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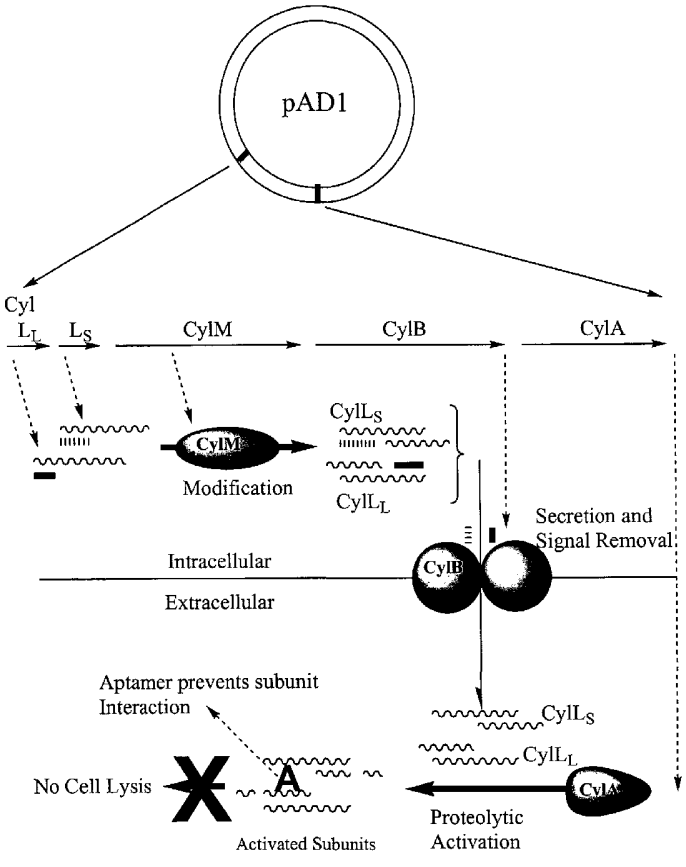
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(54) Title: NUCLEIC ACID MEDIATED INHIBITION OF ENTEROCOCCUS INFECTION AND CYTOLYSIN TOXIN ACTIVITY



(57) Abstract: The present invention relates to nucleic acid aptamers that bind to bacterial proteases such as CylA and methods for their use alone or in combination with other therapies, such as antibiotics. Also disclosed are nucleic acids such as siRNA, antisense, and enzymatic nucleic acid molecules that can modulate the expression of CylA genes. The compounds and methods of the invention are expected to inhibit enterococcus infection and cytolysin activity.

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NUCLEIC ACID MEDIATED INHIBITION OF ENTEROCOCCUS INFECTION AND CYTOLYSIN TOXIN ACTIVITY

This application claims the benefit of U.S. Provisional Application No. 60/389,198 filed June 17, 2002, U.S. Provisional Application No. 60/406,784 filed August 29, 2002, U.S. Provisional Application No. 60/408,378 filed September 5, 2002, U.S. Provisional Application No. 60/409,293 filed September 9, 2002, and U.S. Provisional Application No. 60/440,129 filed January 15, 2003. This application is also a continuation-in-part of US Patent Application No. 10/444,853 filed May 23, 2003 and a continuation-in-part of International Patent Application No. PCT/US03/05346, filed February 20, 2003, which claims the benefit of U.S. Provisional Application No. 60/358,580 filed February 20, 2002, U.S. Provisional Application No. 60/363,124 filed March 11, 2002, U.S. Provisional Application No. 60/386,782 filed June 6, 2002. The instant application claims the priority of all the listed applications, which are hereby incorporated by reference herein in their entireties, including the drawings.

Technical Field Of The Invention

The present invention concerns compounds, compositions, and methods for the study, diagnosis, and treatment of degenerative and disease states related to enterococcus infection and cytolysin toxin activity. Specifically, the invention relates to nucleic acid aptamers used to inhibit protease mediated processing and activation of enterococcus cytolysin toxin.

Background Of The Invention

Enterococci are a leading cause of nosocomial bacteremia, surgical wound infection, endocarditis and urinary tract infection. Antibiotic resistance of *E. faecalis* and *E. faecium* is a growing problem, with some clinical isolates being resistant to all standard therapies. Cytolysin is expressed in the majority of enterococcus strains associated with disease. Cytolysin causes rupture of a variety of target membranes, including bacterial cells, erythrocytes, and other mammalian cells. The toxin has been shown to contribute to the toxicity and lethality of infection in several infection models, and is associated with a five-fold increase in the risk of sudden death from nosocomial bacteremia. Based on mutational

analysis, inhibition of the cytolysin toxin should result in a several log reduction of circulating E. faecalis.

Two cytolysin subunits, $CylL_L$ and $CylL_S$, are ribosomally synthesized and secreted by the bacterial cell in an inactive form. A putative modification protein, ClyM, is thought to be required for the generation of the inactive secreted forms of $CylL_L$ and $CylL_S$ by post-translational modification to include lanthionine residues. The subunits are subsequently activated by extracellular removal of six amino-terminal residues by CylA, a subtilisn-type serine protease. Since this final catalytic event is required for cytolysin activity, occurs extracellularly, and is catalyzed by a class of enzyme for which a substantial body of structural information exists, it represents an ideal therapeutic target.

Ever since antibiotic use became widespread over 50 years ago, bacteria have steadily and routinely developed resistance, resulting in a high degree of vulnerability of our existing treatment arsenal and an unmet medical need. Control of the emergence of resistance will depend on sustained and new approaches to prudent antibiotic use in hospitals and clinics, based in part on improved surveillance for MDR enterococci and on better systems to encourage staff adherence to contact isolation procedures. Equally important is be development of new drugs with narrower spectra of activity aimed at known and potentially new targets. Disruption of the CylA, $CylL_L$ and/or $CylL_S$ via nucleic acid aptamers therefore provides an attractive approach to treating mutidrug resistant (MDR) enterococcus infection.

SUMMARY OF THE INVENTION

One embodiement of the invention provides an isolated nucleic acid molecule that inhibits bacterial subtilisn type serine protease CylA activity. The nucleic acid molecule can be an aptamer or a siNA. The aptamer can specifically bind to CylA, $CylL_L$, and/or $CylL_S$. The nucleic acid molecule can be chemically synthesized and can contain one or more chemically modified nucleotides, one or more nucleic acid sugar modifications, one or more nucleic acid base modifications and/or one or more nucleic acid backbone modifications. The aptamer can specifically bind to a polypeptide sequence having SEQ ID NO: 1.

Another embodiment of the invention provides a composition comprising a nucleic acid molecule of the invention in a pharmaceutically acceptable carrier or diluent.

Even another embodiment of the invention provides a cell comprising a nucleic acid molecule of the invention. The cell can be a mammalian cell, such as a human cell.

Yet another embodiment of the invention provides a method for treating enterococcus infection. The method comprises administering to a subject a nucleic acid molecule of the invention under conditions suitable for the treatment, whereby the enterococcus infection is treated.

Still another embodiment of the invention provides a method for treating nosocomial bacteremia. The method comprises administering to a subject a nucleic acid molecule of the invention under conditions suitable for the treatment, whereby the nosocomial bacteremia is treated.

Another embodiment of the invention provides a method of reducing CylA activity in a cell. The method comprises contacting the cell with a nucleic acid molecule of the invention under conditions suitable for the reduction of CylA activity, whereby CylA activity is reduced.

Even another embodiment of the invention provides a method of reducing $CylL_L$ activity in a cell. The method comprises contacting the cell with a nucleic acid molecule of the invention under conditions suitable for the reduction of $CylL_L$ activity, whereby $CylL_L$ activity is reduced.

Yet another embodiment of the invention provides a method of reducing $CylL_S$ activity in a cell. The method comprises contacting the cell with a nucleic acid molecule of the invention under conditions suitable for the reduction of $CylL_S$ activity, whereby $CylL_S$ activity is reduced.

The present invention relates to nucleic acid molecules such as aptamers, enzymatic nucleic acid molecules, small interfering RNA (siRNA), nucleic acid sensor molecules, allozymes, antisense nucleic acid molecules, 2,5-A nucleic acid chimeras, triplex oligonucleotides, and antisense nucleic acid molecules with nucleic acid cleaving groups, used to inhibit enterococcus infection and toxemia. The invention also relates to nucleic acid molecules directed to disrupt the function of CylA protease activity, such as to prevent proteolytic activation of cytolysin. In particular, the present invention describes the selection and function of nucleic acid molecules, such as aptamers, capable of specifically binding to the bacterial subtilisn type serine protease, CylA, and modulating activity of CylA and/or components thereof. Additionally, the present invention features nucleic acid molecules, such as aptamers, that bind to cytolysin subunits, for example $CylL_L$ and $CylL_S$ and modulate cytolysin activity. These nucleic acid molecules can be used to treat diseases and disorders

associated with enterococcus infection, or as a prophylactic measure to prevent enterococcus infection.

The nucleic acid aptamers of the invention can be used as antibacterial agents. The antibacterial activity of the aptamers of the invention can result from the ability to modulate intracellular processes that involve protein-protein interactions required for proteolytic activation of cytolysin. The antibacterial activity of the aptamers of the invention includes but is not limited to the inhibition of enterococcus cytolysin activation by CylA (see for example Genbank Accession No. AAM21177), and/or interactions with or between $CylL_L$ (see for example Genbank Accession No. A55226) and $CylL_S$ (see for example Genbank Accession No. B55226).

The present invention also features the use of one or more nucleic acid-based techniques for modulating gene expression, such as nucleic acid aptamers, to modulate the activity of CylA $CylL_L$ and/or $CylL_S$. For example, the invention features the use of nucleic acid aptamers to specifically modulate the activity of proteins, such as CylA, $CylL_L$ and/or $CylL_S$, required for cytolysin activation.

In one embodiment, the invention features nucleic acid aptamers directed to disrupt the function of enterococcus CylA or components thereof and prevent CylA mediated proteoltyic activation of enterococcus cytolysin. The nucleic acid aptamers of the invention are designed to interact with CylA, and disrupt the function of CylA or components thereof. Such disruption of the CylA can be effected, for example, by preventing conformational changes to CylA, and/or preventing protein-protein interactions between CylA and the $CylL_L$ and/or $CylL_S$ subunit(s) of cytolysin.

In another embodiment, the invention features nucleic acid aptamers having binding affinity to CylA. In yet another embodiment, the invention features nucleic acid aptamers having binding affinity to peptide sequences having SEQ ID No. 1 (**Table I**) or functional equivalents thereof. In another embodiment, the invention features nucleic acid aptamers having binding affinity to $CylL_L$ and/or $CylL_S$. For example, in certain embodiments, nucleic acid aptamers of the invention can have binding affinity to analogs of the proteins, polypeptides and/or peptides contemplated herein, such analogs can contain one or more amino acid truncations, deletions, insertions or substitutions.

In one embodiment, nucleic acid aptamers of the invention act extracellularly and bind to their CylA, $CylL_L$ and/or $CylL_S$ protein targets outside of cells. Theses nucleic acid aptamers provide an attractive approach to treating enterococcus infection because they are able to act outside of cells or extracellularly.

In another embodiment, the invention features a composition comprising a nucleic acid aptamer of the invention in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a mammalian cell, for example a human cell, comprising a nucleic acid aptamer contemplated by the invention.

In one embodiment, the invention features a method for treatment of enterococcus infection, nosocomial bacteremia, surgical wound infection, endocarditis, meningitis and/or urinary tract infection, comprising administering to a subject a nucleic acid aptamer of the invention under conditions suitable for the treatment.

In another embodiment, the invention features a method of treatment of a subject having a condition associated with enterococcus infection, comprising contacting cells of said subject with a nucleic acid aptamer of the invention under conditions suitable for such treatment. In another embodiment, the invention features a method of treatment of a subject having a condition associated with enterococcus infection, comprising contacting cells of said subject with a nucleic acid aptamer of the invention, and further comprising the use of one or more drug therapies; for example, an antibiotic such as penicillin, ampicillin, vancomycin, novobiocin, doxycycline, clindamycin, clinafloxacin, quinupristin, dalfopristin, cephalosporin, aztreonam, ciprofloxacin, aminoglycoside, metronidazole, fluoroquinolones, streptogramins, oxazolidinones, semisynthetic glycopeptides, glycylcyclines, chloramphenicol or any combination thereof under conditions suitable for said treatment. In another embodiment, the other therapy is administered simultaneously with or separately from the nucleic acid molecule.

In another embodiment, the invention features a method for modulating CylA, $CylL_L$ and/or $CylL_S$ activity in a mammalian cell comprising administering to the cell a nucleic acid molecule of the invention under conditions suitable for the modulation.

In yet another embodiment, the invention features a method of modulating CylA activity, comprising contacting a nucleic acid aptamer of the invention with CylA under conditions suitable for the modulating of the CylA activity.

In yet another embodiment, the invention features a method of modulating $CylL_L$ and/or $CylL_S$ activity, comprising contacting a nucleic acid aptamer of the invention with $CylL_L$ and/or $CylL_S$ under conditions suitable for the modulating of the $CylL_L$ and/or $CylL_S$ activity.

In one embodiment, a nucleic acid molecule of the invention, for example an aptamer, is chemically synthesized. In another embodiment, the nucleic acid molecule of the invention comprises at least one nucleic acid sugar modification. In yet another embodiment, the nucleic acid molecule of the invention comprises at least one nucleic acid base modification. In another embodiment, the nucleic acid molecule of the invention comprises at least one nucleic acid backbone modification.

In one embodiment, the nucleic acid molecule of the invention is an RNA molecule. In another embodiment, the nucleic acid molecule of the invention is a DNA molecule.

In another embodiment, the nucleic acid molecule of the invention comprises at least one 2'-O-alkyl, 2'-alkyl, 2'-alkoxylalkyl, 2'-alkylthioalkyl, 2'-amino, 2'-O-amino, or 2'-halo modification and/or any combination thereof with or without 2'-deoxy and/or 2'-ribo nucleotides. In yet another embodiment, the nucleic acid molecule of the invention comprises all 2'-O-alkyl nucleotides, for example, all 2'-O-allyl nucleotides.

In one embodiment, the nucleic acid molecule of the invention comprises a 5'-cap, 3'-cap, or 5'-3' cap structure, for example an abasic or inverted abasic moiety.

In another embodiment, the nucleic acid molecule of the invention is a linear nucleic acid molecule. In another embodiment, the nucleic acid molecule of the invention is a linear nucleic acid molecule that can optionally form a hairpin, loop, stem-loop, or other secondary structure. In yet another embodiment, the nucleic acid molecule of the invention is a circular nucleic acid molecule.

In one embodiment, the nucleic acid molecule of the invention is a single stranded polynucleotide. In another embodiment, the nucleic acid molecule of the invention is a double-stranded polynucleotide.

In one embodiment, the nucleic acid molecule of the invention comprises an oligonucleotide having between about 3 and about 500 nucleotides. In another embodiment,

the nucleic acid molecule of the invention comprises an oligonucleotide having between about 3 and about 80 nucleotides. In another embodiment, the nucleic acid molecule of the invention comprises an oligonucleotide having between about 3 and about 24 nucleotides. In another embodiment, the nucleic acid molecule of the invention comprises an oligonucleotide having between about 4 and about 16 nucleotides.

In one embodiment, the nucleic acid aptamer of the invention binds to its corresponding ClyA, $CylL_L$ and/or $CylL_S$ target, with a binding affinity of about 100 pM-100 nM or about 20 to 50 nM, for example by non-covalent interaction of the nucleic acid aptamer with a ClyA, $CylL_L$ and/or $CylL_S$ derived protein, polypeptide or peptide sequence, secondary or tertiary structure. In another embodiment, the nucleic acid aptamer of the invention binds to the ClyA, $CylL_L$ and/or $CylL_S$ target with a binding affinity of less than about 20 nM.

In another embodiment, the nucleic acid aptamer of the invention binds irreversibly to the ClyA, $CylL_L$ and/or $CylL_S$ target, for example by covalent attachment of the nucleic acid aptamer to CylA protien, polypeptide, or peptide sequence, secondary or tertiary structure. The covalent attachment can be accomplished by introducing chemical modifications into the nucleic acid aptamer's sequence that are capable of forming covalent bonds to the ClyA, $CylL_L$ and/or $CylL_S$ target sequence.

The present invention also features nucleic acid molecules capable of modulating gene expression, such as enzymatic nucleic acid molecules, small interfering RNA (siRNA), nucleic acid sensor molecules, allozymes, antisense nucleic acid molecules, 2,5-A nucleic acid chimeras, triplex oligonucleotides, and antisense nucleic acid molecules with nucleic acid cleaving groups, which down regulate expression of a sequence encoding a CylA, $CylL_L$ and/or $CylL_S$ nucleic acid molecule (see for example Genbank Accession No. L37110).

The invention also features an enzymatic nucleic acid molecule comprising at least one binding arm wherein one or more of said binding arms comprises a sequence complementary to a sequence derived from a CylA, $CylL_L$ and/or $CylL_S$ encoding nucleic acid molecule, such as Genbank Accession No. L37110.

In one embodiment, an enzymatic nucleic acid molecule of the invention is adapted to treat enterococcus infection, nosocomial bacteremia, surgical wound infection, endocarditis, meningitis and/or urinary tract infection.

In another embodiment, the enzymatic nucleic acid molecule of the invention has an endonuclease activity to cleave RNA having CylA, $CylL_L$ and/or $CylL_S$ sequence.

In yet another embodiment, the enzymatic nucleic acid molecule of the invention is in an Inozyme, Zinzyme, G-cleaver, Amberzyme, DNAzyme Hairpin or Hammerhead configuration.

In one embodiment, an enzymatic nucleic acid molecule of the invention comprises between 12 and 100 bases complementary to a RNA sequence encoding CylA, $CylL_L$ and/or $CylL_S$. In another embodiment, an enzymatic nucleic acid molecule of the invention comprises between 14 and 24 bases complementary to a RNA sequence encoding CylA, $CylL_L$ and/or $CylL_S$.

In one embodiment, the antisense molecule of the invention comprises a sequence complementary to a sequence of CylA, $CylL_L$ and/or $CylL_S$, for example Genbank Accession No. L37110

In one embodiment, the siRNA molecule of the invention comprises a sequence complementary to a sequence of of CylA, $CylL_L$ and/or $CylL_S$, for example Genbank Accession No. L37110.

In yet another embodiment, an enzymatic nucleic acid, siRNA, or antisense nucleic acid molecule of the invention is chemically synthesized. An enzymatic nucleic acid molecule of the invention can comprise at least one 2'-sugar modification, at least one nucleic acid base modification, and/or at least one phosphate backbone modification.

In one embodiment the present invention features a mammalian cell comprising an an enzymatic nucleic acid, siRNA, or antisense nucleic acid molecule of the invention. In one embodiment, the mammalian cell of the invention is a human cell.

The invention features a method of reducing CylA, $CylL_L$ and/or $CylL_S$ activity in a cell comprising contacting the cell with an enzymatic nucleic acid molecule of the invention under conditions suitable for the reduction of CylA, $CylL_L$ and/or $CylL_S$ activity.

The invention features a method of reducing CylA, $CylL_L$ and/or $CylL_S$ activity in a cell comprising contacting the cell with siRNA nucleic acid molecule of the invention under conditions suitable for the reduction of CylA, $CylL_L$ and/or $CylL_S$ activity.

The invention features a method of reducing CylA, $CylL_L$ and/or $CylL_S$ activity in a cell comprising contacting the cell with an antisense nucleic acid molecule of the invention under conditions suitable for the reduction of CylA, $CylL_L$ and/or $CylL_S$ activity.

In one embodiment, methods of treatment contemplated by the invention comprises the use of one or more drug therapies under conditions suitable for the treatment.

The invention features a method of cleaving RNA of a CylA, $CylL_L$, and/or $CylL_S$ gene comprising contacting an enzymatic nucleic acid molecule of the invention with the RNA of a CylA, $CylL_L$, and/or $CylL_S$ gene under conditions suitable for the cleavage. In one embodiment, the cleavage contemplated by the invention is carried out in the presence of a divalent cation, for example Mg2+.

