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(71) Applicants (*for all designated States except US*): MEDICAL UNIVERSITY OF SOUTH CAROLINA [US/US]; 171 Ashley Avenue, Charleston, SC 29425 (US). PENN STATE UNIVERSITY [US/US]; P.O. Box 850, Hershey, PA 17033 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): NORRIS, James, S. [US/US]; 1010 Casseque Province, Mt. Pleasant, SC 29464 (US). SCHMIDT, Michael, G. [US/US]; 1257 Wynnwood Court, Mt. Pleasant, SC 29464 (US). DOLAN, Joseph, W. [US/US]; 1821 Falling Creek Circle, Mt. Pleasant, SC 29464 (US). LONDON, Steven, D. [US/US]; 2075 Hopsewee Lane, Mt. Pleasant, SC 29464 (US). MAY, Harold, D. [US/US]; 729 Verone Place, Mt. Pleasant, SC 29464 (US). CLAWSON, Gary, A. [US/US]; 230 E. Granada Avenue, Hershey, PA 17033 (US).

(74) Agents: SPRATT, Gwendolyn, D. et al.; Needle & Rosenberg, 127 Peachtree Street, N.E., Atlanta, GA 30303 (US).

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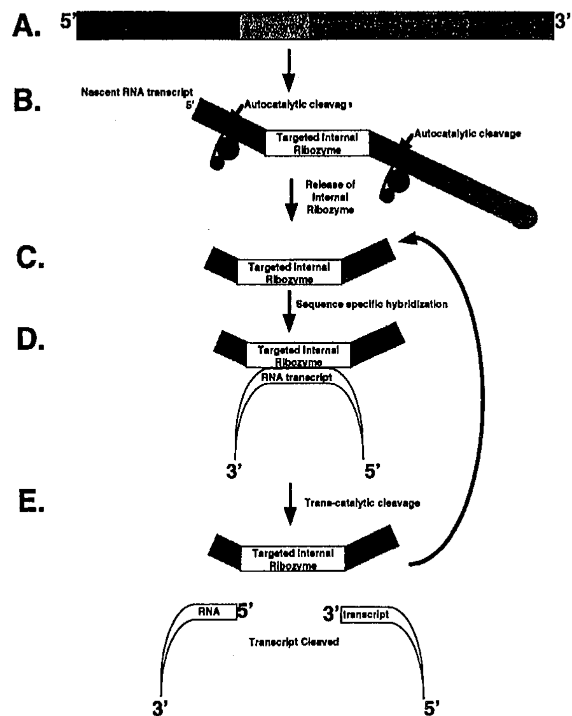
(54) Title: TISSUE SPECIFIC AND TARGET RNA–SPECIFIC RIBOZYMES AS ANTIMICROBIAL THERAPEUTICS AGAINST MICROBIAL PATHOGENS

(57) Abstract

The invention provides compositions comprising the tissue specific and target RNA–specific ribozyme(s) in either a viral delivery system or a biologic liposome preparation, wherein the viral delivery system or a biologic liposome comprises a pathogen–specific promotor upstream from a sequence encoding a triple ribozyme comprising a) a 5' autocatalytically cleaving ribozyme sequence, b) a catalytic ribozyme comprising a target RNA–specific binding site and c) a 3' autocatalytically cleaving ribozyme sequence. The invention also provides methods of treating and/or preventing bacterial infections by administering the compositions of the invention.



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"TISSUE SPECIFIC AND TARGET RNA-SPECIFIC RIBOZYMES AS ANTIMICROBIAL THERAPEUTICS AGAINST MICROBIAL PATHOGENS"

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BACKGROUND

Field of the Invention.

The invention relates to the delivery of ribozymes to microorganisms in order to treat infections. More specifically, the invention relates to the use of virions 10 to package and deliver DNA encoding ribozymes to the an infected patient. Most specifically, the invention relates to the use of target specific virions to package and deliver DNA comprising a target specific promoter and encoding a ribozyme directed to the target organisms 15 nucleic acids.

Background art.

Infectious diseases sicken or kill millions of people each year. Numerous antimicrobial therapies have been 20 designed to target one or several infectious agents. These therapies show varying degrees of success in eradicating infection. However, the failure of many of these therapies to target specific infectious agents has lead to overuse or inappropriate use of the therapies, 25 which in turn has lead to the development of drug resistant microbes. The development of drug resistance in many infectious agents has reduced the efficacy and increased the risk of using the traditional antimicrobial therapies.

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While ribozymes have been known and studied for several years, they have not been used in the treatment

of bacterial infections. There are many reasons for this. A key technical concern in the use of ribozymes as antimicrobial agents is that the ribozyme must be taken up and expressed by the targeted microbe so that the ribozyme(s) can cleave the targeted RNA(s) inside the microorganism. This concern is addressed by the present invention.

SUMMARY OF THE INVENTION

The present invention, RiboZide™, is a microbiocidal agent directed against any viral, bacterial, fungal, or other single or multicellular organism from any known taxonomic family, genus, or species, and from previously unknown, or uncharacterized organism. The present composition of matter has resulted from the development of a new process that delivers a series of ribozymes directed against fundamental and essential cellular processes specific to a targeted microorganism through an inactivated, altered, virus (virion), or abiologic delivery vehicles, capable of delivering a nucleic acid containing the ribozyme(s) into the targeted microorganism. The microorganisms maybe any virus, nonvirus, bacterium, or lower eukaryotes such as fungi, yeast, parasites, protozoa, or other eukaryotes that may be consider normal flora or pathogens of humans, animals, fish, plants or other forms of life.

The present RiboZide™ ribozyme is uniquely suited as an antimicrobial therapeutic in that upon nucleic acid hybridization with the target RNA transcript, the ribozyme-RNA complex achieves a catalytic form that acts as a nuclease to cleave the targeted RNAs. Thus, cleavage

deprives the invading microorganism of essential cellular processes which then kill or render it less fit. This approach offers new and unprecedented advances for antimicrobial therapeutics: (1) the preparation bypasses any *de novo* built-in drug resistance, which sophisticated microbes will be expected to have or develop. (2) cells are generally not capable of counteracting ribozymes delivered into them, (3) microbes have several broad RNA targets that can be attacked in simultaneously with probable synergy, (4) the custom design of the present delivery vehicle can be readily tailored to different families of organisms, (5) the modified delivery vehicle is a non-replicating, artificial construct easy to assemble and manufacture, (6) the preparation can be applied topically or it can be delivered via injection, inhalation, or ingestion, (7) the preparation can be lyophilized and thus confer stability to the antimicrobial therapeutic, (8) the inhalant, ingested or topical form of the antimicrobial therapeutic reduces the immunogenicity of the RiboZide™ preparations as opposed to its parenteral use, and (9) animal test systems exist that enable the evaluation of the RiboZide™ in a measured, incremental fashion to quickly determine the efficacy of the antimicrobial therapeutic agent. Therefore, the combination of the present unique delivery approach and an aggressive mechanism for depriving the microbial cells of essential gene products can achieve the timely defeat of microbes within the host.

The targets of the antimicrobial RiboZide™ therapeutic described herein are the RNAs of invading or normal flora microorganisms. The invention provides the

delivery of a series of ribozymes directed towards essential, housekeeping, or virulence genes of one or a series of candidate microorganisms. A ribozyme is uniquely suited as the active component of the present antimicrobial therapeutic in that it is a catalytic RNA molecule that cleaves RNA in a sequence specific manner. Therefore, the catalytically active component of RiboZide™ contains ribozymes that have been designed to inactivate RNA coding for components of the microbial cell. Inactivation of essential proteins and virulence determinants render the invading microbes inactive or slow their growth, while at the same time, the essential processes of the host are not affected.

At the molecular genetic level the coding sequence for a ribozyme or number of ribozymes may be placed under the control of one or more of the following genetic elements: a naturally occurring strong, intermediate or weak constitutively expressed or regulated promoter from the targeted microorganism, or an artificially contrived constitutively expressed or regulated promoter containing either a strong, intermediate or weak consensus sequence that delivers desired levels of ribozyme expression in the targeted microbe. This genetic information is delivered into the microbe by a either a modified virus or abiologic delivery vehicle. This present RiboZide™ is unique in that it contains sufficient genetic information for expression of the ribozyme(s) and such genetic information necessary and sufficient for its assembly and delivery to the targeted microorganism, but does not include nucleic acids native to the virus. Thus, the virion can serve as a molecular vehicle that delivers the inactivating ribozyme(s). Alternatively, an abiologic delivery system

(e.g., liposomes) can be used to package nucleic acid carrying the genetic elements necessary and sufficient for the proper expression of the ribozyme(s) into liposomes.

5 The integration of this fundamental biological information into the present antimicrobial therapeutic (RiboZide™) can be effective for treating plants, fish, animals and humans exposed to microorganisms.

10 **DESCRIPTION OF THE FIGURES**

Figure 1A shows a schematic of DNA encoding the ribozyme used in the molecular sequence of events in ribozyme maturation and action.

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Figure 1B shows the primary RNA transcript. Autocatalytic cleavage takes place upon completion of transcription.

20 Figure 1C shows the release of the trans acting ribozyme. As a direct result of cleavage of the two cis-acting ribozymes, the internal ribozyme containing a reverse and complementary sequence to the mRNA target is released.

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Figure 1D shows the sequence specific hybridization of the ribozyme. The internal or trans acting ribozyme contains a sequence that is reverse and complementary to the targeted message. The ribozyme is synthesized at a
30 concentration sufficient to locate and hybridize to all or substantially all targeted transcripts.

Figure 1E shows the trans-catalytic cleavage. Upon hybridization of the internal ribozyme to the targeted mRNA transcript, the internal ribozyme achieves a catalytic topology and cleaves the targeted message. Upon cleavage the trans acting ribozyme is released and its activity and function are recycled.

Figure 2A shows *in vitro* packaging of the RiboZide™. Purified double-stranded DNA containing a pathogen-specific promoter, ribozymes, transcriptional termination site and packaging elements is mixed with an appropriate concentration of the *in vitro* packaging extract which contains all of the necessary proteins required for the spontaneous self assembly of viral head around the nucleic acid.

Figure 2B shows the collection and purification of the intact phage from the components in Panel A.

Figure 3A shows *in vivo* packaging of the RiboZide™. Induction of the viral lysogen results in the production the packaging elements that then spontaneously self assembly into a empty virion which are then filled with the ribozyme encoding DNA.

Figure 3B shows the collection and purification of the intact virion from the components in Panel A.

Figure 4 schematic representation of the essential genetic elements necessary and sufficient for the production of an active RiboZide™ in a liposome constituting an abiologic delivery vehicle.

Figure 5 shows the present plasmid, pClip, used to generate the ribozyme encoding DNA construct.

Figure 6 shows several mutations in the ribozyme that
5 effect catalytic activity.

DESCRIPTION OF THE INVENTION

As used in the claims, "a" or "an" can mean one or more, depending upon the context in which it is used.

The present invention provides a virion comprising non-viral DNA. The term "virion" as used herein can be a bacteriophage, a mycovirus, or other virus that attacks a bacteria, fungus, yeast, parasite or virus, which can be modified to deliver ribozyme-encoding DNA. The virion can be modified to be non-replicative. Alternatively, if preferred, it can maintain the replicative ability of the native virus.

