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Review Article

RIBOZYMES: NUCLEIC ACID ENZYMES WITH POTENTIAL PHARMACEUTICAL APPLICATIONS - A REVIEW

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ABSTRACT

The discovery over 30 years ago that RNA molecules called ribozymes are able to catalyze chemical reactions was a breakthrough in biology. Because of their high specificity, wide range of target selection and action before protein translation, the different classes of both natural and artificial ribozymes have been claimed to be used as specific suppressors of gene functions with the additional aim of validating disease-related genes as potential targets for new therapeutic interventions. However, the lack of suitable delivery systems in to target cells still hampers the clinical development of ribozyme-based therapeutics. This review briefly summarizes the ever increasing evidence to the use of ribozymes as innovative nucleic acid-based enzymes along with their classifications, their mechanism in initiating catalytic effect, their design, delivery strategies and their potential pharmaceutical applications and their roles in gene function study and in target validation.

Keywords: Nucleic acid enzymes, Ribozymes, Hammerhead ribozyme, Hairpin ribozyme.

INTRODUCTION

RNA was previously viewed as a mere bridge between DNA (reservoir of all genetic information) and protein (determinants of biological structure, function, and integrity).[1] Its perception as a simple vehicle for deciphering the genetic code to a translatable readout changed in 1982 when RNA molecule with enzymatic properties were first discovered in the laboratory of Thomas Czech, at the University of Colorado.[1,2] Such RNA molecules that catalyze a chemical reaction and retain their structure after the reaction has been completed are called ribozymes.[3] The first naturally occurring ribozyme activity, described by Thomas Czech and colleagues, was discovered in the self-splicing group I intron of *Tetrahymena thermophila*. The intron folds to form an autocatalytic unit which affects its excision that can occur in the absence of any protein co-factor or external energy source.[2, 4] Shortly thereafter, Sidney Altman's group, at Yale University, showed that the RNA component of ribonuclease P (RNAse P), M1 RNA, from *Escherichia coli* was likewise able to process transfer RNA (tRNA) precursors without any protein factors. Thomas Czech and Sidney Altman were awarded the Nobel Prize in Chemistry in 1989 for this work.[2, 5] Ribozymes are nucleic acid enzymes

that catalyze the cleavage and inactivation of other cellular and viral RNA molecules having a specified nucleotide sequence, there by irreversibly inactivating RNA that would otherwise direct the synthesis of a protein.[4, 6] They have site-specific RNA cleavage or ligation activities[7, 8] that can catalyze a variety of reactions in cells.[7, 9] The discovery that RNA can be a catalytically competent molecule has stimulated RNA research considerably. In particular, the ability of ribozymes to cleave RNA in an intermolecular or in trans- mode has created much interest. This property makes ribozymes useful for the sequence-specific cleavage of mRNAs and thus for the inhibition of gene expression and for gene-function analysis.[10]

Ribozymes occur naturally, but can also be artificially designed to target specific sequences in a cis- or a trans- manner.[11] Although there are different types of ribozymes in experimental usage, two types of ribozymes, the hammerhead and hairpin ribozymes, have been extensively studied due to their small size and high cleavage efficiency.[3, 12] Particularly, the modified hammerhead ribozyme that is fewer than 40 nucleotides long and consists of two substrate-binding arms and a catalytic domain is therapeutically the most relevant type of ribozyme.[12] Like other antisense therapeutic agents, ribozymes offer promising potential in the treatment of a variety of diseases ranging from inborn metabolic disorders to viral infections and acquired diseases such as cancer. [9, 13] Currently, there are few examples of ribozymes that are under clinical trails for application to human diseases such as for AIDS and cancer. The first clinical trial using a ribozyme targeted human immunodeficiency virus 1(HIV-1) had shown that an anti-HIV-l gag ribozyme that was delivered intracellularly can interfere with both pre-integration and post-integration events of the HIV replication cycle, by cleaving incoming viral RNA and transcribed mRNAs.[14, 15] They have been also used as tools for pathway elucidation and target validation.[11]

In this review, the ever increasing evidence to the use of ribozymes as innovative nucleic acid-based enzymes is discussed along with their classifications, their mechanism in initiating catalytic effect, their design, delivery strategies and their potential pharmaceutical and biomedical applications.