In another embodiment, the enzymatic nucleic acid molecule, siRNA, or antisense nucleic acid molecule of the invention comprises a cap structure, wherein the cap structure is at the 5'-end, or 3'-end, or both the 5'-end and the 3'-end of the nucleic acid molecule, for example a 3',3'-linked or 5',5'-linked deoxyabasic ribose derivative.

The present invention features an expression vector comprising a nucleic acid sequence encoding at least one enzymatic nucleic acid molecule, siRNA, or antisense nucleic acid molecule of the invention in a manner which allows expression of the nucleic acid molecule.

The invention also features a mammalian cell, for example a human cell, comprising an expression vector contemplated by the invention.

In one embodiment, an expression vector of the invention comprises a nucleic acid sequence encoding two or more enzymatic nucleic acid molecules, siRNAs, or antisense nucleic acid molecules, which may be the same or different.

The present invention features a method for treatment of enterococcus infection, nosocomial bacteremia, surgical wound infection, endocarditis, meningitis and/or urinary tract infection, comprising administering to a subject an enzymatic nucleic acid molecule, siRNA, or antisense nucleic acid molecule of the invention under conditions suitable for the treatment.

In one embodiment, an enzymatic nucleic acid molecule of the invention comprises at least five ribose residues, at least ten 2'-O-methyl modifications, and a 3'- end modification, for example a 3'-3' inverted abasic moiety.

In another embodiment, a nucleic acid molecule of the invention further comprises phosphorothioate linkages on at least three of the 5' terminal nucleotides.

In yet another embodiment, a DNAzyme of the invention comprises at least ten 2'-O-methyl modifications and a 3'-end modification, for example a 3'-3' inverted abasic moiety. In a further embodiment, the DNAzyme of the invention further comprises phosphorothioate linkages on at least three of the 5' terminal nucleotides.

The invention features a composition comprising a nucleic acid molecule of the invention in a pharmaceutically acceptable carrier.

In one embodiment, the invention features a method of administering to a cell, for example a mammalian cell or human cell, a nucleic acid molecule of the invention comprising contacting the cell with enzymatic nucleic acid molecule, siRNA, or antisense nucleic acid molecule under conditions suitable for the administration. The method of administration can be in the presence of a delivery reagent, for example a lipid, cationic lipid, phospholipid, or liposome.

In one embodiment, the invention features a composition comprising a nucleic acid molecule of the invention, in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a composition comprising at least one antibiotic and a nucleic acid molecule of the invention, in a pharmaceutically acceptable carrier or diluent.

In another embodiment, the invention features a method of administering to a cell, for example a mammalian cell or human cell, a nucleic acid molecule of the invention independently or in conjunction with other therapeutic compounds such as antibiotics, comprising contacting the cell with the nucleic acid molecule and the antibiotic under conditions suitable for the administration.

In yet another embodiment, the invention features a method of administering to a cell, for example a mammalian cell or human cell, a nucleic acid molecule of the invention independently or in conjunction with other therapeutic compounds such as aptamers,

enzymatic nucleic acid molecules, antisense molecules, decoys, triplex forming oligonucleotides, 2,5-A chimeras, and/or RNAi, comprising contacting the cell with the nucleic acid molecule(s) of the invention under conditions suitable for the administration.

In another embodiment, administration of a nucleic acid molecule of the invention is administered to a cell or subject in the presence of a delivery reagent, for example a lipid, cationic lipid, phospholipid, or liposome.

In one embodiment, the invention features a method for identifying nucleic acid aptamers having CylA inhibitory activity, comprising: (a) generating a randomized pool of oligonucleotides; (b) combining the oligonucleotides from (a) with CylA in vitro under conditions suitable to allow at least one oligonucleotide to bind to the target CylA peptide; (c) removing non-bound oligonucleotide sequences from (b) under conditions suitable for isolating oligonucleotide sequences from (b) that possess binding affinity to CylA; (d) amplifying the oligonucleotide sequences isolated from (c) under conditions suitable for introducing some degree of mutation into the sequences; and (e) repeating steps (c) and (d) under conditions suitable for isolating one or more nucleic acid aptamers having binding affinity to CylA.

In one embodiment, the invention features a method for identifying nucleic acid aptamers having $CylL_L$ and/or $CylL_S$ inhibitory activity, comprising: (a) generating a randomized pool of oligonucleotides; (b) combining the oligonucleotides from (a) with $CylL_L$ and/or $CylL_S$ in vitro under conditions suitable to allow at least one oligonucleotide to bind to the target $CylL_L$ and/or $CylL_S$ peptide; (c) removing non-bound oligonucleotide sequences from (b) under conditions suitable for isolating oligonucleotide sequences from (b) that possess binding affinity to $CylL_L$ and/or $CylL_S$; (d) amplifying the oligonucleotide sequences isolated from (c) under conditions suitable for introducing some degree of mutation into the sequences; and (e) repeating steps (c) and (d) under conditions suitable for isolating one or more nucleic acid aptamers having binding affinity to $CylL_L$ and/or $CylL_S$.

In the described methods, the random pool of oligonucleotides can comprise DNA and/or RNA, with or without chemically modified nucleotides. When chemically modified nucleotides are used in the method, such modifications can be chosen such that a non-discriminatory polymerase will incorporate the chemically modified nucleotide into the oligonucleotide sequence when generated or amplified. Non-limiting examples of chemically

modified nucleoside triphosphates (NTPs) that can be used in the method of the invention include 2'-deoxy-2'-fluoro, 2'-deoxy-2'-amino, 2'-O-alkyl, and 2'-O-methyl NTPs as well as various base modified NTPs, such as C5-modified pyrimidines, 2,6-diaminopurine, and inosine. The oligonucleotides used in the method can be of fixed or variable length. The target protein, polypeptide, or peptide derived from CylA used in the method of the invention can comprise a synthetic, recombinant or naturally occurring protein, polypeptide, or peptide that is synthesized or isolated from bacterial protein, for example by proteolytic cleavage. The target protein, polypeptide, or peptide can comprise sequence derived from proteins having sequence identical or similar to GenBank Accession No. AAM21177 (CylA), A55226 ($CylL_L$), or B55226 ($CylL_S$) or analogs thereof. For example, the target protein, polypeptide, or peptide can comprise sequences derived from CylA that are essential for protease activity and cytolysin activation, such as SEQ ID NO. 1, and/or analogs thereof. These analogs can contain one or more amino acid truncations, deletions, insertions or substitutions. The conditions used in the method preferably provide nucleic acid aptamers that bind to their respective target in the conformation that the target adopts in its natural state. For example, protein, polypeptide, or peptide targets and binding conditions are chosen such that the isolated aptamer binds to its target site within CylA such that protease activity of the protein is disrupted, such as by preventing intermolecular or intramolecular protein-protein interactions between CylA, $CylL_L$ and/or $CylL_S$. The nucleic acid aptamers thus isolated by methods of the invention can be tested, for example, for an ability to inhibit protease activity using assays described herein.

In another embodiment, the method for identifying nucleic acid aptamers having CylA, $CylL_L$ and/or $CylL_S$ inhibitory properties comprises attaching the target CylA, $CylL_L$ and/or $CylL_S$ protein, polypeptide or peptide sequence to a solid matrix, such as beads, microtiter plate wells, membranes, or chip surfaces. In such a system, the target can be attached to the solid matrix either covalently or non-covalently. In yet another embodiment, the oligonucleotide or nucleic acid aptamer used in a method of the invention can be labeled, either directly or non-directly, for example with a radioactive label, absorption label such as biotin, or a fluorescent label such as fluorescein or rhodamine.

In another embodiment, the invention features the use of nucleic acid aptamers to modulate the activity, expression, or level of proteins required for enterococcus intection and/or cytolysin activity. For example, the invention features the use of nucleic acid

aptamers to specifically modulate the activity of proteins required for CylA protease activity for cytolysin activation.

In one embodiment, nucleic acid molecules of the invention are used to treat enterococcus-infected cells or an enterococcus-infected subject wherein the enterococcus is resistant or the subject does not respond to treatment with current antibiotic/antibacterial therapeutics, either alone or in combination with other therapies under conditions suitable for the treatment.

The term "enterococcus" as used herein refers to bacteria of Enterococci origin, such as *Enterococcus faecalis* and *Enterococcus faecium*.

The term "protease activity" as used herein refers to enzymatic cleavage of peptide bonds, such as removal of terminal amino acid residues from $CylL_L$ and/or $CylL_S$ by CylA (see for example Booth *et al.*, 1996, *Mol Microbiol*, 21, 1175).

The term "binding" as used herein refers to the relative affinity that one molecule has for another molecule, such that the affinity between the two molecules results in the predominance of an associated state between the molecules as compared to a disassociated state. Association between the molecules can result from, for example, hydrogen bonds, van Der-Waals interactions, hydrophobic interactions, ionic interactions, electrostatic interactions and the like.

The term "antibiotic" or "antibacterial" as used herein refers to the ability of a compound to inhibit or reduce bacterial infection of cells.

The term "CylA" as used herein refers to subtilisin-type serine protease, such as Genbank Accession No. AAM21177 or analogs thereof. CylA generally refers to proteins or polypepdides having protease activity involved in cytolysin activation, such as SEQ ID NO. 1, and/or analogs thereof.

The term "$CylL_L$" as used herein, refers to a subunit of cytolysin (see for example Genbank Accession No. A55226) that includes both inactive intracellular forms of $CylL_L$, inactive extracellular forms of $CylL_L$, such as prior to activiation by CylA, and active extracellular forms of $CylL_L$, such as after activation by CylA.

The term "$CylL_S$" as used herein, refers to a subunit of cytolysin (see for example Genbank Accession No. B55226) that includes both inactive intracellular forms of $CylL_S$, inactive extracellular forms of $CylL_S$, such as prior to activiation by CylA, and active extracellular forms of $CylL_S$, such as after activation by CylA.

The term "modulate" as used herein refers to a stimulatory or inhibitory effect on the intracellular or intercellular process of interest relative to the level or activity of such a process in the absense of a nucleic acid molecule of the invention. For example, the level of protease activity between two or more moieties is enhanced or decreased in the presence of a modulator relative to the level of protease activity which occurs between the moieties in the absence of the modulator. In another non-limiting example, the expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits is up regulated or down regulated, such that expression, level, or activity is greater than or less than that observed in the absence of the modulator. For example, the term "modulate" can mean "inhibit," but the use of the word "modulate" is not limited to this definition.

The term "inhibit" as used herein refers to when the activity of CylA, $CylL_L$ and/or $CylL_S$, or level of RNAs or equivalent RNAs encoding one or more protein subunits of CylA, $CylL_L$ and/or $CylL_S$ or functional equivalents thereof, is reduced below that observed in the absence of the nucleic acid of the invention. In one embodiment, inhibition with nucleic acid molecule preferably is below that level observed in the presence of non-binding or an inactive or attenuated molecule that is unable to bind to the same target site. In another embodiment, inhibition of bacterial infection, CylA, $CylL_L$ and/or $CylL_S$ expression or activity, or cytolysin activity with the nucleic acid molecule of the instant invention is greater in the presence of the nucleic acid molecule than in its absence.

The methods of this invention can be used to treat enterococcus infections, which include productive bacterial infection, latent or persistent bacterial infection. The utility can be extended to other species of enterococci that infect non-human animals where such infections are of veterinary importance.

By "aptamer" or "nucleic acid aptamer" as used herein is meant a nucleic acid molecule that binds specifically to a target molecule. The target molecule can be any molecule of interest. For example, the aptamer can be used to bind to a ligand-binding domain of a

protein, thereby preventing interaction of the naturally occurring ligand with the protein. The aptamer can also be used to prevent protein-protein interactions or conformational changes within a protein by binding to a portion of a target protein that interacts with another protein or with another portion of the same protein. This is a non-limiting example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art, see for example Gold *et al.*, 1995, *Annu. Rev. Biochem.*, 64, 763; Brody and Gold, 2000, *J. Biotechnol.*, 74, 5; Sun, 2000, *Curr. Opin. Mol. Ther.*, 2, 100; Kusser, 2000, *J. Biotechnol.*, 74, 27; Hermann and Patel, 2000, *Science,* 287, 820; and Jayasena, 1999, *Clinical Chemistry,* 45, 1628.

By "enzymatic nucleic acid molecule" is meant a nucleic acid molecule that has complementarity in a substrate binding region to a specified gene target, and also has an enzymatic activity which is active to specifically cleave a target RNA or DNA molecule. That is, the enzymatic nucleic acid molecule is able to intermolecularly cleave a RNA or DNA molecule and thereby inactivate a target RNA or DNA molecule. These complementary regions allow sufficient hybridization of the enzymatic nucleic acid molecule to a target RNA molecule and thus permit cleavage. One hundred percent complementarity is preferred, but complementarity as low as 50-75% may also be useful in this invention (see for example Werner and Uhlenbeck, 1995, *Nucleic Acids Research,* 23, 2092-2096; Hammann *et al.,* 1999, *Antisense and Nucleic Acid Drug Dev.,* 9, 25-31). The nucleic acids can be modified at the base, sugar, and/or phosphate groups. The term enzymatic nucleic acid is used interchangeably with phrases such as ribozymes, catalytic RNA, enzymatic RNA, catalytic DNA, aptazyme or aptamer-binding ribozyme, regulatable ribozyme, catalytic oligonucleotides, nucleozyme, DNAzyme, RNA enzyme, endoribonuclease, endonuclease, minizyme, leadzyme, oligozyme or DNA enzyme. All of these terminologies describe nucleic acid molecules with enzymatic activity. The specific enzymatic nucleic acid molecules described in the instant application are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic nucleic acid molecule of this invention is that it have a specific substrate binding site that is complementary to one or more of the target nucleic acid regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart a nucleic acid cleaving activity to the molecule (Cech et al., U.S. Patent No. 4,987,071; Cech et al., 1988, JAMA 260:20 3030-4).

By "nucleic acid molecule" as used herein is meant a molecule comprising nucleotides. The nucleic acid can be single, double, or multiple stranded and can comprise modified or unmodified nucleotides or non-nucleotides or various mixtures and combinations thereof.

By "Inozyme" or "NCH" motif or configuration is meant, an enzymatic nucleic acid molecule comprising a motif as is generally described as NCH Rz in Ludwig *et al.,* International PCT Publication No. WO 98/58058 and US Patent Application Serial No. 08/878,640, which is herein incorporated by reference in its entirety including the drawings. Inozymes possess endonuclease activity to cleave RNA substrates having a cleavage triplet NCH/, where N is a nucleotide, C is cytidine and H is adenosine, uridine or cytidine, and / represents the cleavage site. Inozymes can also possess endonuclease activity to cleave RNA substrates having a cleavage triplet NCN/, where N is a nucleotide, C is cytidine, and / represents the cleavage site

By "G-cleaver" motif or configuration is meant, an enzymatic nucleic acid molecule comprising a motif as is generally described in Eckstein *et al.,* US 6,127,173, which is herein incorporated by reference in its entirety including the drawings, and in Kore *et al.*, 1998, *Nucleic Acids Research* 26, 4116-4120. G-cleavers possess endonuclease activity to cleave RNA substrates having a cleavage triplet NYN/, where N is a nucleotide, Y is uridine or cytidine and / represents the cleavage site. G-cleavers can be chemically modified.

By "zinzyme" motif or configuration is meant, an enzymatic nucleic acid molecule comprising a motif as is generally described in Beigelman *et al.,* International PCT publication No. WO 99/55857 and US Patent Application Serial No. 09/918,728, which is herein incorporated by reference in its entirety including the drawings. Zinzymes possess endonuclease activity to cleave RNA substrates having a cleavage triplet including but not limited to, YG/Y, where Y is uridine or cytidine, and G is guanosine and / represents the cleavage site. Zinzymes can be chemically modified to increase nuclease stability through various substitutions, including substituting 2'-O-methyl guanosine nucleotides for guanosine nucleotides. In addition, differing nucleotide and/or non-nucleotide linkers can be used to substitute the 5'-gaaa-2' loop of the motif. Zinzymes represent a non-limiting example of an enzymatic nucleic acid molecule that does not require a ribonucleotide (2'-OH) group within its own nucleic acid sequence for activity.

By "amberzyme" motif or configuration is meant, an enzymatic nucleic acid molecule comprising a motif as is generally described in Beigelman *et al.,* International PCT publication No. WO 99/55857 and US Patent Application Serial No. 09/476,387, which is herein incorporated by reference in its entirety including the drawings. Amberzymes possess endonuclease activity to cleave RNA substrates having a cleavage triplet NG/N, where N is a nucleotide, G is guanosine, and / represents the cleavage site. Amberzymes can be chemically modified to increase nuclease stability. In addition, differing nucleoside and/or non-nucleoside linkers can be used to substitute the 5'-gaaa-3' loops of the motif. Amberzymes represent a non-limiting example of an enzymatic nucleic acid molecule that does not require a ribonucleotide (2'-OH) group within its own nucleic acid sequence for activity.

By 'DNAzyme' is meant, an enzymatic nucleic acid molecule that does not require the presence of a 2'-OH group within its own nucleic acid sequence for activity. In particular embodiments, the enzymatic nucleic acid molecule can have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. DNAzymes can be synthesized chemically or expressed endogenously *in vivo,* by means of a single stranded DNA vector or equivalent thereof. Non-limiting examples of DNAzymes are generally reviewed in Usman *et al.,* US patent No. 6,159,714, which is herein incorporated by reference in its entirety including the drawings; Chartrand *et al.,* 1995, *NAR* 23, 4092; Breaker *et al.,* 1995, *Chem. Bio.* 2, 655; Santoro *et al.,* 1997, *PNAS* 94, 4262; Breaker, 1999, *Nature Biotechnology,* 17, 422-423; and Santoro *et. al.,* 2000, *J. Am. Chem. Soc.,* 122, 2433-39. The "10-23" DNAzyme motif is one particular type of DNAzyme that was evolved using *in vitro* selection as generally described in Joyce *et al.,* US 5,807,718 and Santoro *et al., supra.* Additional DNAzyme motifs can be selected for using techniques similar to those described in these references, and hence, are within the scope of the present invention.

By "nucleic acid sensor molecule" or "allozyme" as used herein is meant a nucleic acid molecule comprising an enzymatic domain and a sensor domain, where the ability of the enzymatic nucleic acid domain's ability to catalyze a chemical reaction is dependent on the interaction with a target signaling molecule, such as a nucleic acid, polynucleotide, oligonucleotide, peptide, polypeptide, or protein such as CylA. The introduction of chemical modifications, additional functional groups, and/or linkers, to the nucleic acid sensor molecule can provide enhanced catalytic activity of the nucleic acid sensor molecule,

increased binding affinity of the sensor domain to a target nucleic acid, and/or improved nuclease/chemical stability of the nucleic acid sensor molecule, and are hence within the scope of the present invention (see for example Usman *et al.,* US Patent Application No. 09/877,526, George *et al.,* US Patent Nos. 5,834,186 and 5,741,679, Shih *et al.,* US Patent No. 5,589,332, Nathan *et al.,* US Patent No 5,871,914, Nathan and Ellington, International PCT publication No. WO 00/24931, Breaker *et al.,* International PCT Publication Nos. WO 00/26226 and 98/27104, and Sullenger *et al.,* US Patent Application Serial No. 09/205,520).

By "sensor component" or "sensor domain" of the nucleic acid sensor molecule as used herein is meant, a nucleic acid sequence (e.g., RNA or DNA or analogs thereof) that interacts with a target signaling molecule, for example a nucleic acid sequence in one or more regions of a target nucleic acid molecule or more than one target nucleic acid molecule, and which interaction causes the enzymatic nucleic acid component of the nucleic acid sensor molecule to either catalyze a reaction or stop catalyzing a reaction. In the presence of target signaling molecule of the invention, such as CylA, the ability of the sensor component, for example, to modulate the catalytic activity of the nucleic acid sensor molecule, is modulated or diminished. The sensor component can comprise recognition properties relating to chemical or physical signals capable of modulating the nucleic acid sensor molecule via chemical or physical changes to the structure of the nucleic acid sensor molecule. The sensor component can be derived from a naturally occurring nucleic acid binding sequence, for example, RNAs that bind to other nucleic acid sequences *in vivo*. Alternately, the sensor component can be derived from a nucleic acid molecule (aptamer), which is evolved to bind to a nucleic acid sequence within a target nucleic acid molecule. The sensor component can be covalently linked to the nucleic acid sensor molecule, or can be non-covalently associated. A person skilled in the art will recognize that all that is required is that the sensor component is able to selectively modulate the activity of the nucleic acid sensor molecule to catalyze a reaction.