The virion of the present invention can be any bacteriophage which specifically infects a bacterial pathogen of the present invention as well as any virus which can be specifically targeted to infect the pathogen of the present invention. For example, the bacteriophage can include, but is not limited to, those specific for bacterial cells of the following genera: *Bacillus, Campylobacter, Corynebacterium, Enterobacter, Enterococcus, Escherichia, Klebsiella, Mycobacterium, Pseudomonas, Salmonella, Shigella, Staphylococcus, Streptococcus, Vibrio, Streptomyces, Yersinia* and the like (see, e.g., the American Type Culture Collection Catalogue of Bacteria and Bacteriophages, latest edition, Rockville, MD), as well as any other bacteriophages now known or later identified to specifically infect a bacterial pathogen of this invention.

In the present virion, the non-viral DNA can encode a ribozyme. In the present virion, the non-viral DNA can comprises a pathogen-specific promoter upstream from a sequence encoding a triple ribozyme comprising a) a 5′ autocatalytically cleaving ribozyme sequence, b) a catalytic ribozyme comprising a target RNA-specific binding site and c) a 3′ autocatalytically cleaving ribozyme sequence.

The present virion, wherein the non-phage DNA encodes more than one triple ribozyme is also provided. There are several options for constructing the ribozyme encoding sequences: 1) ribozymes directed to different targets in the same pathogen; and 2) multiple copies of the same ribozyme. These be combined in various ways, e.g., multiple copies of DNA encoding 4 different ribozymes in a single construct under one promoter. The promoter can have the chosen level of specificity as described herein.

The virion can contain a nucleic acid encoding at least two different triple ribozymes. The virion can contain a nucleic acid encoding more than one copy of a triple ribozyme. The virion can comprise any ribozyme-encoding nucleic acid, particularly those described herein.

A liposome is provided, comprising a nucleic acid comprising a pathogen-specific promoter upstream from a sequence encoding a triple ribozyme comprising a) a 5′ autocatalytically cleaving ribozyme sequence, b) a catalytic ribozyme comprising a target RNA-specific binding site and c) a 3′ autocatalytically cleaving ribozyme sequence.

The liposome of the invention, wherein the nucleic acid encodes more than one triple ribozyme is provided. The liposome can comprise any ribozyme-encoding nucleic acid, particularly those described herein.

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The viral and liposomal delivery systems of the invention can be used to deliver a nucleic acid comprising a pathogen-specific promoter upstream from a sequence encoding a triple ribozyme comprising a) a 5′ autocatalytically cleaving ribozyme sequence, b) a catalytic ribozyme comprising a target RNA-specific binding site and c) a 3′ autocatalytically cleaving ribozyme sequence.

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The nucleic acid delivered by the virion or liposome can encode more than one triple ribozyme. The nucleic acid can encode at least two different triple ribozymes. The nucleic acid can encode more than one copy of the same triple ribozyme. The nucleic acid can encode combinations of different ribozymes, some or all of which may be encoded in more than one copy.

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The nucleic acid, wherein at least one triple ribozyme is targeted to the *rpoA* transcript of the pathogen is provided. The nucleic acid, wherein at least one triple ribozyme is targeted to the *secA* transcript of the pathogen is provided. The nucleic acid, wherein at least one triple ribozyme is directed to the *dnaG* transcript of the pathogen is provided. The nucleic acid, wherein at least one triple ribozyme is directed to the *ftsZ* transcript of the pathogen is provided. A ribozyme-encoding nucleic acid can encode all or some of the above

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triple ribozymes. The triple ribozymes can all be under the control of a single promoter.

Many examples of the nucleic acid encoding the transacting ribozyme of the triple ribozyme are described herein and in the Sequence Listing (e.g., SEQ ID NOS:8-17).

A method of treating an infection in a subject is provided, comprising administering to the subject a virion comprising DNA comprising a target-specific promoter and encoding a ribozyme, whereby the ribozyme encoded by the non-phage DNA is expressed and the infectious agent is killed or weakened. The infection can be bacterial, fungal, yeast, parasitic, viral or non-viral.

Examples of bacterial pathogens that can be targeted by the present virion construct include, but are not limited to, species of the following genera: *Salmonella, Shigella, Chlamydia, Helicobacter, Yersinia, Bordatella, Pseudomonas, Neisseria, Vibrio, Haemophilus, Mycoplasma, Streptomyces, Treponema, Coxiella, Ehrlichia, Brucella, Pasteurella, Clostridium, Corynebacterium, Listeria, Bacillus, Erysipelothrix, Rhodococcus, Escherichia, Klebsiella, Enterobacter, Serratia, Staphylococcus, Streptococcus, Legionella, Mycobacterium, Proteus, Campylobacter, Enterococcus, Acinetobacter, Morganella, Moraxella, Citrobacter, Rickettsia, Rochlimeae* and any other bacterial species now known or later identified to be pathogenic.

The pathogen of the present invention can also include, but is not limited to pathogenic species of yeast/fungal genera (e.g., *Candida, Cryptococcus, Aspergillus, Trichophyton, Microsporum*)as well as any other yeast or fungus now known or later identified to be pathogenic. Furthermore, the pathogen of the present invention can be a parasite, including, but not limited to, members of the Apicomplexa phylum such as, for example, *Babesia, Toxoplasma, Plasmodium, Eimeria, Isospora, Atoxoplasma, Cystoisospora, Hammondia, Besniotia, Sarcocystis, Frenkelia, Haemoproteus, Leucocytozoon, Theileria, Perkinsus* and *Gregarina* spp.; *Pneumocystis carinii*; members of the Microspora phylum such as, for example, *Nosema, Enterocytozoon, Encephalitozoon, Septata, Mrazekia, Amblyospora, Ameson, Glugea, Pleistophora* and *Microsporidium* spp.; and members of the Ascetospora phylum such as, for example, *Haplosporidium* spp., as well as any other parasite now known or later identified to be pathogenic.

Examples of viral pathogens include, but are not limited to, retroviruses (human immunodeficiency viruses), herpesviruses (herpes simplex virus; Epstein Barr virus; varicella zoster virus), orthomyxoviruses (influenza), paramyxoviruses (measles virus; mumps virus; respiratory syncytial virus), picornaviruses (Coxsackie viruses; rhinoviruses), hepatitis viruses (hepatitis C), bunyaviruses (hantavirus; Rift Valley fever virus), arenaviruses (Lassa fever virus), flaviviruses (dengue fever virus; yellow fever virus; chikungunya virus), adenoviruses, birnaviruses, phleboviruses, caliciviruses, hepadnaviruses, orbiviruses, papovaviruses, poxviruses,

reoviruses, rotaviruses, rhabdoviruses, parvoviruses, alphaviruses, pestiviruses, rubiviruses, filiviruses, coronaviruses and any other virus now known or later identified to be pathogenic.

The virion construct used in this method can comprise any ribozyme-encoding nucleic acid, particularly those described herein targeted to genes of the pathogen. The virion can be a bacteriophage, or other virus selected for its ability to target a specific cell-type, microorganism or animal. The bacteriophage can be lambda, P1 or other phage. When P1 is the virion, the non-viral DNA can further comprise a PAC site is also provided. This construct is preferred when using P1. Alternatively, the virion can be selected because it has a broad range of targets.

A method of treating an infection in a subject is provided, comprising administering to the subject the liposome comprising DNA comprising a target-specific promoter and encoding a ribozyme, whereby the ribozyme encoded by the DNA is expressed and the infectious agent is killed or weakened. The liposome used in this method can comprise any ribozyme-encoding nucleic acid, particularly those described herein targeted to genes of the pathogen. The infection can be bacterial, fungal, yeast, parasitic, viral or non-viral.

A method of delivering a ribozyme to a target (e.g., a pathogen) in a subject is provided, comprising a) generating a virion comprising DNA having a promoter specific for the target (e.g., the pathogen) and encoding the ribozyme; and b) delivering the virion to the subject,

whereby the pathogen-specific promoter directs transcription of the ribozyme in the cells of the pathogen, thereby targeting the ribozyme to the pathogen. The target can be a pathogen, for example, a bacteria,
5 fungus, yeast, parasite, virus or non-viral pathogen.

The above targeting method, wherein the virion is a bacteriophage is provided. The bacteriophage can be lambda, P1 or other phage. The targeting method, wherein
10 the non-viral DNA further comprises a PAC site is also provided. This construct is preferred when using P1.

A method of delivering a ribozyme to a target (e.g., a pathogen) in a subject is provided, comprising a)
15 generating a liposome comprising a promoter and ribozyme encoding sequence; and b) delivering the liposome to the subject, whereby the target-specific promoter directs transcription of the ribozyme in the cells of the target. The target can be a pathogen, for example, a bacteria,
20 fungus, yeast, parasite, virus or non-viral pathogen.

A method of targeted delivery of a ribozyme to a pathogen in a subject, comprising a) generating a virion comprising non-viral DNA of the invention; b) combining it
25 with a liposome; and b) delivering the liposome containing the virion to the subject, whereby liposome enters the eukaryotic cell and releases the virion, which delivers the DNA to the pathogen, whereby the pathogen-specific promoter directs transcription of the ribozyme in the
30 cells of the pathogen.

Ribozymes are catalytic RNA molecules that cleave RNA in a sequence specific manner (3, 5, 24). With the

invention of the present delivery system and construct, they are uniquely suited as antimicrobial compounds in that they possess an intrinsic enzymatic activity under which the ribozyme can act as a nuclease against itself (*cis* acting) or against a different RNA targets (*trans* acting). Catalytic activity of the trans acting ribozyme requires hybridization between the ribozyme and its target at which a catalytic topology is achieved and cleavage occurs. Consequently when three hammerhead ribozymes are assembled in a sequence where the first (5′) and last (3′) ribozyme function as *cis* acting elements, the endonuclease activity of these 5′ and 3′ ribozymes releases the internal ribozyme whose endonuclease specificity has been targeted against an essential RNA molecule of the invading microorganism (Figure 1). Thus, the internal ribozyme if this triple ribozyme functions in *trans* and renders the invading microorganism's RNA(s) inactive through the endonuclease cleavage of the molecule thus destroying the function of the targeted RNA molecule. Expression of the *trans* acting ribozyme can be achieved by placing the coding sequence of the three hammerhead ribozymes under the control of one or more of the following genetic elements: a naturally occurring strong, intermediate or weak constitutively expressed or regulated promoter from the targeted microorganism, or an artificially contrived constitutively expressed or regulated promoter containing either a strong, intermediate or weak consensus sequence that delivers desired levels of ribozyme expression in the targeted microbe. Once delivered and transcribed the *trans* acting ribozyme is lethal against the targeted microorganism for it is synthesized at a concentration sufficient to locate and hybridize to all or substantially

all of the targeted transcripts. Once hybridization of the internal ribozyme has occurred with the targeted mRNA transcript the internal ribozyme achieves a catalytic topology and cleaves the targeted RNA. Following cleavage 5 the ribozyme is available to repeat the process.

Target Selection

The first critical component in the assembly of the RiboZide™ is the selection of appropriate RNA targets. 10 For ribozymes to be effective anti-microbial therapy, it is preferable to target the RNA of, for example, several key proteins, tRNA, rRNA or any other RNA molecule essential for cell viability or fitness, in order to insure complete inactivation and prevent escape of the 15 invading microorganism. For example, four bacterial genes, essential for viability and unrelated in activity, have been selected and are described herein to highlight how the selection of appropriate mRNA targets is carried out for the preferred construction of the RiboZide™ 20 against prokaryotic targets. Cross-genera RNA targets can be used to design a RiboZide™ that can have broad application, modified by the specificity of the promoter.

