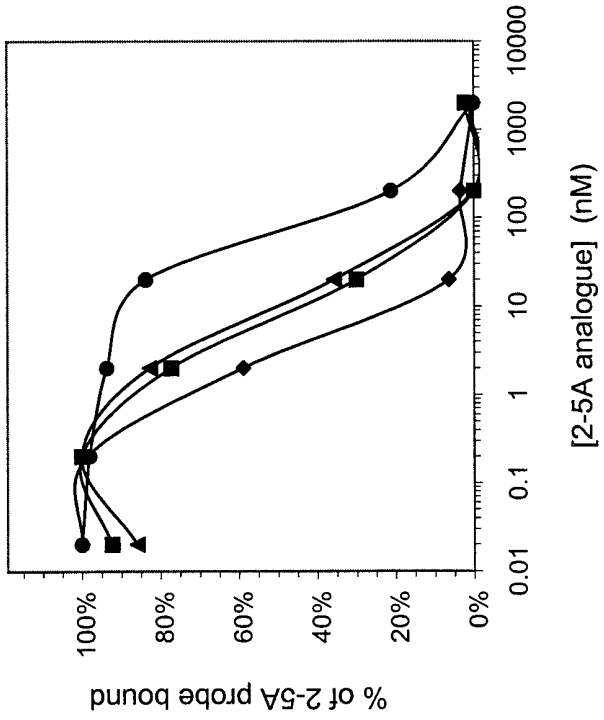

% of 2-5A probe bound
100%
80%
60%
40%
20%
0%
0.01
0.1
1
10
100
1000
10000
[2-5A analogue] (nM)

Fig. 6