By "target molecule" or "target signaling molecule" is meant a molecule capable of interacting with a nucleic acid sensor molecule, specifically a sensor domain of a nucleic acid sensor molecule, in a manner that causes the nucleic acid sensor molecule to be active or inactive. The interaction of the signaling agent with a nucleic acid sensor molecule can result in modification of the enzymatic nucleic acid component of the nucleic acid sensor molecule via chemical, physical, topological, or conformational changes to the structure of the

molecule, such that the activity of the enzymatic nucleic acid component of the nucleic acid sensor molecule is modulated, for example is activated or deactivated. Signaling agents can comprise target signaling molecules such as macromolecules, ligands, small molecules, metals and ions, nucleic acid molecules including but not limited to RNA and DNA or analogs thereof, proteins, peptides, antibodies, polysaccharides, lipids, sugars, microbial or cellular metabolites, pharmaceuticals, and organic and inorganic molecules in a purified or unpurified form, for example CylA, Cytolysisn, and/or peptide sequences such as SEQ ID No: 1 or analogs thereof.

By "sufficient length" is meant a nucleic acid molecule long enough to provide the intended function under the expected condition. For example, a nucleic acid molecule of the invention needs to be of "sufficient length" to provide stable binding to a target site under the expected binding conditions and environment. In another non-limiting example, for the binding arms of an enzymatic nucleic acid, "sufficient length" means that the binding arm sequence is long enough to provide stable binding to a target site under the expected reaction conditions and environment. The binding arms are not so long as to prevent useful turnover of the nucleic acid molecule. By "stably interact" is meant interaction of the oligonucleotides with target, such as a target protein or target nucleic acid (*e.g.,* by forming hydrogen bonds with complementary amino acids or nucleotides in the target under physiological conditions) that is sufficient for the intended purpose (e.g., specific binding to a protein target to disrupt the function of that protein or cleavage of target RNA/DNA by an enzyme).

By "homology" is meant the nucleotide sequence of two or more nucleic acid molecules, or the amino acid sequence of two or more proteins, is partially or completely identical.

By "antisense nucleic acid", it is meant a non-enzymatic nucleic acid molecule that binds to target RNA by means of RNA-RNA or RNA-DNA or RNA-PNA (protein nucleic acid; Egholm *et al.,* 1993 *Nature* 365, 566) interactions and alters the activity of the target RNA (for a review, see Stein and Cheng, 1993 *Science* 261, 1004 and Woolf *et al.,* US patent No. 5,849,902). Typically, antisense molecules are complementary to a target sequence along a single contiguous sequence of the antisense molecule. However, in certain embodiments, an antisense molecule can bind to substrate such that the substrate molecule forms a loop, and/or an antisense molecule can bind such that the antisense molecule forms a loop. Thus, the antisense molecule can be complementary to two or more non-contiguous

substrate sequences or two or more non-contiguous sequence portions of an antisense molecule can be complementary to a target sequence, or both. For a review of current antisense strategies, see Schmajuk *et al.,* 1999, *J. Biol. Chem.,* 274, 21783-21789, Delihas *et al.,* 1997, *Nature,* 15, 751-753, Stein *et al.,* 1997, *Antisense N. A. Drug Dev.,* 7, 151, Crooke, 2000, *Methods Enzymol.,* 313, 3-45; Crooke, 1998, *Biotech. Genet. Eng. Rev.,* 15, 121-157, Crooke, 1997, *Ad. Pharmacol.,* 40, 1-49. Antisense molecules of the instant invention can include 2-5A antisense chimera molecules. In addition, antisense DNA can be used to target RNA by means of DNA-RNA interactions, thereby activating RNase H, which digests the target RNA in the duplex. The antisense oligonucleotides can comprise one or more RNAse H activating region that is capable of activating RNAse H cleavage of a target RNA. Antisense DNA can be synthesized chemically or expressed via the use of a single stranded DNA expression vector or equivalent thereof.

By "RNase H activating region" is meant a region (generally greater than or equal to 4-25 nucleotides in length, preferably from 5-11 nucleotides in length) of a nucleic acid molecule capable of binding to a target RNA to form a non-covalent complex that is recognized by cellular RNase H enzyme (see for example Arrow *et al.,* US 5,849,902; Arrow *et al.,* US 5,989,912). The RNase H enzyme binds to the nucleic acid molecule-target RNA complex and cleaves the target RNA sequence. The RNase H activating region comprises, for example, phosphodiester, phosphorothioate (for example, at least four of the nucleotides are phosphorothiote substitutions; more specifically, 4-11 of the nucleotides are phosphorothiote substitutions), phosphorodithioate, 5'-thiophosphate, or methylphosphonate backbone chemistry or a combination thereof. In addition to one or more backbone chemistries described above, the RNase H activating region can also comprise a variety of sugar chemistries. For example, the RNase H activating region can comprise deoxyribose, arabino, fluoroarabino or a combination thereof, nucleotide sugar chemistry. Those skilled in the art will recognize that the foregoing are non-limiting examples and that any combination of phosphate, sugar and base chemistry of a nucleic acid that supports the activity of RNase H enzyme is within the scope of the definition of the RNase H activating region and the instant invention.

By "2-5A antisense chimera" it is meant, an antisense oligonucleotide containing a 5'-phosphorylated 2'-5'-linked adenylate residue. These chimeras bind to target RNA in a

sequence-specific manner and activate a cellular 2-5A-dependent ribonuclease, which, in turn, cleaves the target RNA (Torrence *et al.,* 1993 *Proc. Natl. Acad. Sci. USA* 90, 1300).

By "triplex nucleic acid" or "triplex oligonucleotide" it is meant a polynucleotide or oligonucleotide that can bind to a double-stranded DNA in a sequence-specific manner to form a triple-strand helix. Formation of such triple helix structure has been shown to modulate transcription of the targeted gene (Duval-Valentin *et al.,* 1992, *Proc. Natl. Acad. Sci.USA,* 89, 504).

The term "short interfering nucleic acid", "siNA", "short interfering RNA", "siRNA", "short interfering nucleic acid molecule", "short interfering oligonucleotide molecule", or "chemically-modified short interfering nucleic acid molecule" as used herein refers to any nucleic acid molecule capable of inhibiting or down regulating gene expression or viral replication, for example by mediating RNA interference "RNAi" or gene silencing in a sequence-specific manner; see for example Bass, 2001, *Nature*, 411, 428-429; Elbashir *et al.,* 2001, *Nature*, 411, 494-498; and Kreutzer *et al.,* International PCT Publication No. WO 00/44895; Zernicka-Goetz *et al.,* International PCT Publication No. WO 01/36646; Fire, International PCT Publication No. WO 99/32619; Plaetinck *et al.,* International PCT Publication No. WO 00/01846; Mello and Fire, International PCT Publication No. WO 01/29058; Deschamps-Depaillette, International PCT Publication No. WO 99/07409; and Li *et al.,* International PCT Publication No. WO 00/44914; Allshire, 2002, *Science,* 297, 1818-1819; Volpe *et al.,* 2002, *Science,* 297, 1833-1837; Jenuwein, 2002, *Science,* 297, 2215-2218; and Hall *et al.,* 2002, *Science,* 297, 2232-2237; Hutvagner and Zamore, 2002, *Science*, 297, 2056-60; McManus *et al.,* 2002, *RNA*, 8, 842-850; Reinhart *et al.,* 2002, *Gene & Dev.,* 16, 1616-1626; and Reinhart & Bartel, 2002, *Science*, 297, 1831). Non limiting examples of siNA molecules of the invention are described in Haeberli *et al.,* USSN (10/444,853), filed May 23, 2003, incorporated by reference herein in its entirety including the drawings. For example the siNA can be a double-stranded polynucleotide molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (i.e. each strand

comprises nucleotide sequence that is complementary to nucleotide sequence in the other strand; such as where the antisense strand and sense strand form a duplex or double stranded structure, for example wherein the double stranded region is about 19 base pairs); the antisense strand comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense strand comprises nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. Alternatively, the siNA is assembled from a single oligonucleotide, where the self-complementary sense and antisense regions of the siNA are linked by means of a nucleic acid based or non-nucleic acid-based linker(s). The siNA can be a polynucleotide with a duplex, asymmetric duplex, hairpin or asymmetric hairpin secondary structure, having self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a separate target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be a circular single-stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof, and wherein the circular polynucleotide can be processed either *in vivo* or *in vitro* to generate an active siNA molecule capable of mediating RNAi. The siNA can also comprise a single stranded polynucleotide having nucleotide sequence complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof (for example, where such siNA molecule does not require the presence within the siNA molecule of nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof), wherein the single stranded polynucleotide can further comprise a terminal phosphate group, such as a 5'-phosphate (see for example Martinez *et al.*, 2002, *Cell.*, 110, 563-574 and Schwarz *et al.*, 2002, *Molecular Cell*, 10, 537-568), or 5',3'-diphosphate. In certain embodiments, the siNA molecule of the invention comprises separate sense and antisense sequences or regions, wherein the sense and antisense regions are covalently linked by nucleotide or non-nucleotide linkers molecules as is known in the art, or are alternately non-covalently linked by ionic interactions, hydrogen bonding, van der waals interactions, hydrophobic intercations, and/or stacking interactions. In certain embodiments, the siNA molecules of the invention comprise nucleotide sequence that is complementary to nucleotide sequence of a target gene. In another embodiment, the siNA

molecule of the invention interacts with nucleotide sequence of a target gene in a manner that causes inhibition of expression of the target gene. As used herein, siNA molecules need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides. In certain embodiments, the short interfering nucleic acid molecules of the invention lack 2'-hydroxy (2'-OH) containing nucleotides. Applicant describes in certain embodiments short interfering nucleic acids that do not require the presence of nucleotides having a 2'-hydroxy group for mediating RNAi and as such, short interfering nucleic acid molecules of the invention optionally do not include any ribonucleotides (e.g., nucleotides having a 2'-OH group). Such siNA molecules that do not require the presence of ribonucleotides within the siNA molecule to support RNAi can however have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. Optionally, siNA molecules can comprise ribonucleotides at about 5, 10, 20, 30, 40, or 50% of the nucleotide positions. The modified short interfering nucleic acid molecules of the invention can also be referred to as short interfering modified oligonucleotides "siMON." As used herein, the term siNA is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi, for example short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering modified oligonucleotide, chemically-modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), and others. In addition, as used herein, the term RNAi is meant to be equivalent to other terms used to describe sequence specific RNA interference, such as post transcriptional gene silencing, translational inhibition, or epigenetics. For example, siNA molecules of the invention can be used to epigenetically silence genes at both the post-transcriptional level or the pre-transcriptional level. In a non-limiting example, epigenetic regulation of gene expression by siNA molecules of the invention can result from siNA mediated modification of chromatin structure to alter gene expression (see, for example, Allshire, 2002, *Science,* 297, 1818-1819; Volpe *et al.,* 2002, *Science,* 297, 1833-1837; Jenuwein, 2002, *Science,* 297, 2215-2218; and Hall *et al.,* 2002, *Science,* 297, 2232-2237).

By "asymmetric hairpin" as used herein is meant a linear siNA molecule comprising an antisense region, a loop portion that can comprise nucleotides or non-nucleotides, and a sense region that comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complimentary nucleotides to base pair with the antisense region and form

a duplex with loop. For example, an asymmetric hairpin siNA molecule of the invention can comprise an antisense region having length sufficient to mediate RNAi in a cell or in vitro system (e.g. about 19 to about 22 nucleotides) and a loop region comprising about 4 to about 8 nucleotides, and a sense region having about 3 to about 18 nucleotides that are complementary to the antisense region. The asymmetric hairpin siNA molecule can also comprise a 5'-terminal phosphate group that can be chemically modified. The loop portion of the asymmetric hairpin siNA molecule can comprise nucleotides, non-nucleotides, linker molecules, or conjugate molecules as described herein.

By "asymmetric duplex" as used herein is meant a siNA molecule having two separate strands comprising a sense region and an antisense region, wherein the sense region comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complimentary nucleotides to base pair with the antisense region and form a duplex. For example, an asymmetric duplex siNA molecule of the invention can comprise an antisense region having length sufficient to mediate RNAi in a cell or in vitro system (e.g. about 19 to about 22 nucleotides) and a sense region having about 3 to about 18 nucleotides that are complementary to the antisense region.

By "gene" it is meant, a nucleic acid that encodes an RNA, for example, nucleic acid sequences including, but not limited to, structural genes encoding a polypeptide.

By "complementarity" is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types. In reference to the nucleic molecules of the present invention, the binding free energy for a nucleic acid molecule with its target or complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., ribozyme cleavage, antisense or triple helix modulation. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner et al., 1987, *CSH Symp. Quant. Biol.* LII pp.123-133; Frier et al., 1986, *Proc. Nat. Acad. Sci.* USA 83:9373-9377; Turner et al., 1987, *J. Am. Chem. Soc.* 109:3783-3785). A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence.

The nucleic acid aptamers that bind to CylA and therefore inactivate CylA mediated activation of cytolysin represent a novel therapeutic approach to treat enterococcus infection, nocosomial bacteremia, surgical wound infection, urinary tract infection, endocarditis, meningitis and related conditions.

In one embodiment of the present invention, an aptamer nucleic acid molecule of the invention is about 4 to about 50 nucleotides in length, in specific embodiments about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides in length. In another embodiment, an enzymatic nucleic acid molecule of the invention, *e.g.,* a ribozyme or DNAzyme, is about 13 to about 100 nucleotides in length, *e.g.,* in specific embodiments about 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, or 38 nucleotides in length. In another embodiment, an antisense nucleic acid molecule, 2,5-A chimera, or triplex oligonucleotide of the invention is about 13 to about 100 nucleotides in length, *e.g.,* in specific embodiments about 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, or 38 nucleotides in length. In another embodiment, a siRNA molecule of the invention is about 18 to about 24 nucleotides in length (such as where each strand of siRNA duplex is about 18 to about 24 nucleotides in length), *e.g.,* in specific embodiments, each strand of the siRNA duplex is about 18, 19, 20, 21, 22, 23, or 24 nucleotides in length. In yet another embodiment, a siRNA molecule of the invention has 2 3'-nucleotide overhangs on each strand of the duplex, for example two thymidine (TT) nucleotide overhangs. In particular embodiments, instead of 100 nucleotides being the upper limit on the length ranges specified above, the upper limit of the length range can be, for example, 30, 40, 50, 60, 70, or 80 nucleotides. Thus, for any of the length ranges, the length range for particular embodiments has lower limit as specified, with an upper limit as specified which is greater than the lower limit. For example, in a particular embodiment, the length range can be 20-50 nucleotides in length. All such ranges are expressly included. Also in particular embodiments, a nucleic acid molecule can have a length which is any of the lengths specified above, for example, 21 nucleotides in length.

Aptamer molecules of the invention are between 4 and 50 nucleotides in length. Exemplary siRNA molecules of the invention are between 18 and 24 nucleotides in length for each strand of the siRNA duplex. In an additional example, enzymatic nucleic acid molecules of the invention are preferably between 15 and 50 nucleotides in length, more

preferably between 25 and 40 nucleotides in length, *e.g.,* 34, 36, or 38 nucleotides in length (for example see Jarvis *et al.,* 1996, *J. Biol. Chem.,* 271, 29107-29112). Exemplary DNAzymes of the invention are preferably between 15 and 40 nucleotides in length, more preferably between 25 and 35 nucleotides in length, *e.g.,* 29, 30, 31, or 32 nucleotides in length (see for example Santoro *et al.,* 1998, *Biochemistry,* 37, 13330-13342; Chartrand *et al.,* 1995, Nucleic Acids Research, 23, 4092-4096). Exemplary antisense molecules of the invention are preferably between 15 and 75 nucleotides in length, more preferably between 20 and 35 nucleotides in length, *e.g.,* 25, 26, 27, or 28 nucleotides in length (see for example Woolf *et al.,* 1992, *PNAS.,* 89, 7305-7309; Milner *et al.,* 1997, *Nature Biotechnology,* 15, 537-541). Exemplary triplex forming oligonucleotide molecules of the invention are preferably between 10 and 40 nucleotides in length, more preferably between 12 and 25 nucleotides in length, *e.g.,* 18, 19, 20, or 21 nucleotides in length (see for example Maher *et al.,* 1990, *Biochemistry,* 29, 8820-8826; Strobel and Dervan, 1990, *Science,* 249, 73-75). Those skilled in the art will recognize that all that is required is that the nucleic acid molecule is of length and conformation sufficient and suitable for the nucleic acid molecule to catalyze a reaction contemplated herein. The length of the nucleic acid molecules of the instant invention are not limiting within the general limits stated.

In one embodiment, the invention provides a method for producing a class of nucleic acid aptamers which exhibit a high degree of specificity for CylA. In another embodiment, the invention provides a method for producing a class of nucleic acid based gene modulating agents which exhibit a high degree of specificity for CylA. The nucleic acid aptamer molecule is preferably targeted to a highly conserved region of CylA such that specific treatment of a disease or condition can be provided with either one or several nucleic acid molecules of the invention. Such nucleic acid molecules can be delivered exogenously to specific tissue or cellular targets as required. Alternatively, the nucleic acid molecules can be expressed from DNA and/or RNA vectors that are delivered to specific cells.

As used herein "cell" is used in its usual biological sense, and does not refer to an entire multicellular organism, e.g., specifically does not refer to a human. The cell can be present in an organism, e.g., birds, plants and mammals such as humans, cows, sheep, apes, monkeys, swine, dogs, and cats. The cell can be prokaryotic (e.g., bacterial cell) or eukaryotic (e.g., mammalian or plant cell).

By "highly conserved nucleic acid binding region" is meant an amino acid sequence of one or more regions in a target protein that does not vary significantly from one generation to the other or from one biological system to the other.

The enzymatic nucleic acid-based modulators of CylA activity are useful for the prevention of the diseases and conditions including enterococcus infection, nocosomial bacteremia, surgical wound infection, urinary tract infection, endocarditis, meningitis, and any other diseases or conditions that are related to the levels of enterococci in a cell or tissue.

By "related to the levels of enterococci" is meant that the reduction of enterococci, specifically CylA activity, and cell entry and/or gene expression (specifically CylA gene) and thus reduction in the activity of CylA in an organism will relieve, to some extent, the symptoms of the disease or condition.

The nucleic acid-based modulators of the invention are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through injection, infusion pump or stent, with or without their incorporation in biopolymers.

In another aspect, the invention provides mammalian cells containing one or more nucleic acid molecules and/or expression vectors of this invention. The one or more nucleic acid molecules can independently be targeted to the same or different sites.

In another aspect of the invention, nucleic acid molecules of the invention are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Nucleic acid expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing nucleic acid molecules of the invention are delivered as described above, and persist in target cells. Alternatively, viral vectors may be used that provide for transient expression of the nucleic acid molecules of the invention. Such vectors might be repeatedly administered as necessary. Once expressed, the nucleic acid molecules of the invention bind to the target protein, RNA and/or DNA and modulate its function or expression. Delivery of nucleic acid expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the subject followed by reintroduction into the

subject, or by any other means that would allow for introduction into the desired target cell. DNA based nucleic acid molecules of the invention can be expressed via the use of a single stranded DNA intracellular expression vector.

By RNA is meant a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" is meant a nucleotide with a hydroxyl group at the 2' position of a β-D-ribo-furanose moiety.

By "vectors" is meant any nucleic acid- and/or viral-based technique used to express and/or deliver a desired nucleic acid.

By "subject" is meant an organism, which is a donor or recipient of explanted cells or the cells themselves. "Subject" also refers to an organism to which the nucleic acid molecules of the invention can be administered. In one embodiment, a subject is a mammal or mammalian cells. In another embodiment, a subject is a human or human cells.