In one embodiment of the invention, the first 25 ribozyme targets an essential transcription factor, the second ribozyme targets an essential general secretory component, the third ribozyme targets an essential component of the primosome required for DNA biosynthesis and the fourth ribozyme targets an enzyme required for 30 cell division. Consequently, the ribozymes are redundant in the fact that they inhibit growth by specifically targeting a fundamental process required for bacterial

growth. Thus, this can minimize the development of resistance to the antimicrobial therapeutic.

The first gene, *rpoA*, produces an essential protein, RpoA or the alpha subunit of RNA core polymerase. RpoA was selected rather than the other components of the RNA polymerase holoenzyme, because it is thought to facilitate the assembly of an active RNA Polymerase enzyme complex. Inactivation of the *rpoA* transcript results in a decrease in the intracellular concentration of the holoenzyme RNA polymerase rendering the cell less able to respond to changes demanded of it once it has invaded a new host. The nucleotide sequence of *rpoA* is known for a large number of microorganisms (>20 genera) and they are readily available from GenBank.

The second ribozyme target can be the mRNA of the *secA* gene from bacteria. The product of this gene is the essential and rate-limiting component of the general secretory pathway in bacteria (2). *SecA* has been found in every prokaryotic cell investigated to date. Additionally, its biosynthesis is translationally coupled to the upstream gene, X (17), presenting a convenient target for a ribozyme. Inhibition or decreased synthesis of *secA* is also sufficient to confer a reduction in viability to the cell (18). Furthermore, as a pathogen responds to changes required of the infectious process a change in the availability of a key protein such as *SecA* will disadvantage the pathogen enabling the host to counteract it. Finally, control over the secretion-responsive expression of *SecA* is at the level of translation (5), and the regulatory sequences within its

polycistronic message have been localized to a region comprised of the end of the upstream gene, X, and the beginning of *secA*. Consequently, inactivation of the transcript by the catalytic cleavage of a ribozyme has profound consequences for the viability of the invading microorganism.

The third ribozyme can target an essential factor for DNA biosynthesis, *DnaG*. Every 1 to 2 seconds, at least 1,000 times for each replication fork within *E. coli*, priming of an Okazaki fragment is repeated as a result of an interaction between the cellular primase *DnaG* (4) and *DnaB* (11). As would be expected of protein required every 1 to 2 seconds during replication, a lesion within *dnaG* or an alteration in its concentration results in an immediate stop phenotype (11, 28). Therefore, inactivation of the *dnaG* message by a ribozyme should have profound cellular consequences in that general priming of the lagging strand is reduced if not eliminated. DnaG is a component of the primosome, a multi-protein complex responsible for priming replication. Any of the components of the primosome, either individually or in any combination, can serve as a target for inactivation of the primosome and, thus, kill the cell. The other components of the primosome are DnaB, DnaC, DnaT, PriA, PriB, and PriC. Thus, the primosome is also sufficiently complex to provide numerous other targets (DnaB, DnaC, DnaT, PriA, PriB and *PriC*) for inactivation by the trans ribozyme.

The fourth target can be *ftsZ*. This gene also encodes an essential protein, FtsZ, that is required for cell division in that it is responsible for the initiation of

separation. FtsZ was selected because its synthesis was under the control of an antisense RNA molecule encoded by the gene *dicF*. Transcription of *dicF* is all that is needed to inhibit the translation of *ftsZ*; thus, overexpression of this antisense molecule is sufficient to cause an inhibition of cell division and a reduction in viability. There is an advantage of using a ribozyme against *ftsZ* over the antisense molecule, *dicF*. Specifically, the ribozyme functions catalytically while *dicF* functions stoichiometrically. Thus, upon cleavage of the *ftsZ* message the ribozyme attacks additional copies of *ftsZ* inhibiting the division of the cell. The nucleotide sequence of *ftsZ* like the other targets selected, is commonly available from GenBank.

It should be clear that any other essential protein of a pathogen can have its message targeted in the present invention, and that determining which proteins are essential can be routinely determined according to standard protocols in the art. In fact, there are over 52,000 viral, 41,000 bacterial and 12,300 fungal sequences deposited in the public section of the Entrez Database at the National Center for Biotechnology Information. Any of these can be used to design the catalytic trans ribozyme of the RiboZide. Thus, RiboZide™ can comprise ribozymes targeted to these other messages.

In addition to targeting mRNA of essential proteins ribozymes may be targeted against other RNA species within the cell. Specifically, appropriate targets in bacteria, fungi and other lower eukarytoes include ribosomal RNA such as Small Subunit RNAs (SSU) or Large Subunit (LSU)

and tRNA molecules required for protein synthesis. As long as the RNA targeted contains a canonical ribozyme cleavage domain the RiboZide™ therapeutic can hybridize and cleave the RNA complimentary RNA and impact on the fitness of the microbial cell. Additionally, over 3000 rRNA species have been sequenced and aligned. This information is available from the Ribosomal Database Project and should facilitate rapid design and adaptation of ribozyme(s) against such targets. For example the 16S rRNA molecule of bacteria is especially attractive in that there are over 4000 copies of the 16S rRNA per cell. Consequently, a reduction in number slows the process of protein synthesis in so far as the 16S rRNA molecule is involved in the process of translational initiation. Thus, a RiboZide™ containing ribozymes directed against mRNA and rRNA impacts the fitness of the offending microorganism.

Promoter Selection

Promoter selection is accomplished using techniques that are available in the art. For example a method is described in the Examples that permits the selection of both controlled and uncontrolled promoters, as well as consensus promoters that can be design for application in the present RiboZide™. The promoter can be a naturally occurring strong, intermediate or weak constitutively expressed or regulated promoter from the targeted microorganism, or an artificially contrived constitutively expressed or regulated promoter containing either a strong, intermediate or weak consensus sequence that delivers desired levels of ribozyme expression in the targeted microbe.

Ribozyme Design

The RiboZide™ ribozyme possesses sufficient catalytic activity to inactivate the RNA of the targeted transcripts. From an antimicrobial perspective, hammerhead-type ribozymes are especially attractive since the molecule inactivates gene expression catalytically through the cleavage of the phosphodiester bond of the mRNA. Furthermore, hammerhead-type ribozymes have been re-engineered to function in an intermolecular or transducer (trans) acting state (6, 27). The catalytic activity of the ribozyme requires a sufficient concentration of the divalent cation, Mg^{+2}, and substrate. The substrate can have any sequence as long as the cleavages site contains the recognition element NUX, where N represents any nucleotide, U corresponds to uracil, and X is any nucleotide except G (8). Ribozymes have been widely demonstrated to function *in vivo* (5,7). The present invention improves the initial design of hammerhead-type ribozymes (25) by constructing a cassette containing two *cis* acting hammerhead ribozymes flanking a ribozyme that inactivates the targeted RNA. Upon transcription the targeted ribozyme is released as a 60-70 base transcript which not only improves its specificity by reducing non-specific interactions but also improves its catalytic activity as well.

This invention includes modifications to the ribozyme described in U.S. Serial No. 08/554,369. The arms of the cis acting ribozymes have been lengthened by 20 bases. The sequence has been modified to enhance the catalytic activity of the cis-acting elements, for example those shown in SEQ ID NOS:18-38. Additional restriction sites

are included that facilitate easier cloning and manufacturing. tRNA elements are present in to the 3′ end of the RiboZide™. The addition of the tRNA elements creates additional structure that improves the stability of RiboZide™ helping it resist nuclease attack. An inverted nucleotide repeat has been inserted into the 3' end of the RiboZide¬. The addition of the inverted repeat, a hairpin loop structure, improves the stability of RiboZide¬ helping it resist nuclease attack.

Assembly of the ribozyme(s) into the RiboZide™

Until the present invention, the therapeutic use of ribozymes in eukaryotes was limited because a convenient and efficient delivery system has not been available. A key to the present invention is the strategies used to deliver the ribozyme to the targeted microorganism. Two separate classes of delivery systems can be manufactured, one biologic in nature and the other abiologic.

The key features of the present invention are the combination of ribozyme with viral delivery and assembly of the virions using a unique combination of plasmid features.

Abiologic Delivery Vehicles

Abiologic delivery of the ribozyme is accomplished by packaging plasmid DNA carrying the gene(s) that codes for the ribozyme(s) into liposomes (Fig 4.). The liposome is be composed of cationic and neutral lipids commonly used to transfect cells *in vitro*. The cationic lipids complex with the plasmid DNA and form liposomes.

The RiboZide that is administered to a subject can further comprise a liposome. Cationic and neutral liposomes are contemplated by this invention. Cationic liposomes can be complexed with the a negatively-charged biologically active molecule (e.g., DNA) by mixing these components and allowing them to charge-associate. Cationic liposomes are particularly useful when the biologically active molecule is a nucleic acid because of the nucleic acids negative charge. Examples of cationic liposomes include lipofectin, lipofectamine, lipofectace and DOTAP (30-32). Procedures for forming cationic liposomes encasing substances are standard in the art (33) and can readily be utilized herein by one of ordinary skill in the art to encase the complex of this invention.

The liposomes may be incorporated into a topical ointment for application or delivered in other forms, such as a solution which can be injected into an abscess or delivered systemically.

Plasmid DNA coding for the ribozymes is used rather than preformed ribozymes for the following reasons. Plasmid DNA allows the targeted cells to produce the ribozyme and, thus, results in a higher delivered dose to the cell than can be expected by delivery of ribozyme RNA via liposome. The DNA also provides specificity of action based on target sequence specificity. The liposomes deliver their DNA to any cell in the area of administration, including cells of the host. The promoter driving the transcription of the ribozyme is specific for the targeted microorganism and, thus, will be inactive in other cell types. Therefore, liposomal delivery of DNA

coding for the ribozyme provides amplification and specificity.

Biologic Delivery Vehicles

5 Not all microorganisms are expected to take up DNA delivered by liposome. Consequently, a biologic delivery system is also required. The biologic delivery vehicle of the RiboZide™ takes advantage of the fact that generalized transducing particles completely lack DNA originating from 10 the viral vector. Instead such particles only contain sequences of host origin. Consequently, the invention uses a biologic assembly of viral head proteins (packaging elements for the antimicrobial therapeutic) around the nucleic acid containing the necessary genetic elements 15 that will insure the desired level of expression of the ribozyme(s)(Figures 2, 3 and 4).

This delivery system consists of a DNA plasmid carrying the gene(s) coding for the ribozyme(s) packaged 20 into viral particles. Specificity is conferred by the promoter driving transcription of the ribozymes and by the host specificity of the viral vehicle. The following description is one example of the system using bacteriophage lambda virions to package DNA carrying 25 ribozymes directed against *Escherichia coli*. Similar strategies are used to generate RiboZides™ capable of delivering ribozymes directed against other microorganisms. The virions used to package the DNA can be species specific, such as the virion derived from the 30 bacteriophage lambda coat, or they can possess a broader host range, such as virion derived from bacteriophage P1. Broad host-range viruses facilitate production of the

anti-microbial agents without the loss of species specificity because species-specific promoters are used to direct the transcription of the ribozymes which are directed against species specific targeted RNA sequences.

One example of construction the present RibZide entails the use of a plasmid carrying the ribozyme gene(s), a plasmid origin of replication, a selectable marker for plasmid maintenance, the minimal lambda origin of replication, and cos sites, which are required for packaging of DNA into lambda virions. This plasmid is maintained in a lambda lysogen that is defective in integration/excision and recombination functions. The defective lysogen provides all of the replication factors needed to activate the lambda origin of replication on the plasmid and all of the structural components needed to form mature virions; however, the lysogen is not able to replicate and package its own DNA into the virions. The lysogen also carries the cI^{857} temperature-sensitive repressor mutation. Induction of the lysogen is described in the Examples. A similar strategy can be used to generate ribozyme-encoding plasmids packaged into bacteriophage P1 virions.

A common bacteriophage of *E. coli*, P1, is an attractive delivery vehicle for the invention, RiboZide for a number of reasons. First and foremost, P1 has a broad intergenera and interspecies range (29). The P1 receptor of *E. coli* is the terminal glucose of the lipopolysaccharide (LPS) core lysergic ring of the bacterial outer membrane (12). Yarmolinsky and Sternberg report that in addition to *E. coli*, this particular phage

has the ability to inject its nucleic acid into a large number (>25) of diverse gram negative bacteria (29). Secondly, P1 can accommodate a significant amount of genetic information, over 2% (100,000 bp) of the DNA of *E. coli* (12). Consequently, gene dosage of the ribozymes can be increased through multiplication of the present ribozyme cassettes, thereby increasing the microbiocidal activity of the RiboZide™. Bacterial strains already exist that can be readily modified to package ribozyme coding DNA *in vivo* by a process similar to that described above. Additionally, a process utilizing i*n vitro* packaging is also possible. *In vitro* packaging can be accomplished through the addition of PAC-sites to the genetic information already present within the ribozyme construct. P1 packaging initiates within one of the P1 PAC genes (23). It has been reported that the active PAC site is contained within a 161 base-pair segment of the P1 EcoR1 fragment 20 (23). Thus, the phage head serves as a molecular syringe that delivers the inactivating ribozyme(s) to the pathogen.

Protection of RiboZide-producing cells

The genes coding for the ribozymes can be toxic to the cells that are needed to produce the ribozyme-carrying virions. When using a broad host-range virus like P1, the organism used to produce the RiboZide™ can be different from the target organism. In this way, the producing strain is resistant to the toxic effects of the ribozymes because the ribozymes are not efficiently expressed in the producing strain, due to species-specific promoter elements, and the ribozymes will not have any target RNA molecules to attack, due to the species-specific sequences

that target the ribozymes. When using a species-specific virus that must be expressed and assembled within a strain of the targeted microorganism, this toxicity becomes a significant concern. The assembly of a RiboZide™ consisting of anti-*E. coli* ribozyme genes packaged in lambda will illustrate the approach used to circumvent the toxicity. The ribozymes directed against RNA species of *E. coli* is expressed from a artificial promoter containing consensus promoter elements. This promoter provides high level transcription of the ribozyme immediately upon infection of targeted cells. In order to prevent the unwanted death of the producing strain of *E. coli*, transcription is repressed in the producing strain by a mechanism not available to the wildtype strains that are targeted for killing. Sequences constituting the DNA binding sites for a heterologous transcription factor are interspersed between the essential activating elements of the ribozyme promoter. Expression of the heterologous transcription factor in the producing strain results in the occlusion of the activating promoter elements and preventing the binding of RNA polymerase. As an example, the gene for the *Saccharomyces cerevisiae* transcription factor Ste12p may be expressed in *E. coli* and bind to its binding sites, the pheromone response element, located within the ribozyme promoter. Ste12p will not be found in wild strains of *E. coli*; therefore, the ribozyme promoter will be accessible to RNA polymerase following delivery of the plasmid to the targeted cells.

An alternative strategy that can protect the producing strain from the toxicity of the ribozymes employs ribozyme-resistant versions of the targeted RNA

molecules. This strategy can be used when the target RNA molecule codes for a protein. The ribozyme target site within the mRNA molecule is mutated by site-directed mutagenesis such that the amino acid sequence of the translated protein does not change but the mRNA sequence no longer serves as a substrate for the ribozyme. For example, hammerhead ribozymes require an NUX sequence within the target mRNA for cleavage to occur. By changing this sequence to something else, the ribozyme will not cleave the mRNA. This type of ribozyme resistant version of the target RNA can be expressed from a plasmid or integrated into the chromosome of the producing strain and thus render this strain resistant to the toxic effects of the ribozyme.

Merrill and co-workers recently reported on the selection of long-circulating bacteriophages as anti-bacterial agents (13). They were able to show that it is possible to select for phage variants that remain refractory to clearance by the reticuloendothelial system (RESONANT) for a period of time sufficient to confer a therapeutic response within an infected animal (13). Specifically, the phage will promptly induce neutralizing antibodies interfering with the phage's ability to attack against the bacteria and opsonins that will restore the vulnerability of the phage to the RESONANT (10). The improvement in the present invention is that a non-replicative delivery system has an advantage in that once the phage coat has injected the nucleic acid into the targeted bacterium, the expression of the RiboZide™ ribozyme will destroy the microbe, as opposed to a lytic infection cycle typical of an intact bacteriophage. Consequently, amplification of the phage coat will not be

an issue and it is less likely that the non-replicative phage delivery system will generate an immune response such that subsequent use of the delivery system would be jeopardized. Moreover, if the patient has been exposed to a resistant pathogenic microbe and the RiboZide™ is effective and neutralizes the invading microbe, then it is expected that the microbial antigens liberated as a result of the action of the RiboZide™, will illicit sufficient humoral immunity and cell-mediated immunity to confer protection against subsequent attacks.

Administration

Parenteral administration, if used, is generally characterized by injection (intravenous, intradermal, subcutaneous and intramuscular). Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution of suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves use of a slow release or sustained release system such that a constant level of dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which is incorporated by reference herein.

Suitable carriers for parenteral administration of the substance in a sterile solution or suspension can include sterile saline that can contain additives, such as ethyl oleate or isopropyl myristate, and can be injected, for example, intravenously, as well as into subcutaneous or intramuscular tissues.

Topical administration can be by creams, gels, suppositories and the like. *Ex vivo* (extracorporeal) delivery can be as typically used in other contexts. Oral administration is also contemplated.

Suitable carriers for oral administration include one or more substances which can also act as flavoring agents, lubricants, suspending agents, or as protectants. Suitable solid carriers include calcium phosphate, calcium carbonate, magnesium stearate, sugars, starch, gelatin, cellulose, carboxypolymethylene, or cyclodextrans. Suitable liquid carriers can be water, pyrogen free saline, pharmaceutically accepted oils, or a mixture of any of these. The liquid can also contain other suitable pharmaceutical additions such as buffers, preservatives, flavoring agents, viscosity or osmo-regulators, stabilizers or suspending agents. Examples of suitable liquid carriers include water with or without various additives, including carboxypolymethylene as a pH-regulated gel.

The RiboZide™ can be administered to the subject in amounts sufficient to produce an antibiotic effect or to inhibit or reduce the activity of the target pathogen. Optimal dosages used will vary according to the individual, on the basis of age, size, weight, condition, etc, as well as the particular modulating effect being induced. One skilled in the art will realize that dosages are best optimized by the practicing physician and methods for determining dosage are described, for example, in *Remington's Pharmaceutical Sciences* [Martin, E.W. (ed.) *Remington's Pharmaceutical Sciences*, latest edition Mack

Publishing Co., Easton, PA.]. Treatment can be at intervals and can be continued for an indefinite period of time, as indicated by monitoring of the signs, symptoms and clinical parameters associated with a particular
5 infection. The parameters associated with infection are well known for many pathogens and can be routinely assessed during the course treatment.

EXAMPLES

10

Promoter Selection

Promoters specific for the target (e.g., a specific pathogen, genus, etc.) in question can be selected by screening genomic sequences for the ability to activate a
15 promoterless reporter gene. The promoterless reporter gene is based on the strategy developed for use with plasmid pMC1871 (Casadaban MJ et al Meth. Enzymol. 100, 293 (1983)). For non-viral pathogens, plasmid capable of stable replication and maintenance in the microorganism
20 understudy is modified by standard molecular biology techniques to carry the coding region of a reporter gene (Sambrook et al. latest eddition). The reporter gene can be any of a number of standard reporter genes including but not limited to the lacZ gene of E. coli, which codes
25 for β-galactosidase. Total genomic DNA is isolated from cells of the pathogen, cleaved with restriction endonucleases to yield fragments of a few hundred basepairs on average. These fragments are then ligated into a unique restriction endonuclease cleavage site at
30 the 5' end of the reporter gene coding region, creating a library of plasmids. The library is then transformed into the pathogen by standard techniques and the resulting

transformants are screened for expression of the reporter gene. In the case of lacZ, the transformants can be plated onto medium containing the chromogenic °-galactosidase substrate X-Gal (5-bromo-4-chloro-3-indolyl-°-D-galactoside). Transformants that contain a plasmid with an insert carrying a promoter will express β-galactosidase and will turn blue on X-Gal plates. The intensity of the blue color is relative to the level of expression; promoters of different strength can be selected based on the intensity of the blue color.

The above-described screening procedure can be modified to identify regulated promoters. For example, promoters that are regulated by carbon source availability can be screened on plates that contain different carbon sources. Other modifications are possible and will depend, in part, on the organism in question. To test for species-specificity, the identified promoters are transferred to promoterless reporter plasmids capable of replication and maintenance in a different organism. Truly species-specific promoters will not activate the expression of the reporter gene in any other species. Obvious modifications can be used to identify and test artificial promoters composed of synthetic oligonucleotides inserted into the promoterless reporter plasmid.

Biologic Delivery.

The present example entails the use of a plasmid carrying the ribozyme gene(s), a plasmid origin of replication, a selectable marker for plasmid maintenance,

the minimal lambda origin of replication, and cos sites, which are required for packaging of DNA into lambda virions. This plasmid is maintained in a lambda lysogen that is defective in integration/excision and recombination functions. The defective lysogen provides all of the replication factors needed to activate the lambda origin of replication on the plasmid and all of the structural components needed to form mature virions; however, the lysogen is not able to replicate and package its own DNA into the virions. The lysogen also carries the cI^{857} temperature-sensitive repressor mutation. Induction of the lysogen by temperature shift to 42°C or by other means, such as exposure to $5J/m^2$ of ultraviolet radiation will mobilize the plasmid and result in its replication and packaging into lambda virions (Fig. 3). The virions can then be harvested, purified free of *E. coli proteins* and be used to deliver the ribozyme gene(s) to *E. coli*.

Abiologic Delivery.

Abiologic delivery of the RiboZide¬ is accomplished with ribozyme(s) constructs that have been engineered to be expressed within the targeted tissue. Briefly, the genetic element containing the promoter and ribozyme(s) are complexed with cationic liposomes (Lipofectamine--Gibco BRL) in a 1:10 ratio and are introduced into test animals by either single or multiple injection of 0.2 ml total volume nucleic acid-liposome mixture.

In Vivo Testing

Following the demonstration that RiboZides¬ have an in vitro biological activity (either directly on bacterial cultures or in an infectious tissue culture cell assay system), the effectiveness of the RiboZides¬ is shown in an in vivo model system. To demonstrate the efficacy of RiboZides¬ in vivo, experimental animal model systems are utilized. For an initial demonstration of the efficacy of the RiboZides¬ in vivo, mice are infected with a microbial pathogen which has previously been shown to be sensitive to the RiboZide¬ construct(s) and the effect of RiboZides administered in vivo is determined. In the first series of in vivo trials, one determines the effectiveness of RiboZides¬ at preventing an acute infection in a murine model system when the RiboZide¬ is added directly to the microbe prior to administration in vivo.