The nucleic acid molecules of the instant invention, individually, or in combination or in conjunction with other drugs, can be used to treat diseases or conditions discussed herein. For example, to treat a disease or condition associated with the levels of enterococci, the nucleic acid molecules can be administered to a subject or can be administered to other appropriate cells evident to those skilled in the art, individually or in combination with one or more drugs under conditions suitable for the treatment.

In a further embodiment, the described molecules, such as aptamers, siRNA, antisense, or enzymatic nucleic acids, can be used in combination with other known treatments to treat conditions or diseases discussed above. For example, the described molecules could be used in combination with one or more known therapeutic agents, for example, an antibiotic such as penicillin, ampicillin, vancomycin, novobiocin, doxycycline, clindamycin, clinafloxacin, quinupristin, dalfopristin, cephalosporin, aztreonam, ciprofloxacin, aminoglycoside, metronidazole, fluoroquinolones, streptogramins, oxazolidinones, semisynthetic glycopeptides, glycylcyclines, chloramphenicol or any combination thereof under conditions suitable for said treatment.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

First the drawings will be described briefly.

Drawings

Figure 1 shows a scheme which outlines the steps involved in cytolysin expression and processing. Cytolysin precursors $CylL_L$ and $CylL_S$ are ribosomally synthesized and modified by a putative modification protein, CylM, that modifies and $CylL_S$ such that they can be later activated (see for example Booth *et al.,* 1996, *Mol Microbiol,* 21, 1175). Modified $CylL_L$ and $CylL_S$ are secreted by CylB and activated by proteolytic trimming of 6 terminal amino acids by CylA.

Figure 2 shows a non-limiting example of inhibition of cytolysin processing via aptamer mediated disruption of CylA. Aptamer binding to CylA prevents proteolytic processing of modified $CylL_L$ and $CylL_S$, thus preventing activation of cytolysin and subsequent cell lysis.

Figure 3 shows a non-limiting example of inhibition of cytolysin activity via aptamer mediated disruption of $CylL_L$ and $CylL_S$ interaction. Aptamer binding to $CylL_L$ and/or $CylL_S$ prevents functional activity of cytolysin, thus preventing subsequent cell lysis.

Mechanism of action of Nucleic Acid Molecules of the Invention

Aptamer: Nucleic acid aptamers can be selected to specifically bind to a particular ligand of interest (see for example Gold *et al.,* US 5,567,588 and US 5,475,096, Gold *et al.,* 1995, *Annu. Rev. Biochem.,* 64, 763; Brody and Gold, 2000, *J. Biotechnol.,* 74, 5; Sun, 2000, *Curr. Opin. Mol. Ther.,* 2, 100; Kusser, 2000, *J. Biotechnol.,* 74, 27; Hermann and Patel, 2000, *Science,* 287, 820; and Jayasena, 1999, *Clinical Chemistry,* 45, 1628). For example, the use of in vitro selection can be applied to evolve nucleic acid aptamers with binding specificity for CylA. Nucleic acid aptamers can include chemical modifications and linkers as described herein. Nucleic apatmers of the invention can be double stranded or single stranded and can comprise one distinct nucleic acid sequence or more than one nucleic acid sequences complexed with one another. Aptamer molecules of the invention that bind to CylA, can modulate the protease activity of CylA and subsequent activation of cytolysin, and therefore modulate the acute toxicity accociated with enterococcal infection.

Antisense: Antisense molecules can be modified or unmodified RNA, DNA, or mixed polymer oligonucleotides and primarily function by specifically binding to matching sequences resulting in modulation of peptide synthesis (Wu-Pong, Nov 1994, *BioPharm*, 20-33). The antisense oligonucleotide binds to target RNA by Watson Crick base-pairing and blocks gene expression by preventing ribosomal translation of the bound sequences either by steric blocking or by activating RNase H enzyme. Antisense molecules may also alter protein synthesis by interfering with RNA processing or transport from the nucleus into the cytoplasm (Mukhopadhyay & Roth, 1996, *Crit. Rev. in Oncogenesis* 7, 151-190).

In addition, binding of single stranded DNA to RNA may result in nuclease degradation of the heteroduplex (Wu-Pong, *supra*; Crooke, *supra*). To date, the only backbone modified DNA chemistry which will act as substrates for RNase H are phosphorothioates, phosphorodithioates, and borontrifluoridates. Recently, it has been reported that 2'-arabino and 2'-fluoro arabino- containing oligos can also activate RNase H activity.

A number of antisense molecules have been described that utilize novel configurations of chemically modified nucleotides, secondary structure, and/or RNase H substrate domains (Woolf *et al.*, US 5,989,912; Thompson *et al.*, USSN 60/082,404 which was filed on April 20, 1998; Hartmann *et al.*, USSN 60/101,174 which was filed on September 21, 1998) all of these are incorporated by reference herein in their entirety.

Antisense DNA can be used to target RNA by means of DNA-RNA interactions, thereby activating RNase H, which digests the target RNA in the duplex. Antisense DNA can be chemically synthesized or can be expressed via the use of a single stranded DNA intracellular expression vector or the equivalent thereof.

Triplex Forming Oligonucleotides (TFO): Single stranded oligonucleotide can be designed to bind to genomic DNA in a sequence specific manner. TFOs can be comprised of pyrimidine-rich oligonucleotides which bind DNA helices through Hoogsteen Base-pairing (Wu-Pong, *supra*). In addition, TFOs can be chemically modified to increase binding affinity to target DNA sequences. The resulting triple helix composed of the DNA sense, DNA antisense, and TFO disrupts RNA synthesis by RNA polymerase. The TFO mechanism can result in gene expression or cell death since binding may be irreversible (Mukhopadhyay & Roth, *supra*)

2'-5' Oligoadenylates: The 2-5A system is an interferon-mediated mechanism for RNA degradation found in higher vertebrates (Mitra *et al.*, 1996, *Proc Nat Acad Sci USA* 93, 6780-6785). Two types of enzymes, 2-5A synthetase and RNase L, are required for RNA cleavage. The 2-5A synthetases require double stranded RNA to form 2'-5' oligoadenylates (2-5A). 2-5A then acts as an allosteric effector for utilizing RNase L, which has the ability to cleave single stranded RNA. The ability to form 2-5A structures with double stranded RNA makes this system particularly useful for modulation of viral replication.

(2'-5') oligoadenylate structures can be covalently linked to antisense molecules to form chimeric oligonucleotides capable of RNA cleavage (Torrence, *supra*). These molecules putatively bind and activate a 2-5A-dependent RNase, the oligonucleotide/enzyme complex then binds to a target RNA molecule which can then be cleaved by the RNase enzyme. The covalent attachment of 2'-5' oligoadenylate structures is not limited to antisense applications, and can be further elaborated to include attachment to nucleic acid molecules of the instant invention.

Enzymatic Nucleic Acid: Several varieties of naturally occurring enzymatic RNAs are presently known (Doherty and Doudna, 2001, *Annu. Rev. Biophys. Biomol. Struct.*, 30, 457-475; Symons, 1994, *Curr. Opin. Struct. Biol.*, 4, 322-30). In addition, several *in vitro* selection (evolution) strategies (Orgel, 1979, *Proc. R. Soc. London,* B 205, 435) have been used to evolve new nucleic acid catalysts capable of catalyzing cleavage and ligation of phosphodiester linkages (Joyce, 1989, *Gene,* 82, 83-87; Beaudry *et al.,* 1992, *Science* 257, 635-641; Joyce, 1992, *Scientific American* 267, 90-97; Breaker *et al.,* 1994, *TIBTECH* 12, 268; Bartel *et al.,* 1993, *Science* 261:1411-1418; Szostak, 1993, *TIBS* 17, 89-93; Kumar *et al.,* 1995, *FASEB J.,* 9, 1183; Breaker, 1996, *Curr. Op. Biotech.*, 7, 442; Santoro *et al.,* 1997, *Proc. Natl. Acad. Sci.,* 94, 4262; Tang *et al.,* 1997, *RNA* 3, 914; Nakamaye & Eckstein, 1994, *supra*; Long & Uhlenbeck, 1994, supra; Ishizaka et al., 1995, *supra*; Vaish *et al.,* 1997, *Biochemistry* 36, 6495). Each can catalyze a series of reactions including the hydrolysis of phosphodiester bonds in *trans* (and thus can cleave other RNA molecules) under physiological conditions.

Nucleic acid molecules of this invention can block CylA protein expression, specifically, can be used to treat disease or diagnose disease associated with the levels of enterococci.

The enzymatic nature of an enzymatic nucleic acid has significant advantages, such as the concentration of nucleic acid necessary to affect a therapeutic treatment is low. This advantage reflects the ability of the enzymatic nucleic acid molecule to act enzymatically. Thus, a single enzymatic nucleic acid molecule is able to cleave many molecules of target RNA. In addition, the enzymatic nucleic acid molecule is a highly specific modulator, with the specificity of modulation depending not only on the base-pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can be chosen to completely eliminate catalytic activity of an enzymatic nucleic acid molecule.

Nucleic acid molecules having an endonuclease enzymatic activity are able to repeatedly cleave other separate RNA molecules in a nucleotide base sequence-specific manner. With proper design and construction, such enzymatic nucleic acid molecules can be targeted to any RNA transcript, and efficient cleavage achieved *in vitro* (Zaug *et al.*, 324, *Nature* 429 1986; Uhlenbeck, 1987 *Nature* 328, 596; Kim et al., 84 *Proc. Natl. Acad. Sci. USA* 8788, 1987; Dreyfus, 1988, *Einstein Quart. J. Bio. Med.*, 6, 92; Haseloff and Gerlach, 334 *Nature* 585, 1988; Cech, 260 *JAMA* 3030, 1988; and Jefferies et al., 17 *Nucleic Acids Research* 1371, 1989; Chartrand *et al.*, 1995, *Nucleic Acids Research* 23, 4092; Santoro *et al.*, 1997, *PNAS* 94, 4262).

Because of their sequence specificity, *trans*-cleaving enzymatic nucleic acid molecules show promise as therapeutic agents for human disease (Usman & McSwiggen, 1995 *Ann. Rep. Med. Chem.* 30, 285-294; Christoffersen and Marr, 1995 *J. Med. Chem.* 38, 2023-2037). Enzymatic nucleic acid molecule can be designed to cleave specific RNA targets within the background of cellular RNA. Such a cleavage event renders the RNA non-functional and abrogates protein expression from that RNA. In this manner, synthesis of a protein associated with a disease state can be selectively modulated (Warashina *et al.*, 1999, *Chemistry and Biology,* 6, 237-250.

The present invention also features nucleic acid sensor molecules or allozymes having sensor domains comprising nucleic acid decoys and/or aptamers of the invention. Interaction of the nucleic acid sensor molecule's sensor domain with a molecular target, such CylA or cytolysin, can activate or inactivate the enzymatic nucleic acid domain of the nucleic acid sensor molecule, such that the activity of the nucleic acid sensor molecule is modulated in the presence of the target-signaling molecule. The nucleic acid sensor molecule can be designed

to be active in the presence of the target molecule or alternately, can be designed to be inactive in the presence of the molecular target. For example, a nucleic acid sensor molecule is designed with a sensor domain comprising an aptamer with binding specificity for CylA. In a non-limiting example, interaction of the CylA with the sensor domain of the nucleic acid sensor molecule can activate the enzymatic nucleic acid domain of the nucleic acid sensor molecule such that the sensor molecule catalyzes a reaction, for example cleavage of CylA RNA. In this example, the nucleic acid sensor molecule is activated in the presence of CylA, and can be used as a therapeutic to treat enterococcus infection. Alternately, the reaction can comprise cleavage or ligation of a labeled nucleic acid reporter molecule, providing a useful diagnostic reagent to detect the presence of enterococci in a system.

RNA interference: RNA interference refers to the process of sequence specific post transcriptional gene silencing in animals mediated by short interfering RNAs (siRNA) (Fire *et al.,* 1998, *Nature,* 391, 806). The corresponding process in plants is commonly referred to as post transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post transcriptional gene silencing is thought to be an evolutionarily conserved cellular defense mechanism used to prevent the expression of foreign genes which is commonly shared by diverse flora and phyla (Fire *et al.,* 1999, *Trends Genet.,* 15, 358). Such protection from foreign gene expression may have evolved in response to the production of double stranded RNAs (dsRNA) derived from viral infection or the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response though a mechanism that has yet to be fully characterized. This mechanism appears to be different from the interferon response that results from dsRNA mediated activation of protein kinase PKR and 2',5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNA) (Berstein *et al.,* 2001, *Nature,* 409, 363). Short interfering RNAs derived from dicer activity are typically about 21-23 nucleotides in length and comprise about 19 base pair duplexes. Dicer has also been implicated in the excision of 21 and 22 nucleotide small temporal RNAs (stRNA) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner *et al.,* 2001, *Science,* 293, 834). The RNAi response also features an endonuclease complex

containing a siRNA, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single stranded RNA having sequence homologous to the siRNA. Cleavage of the target RNA takes place in the middle of the region complementary to the guide sequence of the siRNA duplex (Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188).

Short interfering RNA mediated RNAi has been studied in a variety of systems. Fire *et al.*, 1998, *Nature,* 391, 806, were the first to observe RNAi in *C. Elegans*. Wianny and Goetz, 1999, *Nature Cell Biol.*, 2, 70, describes RNAi mediated by dsRNA in mouse embryos. Hammond *et al.*, 2000, *Nature,* 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir *et al.*, 2001, *Nature,* 411, 494, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells. Recent work in Drosophila embryonic lysates has revealed certain requirements for siRNA length, structure, chemical composition, and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21 nucleotide siRNA duplexes are most active when containing two nucleotide 3'-overhangs. Furthermore, substitution of one or both siRNA strands with 2'-deoxy or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of 3'-terminal siRNA nucleotides with deoxy nucleotides was shown to be tolerated. Mismatch sequences in the center of the siRNA duplex were also shown to abolish RNAi activity. In addition, these studies also indicate that the position of the cleavage site in the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA (Nykanen *et al.*, 2001, *Cell,* 107, 309), however siRNA molecules lacking a 5'-phosphate are active when introduced exogenously, suggesting that 5'-phosphorylation of siRNA constructs may occur *in vivo*.

Synthesis of Nucleic acid Molecules

Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small nucleic acid motifs ("small" refers to nucleic acid motifs no more than 100 nucleotides in length, preferably no more than 80 nucleotides in length, and most preferably no more than 50 nucleotides in length; *e.g.*, decoy nucleic acid molecules, aptamer nucleic acid molecules antisense nucleic acid molecules, enzymatic nucleic acid molecules) are

preferably used for exogenous delivery. The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of protein and/or RNA structure. Exemplary molecules of the instant invention are chemically synthesized, and others can similarly be synthesized.

Oligonucleotides (e.g., DNA oligonucleotides) are synthesized using protocols known in the art, for example as described in Caruthers *et al.*, 1992, *Methods in Enzymology* 211, 3-19, Thompson *et al.*, International PCT Publication No. WO 99/54459, Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677-2684, Wincott *et al.*, 1997, *Methods Mol. Bio.*, 74, 59, Brennan *et al.*, 1998, *Biotechnol Bioeng.*, 61, 33-45, and Brennan, US patent No. 6,001,311. All of these references are incorporated herein by reference. The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μmol scale protocol with a 2.5 min coupling step for 2'-O-methylated nucleotides and a 45 sec coupling step for 2'-deoxy nucleotides. **Table II** outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 μmol scale can be performed on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 μL of 0.11 M = 6.6 μmol) of 2'-O-methyl phosphoramidite and a 105-fold excess of S-ethyl tetrazole (60 μL of 0.25 M = 15 μmol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 22-fold excess (40 μL of 0.11 M = 4.4 μmol) of deoxy phosphoramidite and a 70-fold excess of S-ethyl tetrazole (40 μL of 0.25 M = 10 μmol) can be used in each coupling cycle of deoxy residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); and oxidation solution is 16.9 mM I_2, 49 mM pyridine, 9% water in THF (PERSEPTIVE™). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of

phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide, 0.05 M in acetonitrile) is used.

Deprotection of the DNA-based oligonucleotides is performed as follows: the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to –20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H2O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder.

The method of synthesis used for normal RNA including certain decoy nucleic acid molecules and enzymatic nucleic acid molecules follows the procedure as described in Usman *et al.,* 1987, *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.,* 1990, *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.,* 1995, *Nucleic Acids Res.* 23, 2677-2684 Wincott *et al.,* 1997, *Methods Mol. Bio.*, 74, 59, and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μmol scale protocol with a 7.5 min coupling step for alkylsilyl protected nucleotides and a 2.5 min coupling step for 2'-O-methylated nucleotides. **Table II** outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 μmol scale can be done on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 μL of 0.11 M = 6.6 μmol) of 2'-O-methyl phosphoramidite and a 75-fold excess of S-ethyl tetrazole (60 μL of 0.25 M = 15 μmol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 66-fold excess (120 μL of 0.11 M = 13.2 μmol) of alkylsilyl (ribo) protected phosphoramidite and a 150-fold excess of S-ethyl tetrazole (120 μL of 0.25 M = 30 μmol) can be used in each coupling cycle of ribo residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation

solution is 16.9 mM I_2, 49 mM pyridine, 9% water in THF (PERSEPTIVE™). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide0.05 M in acetonitrile) is used.

Deprotection of the RNA is performed using either a two-pot or one-pot protocol. For the two-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to –20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H2O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder. The base deprotected oligoribonucleotide is resuspended in anhydrous TEA/HF/NMP solution (300 µL of a solution of 1.5 mL N-methylpyrrolidinone, 750 µL TEA and 1 mL TEA•3HF to provide a 1.4 M HF concentration) and heated to 65 °C. After 1.5 h, the oligomer is quenched with 1.5 M NH_4HCO_3.

Alternatively, for the one-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 33% ethanolic methylamine/DMSO: 1/1 (0.8 mL) at 65 °C for 15 min. The vial is brought to r.t. TEA•3HF (0.1 mL) is added and the vial is heated at 65 °C for 15 min. The sample is cooled at –20 °C and then quenched with 1.5 M NH_4HCO_3.

For purification of the trityl-on oligomers, the quenched NH_4HCO_3 solution is loaded onto a C-18 containing cartridge that had been prewashed with acetonitrile followed by 50 mM TEAA. After washing the loaded cartridge with water, the RNA is detritylated with 0.5% TFA for 13 min. The cartridge is then washed again with water, salt exchanged with 1 M NaCl and washed with water again. The oligonucleotide is then eluted with 30% acetonitrile.

Inactive hammerhead ribozymes or binding attenuated control (BAC) oligonucleotides are synthesized by substituting a U for G_5 and a U for A_{14} (numbering from Hertel, K. J., *et al.*, 1992, Nucleic Acids Res., 20, 3252). Similarly, one or more nucleotide substitutions can

be introduced in other nucleic acid molecules, such as aptamers, to inactivate the molecule and such molecules can serve as a negative control.

The average stepwise coupling yields are typically >98% (Wincott *et al.,* 1995 *Nucleic Acids Res.* 23, 2677-2684). Those of ordinary skill in the art will recognize that the scale of synthesis can be adapted to be larger or smaller than the example described above including but not limited to 96-well format, all that is important is the ratio of chemicals used in the reaction.

Alternatively, the nucleic acid molecules of the present invention can be synthesized separately and joined together post-synthetically, for example, by ligation (Moore *et al.,* 1992, *Science* 256, 9923; Draper *et al.,* International PCT publication No. WO 93/23569; Shabarova *et al.,* 1991, *Nucleic Acids Research* 19, 4247; Bellon *et al.,* 1997, *Nucleosides & Nucleotides*, 16, 951; Bellon *et al.,* 1997, *Bioconjugate Chem.* 8, 204).

The nucleic acid molecules of the present invention can be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-flouro, 2'-*O*-methyl, 2'-H (for a review see Usman and Cedergren, 1992, *TIBS* 17, 34; Usman *et al.,* 1994, *Nucleic Acids Symp. Ser.* 31, 163). Nucleic acid molecules of the invention can be purified by gel electrophoresis using general methods or can be purified by high pressure liquid chromatography (HPLC; see Wincott *et al., supra*, the totality of which is hereby incorporated herein by reference) and re-suspended in water.