The next series of trials determine whether the administration of RiboZides¬ after infection is effective at preventing an acute bacterial infections. In addition to the clinical status of infected mice, tissues obtained at necropsy are examined histologically and the presence of replicating microorganism in tissue samples is determined by standard methodology. Animals can be infected by various routes (systemic and/or mucosal) and the RiboZides¬ delivered over time after infection by systemic and/or mucosal routes. Both abiologic as well as biological delivery of RiboZides¬ is used. The demonstration of a positive effect of the RiboZides¬ in controlled experimental model system provides compelling evidence for the efficacy of the preparation and

determines whether or not the preparation warrants evaluation under conditions of standard clinical trials.

Development and Testing of the Catalytic Component of the RiboZideJ

The following is a routine approach for designing, manufacturing and testing of the ribozymes that are incorporated into the RiboZideJ invention.

The catalytic component of RiboZideJ invention is/are transacting internal targeted ribozyme(s)(ITRz). To facilitate construction of this critical and catalytic component, the targeted triple ribozyme (TRz) containing a double ribozyme cassette was developed: This artifically contrived genetic element consists of autocatalytic, self-cleaving 5' and 3' ribozymes (Figure 5), with a cloning region (denoted by the box entitled *Targeted Ribozyme)* between them. This double ribozyme cassette is then placed within a series of expression vectors that were either constructed (pClip), purchased from commercial vendors (pBluescriptII, Stratagene; pCRII, InVitrogen; pET-30a-c; pBACsurf-1, pIE1 and pIE4, Novagen) and used intact or modified as necessary to confer the desired activity within the RiboZideJ. pClip (the genetic element described in Figure 5) is a modification of pBluescript, wherein the cassett shown is dropped into the Not I site in pBluescript. The targeted ribozyme (transacting catalytic ribozyme) is dropped into the Bgl II site (TGCTCT).

An internal targeted ribozyme (ITRz) is synthesized as reverse complementary overlapping oligodeoxynucleotides, which are designed in such a way that when annealed they form single stranded ends

identical to those produced by digestion with the restriction endonuclease contained with the region between the two cis-acting ribozymes. In this particular example the restriction endonuclease recognition site is that recognized by Bgl II. Essentially any RNA can be targeted: specificity is conferred by selecting sequences for the ribozyme that are reverse and complementary to sequences flanking the chosen cleavage site in the targeted RNA molecule. These internal targeted ribozymes are then cloned into the cloning region within the double ribozyme cassette (Figure 1) to produce the targeted ribozyme. Internal targeted ribozymes to prokaryotic sequences have been constructed including, but not limited to, *Escherichia coli*: *secA* (EcoSecA, AE000119 U00096), gene X (EcoSecA, AE000119 U00096) *ftsZ* (AE000119;U00096), *dnaG* (AE000388 U00096), *rpoA*(AE000407 U00096) and tRNA-asp (X14007), *Streptomyces lividins secA* (Z50195), *Enterococcus faecalis, ftsZ* (U94707), *Pseudomonas putida*, *dnaG* (U85774), *Streptomyces coelicolor rpoA* (X92107), *Staphylococcus warneri* tRNA-Asp (X66089 S42075).

SEQ ID NOS:1-7 are examples of the transacting ribosyme sequences.

The following sequences are for ribozymes directed against the targets described. The naming system refers to the target cytosine in the GUC motif. It is the nucleotides number from the referenced sequence (accession number indicated). Ribozymes directed against secA targets have restriction sites for Bgl II on both ends. All other inserts have Bgl II (5 end) and Sty I (3 end) restriction sites for use in the new vector. Antisense arms are boldfaced.

Escherichia coli

ftsZ target (ACCESSION: AE000119 U00096)

105 AGATCTAAA**CGCCGAT**CTGATGAGTCCGTGAGGACGAA**ACTTTAA**AAACCAAGG

713 AGATCTAAA**CATCTCA**CTGATGAGTCCGTGAGGACGAA**ACATTACG**AAACCAAGG

5 1131 AGATCTAAA**TCATTCAC**CTGATGAGTCCGTGAGGACGAA**ACTTTAGC**AAACCAAGG (SEQ ID NO:8)

secA target (ACCESSION: AE000119 U00096)

84 AGATCTAAA**AAAAAAC**CTGATGAGTCCGTGAGGACGAA**ACTGGTT**AAAAGATCT

10 707 AGATCTAAA**TTATCCA**CTGATGAGTCCGTGAGGACGAA**ACGGGCG**AAAAGATCT

856 AGATCTAAA**TCGTTAC**CTGATGAGTCCGTGAGGACGAA**ACTACCG**AAAAGATCT

894 AGATCTAAA**TGATGTT**CTGATGAGTCCGTGAGGACGAA**ACCACTT**AAAAGATCT

979 AGATCTAAA**TTTTCCA**CTGATGAGTCCGTGAGGACGAA**ACGTGC**AAAAAGATCT

1282 AGATCTAAA**TTGATACC**CTGATGAGTCCGTGAGGACGAA**ACAGTCAG**AAAAGATCT

15 2216 AGATCTAAA**TTCGTTT**CTGATGAGTCCGTGAGGACGAA**ACACCAC**AAAAGATCT (SEQ ID NO:9)

dnaG target (ACCESSION: AE000388 U00096)

5344 AGATCTAAA**CGTTAGT**CTGATGAGTCCGTGAGGACGAA**ACCAACA**AAACCAAGG

20 5903 AGATCTAAA**GGCATCA**CTGATGAGTCCGTGAGGACGAA**ACTGTTA**AAACCAAGG

6336 AGATCTAAA**CCACATC**CTGATGAGTCCGTGAGGACGAA**ACAGTTT**AAACCAAGG (SEQ ID NO:10)

rpoA target (ACCESSION: AE000407 U00096)

25 8308 AGATCTAAA**AGAGCG**CTGATGAGTCCGTGAGGACGAA**ACAGTCA**AAACCAAGG

8494 AGATCTAAA**TTTCGAT**CTGATGAGTCCGTGAGGACGAA**ACCAGCT**AAACCAAGG

8737 AGATCTAAA**CGATTTC**CTGATGAGTCCGTGAGGACGAA**ACATCACC**AAACCAAGG (SEQ ID NO: 11)

30 *tRNA-Asp* target (directed against GUC anticodon loop. Accession: X14007)

172 AGATCTAAA**TGCGT**CTGATGAGTCCGTGAGGACGAA**ACAGGCAGGTA**AAACCAAGG (SEQ ID NO:12)

35

Streptomyces lividans

secA target (ACCESSION: Z50195)

1080

5 AGATCTAAACTCGTCCTGATGAGTCCGTGAGGACGAAACGATCAAAACCAAGG

2033

AGATCTAAAGGGCGCTGATGAGTCCGTGAGGACGAAACGCGAAAACCAAGG

2556

AGATCTAAAGTACTCCTGATGAGTCCGTGAGGACGAAACCAGCGAAACCAAGG (SEQ
10 ID NO: 13)

Enterococcus faecalis

ftsZ target (ACCESSION: U94707)

15 10805

AGATCTAAAACTAAATGCTGATGAGTCCGTGAGGACGAAACGAGTTAAAACCAAGG

11182

AGATCTAAAGTTTAATAACTGATGAGTCCGTGAGGACGAAACTTGTTCAAACCAAGG

11512

20 AGATCTAAAACTTTTGCTGATGAGTCCGTGAGGACGAAACGTGTATAAACCAAGG
(SEQ ID NO: 14)

Pseudomonas putida

dnaG target (ACCESSION: U85774)

25

222

AGATCTAAAGGTCCATCTGATGAGTCCGTGAGGACGAAACAAAGCAAACCAAGG

986

AGATCTAAACAGGTTCCTGATGAGTCCGTGAGGACGAAACAATGTAAACCAAGG

30 1891

AGATCTAAATCGCTTTCTGATGAGTCCGTGAGGACGAAACGTGATAAACCAAGG
(SEQ ID NO:15)

Streptomyces coelicolor
rpoA target (ACCESSION: X92107)

290
AGATCTAAAGCTCGATCTGATGAGTCCGTGAGGACGAAACGAACCAAACCAAGG
716
AGATCTAAACGAGTCCTGATGAGTCCGTGAGGACGAAACCGGGAAACCAAGG
1099
AGATCTAAAGTCGATGCTGATGAGTCCGTGAGGACGAAACTTCGCAAACCAAGG
(SEQ ID NO:16)

Staphylococcus warneri
tRNA-Asp target
(directed against GUC anticodon loop. Accession: X66089 S42075)

62
AGATCTAAATGCGTCTGATGAGTCCGTGAGGACGAAACAGGCAGGCGAAACCAAGG
(SEQ ID NO:17)

The utility of the design using eukaryotic sequences has also been evaluated; a) repetitive B2 transcripts (B2); b) RNA polymerase I (polI); c) Hepatitis B virus (HBV); d) Sonic Hedgehog (SH); e) Human Papillomavirus E6/E7 protein (HPV); f) RNA polymerase II (polII); g) Insulin-like Growth Factor 1 (IGF1); h) retinoblastoma protein (RB); i) and j) Multicatalytic Proteinase alpha-subunits C3 and C9 (C3 and C9, respectively); k) telomerase (tel); l) Transforming growth factor beta (TGFß); m) catalase (CAT); n) Peroxisome proliferation associated receptor (PpaRα); and o) Cytochrome P_{450} 1E1 (p4501E1). Target RNAs (with locus names and accession numbers) as well as the selected target sites are

presented (Table 1), as are the sequences of these ITRz (SEQ ID NOS:18-36).

TABLE 1. Summary of Targeted RNAs and Target Sites.

Target RNA	EMBL Locus	Accession	Target Site	Functional Testing	
				in vitro	in vivo
pol II	HSRNAP14K	Z27113	GTC_{83}	ND	ND
HBV	XXHEPAV	X02496	GTC_{438}	IP	+
RB	MUSPP105RB	M26391	GTC_{264}	+	+
IGF1	HUMIGF1B	M37484	GTC_{185}	ND	ND
SH	MMEVX1	X54239	GTC_{558}	IP	IP
Pol I	MUSRPA40	D31966	GTC_{458}	+	+
HPV	PPH16	K02718	GTT_{108}	IP	+
C3	RATC3AA	J02897	GTT_{22}	+	+
C9	RNPTSC9	X533304	GTC_{101}	+	+
B2	B2-Consensus	##	GTT_{24}	+	+
Tel	MMU33831	U33831	CTA_{63}	ND	ND

##see Clawson, G. et al. Cell Growth Diff. 7:635-646 (1996).

Multiple target sites have been selected for TGFb, CAT, PpaRa, and p4501E1 (not shown). All of these ribozymes (with the exception of TGFb) have been functionally tested in vitro. Ribozymes targeted to catalase have also been tested in vivo. In vitro testing refers to target cutting. In vivo testing refers to cell culture experiments or transgenic animals (for polI). IP, in progress. ND, not yet determined. +, substantially decreased target RNA (and/or protein).