Optimizing Activity of the nucleic acid molecule of the invention.

Chemically synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) can prevent their degradation by serum ribonucleases, which can increase their potency (see *e.g.,* Eckstein *et al.*, International Publication No. WO 92/07065; Perrault *et al.*, 1990 *Nature* 344, 565; Pieken et al., 1991, *Science* 253, 314; Usman and Cedergren, 1992, *Trends in Biochem. Sci.* 17, 334; Usman *et al.*, International Publication No. WO 93/15187; and Rossi *et al.*, International Publication No. WO 91/03162; Sproat, US Patent No. 5,334,711; Gold *et al.,* US 6,300,074; and Burgin *et al., supra;* all of which are incorporated by reference herein). All of the above references describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of the nucleic acid molecules described herein. Modifications that enhance their efficacy in cells, and removal of bases

from nucleic acid molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are desired.

There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-flouro, 2'-*O*-methyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992, *TIBS.* 17, 34; Usman *et al.,* 1994, *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.,* 1996, *Biochemistry,* 35, 14090). Sugar modification of nucleic acid molecules have been extensively described in the art (see Eckstein *et al., International Publication* PCT No. WO 92/07065; Perrault *et al. Nature,* 1990, *344,* 565-568; Pieken *et al. Science,* 1991, *253,* 314-317; Usman and Cedergren, *Trends in Biochem. Sci.* , 1992, *17,* 334-339; Usman *et al. International Publication* PCT No. WO 93/15187; Sproat, *US Patent* No. 5,334,711 and Beigelman *et al.,* 1995, *J. Biol. Chem.,* 270, 25702; Beigelman *et al.,* International PCT publication No. WO 97/26270; Beigelman *et al.,* US Patent No. 5,716,824; Usman *et al.,* US patent No. 5,627,053; Woolf *et al.,* International PCT Publication No. WO 98/13526; Thompson *et al.,* USSN 60/082,404 which was filed on April 20, 1998; Karpeisky *et al.,* 1998, *Tetrahedron Lett.,* 39, 1131; Earnshaw and Gait, 1998, *Biopolymers (Nucleic Acid Sciences),* 48, 39-55; Verma and Eckstein, 1998, *Annu. Rev. Biochem.,* 67, 99-134; and Burlina *et al.,* 1997, *Bioorg. Med. Chem.,* 5, 1999-2010; all of the references are hereby incorporated in their totality by reference herein). Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into ribozymes without modulating catalysis, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein to modify the nucleic acid molecules of the instant invention.

While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorothioate, and/or 5'-methylphosphonate linkages improves stability, excessive modifications can cause some toxicity. Therefore, when designing nucleic acid molecules, the amount of these internucleotide linkages should be minimized. The reduction in the concentration of these linkages should lower toxicity, resulting in increased efficacy and higher specificity of these molecules.

Nucleic acid molecules having chemical modifications that maintain or enhance activity are provided. Such a nucleic acid is also generally more resistant to nucleases than an unmodified nucleic acid. Accordingly, the *in vitro* and/or *in vivo* activity should not be significantly lowered. In cases in which modulation is the goal, therapeutic nucleic acid molecules delivered exogenously should optimally be stable within cells until translation of the target RNA has been modulated long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days depending upon the disease state. Improvements in the chemical synthesis of RNA and DNA (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677; Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-19 (incorporated by reference herein)) have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability, as described above.

In one embodiment, nucleic acid molecules of the invention include one or more G-clamp nucleotides. A G-clamp nucleotide is a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine within a duplex, see for example Lin and Matteucci, 1998, *J. Am. Chem. Soc.*, 120, 8531-8532. A single G-clamp analog substation within an oligonucleotide can result in substantially enhanced helical thermal stability and mismatch discrimination when hybridized to complementary oligonucleotides. The inclusion of such nucleotides in nucleic acid molecules of the invention results in both enhanced affinity and specificity to nucleic acid targets. In another embodiment, nucleic acid molecules of the invention include one or more LNA "locked nucleic acid" nucleotides such as a 2', 4'-C mythylene bicyclo nucleotide (see for example Wengel *et al.*, International PCT Publication No. WO 00/66604 and WO 99/14226).

In another embodiment, the invention features conjugates and/or complexes of nucleic acid molecules targeting CylA. Such conjugates and/or complexes can be used to facilitate delivery of molecules into a biological system, such as a cell. The conjugates and complexes provided by the instant invention can impart therapeutic activity by transferring therapeutic compounds across cellular membranes, altering the pharmacokinetics, and/or modulating the localization of nucleic acid molecules of the invention. The present invention encompasses the design and synthesis of novel conjugates and complexes for the delivery of molecules, including, but not limited to, small molecules, lipids, phospholipids, nucleosides, nucleotides, nucleic acids, antibodies, toxins, negatively charged polymers and other polymers, for

example proteins, peptides, hormones, carbohydrates, polyethylene glycols, or polyamines, across cellular membranes. In general, the transporters described are designed to be used either individually or as part of a multi-component system, with or without degradable linkers. These compounds are expected to improve delivery and/or localization of nucleic acid molecules of the invention into a number of cell types originating from different tissues, in the presence or absence of serum (see Sullenger and Cech, US 5,854,038). Conjugates of the molecules described herein can be attached to biologically active molecules via linkers that are biodegradable, such as biodegradable nucleic acid linker molecules.

The term "biodegradable nucleic acid linker molecule" as used herein, refers to a nucleic acid molecule that is designed as a biodegradable linker to connect one molecule to another molecule, for example, a biologically active molecule. The stability of the biodegradable nucleic acid linker molecule can be modulated by using various combinations of ribonucleotides, deoxyribonucleotides, and chemically modified nucleotides, for example, 2'-O-methyl, 2'-fluoro, 2'-amino, 2'-O-amino, 2'-C-allyl, 2'-O-allyl, and other 2'-modified or base modified nucleotides. The biodegradable nucleic acid linker molecule can be a dimer, trimer, tetramer or longer nucleic acid molecule, for example, an oligonucleotide of about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length, or can comprise a single nucleotide with a phosphorus-based linkage, for example, a phosphoramidate or phosphodiester linkage. The biodegradable nucleic acid linker molecule can also comprise nucleic acid backbone, nucleic acid sugar, or nucleic acid base modifications.

The term "biodegradable" as used herein, refers to degradation in a biological system, for example enzymatic degradation or chemical degradation.

The term "biologically active molecule" as used herein, refers to compounds or molecules that are capable of eliciting or modifying a biological response in a system. Non-limiting examples of biologically active molecules contemplated by the instant invention include therapeutically active molecules such as antibodies, hormones, antivirals, peptides, proteins, chemotherapeutics, small molecules, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, 2,5-A chimeras, siRNA, dsRNA, allozymes, aptamers, decoys and analogs thereof. Biologically active molecules of the invention also include molecules capable of modulating the pharmacokinetics and/or pharmacodynamics of other biologically active

molecules, for example, lipids and polymers such as polyamines, polyamides, polyethylene glycol and other polyethers.

The term "phospholipid" as used herein, refers to a hydrophobic molecule comprising at least one phosphorus group. For example, a phospholipid can comprise a phosphorus-containing group and saturated or unsaturated alkyl group, optionally substituted with OH, COOH, oxo, amine, or substituted or unsubstituted aryl groups.

Therapeutic nucleic acid molecules of the invention delivered exogenously optimally are stable within cells such that therapeutic activity is achieved. The nucleic acid molecules can therefore be designed such that they resistant to nucleases and function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of nucleic acid molecules described in the instant invention and in the art have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability as described above.

In yet another embodiment, nucleic acid molecules having chemical modifications that maintain or enhance enzymatic activity and/or nuclease stability are provided. Such nucleic acids are also generally more resistant to nucleases than unmodified nucleic acids. Thus, *in vitro* and/or *in vivo* the activity should not be significantly lowered. As exemplified herein, such nucleic acid molecules are useful *in vitro* and/or *in vivo* even if activity over all is reduced 10 fold (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090).

Use of the nucleic acid-based molecules of the invention will lead to better treatment of the disease progression by affording the possibility of combination therapies (*e.g.*, multiple nucleic acid molecules targeted to different genes; nucleic acid molecules coupled with known small molecule modulators; or intermittent treatment with combinations of molecules (including different motifs) and/or other chemical or biological molecules. The treatment of subjects with nucleic acid molecules may also include combinations of different types of nucleic acid molecules.

In another aspect the nucleic acid molecules comprise a 5' and/or a 3'- cap structure.

By "cap structure" is meant chemical modifications, which have been incorporated at either terminus of the oligonucleotide (see, for example, Wincott *et al.*, WO 97/26270, incorporated by reference herein). These terminal modifications protect the nucleic acid

molecule from exonuclease degradation, and may help in delivery and/or localization within a cell. The cap may be present at the 5'-terminus (5'-cap) or at the 3'-terminal (3'-cap) or may be present on both termini. In non-limiting examples: the 5'-cap is selected from the group comprising inverted abasic residue (moiety); 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide, 4'-thio nucleotide; carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety (for more details, see Wincott *et al.*, International PCT publication No. WO 97/26270, incorporated by reference herein).

In yet another preferred embodiment, the 3'-cap is selected from a group comprising, 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate; 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Iyer, 1993, *Tetrahedron* 49, 1925; incorporated by reference herein).

By the term "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine.

An "alkyl" group refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 12 carbons.

More preferably, it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkyl group can be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2 or $N(CH_3)_2$, amino, or SH. The term also includes alkenyl groups that are unsaturated hydrocarbon groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has 1 to 12 carbons. More preferably, it is a lower alkenyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkenyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2, halogen, $N(CH_3)_2$, amino, or SH. The term "alkyl" also includes alkynyl groups that have an unsaturated hydrocarbon group containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has 1 to 12 carbons. More preferably, it is a lower alkynyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkynyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2 or $N(CH_3)_2$, amino or SH.

Such alkyl groups may also include aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester groups. An "aryl" group refers to an aromatic group that has at least one ring having a conjugated pi electron system and includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which may be optionally substituted. The preferred substituent(s) of aryl groups are halogen, trihalomethyl, hydroxyl, SH, OH, cyano, alkoxy, alkyl, alkenyl, alkynyl, and amino groups. An "alkylaryl" group refers to an alkyl group (as described above) covalently joined to an aryl group (as described above). Carbocyclic aryl groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted. Heterocyclic aryl groups are groups having from 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted. An "amide" refers to an -C(O)-NH-R, where R is either alkyl, aryl, alkylaryl or hydrogen. An "ester" refers to an -C(O)-OR', where R is either alkyl, aryl, alkylaryl or hydrogen.

By "nucleotide" as used herein is as recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the

1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see, for example, Usman and McSwiggen, *supra*; Eckstein *et al.,* International PCT Publication No. WO 92/07065; Usman *et al.,* International PCT Publication No. WO 93/15187; Uhlman & Peyman, *supra,* all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art as summarized by Limbach *et al.,* 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of base modifications that can be introduced into nucleic acid molecules include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-methyluridine), propyne, and others (Burgin *et al.,* 1996, *Biochemistry*, 35, 14090; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents; such bases may be used at any position, for example, within the catalytic core of a nucleic acid decoy molecule and/or in the substrate-binding regions of the nucleic acid molecule.

In one embodiment, the invention features modified nucleic acids, for example aptamers, siRNA, antisense, and enzymatic nucleic acid moelcules with phosphate backbone modifications comprising one or more phosphorothioate, phosphorodithioate, methylphosphonate, morpholino, amidate carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and/or alkylsilyl, substitutions. For a review of oligonucleotide backbone modifications, see Hunziker and Leumann, 1995, *Nucleic Acid Analogues: Synthesis and Properties,* in *Modern Synthetic Methods,* VCH, 331-417, and Mesmaeker *et al.,* 1994, *Novel Backbone Replacements for Oligonucleotides,* in *Carbohydrate Modifications in Antisense Research,* ACS, 24-39.

By "abasic" is meant sugar moieties lacking a base or having other chemical groups in place of a base at the 1' position, (for more details, see Usman *et al.,* US 5,891,683 and Matulic-Adamic *et al.,* US 5,998,203).

By "unmodified nucleoside" is meant one of the bases adenine, cytosine, guanine, thymine, uracil joined to the 1' carbon of β-D-ribo-furanose.

By "modified nucleoside" is meant any nucleotide base which contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate.

In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant 2'-NH_2 or 2'-*O*- NH_2, which may be modified or unmodified. Such modified groups are described, for example, in Eckstein *et al.,* U.S. Patent 5,672,695 and Matulic-Adamic *et al.,* WO 98/28317.

Various modifications to nucleic acid (*e.g.,* aptamer, siRNA, antisense and enzymatic nucleic acid) structure can be made to enhance the utility of these molecules. Such modifications will enhance shelf-life, half-life *in vitro,* stability, and ease of introduction of such oligonucleotides to the target site, *e.g.,* to enhance penetration of cellular membranes, and confer the ability to recognize and bind to targeted cells.

Administration of Nucleic Acid Molecules

Methods for the delivery of nucleic acid molecules are described in Akhtar *et al.,* 1992, *Trends Cell Bio.*, 2, 139; *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, ed. Akhtar, 1995, Maurer *et al.,* 1999, *Mol. Membr. Biol.,* 16, 129-140; Hofland and Huang, 1999, *Handb. Exp. Pharmacol.,* 137, 165-192; and Lee *et al.,* 2000, *ACS Symp. Ser.,* 752, 184-192, all of which are incorporated herein by reference. Sullivan *et al.*, PCT WO 94/02595, further describes the general methods for delivery of enzymatic nucleic acid molecules. These protocols can be utilized for the delivery of virtually any nucleic acid molecule. Nucleic acid molecules can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors (O'Hare and Normand, International PCT Publication No. WO 00/53722). Alternatively, the nucleic acid/vehicle combination is locally delivered by direct injection or by use of an infusion pump. Direct injection of the nucleic acid molecules of the invention, whether subcutaneous, intramuscular, or intradermal, can take place using standard needle and syringe methodologies, or by needle-free technologies such as those described in Conry *et al.,* 1999, *Clin. Cancer Res.,* 5, 2330-2337 and Barry *et al.,* International PCT Publication No. WO

99/31262. The molecules of the instant invention can be used as pharmaceutical agents. Pharmaceutical agents prevent, modulate the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state in a subject.

Thus, the invention features a pharmaceutical composition comprising one or more nucleic acid(s) of the invention in an acceptable carrier, such as a stabilizer, buffer, and the like. The negatively charged polynucleotides of the invention can be administered (*e.g.*, RNA, DNA or protein) and introduced into a subject by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The compositions of the present invention may also be formulated and used as tablets, capsules or elixirs for oral administration, suppositories for rectal administration, sterile solutions, suspensions for injectable administration, and the other compositions known in the art.

The present invention also includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds, *e.g.*, acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, *e.g.*, systemic administration, into a cell or subject, including for example a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation from reaching a target cell (*i.e.*, a cell to which the negatively charged nucleic acid is desirable for delivery). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms that prevent the composition or formulation from exerting its effect.

By "systemic administration" is meant *in vivo* systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include, without limitation: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes expose the desired negatively charged

polymers, *e.g.*, nucleic acids, to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation that can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach may provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells, such as cancer cells.

By "pharmaceutically acceptable formulation" is meant, a composition or formulation that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity. Nonlimiting examples of agents suitable for formulation with the nucleic acid molecules of the instant invention include: P-glycoprotein inhibitors (such as Pluronic P85), which can enhance entry of drugs into the CNS (Jolliet-Riant and Tillement, 1999, *Fundam. Clin. Pharmacol.*, 13, 16-26); biodegradable polymers, such as poly (DL-lactide-coglycolide) microspheres for sustained release delivery after intracerebral implantation (Emerich, DF *et al*, 1999, *Cell Transplant*, 8, 47-58) (Alkermes, Inc. Cambridge, MA); and loaded nanoparticles, such as those made of polybutylcyanoacrylate, which can deliver drugs across the blood brain barrier and can alter neuronal uptake mechanisms (*Prog Neuropsychopharmacol Biol Psychiatry*, 23, 941-949, 1999). Other non-limiting examples of delivery strategies for the nucleic acid molecules of the instant invention include material described in Boado *et al.*, 1998, *J. Pharm. Sci.*, 87, 1308-1315; Tyler *et al.*, 1999, *FEBS Lett.*, 421, 280-284; Pardridge *et al.*, 1995, *PNAS USA.*, 92, 5592-5596; Boado, 1995, *Adv. Drug Delivery Rev.*, 15, 73-107; Aldrian-Herrada *et al.*, 1998, *Nucleic Acids Res.*, 26, 4910-4916; and Tyler *et al.*, 1999, *PNAS USA.*, 96, 7053-7058.

The invention also features the use of the composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer a method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic *et al. Chem. Rev.* 1995, 95, 2601-2627; Ishiwata *et al.*, *Chem. Pharm. Bull.* 1995, 43, 1005-1011).

Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic *et al., Science* 1995, 267, 1275-1276; Oku *et al.*,1995, *Biochim. Biophys. Acta*, 1238, 86-90). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu *et al., J. Biol. Chem.* 1995, 42, 24864-24870; Choi *et al.,* International PCT Publication No. WO 96/10391; Ansell *et al.,* International PCT Publication No. WO 96/10390; Holland *et al.,* International PCT Publication No. WO 96/10392). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen.

The present invention also includes compositions prepared for storage or administration, which include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co. (A.R. Gennaro edit. 1985) hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents may be provided. These include sodium benzoate, sorbic acid and esters of *p*-hydroxybenzoic acid. In addition, antioxidants and suspending agents may be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence of, or treat (alleviate a symptom to some extent, preferably all of the symptoms) a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors that those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer.

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The nucleic acid molecules of the invention and formulations thereof can be administered orally, topically, parenterally, by inhalation or spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and/or vehicles. The term parenteral as used herein includes percutaneous, subcutaneous, intravascular (*e.g.*, intravenous), intramuscular, or intrathecal injection or infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a nucleic acid molecule of the invention and a pharmaceutically acceptable carrier. One or more nucleic acid molecules of the invention can be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants, and if desired other active ingredients. The pharmaceutical compositions containing nucleic acid molecules of the invention can be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

Compositions intended for oral use can be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions can contain one or more such sweetening agents, flavoring agents, coloring agents or preservative agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients that are suitable for the manufacture of tablets. These excipients can be, for example, inert diluents; such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn

starch, or alginic acid; binding agents, for example starch, gelatin or acacia; and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets can be uncoated or they can be coated by known techniques. In some cases such coatings can be prepared by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monosterate or glyceryl distearate can be employed.

Formulations for oral use can also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

Aqueous suspensions contain the active materials in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropyl-methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents can be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions can also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.

Oily suspensions can be formulated by suspending the active ingredients in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions can contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents can be added to provide palatable oral preparations. These compositions can be preserved by the addition of an anti-oxidant such as ascorbic acid.

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents or suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, can also be present.

Pharmaceutical compositions of the invention can also be in the form of oil-in-water emulsions. The oily phase can be a vegetable oil or a mineral oil or mixtures of these. Suitable emulsifying agents can be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, for example sorbitan monooleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions can also contain sweetening and flavoring agents.

Syrups and elixirs can be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol, glucose or sucrose. Such formulations can also contain a demulcent, a preservative and flavoring and coloring agents. The pharmaceutical compositions can be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents that have been mentioned above. The sterile injectable preparation can also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be employed including synthetic mono-or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The nucleic acid molecules of the invention can also be administered in the form of suppositories, *e.g.*, for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable non-irritating excipient that is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials include cocoa butter and polyethylene glycols.

Nucleic acid molecules of the invention can be administered parenterally in a sterile medium. The drug, depending on the vehicle and concentration used, can either be suspended or dissolved in the vehicle. Advantageously, adjuvants such as local anesthetics, preservatives and buffering agents can be dissolved in the vehicle.