B2	**TGCTCTT** CTGATGAGTCCGTGAGGACGAAA **CCGCCTGA** (SEQ ID NO:18)
Pol 1	**TTCAAAGA** CTGATGAGTCCGTGAGGACGAAA **CGAGGATC** (SEQ ID NO:19)
Sonic Hedgehog	**GTCCAT** CTGATGAGTCCGTGAGGACGAAA **CCGGC** (SEQ ID NO:20)
HBV	**ATTAGAG** CTGATGAGTCCGTGAGGACGAAA **CAAACG** (SEQ ID NO:21)
HPV	**GTCCTGA** CTGATGAGTCCGTGAGGACGAAA **CATTGCA** (SEQ ID NO:22)
Pol III	**TCCGTTGTCT** CTGATGAGTCCGTGAGGACGAAA **CATGACACCGA** (SEQ ID NO:23)
IGF-1	**GCGAGGAG** CTGATGAGTCCGTGAGGACGAAA **CATGGTGT** (SEQ ID NO:24)
RB	**AACTTTT** CTGATGAGTCCGTGAGGACGAAA **CATAATG** (SEQ ID NO:25)
C3	**TCGAAGCTGT** CTGATGAGTCCGTGAGGACGAAA **CCGCGTTGA** (SEQ ID NO:26)
TEL	**ATCAGGGT** CTGATGAGTCCGTGAGGACGAAA **GGTGCC** (Seq ID NO:27)
C9	**TCTTCGA** CTGATGAGTCCGTGAGGACGAAA **CATGGCT** (SEQ ID NO:28)
TGFß-1	**TAGCACA** CTGATGAGTCCGTGAGGACGAAA **CGTTTGA** (SEQ ID NO:29)
CAT/#13	**TGCAATA** CTGATGAGTCCGTGAGGACGAAA **CTGCCT** (SEQ ID NO:30)
CAT/#15	**AAGTCAT** CTGATGAGTCCGTGAGGACGAAA **CCTGGA** (SEQ ID NO:31)
PpaRa/#2	**GATAAGG** CTGATGAGTCCGTGAGGACGAAA **CTTTCC** (SEQ ID NO:32)
PpaRa/#8	**CATATTC** CTGATGAGTCCGTGAGGACGAAA **CACTCG** (SEQ ID NO:33)
PpaRa/#14	**TCATGTAT** CTGATGAGTCCGTGAGGACGAAA **CAAAAGG** (SEQ ID NO:34)
P4501E1/#2	**GGTTAAA** CTGATGAGTCCGTGAGGACGAAA **CTTGGG** (SEQ ID NO:35)
P4501E1/#8	**GTCCAGT** CTGATGAGTCCGTGAGGACGAAA **CTTAAG** (Seq ID NO:36)

For many of these constructs, "mutants" have also been created by substituting an A for a G, or a G for an A, at nucleotides (denoted within circles, Figure 6) which are absolutely required for catalytic activity. These "mutants" allow us to document that the efficiency of destruction of the targeted RNAs is due to ribozyme catalytic activity and not to antisense effects.

Characterization and Validation of the TRz construct

Typically, once the recombinant plasmid has been created the TRz constructs are isolated from the bacterium their nucleotide sequence is determined to confirm their

identities and to document their orientation within the vector. The constructs are then transcribed *in vitro* using SP6 and T7 RNA polymerases with ^{32}P-CTP. When transcribed in the "sense" orientation, all of these TRz constructs should be "self-liberating"; that is, the 5' and 3' self-cleaving ribozymes should work effectively, freeing the ITRz during (or immediately after) transcription (Figure 1c). The 5' liberated ribozyme (whose only function is self-cleavage, liberating the 5' end of the ITRz) is associated with relatively short stretches of vector sequences and the 3' self-cleaving ribozyme (whose only function is self-cleavage, liberating the 3' end of the ITRz) remains associated with long vector sequences. The liberated ITRz achieves its catalytic topology upon hybridization with the targeted sequence. Transcription of all of these TRz in the "antisense" direction should not result in self-cleavage.

***In vitro* evaluation**

Upon validation of the TRz construct, the self-liberating TRz should be evaluated for their ability to effectively cut their targeted RNAs. Appropriate regions of the targeted RNAs are generally cloned using cellular RNA and reverse transcription/polymerase chain reaction amplifications. In some cases, cloned full-length cellular RNAs can also used. The identities of the constructs used for transcription of target RNAs are also confirmed by sequencing. Target RNAs are then synthesized *in vitro* using the appropriate T7/SP6 RNA polymerase with ^{32}P-CTP, and are subsequently gel-purified. A preparation of the TRz under evaluation is then synthesized without ^{32}P-CTP. The TRz preparation is then mixed with their an appropriate concentration of radiometrically ^{32}P-labeled target or substrate RNA

(^{32}P-labeled target RNAs and unlabeled TRz preparations are mixed at a 10:1 molar ratio) and is incubated for various lengths of time. Following incubations, the RNA is examined using polyacrylamide gel electrophoresis (PAGE) and autoradiography. All of the constructs tested should be able to cleave their target RNAs. In general, the data show an approximate catalytic rate of 0.2 cleavages/ribozyme-min.

***In vivo* evaluation**

The TRz should then be evaluated with intact cells. The TRz cassette is excised from the parental plasmid and is then placed into an appropriate expression vector. Vectors utilized include (but are not limited to) the LacSwitch vector (from Stratagene), which is an IPTG-inducible system, and the TetSplice vector (from Gibco-BRL), which is a tetracycline-inducible system.

The TRz constructs in these expression vectors were then transfected into cells using standard techniques. Cell types used in transfections have included E. coli, human CaSki cervical carcinoma cells, SV-40 immortalized rat hepatocytes, and mouse fibroblasts. In transient transfection analyses, all constructs tested produced substantial reductions in their respective target RNAs.

1. The secA targeted TRz construct against the *secA* gene of *E. coli* is in the vector pClip, which is a variation of the generalized cloning vector, pBluescript of Stratagene. The plasmid containing the construct was transformed into competent bacterial cells and cells containing the plasmid with the TRz were selected by using the antibiotic selectable marker within the vector,. pClip. Upon induction of the promoter with isopropyl-ß-D

thiogalactoside, (IPTG) the effect of ribozyme expression is monitored by standard bacterial viable counts. A reduction in total viable cells is an indication of synthesis and catalytic activity of the TRz against the essential target.

2. The polI-targeted TRz construct (in LacSwitch vector) was used in transfections of SV40-immortalized rat hepatocytes (CWSV1 cells), and stably transfected cell populations were obtained. Cells not transfected with the antibiotic resistance plasmid were all dead by day 5, indicating that the antibiotic selection procedure was effective. Growth of cells transfected with the double ribozyme (as a control, with no cellular RNA target), and of cells expressing the catalytically inactive "mutant" polI TRz, was unaffected. However, growth of cells expressing the polI-targeted TRz was depressed by nearly 90%.. Concurrently, the mRNA for polI was decreased by at least 70% from polI mRNA levels in the cells expressing the mutant polI TRz. Since expression of the TRz was essentially equivalent for the two TRz, this clearly documents that the effects on cell growth are due to TRz catalytic activity and not to antisense effects. In other experiments, expression of the polI-TRz in LacSwitch resulted in cell death with mouse fibroblast cell populations.

3. The RB-targeted TRz (in the tetracycline-inducible TetSplice vector system) was used in transfections with CWSV1 cells, and stably transfected cells were selected using G418 antibiotic in the presence of tetracycline, and individual clones were harvested and used (expression of RB-targeted TRz is "off" in the presence of tetracycline, and is "on" in the absence of tetracycline). Expression

of the RB-targeted TRz had no effect on cell growth, as expected. Expression of RB mRNA was substantially reduced to below detectable levels by Northern blot analysis. To extend this result, metabolic labeling of proteins was performed using ^{35}S-methionine, and immunoprecipitations were performed using an antibody directed against RB. The data show that RB protein levels were reduced by 75%, comparing clonal cell populations grown in the presence vs. absence of tetracycline, even after only 18h of induction of RB-targeted TRz expression (removal of tetracycline). This shows that the effects of the reduction in RB RNA levels also extends to the production of the RB protein. In addition, the (+)-tetracycline sample provides an ideal control, since it represents the exact same clonal cell line. Essentially any inducible vector system can be used in parallel fashion.

4. The B2-targeted TRz (in the IPTG-inducible LacSwitch vector) was used in transfections of CWSV1 cells, and antibiotic selection was used to obtain a number of individual clones. Reductions in cytoplasmic B2 RNA levels of up to 80% were observed by Northern blot analysis, and growth of transfected clones was reduced in parallel. In fact, a linear relationship between growth rate and B2 RNA levels was observed. The reductions in B2 transcripts paralleled the level of B2-targeted TRz expression (as determined by slot-blot analysis). The B2 target RNA is of additional interest, because B2 transcripts are not translated (i.e., they are not mRNAs) and they are abundant, highly-structured RNAs.

Other TRz constructs have also been successfully tested using this methodology (including C9 in the

tetracycline-inducible system, and CAT, BRAC1 and Albumin driven by the albumin promoter with HepG2 cells).

Throughout this application various publications are referenced by numbers within parentheses. Full citations for these publications are shown below. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

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SEQUENCE LISTING

5 (1) GENERAL INFORMATION

(i) APPLICANT: MEDICAL UNIVERSITY OF SOUTH CAROLINA
171 Ashley Avenue
Charleston, SC 29425

10

PENN STATE UNIVERSITY
P. O. Box 850
Hershey, PA 17033

15 Norris, James S.
Schmidt, Michael G.
Dolan, Joseph W.
London, Steven D.
May, Harold D.
20 Clawson, Gary A.

(ii) TITLE OF THE INVENTION: TISSUE SPECIFIC AND TARGET
RNA-SPECIFIC RIBOZYMES AS ANTIMICROBIAL TISSUE SPECIFIC AND
TARGET RNA-SPECIFIC RIBOZYMES AS
25ANTIMICROBIAL THERAPEUTICS AGAINST MICROBIAL PATHOGENS

(iii) NUMBER OF SEQUENCES: 36

30 (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: NEEDLE & ROSENBERG, P.C.
(B) STREET: 127 Peachtree Street, N.E., Suite 1200
(C) CITY: Atlanta
(D) STATE: GA
35 (E) COUNTRY: USA
(F) ZIP: 30303-1811

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
40 (B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
45 (A) APPLICATION NUMBER:
(B) FILING DATE: 02 DEC 1997
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
50 (A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
55 (A) NAME: Spratt, Gwendolyn DD
(B) REGISTRATION NUMBER: 36,016
(C) REFERENCE/DOCKET NUMBER: 19070.0049/P

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 404 688 0770
(B) TELEFAX: 404 688 9880
(C) TELEX:

5 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 165 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 380 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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GATGAGTCCG TGAGGACGAA ACCACTTAAA AGATCTAGAT CTAAATTTTC CACTGATGAG    240
TCCGTGAGGA CGAAACGTGC AAAAAGATCT AGATCTAAAT TGATACCCTG ATGAGTCCGT    300
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(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 162 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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AGATCTAAAC GTTAGTCTGA TGAGTCCGTG AGGACGAAAC CAACAAAACC AAGGAGATCT     60
AAAGGCATCA CTGATGAGTC CGTGAGGACG AAACTGTTAA AACCAAGGAG ATCTAAACCA    120
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  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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TTCCTGATGA GTCCGTGAGG ACGAAACATC ACCAAACCAA GG                       162
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  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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(2) INFORMATION FOR SEQ ID NO:13:

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  (A) LENGTH: 157 base pairs
  (B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

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TGATGAGTCC GTGAGGACGA AACCAGCGAA ACCAAGG                            157
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(A) LENGTH: 168 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

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CTAAAGTTTA ATAACTGATG AGTCCGTGAG GACGAAACTT GTTCAAACCA AGGAGATCTA   120
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(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 162 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

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(A) LENGTH: 160 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

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(2) INFORMATION FOR SEQ ID NO:17:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

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(2) INFORMATION FOR SEQ ID NO:20:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

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(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

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(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GCGAGGAGCT GATGAGTCCG TGAGGACGAA ACATGGTGT 39

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

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(2) INFORMATION FOR SEQ ID NO:26:

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(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

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(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ATCAGGGTCT GATGAGTCCG TGAGGACGAA AGGTGCC 37

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TCTTCGACTG ATGAGTCCGT GAGGACGAAA CATGGCT 37

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TAGCACACTG ATGAGTCCGT GAGGACGAAA CGTTTGA 37

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TGCAATACTG ATGAGTCCGT GAGGACGAAA CTGCCT 36

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AAGTCATCTG ATGAGTCCGT GAGGACGAAA CCTGGA 36

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GATAAGGCTG ATGAGTCCGT GAGGACGAAA CTTTCC 36

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CATATTCCTG ATGAGTCCGT GAGGACGAAA CACTCG 36

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TCATGTATCT GATGAGTCCG TGAGGACGAA ACAAAAGG 38

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GGTTAAACTG ATGAGTCCGT GAGGACGAAA CTTGGG 36

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GTCCAGTCTG ATGAGTCCGT GAGGACGAAA CTTAAG 36

What is claimed is:

1. A virion, modified to be non-replicative and comprising non-viral DNA.

2. The virion of claim 1, wherein the non-viral DNA encodes a ribozyme.

3. The virion of claim 2, wherein the non-viral DNA comprises a pathogen-specific promoter upstream from a sequence encoding a triple ribozyme comprising a) a 5′ autocatalytically cleaving ribozyme sequence, b) a catalytic ribozyme comprising a target RNA-specific binding site and c) a 3′ autocatalytically cleaving ribozyme sequence.

4. The virion of claim 3, wherein the non-viral DNA encodes more than one triple ribozyme.

5. A liposome comprising a nucleic acid comprising a pathogen-specific promoter upstream from a sequence encoding a triple ribozyme comprising a) a 5′ autocatalytically cleaving ribozyme sequence, b) a catalytic ribozyme comprising a target RNA-specific binding site and c) a 3′ autocatalytically cleaving ribozyme sequence.

6. The liposome of claim 5, wherein the nucleic acid encodes more than one triple ribozyme.

7. A nucleic acid comprising a pathogen-specific promoter upstream from a sequence encoding a triple ribozyme comprising a) a 5′ autocatalytically cleaving

ribozyme sequence, b) a catalytic ribozyme comprising a target RNA-specific binding site and c) a 3′ autocatalytically cleaving ribozyme sequence.

8. The nucleic acid of claim 7, encoding more than one triple ribozyme.

9. The nucleic acid of claim 8, encoding at least two different triple ribozymes.

10. The nucleic acid of claim 8, encoding more than one copy of a triple ribozyme.

11. The nucleic acid of any of claims 7, 8, 9, 10, 12, 13 or 14, wherein at least one triple ribozyme is targeted to the *rpoA* transcript of the pathogen.

12. The nucleic acid of any of claims 7, 8, 9, 10, 11, 13 or 14 wherein at least one triple ribozyme is targeted to the *secA* transcript of the pathogen.

13. The nucleic acid of any of claims 7, 8, 9, 10, 11, 12 or 14, wherein at least one triple ribozyme is directed to the *dnaG* transcript of the pathogen.

14. The nucleic acid of any of claims 7, 8, 9, 10, 11, 12 or 13 wherein at least one triple ribozyme is directed to the *ftsZ* transcript of the pathogen.

15. A method of treating an infection in a subject, comprising administering to the subject the virion of any of claims 2, 3 or 4, whereby the ribozyme encoded by the

non-phage DNA is expressed and the infectious agent is killed or weakened.

16. A method of treating an infection in a subject, comprising administering to the subject the liposome of claim 5 or 6, whereby the ribozyme encoded by the DNA is expressed and the infectious agent is killed or weakened.

17. A method of targeted delivery of a ribozyme to a pathogen in a subject, comprising

a) generating a virion of any of claims 2, 3 or 4; and

b) delivering the virion to the subject, whereby the pathogen-specific promoter directs transcription of the ribozyme in the cells of the pathogen.

18. The method of claim 17, wherein the virion is from bacteriophage lambda.

19. The method of claim 17, wherein the virion is from bacteriophage P1.

20. The method of claim 19, wherein the non-phage DNA further comprises a PAC site.

21. A method of targeted delivery of a ribozyme to a pathogen in a subject, comprising

a) generating a liposome of claim 5 or 6; and

b) delivering the liposome to the subject, whereby the pathogen-specific promoter directs transcription of the ribozyme in the cells of the pathogen.

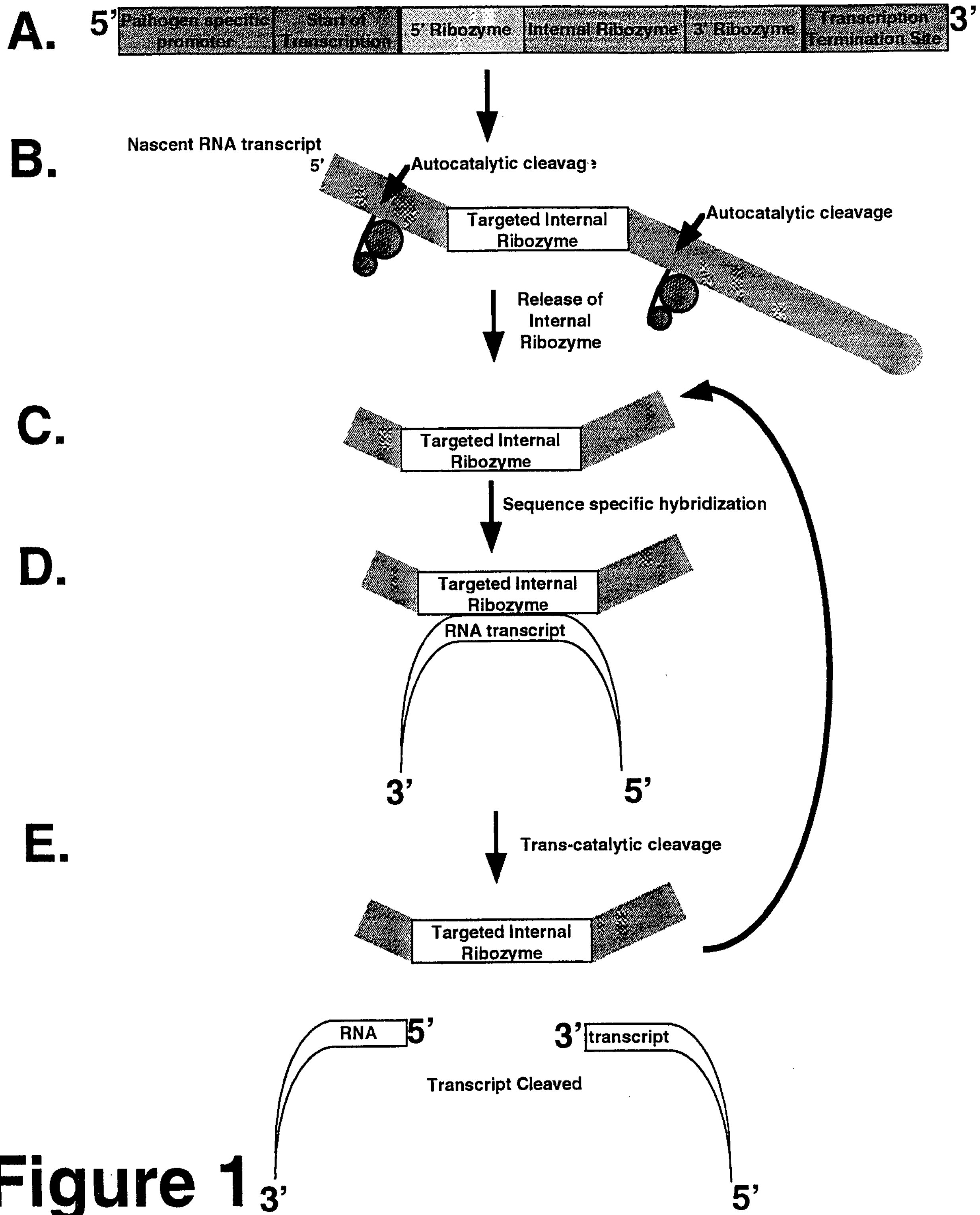
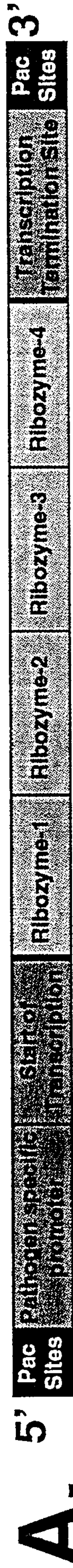
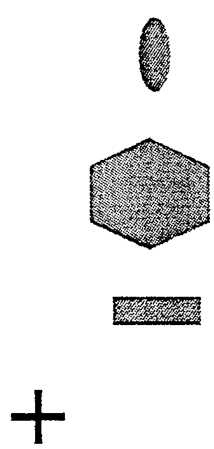


Figure 1

A.



+



In vitro Packaging Extract

B.

Self Assembly

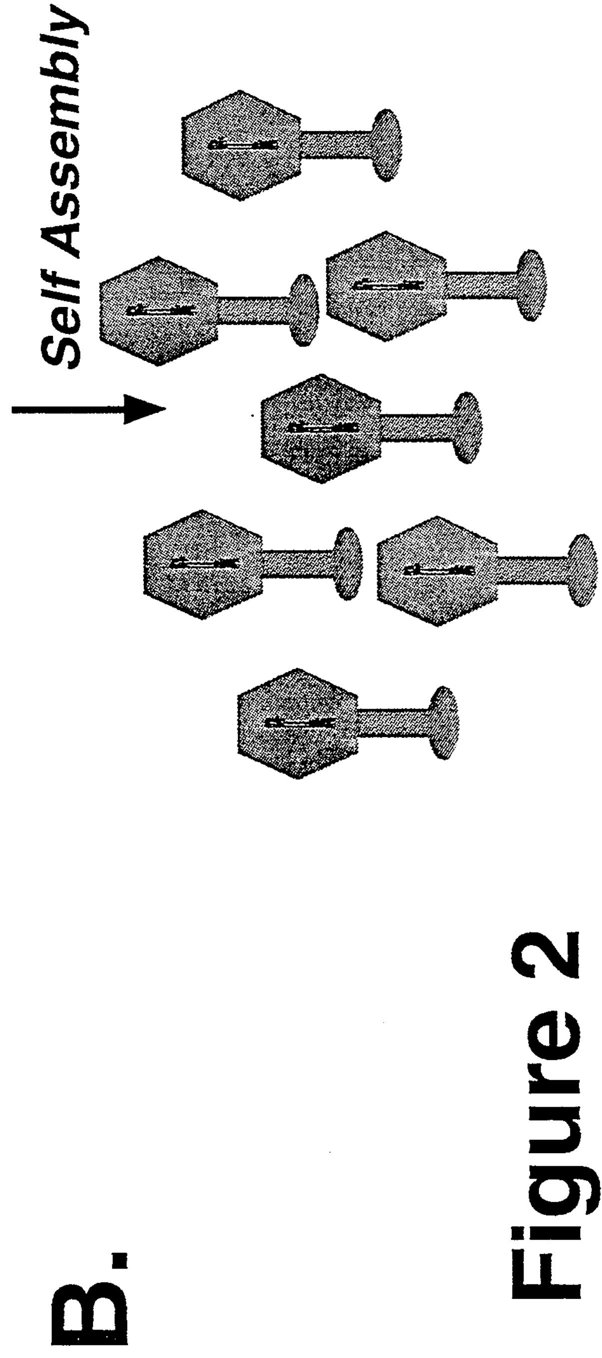


Figure 2

A.