Dosage levels of the order of from about 0.1 mg to about 140 mg per kilogram of body weight per day are useful in the treatment of the above-indicated conditions (about 0.5 mg to about 7 g per subject per day). The amount of active ingredient that can be combined with the carrier materials to produce a single dosage form varies depending upon the host treated and the particular mode of administration. Dosage unit forms generally contain between from about 1 mg to about 500 mg of an active ingredient.

It is understood that the specific dose level for any particular subject depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

For administration to non-human animals, the composition can also be added to the animal feed or drinking water. It can be convenient to formulate the animal feed and drinking water compositions so that the animal takes in a therapeutically appropriate quantity of the composition along with its diet. It can also be convenient to present the composition as a premix for addition to the feed or drinking water.

The nucleic acid molecules of the present invention may also be administered to a subject in combination with other therapeutic compounds to increase the overall therapeutic effect. The use of multiple compounds to treat an indication may increase the beneficial effects while reducing the presence of side effects.

In one embodiment, the invention compositions suitable for administering nucleic acid molecules of the invention to specific cell types, such as hepatocytes. For example, the asialoglycoprotein receptor (ASGPr) (Wu and Wu, 1987, *J. Biol. Chem.* 262, 4429-4432) is unique to hepatocytes and binds branched galactose-terminal glycoproteins, such as asialoorosomucoid (ASOR). Binding of such glycoproteins or synthetic glycoconjugates to the receptor takes place with an affinity that strongly depends on the degree of branching of the oligosaccharide chain, for example, triatennary structures are bound with greater affinity than biatenarry or monoatennary chains (Baenziger and Fiete, 1980, *Cell,* 22, 611-620;

Connolly *et al.,* 1982, *J. Biol. Chem.,* 257, 939-945). Lee and Lee, 1987, *Glycoconjugate J.,* 4, 317-328, obtained this high specificity through the use of N-acetyl-D-galactosamine as the carbohydrate moiety, which has higher affinity for the receptor, compared to galactose. This "clustering effect" has also been described for the binding and uptake of mannosyl-terminating glycoproteins or glycoconjugates (Ponpipom *et al.,* 1981, *J. Med. Chem.,* 24, 1388-1395). The use of galactose and galactosamine based conjugates to transport exogenous compounds across cell membranes can provide a targeted delivery approach to the treatment of liver disease such as HBV infection or hepatocellular carcinoma. The use of bioconjugates can also provide a reduction in the required dose of therapeutic compounds required for treatment. Furthermore, therapeutic bioavialability, pharmacodynamics, and pharmacokinetic parameters can be modulated through the use of nucleic acid bioconjugates of the invention.

Alternatively, certain of the nucleic acid molecules of the instant invention can be expressed within cells from eukaryotic promoters (*e.g.,* Izant and Weintraub, 1985, *Science,* 229, 345; McGarry and Lindquist, 1986, *Proc. Natl. Acad. Sci.*, USA 83, 399; Scanlon *et al.,* 1991, *Proc. Natl. Acad. Sci. USA*, 88, 10591-5; Kashani-Sabet *et al.,* 1992, *Antisense Res. Dev.,* 2, 3-15; Dropulic *et al.,* 1992, *J. Virol.,* 66, 1432-41; Weerasinghe *et al.,* 1991, *J. Virol.,* **65**, 5531-4; Ojwang *et al.,* 1992, *Proc. Natl. Acad. Sci. USA*, 89, 10802-6; Chen *et al.,* 1992, *Nucleic Acids Res.,* 20, 4581-9; Sarver *et al.,* 1990 *Science,* 247, 1222-1225; Thompson *et al.,* 1995, *Nucleic Acids Res.,* 23, 2259; Good *et al.,* 1997, *Gene* Therapy, 4, 45; all of these references are hereby incorporated in their totalities by reference herein). Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a ribozyme (Draper *et al.,* PCT WO 93/23569, and Sullivan *et al.,* PCT WO 94/02595; Ohkawa *et al.,* 1992, *Nucleic Acids Symp. Ser.,* 27, 15-6; Taira *et al.,* 1991, *Nucleic Acids Res.,* 19, 5125-30; Ventura *et al.,* 1993, *Nucleic Acids Res.,* 21, 3249-55; Chowrira *et al.,* 1994, *J. Biol. Chem.,* 269, 25856; all of these references are hereby incorporated in their totality by reference herein).

In another aspect of the invention, RNA molecules of the present invention are preferably expressed from transcription units (see, for example, Couture *et al.,* 1996, *TIG.,* 12, 510) inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Ribozyme expressing viral vectors could be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably,

the recombinant vectors capable of expressing the nucleic acid molecules are delivered as described above, and persist in target cells. Alternatively, viral vectors may be used that provide for transient expression of nucleic acid molecules. Such vectors might be repeatedly administered as necessary. Once expressed, the nucleic acid molecule binds to the target mRNA. Delivery of nucleic acid molecule expressing vectors could be systemic, such as by intravenous or intra-muscular administration, by administration to target cells ex-planted from the subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell (for a review see Couture *et al.*, 1996, *TIG.*, 12, 510). In addition, nucleic acid molecules of the invention can be expressed from a bacterial expression vector, for example by cloning the desired nucleic acid sequence into the enterococcal pAD1 plasmid or another suitable bacterial vector.

In one aspect, the invention features an expression vector comprising a nucleic acid sequence encoding at least one of the nucleic acid molecules of the instant invention. The nucleic acid sequence encoding the nucleic acid molecule of the instant invention is operably linked in a manner which allows expression of that nucleic acid molecule.

In another aspect the invention features an expression vector comprising: a) a transcription initiation region (*e.g.*, eukaryotic pol I, II or III initiation region); b) a transcription termination region (*e.g.*, eukaryotic pol I, II or III termination region); c) a nucleic acid sequence encoding at least one of the nucleic acid catalyst of the instant invention; and wherein said sequence is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. The vector may optionally include an open reading frame (ORF) for a protein operably linked on the 5' side or the 3'-side of the sequence encoding the nucleic acid catalyst of the invention; and/or an intron (intervening sequences).

Transcription of the nucleic acid molecule sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990, *Proc. Natl. Acad. Sci. U S A*, 87, 6743-7; Gao and Huang 1993, *Nucleic Acids Res.*, 21, 2867-72; Lieber et al., 1993,

Methods Enzymol., 217, 47-66; Zhou et al., 1990, *Mol. Cell. Biol.*, 10, 4529-37). All of these references are incorporated by reference herein. Several investigators have demonstrated that nucleic acid molecules, such as ribozymes expressed from such promoters can function in mammalian cells (e.g. Kashani-Sabet et al., 1992, *Antisense Res. Dev.*, 2, 3-15; Ojwang et al., 1992, *Proc. Natl. Acad. Sci. U S A*, 89, 10802-6; Chen *et al.*, 1992, *Nucleic Acids Res.*, 20, 4581-9; Yu et al., 1993, *Proc. Natl. Acad. Sci. U S A*, 90, 6340-4; L'Huillier *et al.*, 1992, *EMBO J.*, 11, 4411-8; Lisziewicz *et al.*, 1993, *Proc. Natl. Acad. Sci. U. S. A*, 90, 8000-4; Thompson *et al.*, 1995, *Nucleic Acids Res.*, 23, 2259; Sullenger & Cech, 1993, *Science,* 262, 1566). More specifically, transcription units such as the ones derived from genes encoding U6 small nuclear (snRNA), transfer RNA (tRNA) and adenovirus VA RNA are useful in generating high concentrations of desired RNA molecules such as ribozymes in cells (Thompson *et al., supra*; Couture and Stinchcomb, *1996, supra*; Noonberg *et al.*, 1994, *Nucleic Acid Res.*, 22, 2830; Noonberg *et al.*, US Patent No. 5,624,803; Good *et al.*, 1997, *Gene Ther.*, 4, 45; Beigelman *et al.*, International PCT Publication No. *WO 96/18736*; all of these publications are incorporated by reference herein). The above ribozyme transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, *1996, supra).*

In yet another aspect, the invention features an expression vector comprising nucleic acid sequence encoding at least one of the nucleic acid molecules of the invention, in a manner that allows expression of that nucleic acid molecule. The expression vector comprises in one embodiment; a) a transcription initiation region; b) a transcription termination region; c) a nucleic acid sequence encoding at least one said nucleic acid molecule; and wherein said sequence is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an open reading frame; d) a nucleic acid sequence encoding at least one said nucleic acid molecule, wherein said sequence is operably linked to the 3'-end of said open reading frame; and wherein said sequence is operably linked to said initiation region, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In yet another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription

termination region; c) an intron; d) a nucleic acid sequence encoding at least one said nucleic acid molecule; and wherein said sequence is operably linked to said initiation region, said intron and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) an open reading frame; e) a nucleic acid sequence encoding at least one said nucleic acid molecule, wherein said sequence is operably linked to the 3'-end of said open reading frame; and wherein said sequence is operably linked to said initiation region, said intron, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

Examples:

The following are non-limiting examples showing the selection, isolation, synthesis and activity of nucleic acids of the instant invention.

Example 1: Identification of Aptamers that specifically bind to CylA

A nucleic acid aptamer that selectively binds CylA is provided in accordance with the present invention. The binding affinity of the aptamer for CylA is preferably represented by the dissociation constant of about 20 nanomolar (nM) or less, and more preferably about 10 nM or less. In one embodiment, the Kd of the aptamer and CylA target is established using a double filter nitrocellulose filter binding assay such as that disclosed by Wong and Lohman, 1993, *PNAS USA*, 90, 5428-5432.

Generally, the method for isolating aptamers of the invention having specificity for CylA comprises: (a) preparing a candidate mixture of potential oligonucleotide ligands for CylA wherein the candidate mixture is complex enough to contain at least one oligonucleotide ligand for CylA or a peptide derivative thereof (the CylA target); (b) contacting the candidate mixture with the CylA target under conditions suitable for at least one oligonucleotide in the candidate mixture to bind to the CylA target; (c) removing unbound oligonucleotides from the candidate mixture; (d) collecting the oligonucleotide ligands that are bound to the CylA target to produce a first collected mixture of oligonucleotide ligands; (e) contacting the mixture from (d) with the CylA target under more stringent binding conditions than in (b), wherein oligonucleotide ligands having increased affinity to the CylA target relative to the first collected mixture of (d); (f) removing unbound

oligonucleotides from (e); and (g) collecting the oligonucleotide ligands that are bound to the CylA target to produce a second collected mixture of oligonucleotide ligands to thereby identify oligonucleotides having specificity for CylA. The method can comprise additional steps in which the oligonucleotides isolated in the first or second collected mixture are enriched or expanded by any suitable technique, such as amplification or mutagenesis, prior to contacting the first collected oligonucleotide mixture with the target under the higher stringency conditions, after collecting the oligonucleotides that bound to the target under the higher stringency conditions, or both. Optionally, the contacting and expanding or enriching steps are repeated as necessary to produce the desired aptamer. Thus, it is possible that the second collected oligonucleotide mixture can comprise a single aptamer. The conditions used to affect the stringency of binding used in the method can include varying reaction conditions used for binding, for example the composition of a buffer, temperature, time, and concentration of the components used for binding can be optimized for the desired level of stringency.

In vitro Selection

In a non-limiting example, aptamers having binding specificity for a CylA target are isolated by applying the method under the following conditions. First, the CylA target is attached to a solid matrix such as a bead or chip surface by means of a covalent (eg. amide or morpholino bond) or non-covalent (eg. biotin/streptavidin) linkage. The CylA target can comprise the entire isolated CylA polypeptide or an isolated peptide sequence derived therefrom. The isolated peptide sequence can be, for example, synthesized or isolated by protein digest.

A random pool of DNA oligomers is synthesized where the 5' and 3' proximal ends are fixed sequences used for amplification and the central region consists of randomized positions. Ten picomoles of template are PCR amplified for 8 cycles in the initial round. Copy DNA of the selected pool of RNA from subsequent rounds of amplification are PCR amplified 18 cycles. PCR reactions are carried out in a 50 microliter volume containing 200 picomoles of each primer, 2 mM final concentration dNTP's, 5 units of Thermus aquaticus DNA polymerase (Perkin Elmer Cetus) in a PCR buffer (10 mM Tris-Cl pH 8.4, 50 mM KCl, 7.5 mM $MgCl_2$, 0.05 mg/ml BSA). Primers are annealed at 58°C for 20 seconds and extended at 74°C for 2 minutes. Denaturation can occur at 93°C for 30 seconds.

Products from PCR amplification are used for T7 *in vitro* transcription in a 200 microliter reaction volume. T7 transcripts are purified from an 8 percent, 7M Urea polyacrylamide gel and eluted by crushing gel pieces in a Sodium Acetate/EDTA solution. For each round of amplification, 50 picomoles of the selected pool of RNA is phosphatased for 30 minutes using Calf Intestinal Alkaline Phosphatase. The reaction is then phenol extracted 3 times and chloroform extracted once, then ethanol precipitated. 25 picomoles of this RNA is 5' end-labeled using gamma ^{32}P ATP with T4 polynucleotide kinase for 30 minutes. Kinased RNA is gel purified and a small quantity (about 150 fmoles; 100,000 cpm) is used along with 250 picomoles of cold RNA to follow the fraction of RNA bound to CylA and retained on nitrocellulose filters during the separation step of the method. Typically a protein concentration is used that binds one to five percent of the total input RNA. A control (without protein) is used to determine the background which is typically 0.1% of the total input. Selected RNA is eluted from the filter by extracting three times with water saturated phenol containing 2% lauryl sulfate (SDS), 0.3M NaOAc and 5 mM EDTA followed by a chloroform extraction. Twenty five percent of this RNA is then used to synthesize cDNA for PCR amplification.

Selection with Non-Amplifiable Competitor RNA

In a non-limiting example, selections are performed using two buffer conditions where the only difference between the buffers is sodium concentration (250 mM NaCl or 500 mM NaCl). Two different buffer conditions are used to increase stringency (with the higher salt concentration being more stringent) and to determine whether different ligands can be obtained. After 10 rounds of amplification, the binding constant of the selected pool can decrease by about an order of magnitude and can remain constant for the next two additional rounds. Competitor RNA is not used in the first 12 rounds. After this round, the pool is split and selection carried out in the presence and absence (control) of competitor RNA. For rounds 12 through 18, a 50-fold excess of a non-amplifiable random pool of RNA is present during selection to compete with non-specific low-affinity binders that may survive and thus be amplified. The competitor RNA, which had a 30N random region, is made as described above for the amplifiable pool RNA; however, the competitor RNA has different primer annealing sequences. Thus, the competitor RNA does not survive the cDNA synthesis or PCR amplification steps. It would be apparent to one skilled in the art that other primer sequences could be used as long as they are not homologous to those used for the pool RNA.

The use of competitor RNA can increase the affinity of the selected pool by several orders of magnitude.

Cloning and Sequencing

In a non-limiting example, PCR amplified DNA from the last round selected-pool of RNA is phenol and chloroform extracted and ethanol precipitated. The extracted PCR DNA is then digested using Bam HI and Hind III restriction enzymes and sub-cloned into pUC18. DNAs are phenol and chloroform extracted following digestion. Ligation is carried out at room temperature for two hours after which time the reaction is phenol and chloroform extracted and used to electroporate competent cells. Fifty transformants from the selections using competitor RNA at both NaCl concentrations are picked and their DNAs sequenced.

Binding Assays

In a non-limiting example, binding assays are performed by adding 5 microliters of CylA protein, at the appropriate concentrations (i.e., ranging from 2 x 10^{-6} with 3 fold dilutions to 9 x 10^{-9} for 250 mM NaCl and 0.5 x 10^{-7} with 3 fold dilutions to 2 x 10^{-10} for 50 mM NaCl), to 45 ul of binding buffer (50 mM Na-HEPES pH 7.5, 250 mM NaCl, 2 mM DTT, 10 mM $MnCl_2$, 5 mM CHAPS) on ice, then adding 50,000 cpm of kinased RNA (<200 fmoles) in a volume of 3 to 4 microliters. This mix is incubated at 37°C for 20 minutes. The reactions are then passed over nitrocellulose filters, which are pre-equilibrated in buffer, and washed with a 50 mM Tris-Cl pH 7.5 solution. Filters are dried and counted.

General Considerations in Aptamer Selection

When a consensus sequence is identified, oligonucleotides that contain that sequence can be made by conventional synthetic or recombinant techniques. These aptamers can also function as target-specific aptamers of this invention. Such an aptamer can conserve the entire nucleotide sequence of an isolated aptamer, or can contain one or more additions, deletions or substitutions in the nucleotide sequence, as long as a consensus sequence is conserved. A mixture of such aptamers can also function as target-specific aptamers, wherein the mixture is a set of aptamers with a portion or portions of their nucleotide sequence being random or varying, and a conserved region that contains the consensus sequence. Additionally, secondary aptamers can be synthesized using one or more of the modified

bases, sugars and linkages described herein using conventional techniques and those described herein.

In some embodiments of this invention, aptamers can be sequenced or mutagenized to identify consensus regions or domains that are participating in aptamer binding to target, and/or aptamer structure. This information is used for generating second and subsequent pools of aptamers of partially known or predetermined sequence. Sequencing used alone or in combination with the retention and selection processes of this invention, can be used to generate less diverse oligonucleotide pools from which aptamers can be made. Further selection according to these methods can be carried out to generate aptamers having preferred characteristics for diagnostic or therapeutic applications. That is, domains that facilitate, for example, drug delivery could be engineered into the aptamers selected according to this invention.

Although this invention is directed to making aptamers using screening from pools of non-predetermined sequences of oligonucleotides, it also can be used to make second-generation aptamers from pools of known or partially known sequences of oligonucleotides. A pool is considered diverse even if one or both ends of the oligonucleotides comprising it are not identical from one oligonucleotide pool member to another, or if one or both ends of the oligonucleotides comprising the pool are identical with non-identical intermediate regions from one pool member to another. Toward this objective, knowledge of the structure and organization of the target protein can be useful to distinguish features that are important for biochemical pathway inhibition or biological response generation in the first generation aptamers. Structural features can be considered in generating a second (less random) pool of oligonucleotides for generating second round aptamers:

Those skilled in the art will appreciate that comparisons of the complete or partial amino acid sequences of the purified protein target to identify variable and conserved regions is useful. Comparison of sequences of aptamers made according to this invention provides information about the consensus regions and consensus sequences responsible for binding. It is expected that certain nucleotides will be rigidly specified and certain positions will exclusively require certain bases. Likewise, studying localized regions of a protein to identify secondary structure can be useful. Localized regions of a protein can adopt a number of different conformations including beta strands, alpha helices, turns (induced principally by proline or glycine residues) or random structure. Different regions of a polypeptide interact

with each other through hydrophobic and electrostatic interactions and also by formation of salt bridges, disulfide bridges, etc. to form the secondary and tertiary structures. Defined conformations can be formed within the protein organization, including beta sheets, beta barrels, and clusters of alpha helices.

It sometimes is possible to determine the shape of a protein target or portion thereof by crystallography X ray diffraction or by other physical or chemical techniques known to those skilled in the art. Many different computer programs are available for predicting protein secondary and tertiary structure, the most common being those described in Chou and Fasman, 1978, *Biochemistry*, 13, 222-245, and Gamier *et al.*, 1978, *J. Mol. Biol,* 120, 97-120. Generally, these and other available programs are based on the physical and chemical properties of individual amino acids (hydrophobicity, size, charge and presence of side chains) and on the amino acids' collective tendency to form identifiable structures in proteins whose secondary structure has been determined. Many programs attempt to weight structural data with their known influences. For example, amino acids such as proline or glycine are often present where polypeptides have turns. Long stretches of hydrophobic amino acids (as determined by hydropathy plot), usually have a strong affinity for lipids.

Data obtained by the methods described above and by other conventional methods and tools can be correlated with the presence of particular sequences of nucleotides in the first and second generation aptamers to engineer second and third generation aptamers. Further, according to this invention, second generation aptamers can be identified simply by sequentially screening from pools of oligonucleotides having more predetermined sequences than the pools used in earlier rounds of selection.

These methods can be used to design optimal binding sequences for any desired protein target (which can be portions of aptamers or entire aptamers) and/or to engineer into aptamers any number of desired targeted functions or features. Optimal binding sequences will be those which exhibit high relative affinity for target, i.e., affinity measured in Kd in at least in the nanomolar range, and, for certain drug applications, the nanomolar or picomolar range. In practicing this invention, studying the binding energies of aptamers using standard methods known generally in the art can be useful. Generally, consensus regions can be identified by comparing the conservation of nucleotides for appreciable enhancement in binding.

Structural knowledge can be used to engineer aptamers made according to this invention. For example, stem structures in the aptamer pool can be vital components in some embodiments where increased aptamer rigidity is desired. According to the teachings of the instant invention, a randomly generated pool of oligonucleotides having the stem sequences can be generated. After aptamers are identified which contain the stem (i.e., use the stem in primers), one can put cross-linkers in the stem to covalently fix the stem in the aptamer structure. Cross-linkers also can be used to fix an aptamer to a target. Once an aptamer has been identified, it can be used, either by linkage to, or use in combination with, other aptamers identified according to these methods. One or more aptamers can be used in this manner to bind to one or more targets.

Techniques used in optimizing Aptamer binding

In order to produce nucleic acid aptamers desirable for use as a pharmaceutical composition, it is preferred that the nucleic acid aptamer binds to the target in a manner capable of achieving the desired effect on the target; be as small as possible to obtain the desired effect; be as stable as possible; and be a specific ligand to the chosen target. In most, if not all, situations it is preferred that the nucleic acid ligand has the highest possible affinity to the target. Modifications or derivatizations of the ligand that confer resistance to degradation and clearance *in situ* during therapy, the capability to cross various tissue or cell membrane barriers, or any other accessory properties that do not significantly interfere with affinity for the target molecule can also be provided as improvements.

One of the uses of nucleic acid ligands derived by *in vitro* selection or another approach is to find ligands that alter target molecule function. Thus, it is a good procedure to first assay for inhibition or enhancement of function of the target protein. One could even perform such functional tests of the combined ligand pool prior to cloning and sequencing. Assays for the biological function of the chosen target are generally available and known to those skilled in the art, and can be easily performed in the presence of the nucleic acid ligand to determine if inhibition occurs.

Enrichment can supply a number of cloned ligands of probable variable affinity for the target molecule. Sequence comparisons can yield consensus secondary structures and primary sequences that allow grouping of the ligand sequences into motifs. Although a single ligand sequence (with some mutations) can be found frequently in the total population of cloned

sequences, the degree of representation of a single ligand sequence in the cloned population of ligand sequences cannot absolutely correlate with affinity for the target molecule. Therefore mere abundance is not the sole criterion for judging "winners" after *in vitro* selection and binding assays for various ligand sequences (adequately defining each motif that is discovered by sequence analysis) are required to weigh the significance of the consensus arrived at by sequence comparisons. The combination of sequence comparison and affinity assays should guide the selection of candidates for more extensive ligand characterization.

An important step for determining the length of sequence relevant to specific affinity is to establish the boundaries of that information within a ligand sequence. This is conveniently accomplished by selecting end-labeled fragments from hydrolyzed pools of the ligand of interest so that 5' and 3' boundaries of the information can be discovered. To determine a 3' boundary, one can perform a large-scale in vitro transcription of the amplified aptamer sequence, gel purify the RNA using UV shadowing on an intensifying screen, phosphatasing the purified RNA, phenol extracting extensively, labeling by kinase reactions with ^{32}P, and gel purification of the labeled product (for example by using a film of the gel as a guide). The resultant product can then be subjected to pilot partial digestions with RNase T1 (varying enzyme concentration and time, at 50°C. in a buffer of 7M urea, 50 mM sodium citrate pH 5.2) and alkaline hydrolysis (at 50 mM NaC03, adjusted to pH 9.0 by prior mixing of 1 M bicarbonate and carbonate solutions; test over ranges of 20 to 60 minutes at 95°C). Once optimal conditions for alkaline hydrolysis are established (so that there is an even distribution of small to larger fragments) one can scale up to provide enough material for selection by the target (for example on nitrocellulose filters). Binding assays can be performed which vary target protein concentration from the lowest saturating protein concentration to that protein concentration at which approximately 10% of RNA is bound as determined by the binding assays for the ligand. One can vary target concentration by increasing volume rather than decreasing the absolute amount of target; this provides a good signal to noise ratio as the amount of RNA bound to the filter is limited by the absolute amount of target. The RNA is eluted as, for example, in *in vitro* selection and then run on a denaturing gel with T1 partial digests so that the positions of hydrolysis bands can be related to the ligand sequence.

The 5' boundary can be similarly determined. Large-scale in vitro transcriptions are purified as described herein. There are two methods for labeling the 3' end of the RNA. One

method is to kinase Cp with ^{32}P (or purchase ^{32}P-Cp) and ligate to the purified RNA with RNA ligase. The labeled RNA is then purified and subjected to very identical protocols. An alternative is to subject unlabeled RNAs to partial alkaline hydrolyses and extend an annealed, labeled primer with reverse transcriptase as the assay for band positions. One of the advantages over pCp labeling is the ease of the procedure, the more complete sequencing ladder (by dideoxy chain termination sequencing) with which one can correlate the boundary, and increased yield of assayable product. A disadvantage is that the extension on eluted RNA sometimes contains artifactual stops, so it can be important to control by spotting and eluting starting material on nitrocellulose filters without washes and assaying as the input RNA. Using techniques as described herein, it is possible to find the boundaries of the sequence information required for high affinity binding to the target.

Assessment of Nucleotide Contributions to Aptamer Target Binding Affinity

Once a minimal high affinity ligand sequence is identified, the sequence can be used to identify the nucleotides within the boundaries that are critical to the interaction with the target molecule. One method is to create a new random template in which all of the nucleotides of a high affinity ligand sequence are partially randomized or blocks of randomness are interspersed with blocks of complete randomness for use in an *in vitro* selection method for example, preferably a modified *in vitro* selection method as disclosed herein. Such "secondary" *in vitro* selections produce a pool of ligand sequences in which critical nucleotides or structures are absolutely conserved, less critical features preferred, and unimportant positions unbiased. Secondary *in vitro* selections can thus help to further elaborate a consensus that is based on relatively few ligand sequences. In addition, even higher-affinity ligands can be provided whose sequences were unexplored in the original *in vitro* selection.

Another method is to test oligo-transcribed variants (i.e. nucleotide substitution) where the consensus sequence can be unclear. An additional useful set of techniques are inclusively described as chemical modification experiments. Such experiments can be used to probe the native structure of RNAs, by comparing modification patterns of denatured and non-denatured states. The chemical modification pattern of an RNA ligand that is subsequently bound by target molecule can be different from the native pattern, indicating potential changes in structure upon binding or protection of groups by the target molecule. In addition, RNA ligands can fail to be bound by the target molecule when modified at positions critical

to either the bound structure of the ligand or critical to interaction with the target molecule. Such experiments in which these positions are identified are described as "chemical modification interference" experiments.

There are a variety of available reagents to conduct such experiments that are known to those skilled in the art (see for example, Ehresmann *et al.,* 1987, *Nuc. Acids. Res.*, 15, 9109-9128). Chemicals that modify bases can be used to modify ligand RNAs. A pool is bound to the target at varying concentrations and the bound RNAs recovered (such as in the boundary experiments) and the eluted RNAs analyzed for the modification. An assay can be by subsequent modification-dependent base removal and aniline scission at the baseless position or by reverse transcription assay of sensitive (modified) positions. In such assays, bands (indicating modified bases) in unselected RNAs, appear that disappear relative to other bands in target protein-selected RNAs. Similar chemical modifications with ethyl nitrosourea, or via mixed chemical or enzymatic synthesis with, for example, 2'-methoxys on ribose or phosphorothioates can be used to identify essential atomic groups on the oligonucleotide backbone. In experiments with 2'-methoxy vs. 2'-OH mixtures, the presence of an essential OH group can result in enhanced hydrolysis relative to other positions in molecules that have been stringently selected by the target.

Comparisons of the intensity of bands for bound and unbound ligands can reveal not only modifications that interfere with binding, but also modifications that enhance binding. A ligand can be made with precisely that modification and tested for the enhanced affinity. Thus chemical modification experiments can be a method for exploring additional local contacts with the target molecule, just as walking experiments (see below) are for additional nucleotide level contacts with adjacent domains.

A consensus of primary and secondary structures that enables the chemical or enzymatic synthesis of oligonucleotide ligands whose design is based on that consensus is provided herein via an *in vitro* selection method, preferably a modified *in vitro* selection method as disclosed herein. Because the replication machinery of *in vitro* selection requires that rather limited variation at the subunit level (ribonucleotides, for example), such ligands imperfectly fill the available atomic space of a target molecule's binding surface. However, these ligands can be thought of as high-affinity scaffolds that can be derivatized to make additional contacts with the target molecule. In addition, the consensus contains atomic group descriptors that are pertinent to binding and atomic group descriptors that are coincidental to

the pertinent atomic group interactions. Such derivatization does not exclude incorporation of cross-linking agents that will give specifically directly covalent linkages to the target protein. Such derivatization analyses can be performed at but are not limited to the 2' position of the ribose, and thus can also include derivatization at any position in the base or backbone of the nucleotide ligand.

The present invention thus includes nucleic acid ligands wherein certain chemical modifications have been made in order to increase the *in vivo* stability of the ligand or to enhance or mediate the delivery of the ligand. Examples of such modifications include chemical substitutions at the ribose and/or phosphate positions of a given RNA sequence. A logical extension of this analysis is a situation in which one or a few nucleotides of the polymeric ligand are used as a site for chemical derivative exploration. The rest of the ligand serves to anchor in place this monomer (or monomers) on which a variety of derivatives are tested for non-interference with binding and for enhanced affinity. Such explorations can result in small molecules that mimic the structure of the initial ligand framework, and have significant and specific affinity for the target molecule independent of that nucleic acid framework. Such derivatized subunits, which can have advantages with respect to mass production, therapeutic routes of administration, delivery, clearance or degradation than the initial ligand, can become the therapeutic and can retain very little of the original ligand. Thus, the aptamer ligands of the present invention can allow directed chemical exploration of a defined site on the target molecule known to be important for the target function.

Walking Experiments

After a minimal consensus ligand sequence has been determined for a given target, it is possible to add random sequence to the minimal consensus ligand sequence and evolve additional contacts with the target, perhaps to separate but adjacent domains. This procedure has been referred to in the art as "walking". A walking experiment can involve two experiments performed sequentially. A new candidate mixture is produced in which each of the members of the candidate mixture has a fixed nucleic acid region that corresponds to a nucleic acid ligand of interest. Each member of the candidate mixture also contains a randomized region of sequences. According to this method it is possible to identify what are referred to as "extended" nucleic acid ligands, which contain regions that can bind to more than one binding domain of a target.

Covariance Analysis

In conjunction with empirical methods for determining the three dimensional structure of nucleic acids, computer modeling methods for determining structure of nucleic acid ligands can also be employed. Secondary structure prediction is a useful guide to correct sequence alignment. It is also a highly useful stepping-stone to correct 3D structure prediction, by constraining a number of bases into A-form helical geometry.

Tables of energy parameters for calculating the stability of secondary structures exist. Although early secondary structure prediction programs attempted to simply maximize the number of base-pairs formed by a sequence, most current programs seek to find structures with minimal free energy as calculated by these thermodynamic parameters. There are two problems in this approach that should be borne in mind. First, the thermodynamic rules are inherently inaccurate, typically to 10% or so, and there are many different possible structures lying within 10% of the global energy minimum. Second, the actual secondary structure need not lie at a global energy minimum, depending on the kinetics of folding and synthesis of the sequence. Nonetheless, for short sequences, these caveats are of minor importance because there are so few possible structures that can form.

The brute force predictive method is a dot-plot: make an N by N plot of the sequence against itself, and mark an X everywhere a base pair is possible. Diagonal runs of X's mark the location of possible helices. Exhaustive tree-searching methods can then search for all possible arrangements of compatible (i.e., non-overlapping) helices of length L or more; energy calculations can be done for these structures to rank them as more or less likely. The advantages of this method are that all possible topologies, including pseudoknotted conformations, can be examined, and that a number of suboptimal structures are automatically generated as well. An elegant predictive method, and currently the most used, is the Zuker program. Zuker, 1989, *Science*, 244, 48-52. Originally based on an algorithm developed by Ruth Nussinov, the Zuker program makes a major simplifying assumption that no pseudoknotted conformations will be allowed. This permits the use of a dynamic programming approach that runs in time proportional to only N3 to N4, where N is the length of the sequence. The Zuker program is the only program capable of rigorously dealing with sequences of than a few hundred nucleotides, so it has come to be the most commonly used by biologists. However, the inability of the Zuker program to predict pseudoknotted conformations is a serious consideration. Where pseudoknotted RNA structures are suspected

or are recognized by eye, a brute-force method capable of predicting pseudoknotted conformations should be employed.

A central element of comparative sequence analysis is sequence covariations. A covariation is when the identity of one position depends on the identity of another position; for instance, a required Watson-Crick base pair shows strong covariation in that knowledge of one of the two positions gives absolute knowledge of the identity at the other position. Covariation analysis has been used previously to predict the secondary structure of RNAs for which a number of related sequences sharing a common structure exist, such as tRNA, rRNAs, and group I introns. It is now apparent that covariation analysis can be used to detect tertiary contacts as well. Stormo and Gutell, 1992, *Nucleic Acids Research,* 29, 5785-5795 have designed and implemented an algorithm that precisely measures the amount of covariations between two positions in an aligned sequence set. The program is called "MIXY"-Mutual Information at position X and Y. Consider an aligned sequence set. In each column or position, the frequency of occurrence of A, C, G, U, and gaps is calculated. This frequency is called f(bx), the frequency of base b in column x. Considering two columns at once, the frequency that a given base b appears in column x is f(bx) and the frequency that a given base b appears in column y is f(by). If position x and position y do not care about each other's identity-that is, the positions are independent; there is no covariation-the frequency of observing bases bx and by at position x and y in any given sequence should be just f(bxby)=f(bx)f(by). If there are substantial deviations of the observed frequencies of pairs from their expected frequencies, the positions are said to covary.

The amount of deviation from expectation can be quantified with an information measure M(x,y), the mutual information of x and y.

$$M(x,y) = \sum_{b_x b_y} f(b_x b_y) \mathrm{In} \frac{f(b_x b_y)}{f(b_x) f(b_y)}$$

M(x,y) can be described as the number of bits of information one learns about the identity of position y from knowing just the identity of position x. If there is no covariation, M(x,y) is zero; larger values of M(x,y) indicate strong covariation. Covariation values can be used to develop three-dimensional structural predictions.

In some ways, the problem is similar to that of structure determination by NMR. Unlike crystallography, which in the end yields an actual electron density map, NMR yields a set of interatomic distances. Depending on the number of interatomic distances one can get, there can be one, few, or many 3D structures with which they are consistent. Mathematical techniques had to be developed to transform a matrix of interatomic distances into a structure in 3D space. The two main techniques in use are distance geometry and restrained molecular dynamics.

Distance geometry is the more formal and purely mathematical technique. The interatomic distances are considered to be coordinates in an N-dimensional space, where N is the number of atoms. In other words, the "position" of an atom is specified by N distances to all the other atoms, instead of the three (x,y,z) coordinates typically considered. Interatomic distances between every atom are recorded in an N-by-N distance matrix. A complete and precise distance matrix is easily transformed into a 3 by N Cartesian coordinates, using matrix algebra operations. The trick of distance geometry as applied to NMR is dealing with incomplete (only some of the interatomic distances are known) and imprecise data (distances are known to a precision of only a few angstroms at best). Much of the time of distance geometry-based structure calculation is thus spent in pre-processing the distance matrix, calculating bounds for the unknown distance values based on the known ones, and narrowing the bounds on the known ones. Usually, multiple structures are extracted from the distance matrix that are consistent with a set of NMR data; if they all overlap nicely, the data were sufficient to determine a unique structure. Unlike NMR structure determination, covariance gives only imprecise distance values; but also only probabilistic rather than absolute knowledge about whether a given distance constraint should be applied.

Restrained molecular dynamics can also be employed, albeit in a more ad hoc manner. Given an empirical force field that attempts to describe the forces that all the atoms feel (van der Waals, covalent bonding lengths and angles, electrostatics), one can simulate a number of femtosecond time steps of a molecule's motion, by assigning every atom at a random velocity (from the Boltzmann distribution at a given temperature) and calculating each atom's motion for a femtosecond using Newtonian dynamical equations; that is "molecular dynamics". In restrained molecular dynamics, one assigns extra ad hoc forces to the atoms when they violate specified distance bounds.

With respect to RNA aptamers, the probabilistic nature of data with restrained molecular dynamics can be addressed. The covariation values can be transformed into artificial restraining forces between certain atoms for certain distance bounds; varying the magnitude of the force according to the magnitude of the covariance. NMR and covariance analysis generates distance restraints between atoms or positions, which are readily transformed into structures through distance geometry or restrained molecular dynamics. Another source of experimental data which can be utilized to determine the three dimensional structures of nucleic acids is chemical and enzymatic protection experiments, which generate solvent accessibility restraints for individual atoms or positions.

Indications

Particular degenerative and disease states that can be associated with enterococci include but are not limited to enterococcus infection, nocosomial bacteremia, surgical wound infection, urinary tract infection, endocarditis, meningitis, and any other diseases or conditions that are related to or will respond to the levels of CylA in a cell or tissue, alone or in combination with other therapies

The present body of knowledge in enterococcal research indicates the need for methods to assay enterococcus activity and for compounds that can regulate enterococcus infection for research, diagnostic, and therapeutic use.

The use of antibiotics represents a non-limiting example of other therapies that can be combined with the nucleic acid moelcules of the invention for the treatment of diseases and conditions described herein. Examples of such compounds include, penicillin, ampicillin, vancomycin, novobiocin, doxycycline, clindamycin, clinafloxacin, quinupristin, dalfopristin, cephalosporin, aztreonam, ciprofloxacin, aminoglycoside, metronidazole, fluoroquinolones, streptogramins, oxazolidinones, semisynthetic glycopeptides, glycylcyclines, chloramphenicol or any combination thereof. Those skilled in the art will recognize that other drug compounds and therapies can be similarly be readily combined with the nucleic acid molecules of the instant invention are hence within the scope of the instant invention.

Diagnostic uses

The aptamers of the invention can be used to detect the presence or absence of the target substances to which they specifically bind, such as CylA. Such diagnostic tests are

conducted by contacting a sample with the aptamer to obtain a complex that is then detected by conventional techniques known in the art. For example, the aptamers can be labeled using radioactive, fluroescent, or chomogenic labels. Interaction of labeled aptamer with the target can result in the detection of the target molecule via an ELISA type assay or sandwich assay, or by other means known in the art. Alternately, the aptamers of the invention can be used to separate or isolate molecules that specifically bind to the aptamer. For example, by coupling the aptamers to a solid support, target molecules which bind to the aptamers can be recovered via affinity chromatography or analyzed by standard means known in the art.

The enzymatic nucleic acid molecules of this invention and siRNA can be used as diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of enterococci in a cell. The close relationship between enzymatic nucleic acid molecule activity and the structure of the target RNA allows the detection of mutations in any region of the molecule which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple enzymatic nucleic acid molecules described in this invention, one can map nucleotide changes which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with enzymatic nucleic acid molecules can be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of disease. In this manner, other genetic targets can be defined as important mediators of the disease. These experiments can lead to better treatment of the disease progression by affording the possibility of combinational therapies (*e.g.*, multiple enzymatic nucleic acid molecules targeted to different genes, enzymatic nucleic acid molecules coupled with known small molecule inhibitors, or intermittent treatment with combinations of enzymatic nucleic acid molecules and/or other chemical or biological molecules). Other *in vitro* uses of enzymatic nucleic acid molecules of this invention are well known in the art, and include detection of the presence of mRNAs associated with enterococcus-related condition. Such RNA is detected by determining the presence of a cleavage product after treatment with an enzymatic nucleic acid molecule using standard methodology.