B.

In vivo Packaging

+

Self Assembly and Autolysis

Figure 3

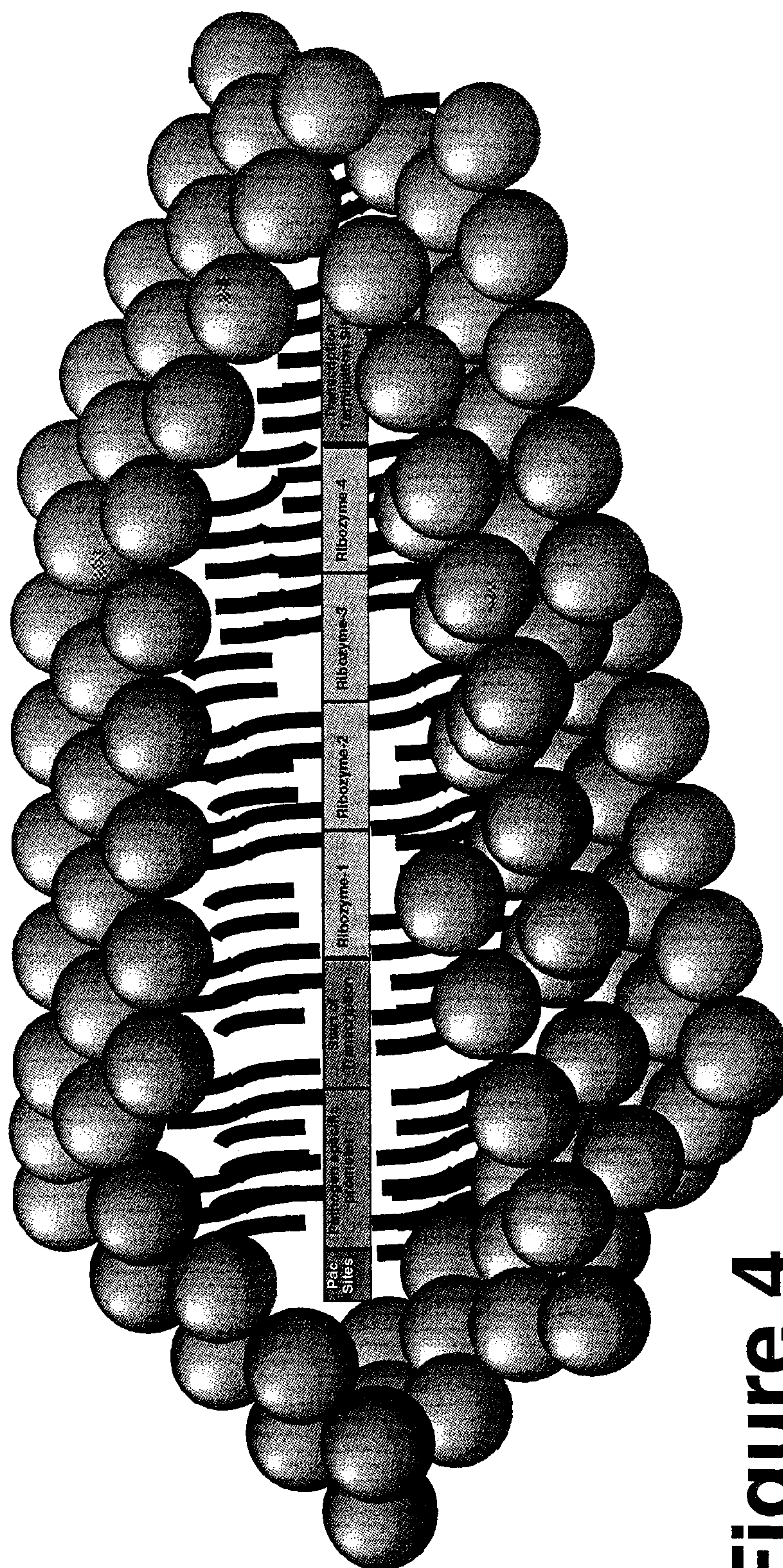


Figure 4

5'Cleavage Site
Targeted Ribozyme
3'Cleavage Site
Bgl II
TACCGTCAGCTCGAGCTCAGATCTGGATCCGTCGACGGA
ATGGCA TCGAGCT
CTAGGCA CTGCCT
Transcription
5'
Promoter
GCGGCCGC
Not I
TGCAGCGGCCGC
Not I
Termination Signal
3'

Figure 5

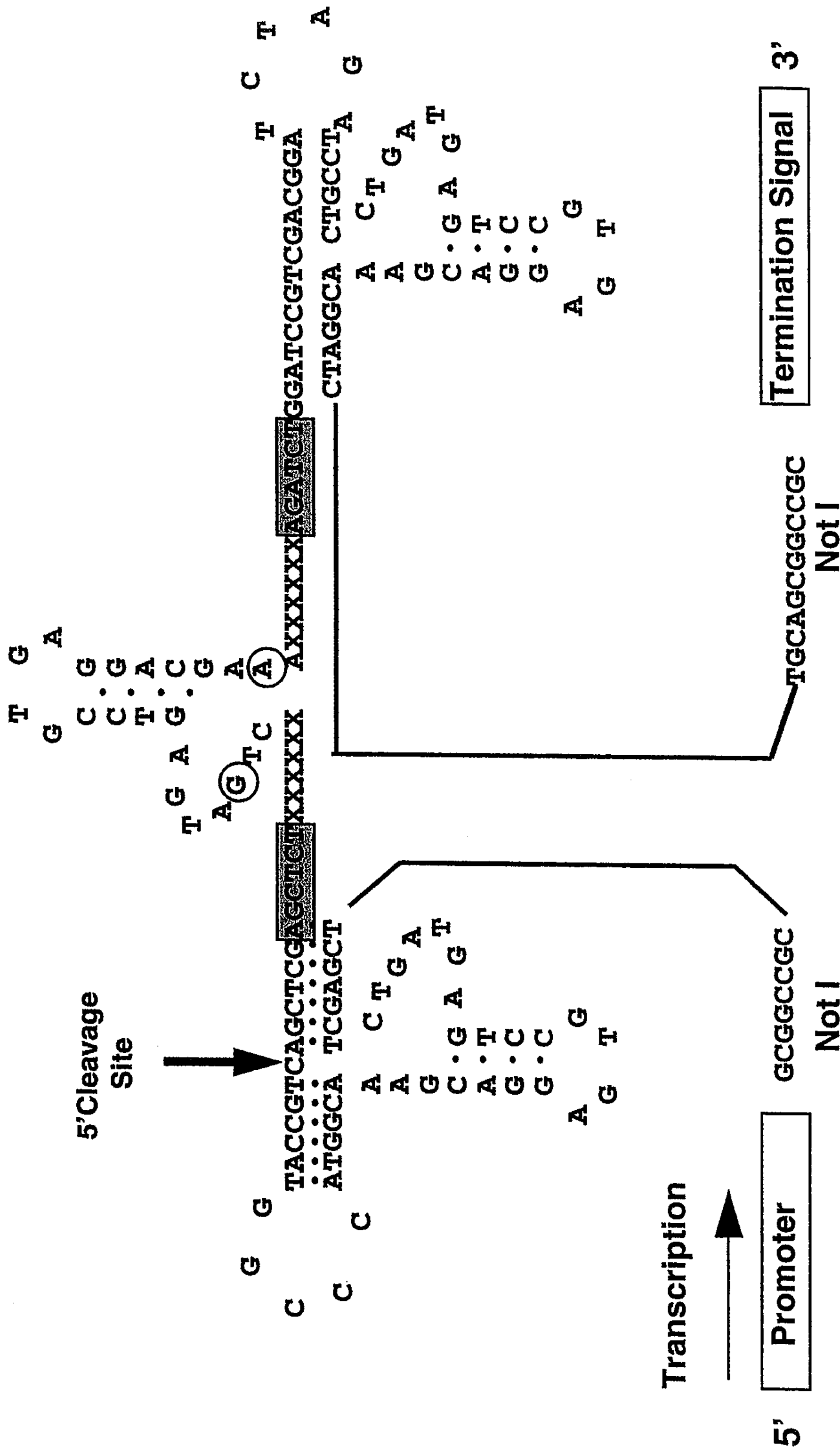


Figure 6

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/21922

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/88 C12N9/00 A61K48/00

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)
IPC 6 C12N A61K

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 practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 5 500 357 A (TAIRA KAZUNARI ET AL) 19 March 1996 In particular Figure 7B and see the whole document ---	7-10 3-6, 11-14
E	WO 97 17433 A (UNIV SOUTH CAROLINA) 15 May 1997 cited in the application see the whole document ---	1-21
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
14 April 1998	15.05.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hix, R

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/21922

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TAIRA K ET AL: "CONSTRUCTION OF SEVERAL KINDS OF RIBOZYMES THEIR REACTIVITIES AND UTILITIES" GENE REGULATION, BIOLOGY OF ANTISENSE RNA AND DNA, 1 January 1992, ERICKSON R P;IZANT J G, pages 35-54, XP002002021	7-10
Y	see in particular Figure 5B and see the whole document	3-6, 11-14

X	OHKAWA J ET AL: "ACTIVITIES OF HIV-RNA TARGETED RIBOZYMES TRANSCRIBED FROM A 'SHOT-GUN' TYPE RIBOZYME-TRIMMING PLASMID" NUCLEIC ACIDS SYMPOSIUM SERIES, vol. 27, 1 January 1992, page 15/16 XP000568246	7-10
Y	see in particular Figure 1b see the whole document	3-6, 11-14

X	C. ZHOU ET AL.: "Expression of Hammerhead ribozymes by retroviral vectors to inhibit HIV-1 replication: Comparison of RNA levels and viral inhibition." ANTISENSE AND NUCLEIC ACID DRUG DEVELOPMENT, vol. 6, 1996, pages 17-24, XP002062053	1,2
Y	see the whole document	3-6, 11-14

Y	WO 95 07923 A (UNIV TEMPLE) 23 March 1995 see the whole document	5,6

A	R. E. CHRISTOFFERSEN ET AL.: "Ribozymes as Human Therapeutic Agents." JOURNAL OF MEDICINAL CHEMISTRY, vol. 38, no. 12, 9 June 1995, pages 2023-2037, XP002062054 cited in the application see the whole document	

A	S. M. SULLIVAN : "Development of Ribozymes for gene therapy" THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, vol. 103, no. 5, November 1994, pages 85S-89S, XP002062055 cited in the application see the whole document	

1

INTERNATIONAL SEARCH REPORT

Inte...ational application No.
PCT/US 97/21922

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 15 to 21 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna al Application No
PCT/US 97/21922

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5500357 A	19-03-96	JP 2580512 B	12-02-97
		JP 5192151 A	03-08-93
WO 9717433 A	15-05-97	AU 7727296 A	29-05-97
WO 9507923 A	23-03-95	US 5635385 A	03-06-97
		AU 7720394 A	03-04-95