In a specific example, enzymatic nucleic acid molecules which cleave only wild-type or mutant forms of the target RNA are used for the assay. The first enzymatic nucleic acid molecule is used to identify wild-type RNA present in the sample and the second enzymatic nucleic acid molecule is used to identify mutant RNA in the sample. As reaction controls,

synthetic substrates of both wild-type and mutant RNA are cleaved by both enzymatic nucleic acid molecules to demonstrate the relative enzymatic nucleic acid molecule efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus each analysis requires two enzymatic nucleic acid molecules, two substrates and one unknown sample which is combined into six reactions. The presence of cleavage products is determined using an RNAse protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the phenotype (*i.e.*, enterococcus) is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels will be adequate and will decrease the cost of the initial diagnosis. Higher mutant form to wild-type ratios are correlated with higher risk whether RNA levels are compared qualitatively or quantitatively. The use of enzymatic nucleic acid molecules in diagnostic applications contemplated by the instant invention is more fully described in George *et al.*, US Patent Nos. 5,834,186 and 5,741,679, Shih *et al.*, US Patent No. 5,589,332, Nathan *et al.*, US Patent No 5,871,914, Nathan and Ellington, International PCT publication No. WO 00/24931, Breaker *et al.*, International PCT Publication Nos. WO 00/26226 and 98/27104, and Sullenger *et al.*, International PCT publication No. WO 99/29842.

All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments, optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the description and the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Table I: CylA sequence (Enterococcus faecalis, Accession AAM21177)

```
  1 mkkrgltyil isyiflilgt tgyasdlsnn isffidnsqt taieeiesel ssekvdyiqe
 61 iglvsfknld dsdrkfigky fnvsegkklp dfkpeevnss ilninilnkd fksfnwpykk
121 ilshidpvke qlgkditial idsgidrlhp nlqdnnlrlk nhvndielde yghgtqvagv
181 idtiaprvnl nsykvmdgtd gnsinmlkai vdatndqvdi invslgsykn meidderftv
241 eafrkavnya rknnilivas agnesrdist gnekhipggl esvitvgatk ksgdiadysn
301 ygsnvsiygp aggygdnyki tgqidaremm mtyyptslvs plgkaadfpd gytlsfgtsl
361 atpevsaala aimsknvdns kdsnevlntl fenadsfidk nsmlkykevr ik
```

(SEQ ID No: 1)

Table II:

A. 2.5 µmol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	6.5	163 µL	45 sec	2.5 min	7.5 min
S-Ethyl Tetrazole	23.8	238 µL	45 sec	2.5 min	7.5 min
Acetic Anhydride	100	233 µL	5 sec	5 sec	5 sec
N-Methyl Imidazole	186	233 µL	5 sec	5 sec	5 sec
TCA	176	2.3 mL	21 sec	21 sec	21 sec
Iodine	11.2	1.7 mL	45 sec	45 sec	45 sec
Beaucage	12.9	645 µL	100 sec	300 sec	300 sec
Acetonitrile	NA	6.67 mL	NA	NA	NA

B. 0.2 µmol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	15	31 µL	45 sec	233 sec	465 sec
S-Ethyl Tetrazole	38.7	31 µL	45 sec	233 min	465 sec
Acetic Anhydride	655	124 µL	5 sec	5 sec	5 sec
N-Methyl Imidazole	1245	124 µL	5 sec	5 sec	5 sec
TCA	700	732 µL	10 sec	10 sec	10 sec
Iodine	20.6	244 µL	15 sec	15 sec	15 sec
Beaucage	7.7	232 µL	100 sec	300 sec	300 sec
Acetonitrile	NA	2.64 mL	NA	NA	NA

C. 0.2 µmol Synthesis Cycle 96 well Instrument

Reagent	Equivalents:DNA/ 2'-O-methyl/Ribo	Amount: DNA/2'-O-methyl/Ribo	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time* Ribo
Phosphoramidites	22/33/66	40/60/120 µL	60 sec	180 sec	360sec
S-Ethyl Tetrazole	70/105/210	40/60/120 µL	60 sec	180 min	360 sec
Acetic Anhydride	265/265/265	50/50/50 µL	10 sec	10 sec	10 sec
N-Methyl Imidazole	502/502/502	50/50/50 µL	10 sec	10 sec	10 sec
TCA	238/475/475	250/500/500 µL	15 sec	15 sec	15 sec
Iodine	6.8/6.8/6.8	80/80/80 µL	30 sec	30 sec	30 sec
Beaucage	34/51/51	80/120/120	100 sec	200 sec	200 sec
Acetonitrile	NA	1150/1150/1150 µL	NA	NA	NA

- Wait time does not include contact time during delivery.

CLAIMS

What we claim is:

1. A nucleic acid molecule that inhibits bacterial subtilisn type serine protease CylA activity.

2. The nucleic acid molecule of claim 1, wherein said nucleic acid molecule is an aptamer.

3. The nucleic acid molecule of claim 1, wherein said nucleic acid molecule is a siNA.

4. The aptamer of claim 2, wherein said aptamer specifically binds to CylA.

5. The aptamer of claim 2, wherein said aptamer specifically binds to $CylL_L$.

6. The aptamer of claim 2, wherein said aptamer specifically binds to $CylL_S$.

7. The nucleic acid molecule of claim 1, wherein said nucleic acid molecule is chemically synthesized.

8. The nucleic acid molecule of claim 1, wherein said nucleic acid molecule contains one or more chemically modified nucleotides.

9. The nucleic acid molecule of claim 1, wherein said nucleic acid molecule comprises one or more nucleic acid sugar modifications.

10. The nucleic acid molecule of claim 1, wherein said nucleic acid molecule comprises one or more nucleic acid base modifications.

11. The nucleic acid molecule of claim 1, wherein said nucleic acid molecule comprises one or more nucleic acid backbone modifications.

12. The aptamer of claim 2, wherein said aptamer specifically binds to a polypeptide sequence having SEQ ID NO: 1.

13. A composition comprising the nucleic acid molecule of claim 1 in a pharmaceutically acceptable carrier or diluent.

14. A cell comprising the nucleic acid molecule of claim 1.

15. The cell of claim 14, wherein said cell is a mammalian cell.

16. The cell of claim 15, wherein said cell is a human cell.

17. A method for treating enterococcus infection, comprising administering to a subject the nucleic acid molecule of claim 1 under conditions suitable for the treatment, whereby the enterococcus infection is treated.

18. A method for treating nosocomial bacteremia, comprising administering to a subject the nucleic acid molecule of claim 1 under conditions suitable for the treatment, whereby the nosocomial bacteremia is treated.

19. A method of reducing CylA activity in a cell, comprising contacting the cell with the nucleic acid molecule of claim 1 under conditions suitable for the reduction of CylA activity, whereby CylA activity is reduced.

20. A method of reducing $CylL_L$ activity in a cell, comprising contacting the cell with the nucleic acid molecule of claim 1 under conditions suitable for the reduction of $CylL_L$ activity, whereby $CylL_L$ activity is reduced.

21. A method of reducing $CylL_S$ activity in a cell, comprising contacting the cell with the nucleic acid molecule of claim 1 under conditions suitable for the reduction of $CylL_S$ activity, whereby $CylL_S$ activity is reduced.

Figure 1

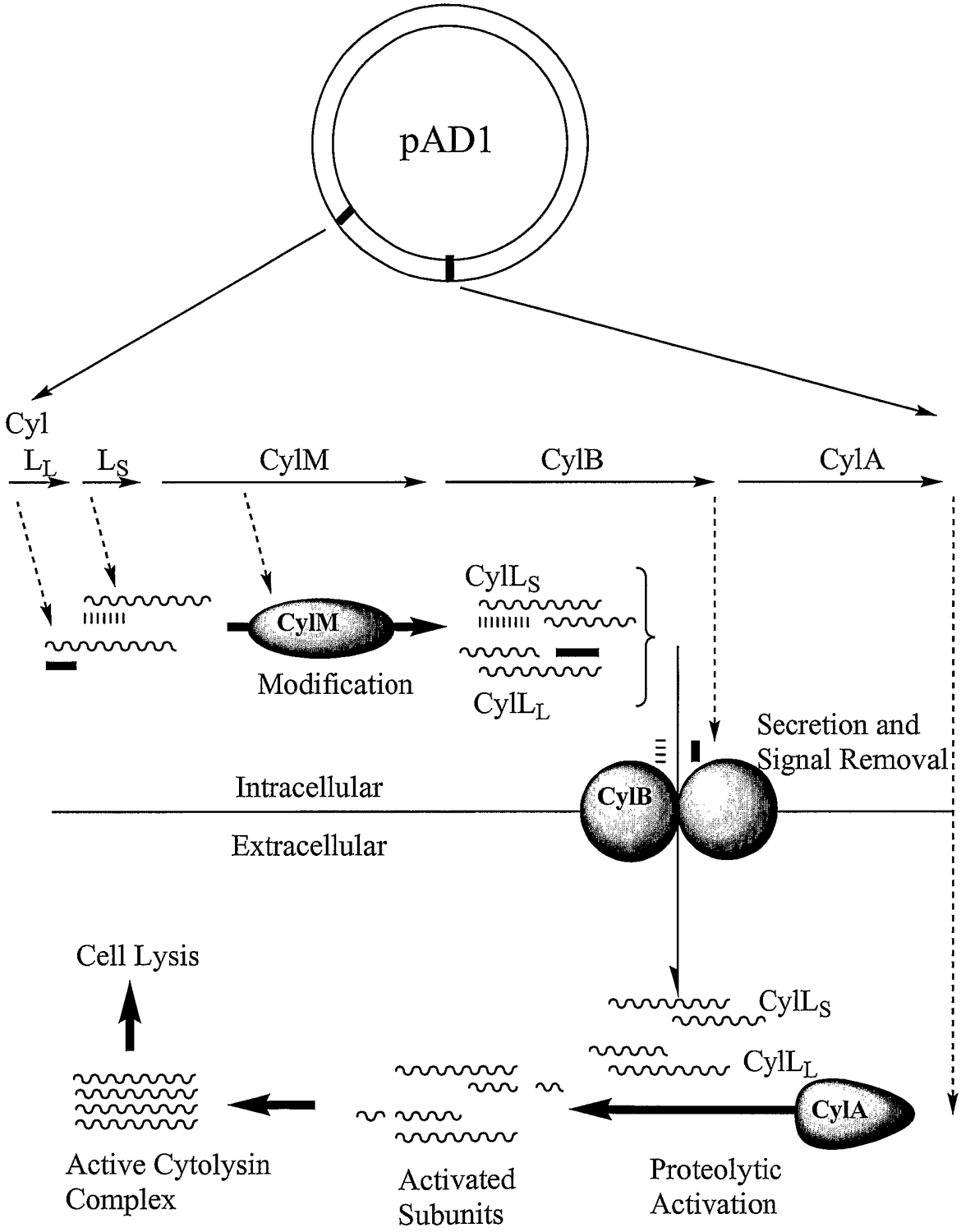

pAD1
Cyl
L_L
L_S
CylM
CylB
CylA
CylM
Modification
$CylL_S$
$CylL_L$
Secretion and
Signal Removal
Intracellular
Extracellular
CylB
Cell Lysis
$CylL_S$
$CylL_L$
CylA
Active Cytolysin
Complex
Activated
Subunits
Proteolytic
Activation

Figure 2

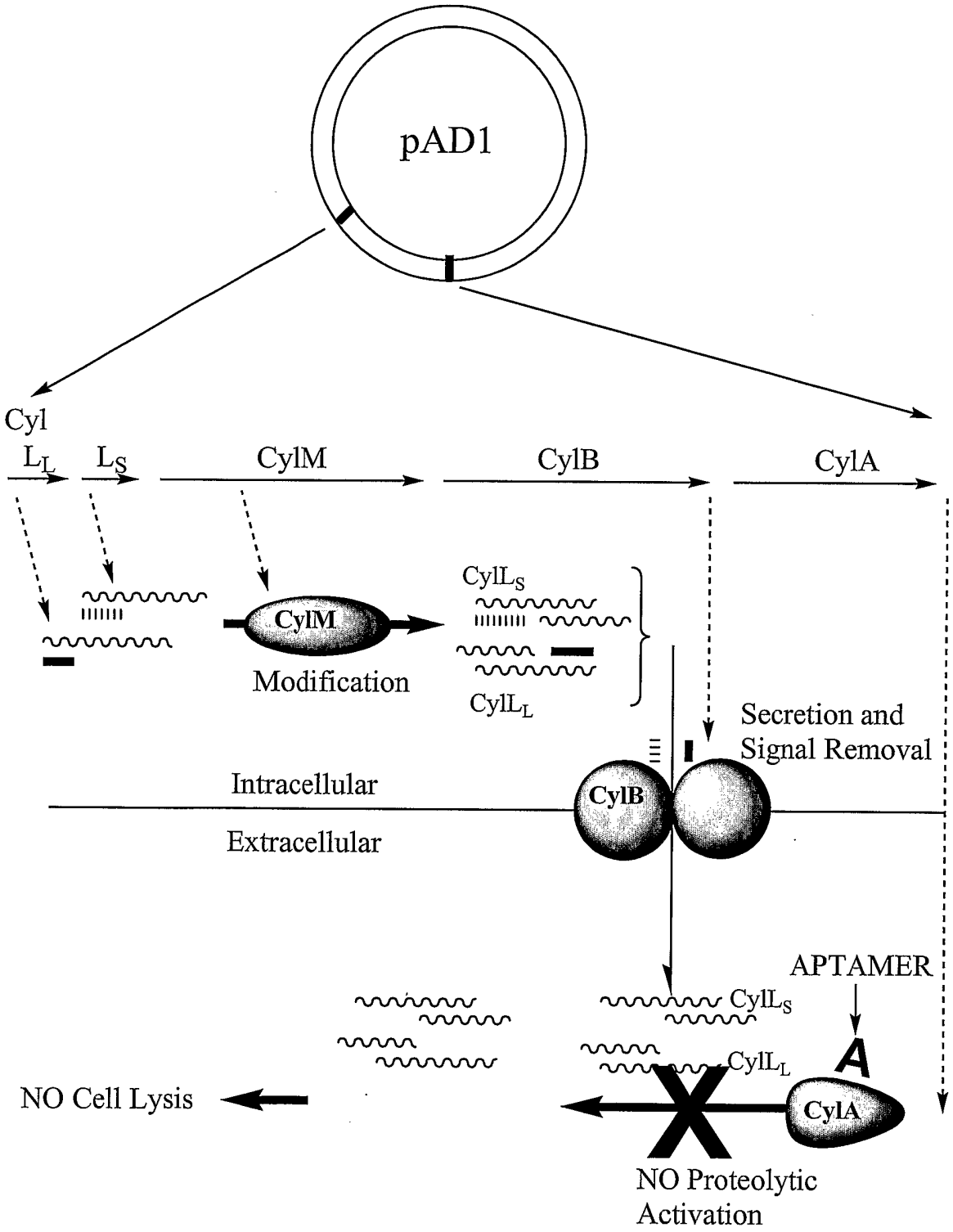
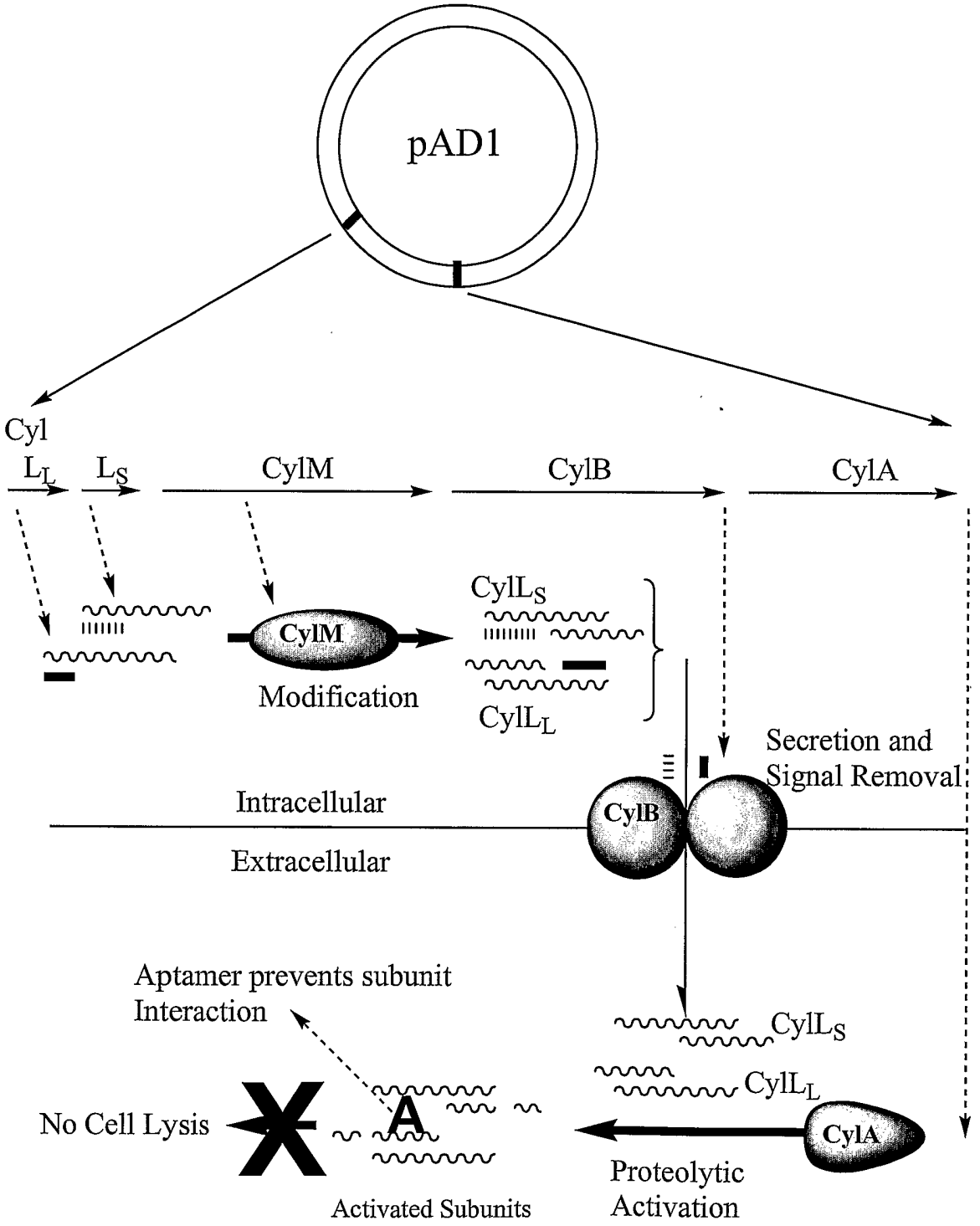

pAD1
Cyl
L_L
L_S
CylM
CylB
CylA
CylM
Modification
$CylL_S$
$CylL_L$
Secretion and
Signal Removal
Intracellular
Extracellular
CylB
APTAMER
$CylL_S$
$CylL_L$
A
CylA
NO Cell Lysis
NO Proteolytic
Activation

Figure 3


pAD1
Cyl
L_L
L_S
CylM
CylB
CylA
CylM
Modification
$CylL_S$
$CylL_L$
Secretion and
Signal Removal
Intracellular
Extracellular
CylB
Aptamer prevents subunit
Interaction
$CylL_S$
$CylL_L$
No Cell Lysis
A
CylA
Proteolytic
Activation
Activated Subunits

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/18911

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/04
US CL : 536/24.5; 514/44; 435/375

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. : 536/24.5; 514/44; 435/375

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)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Database CAPLUS on STN, AN:1992:230597, SEGARRA et al. 'Molecular characterization of the Enterococcus faecalis cytolysin activator.' Infection and Immunity. 1991, Vol. 59, No. 4, pages 1239-46, see Abstract.	1-21
A,T	US 6,617,156 B1 (DOUCETTE-STAMM et al) 9 September 2003 (09.09.2003), column 8, lines 35-42.	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search
03 November 2003 (03.11.2003)

Date of mailing of the international search report
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Form PCT/ISA/210 (second sheet) (July 1998)

Continuation of B. FIELDS SEARCHED Item 3:
CAplus, Medline, Biosis, USPATFULL, PCTFULL, Derwent, EPO, JPO, WPIDS
search terms: subtilisn, subtilisin, cytolysin, cylA, serine, protease