RIBOZYMES

Ribozymes as Nucleic Acid Enzymes

The function of nucleic acids has been an endless source of discovery and invention that has drastically enhanced our appreciation of DNA and RNA as multifaceted polymers. It has been widely known that such functional nucleic acids can either be found in nature or isolated from pools of random nucleic acids via '*in vitro* selection' or systemic evolution of ligands by exponential enrichment ('SELEX') techniques.[16] The availability of many natural functional nucleic acids such as ribozymes, microRNAs, siRNAs and riboswitches, and the artificial ones including aptamers, ribozymes, siRNAs and deoxyribozymes has opened a new horizon for the development of 'smart' molecules for a variety of chemical and biological applications.[16, 17]

Since the discovery of the first natural ribozyme more than 30 years ago, it has become clear that nucleic acids are not only the static depository of genetic information, but also possess intriguing catalytic activity. The term 'nucleic acid enzyme' is used to identify nucleic acids that have catalytic activity.[16, 18] The number of reactions catalyzed by engineered nucleic acid enzymes is growing continuously. The versatility of these catalysts supports the idea of an ancestral world based on RNA predating the emergence of proteins, and also drives many studies towards practical applications for nucleic acid enzymes.[16, 18, 19] Among the 'smart' molecules that act as nucleic acid-based catalysts, ribozymes are the predominant nucleic acid enzymes, which can catalyze the range of chemical reactions. A quick perusal of the literature reveals that many of ribozymes are found in nature and mediate phosphodiester bond cleavage and formation and

peptide bond formation. Furthermore, artificial ribozymes have been also generated via *'in vitro* selection' or 'SELEX' techniques.[16, 17] The realization that nucleic acid enzymes such as ribozymes are highly useful molecular tools has made the study of functional nucleic acids a very important part of chemical biology. The flexibility offered by nucleic acids (stability, ease of immobilization and susceptibility to various chemical modifications and labeling) has motivated molecular scientists worldwide to seek innovative applications of these compounds. [20]

Historical Back Ground of Ribozymes

All known enzymes were considered as proteins until the discovery of some RNA molecules that can act as enzymes in 1980s.[21] The catalytic RNA molecules (or ribozymes) were first discovered in the laboratory of Thomas Czech, at the University of Colorado, in 1982. Czech`s research team found that the ribosomal RNA precursor from *Tetrahymena thermophila* contained an intron, a non encoding sequence that interrupts the gene that was capable of excising itself *in vitro*, without any protein co-factor or external source of energy.[2, 4] Shortly thereafter, Altman's group, at Yale University, showed that the RNA component of RNase P, M1 RNA, from *Escherichia coli* was likewise able to process tRNA precursors without any protein co-factors. Czech and Altman shared the Nobel Prize in Chemistry in 1989 for this work.[2]

The discovery in 1982 that an intron of the protozoan tetrahymena can excise itself from precursor ribosomal RNA (rRNA) in the absence of protein co-factor challenged the belief that only proteins can act as biological catalysts. The new field of RNA enzymology emerged and catalytic RNAs have been shown to fold up to form complex three-dimensional surfaces that can bind specific substrates and break and join RNA chains.[21, 22] Thus, the discovery of RNA catalysis provided a paradigm shift in biology, insight into the evolution of life on the planet and a challenge to understand its mechanistic origins.[23] In general, the discovery of a vast repertory of naturally occurring ribozymes in contemporary cells, and the crucial importance of their reactions, largely lend support to the credibility of an RNA world and to speculations that nucleic acids were the original biocatalysts. Roles for RNA catalysis in the modern protein world can be found in the regulation of gene expression and in protein synthesis, demonstrating that RNA plays a central role in all fundamental processes in present-day cells. All these findings strongly suggest not only that an RNA world could have existed, but also that it still lurks within modern living forms.[24] Now a day, there are different varieties of naturally occurring and/or *in vitro* selected ribozymes for chemical, biological and claimed pharmaceutical and medical applications.

Classes of Ribozymes

Several classes of ribozymes occur in nature, each distinguished by characteristic structures and reaction mechanisms. They are broadly grouped into two classes based on their size and reaction mechanisms: large and small ribozymes. The first group consists of the self-splicing group I and group II introns as well as the RNA component of RNase P, whereas the latter group includes the hammerhead, hairpin, hepatitis delta ribozymes and varkud satellite RNA. Large ribozymes consist of several hundreds up to 3000 nucleotides and they generate reaction products with a free 3'-hydroxyl and 5'-phosphate group. In contrast, small catalytically active nucleic acids which range from 30 to 150 nucleotides in length generate products with a 2'-3'-cyclic phosphate and a 5'-hydroxyl group.[25, 26]

Transfer of phosphate groups can be catalyzed by ribozymes through two types of chemical reactions differing in their products. On this basis, natural ribozymes can be classified into two different groups: 1) the self-cleaving RNAs which include the hammerhead, hairpin, hepatitis delta virus, Varkud satellite and glmS ribozymes and 2) the self-splicing ribozymes that are the group I and II introns, the group-I-like cleavage ribozyme, branching ribozyme and RNase P.[24, 27] In addition, many artificial ribozymes able to catalyze various reactions have been obtained, but most studies regarding the RNA world aimed to

select those ribozymes capable of self-replication, adopting cofactors to assist catalysis and creating activated chemical intermediates that could participate in entirely new pathways such as translation.[24]

Group I introns

The first hint of catalytic activity in RNA molecules concerned a group I intron found in the precursor of *Tetrahymena thermophila* large subunit rRNA.[28] Group I introns have also been found to interrupt a large variety of tRNAs, mRNAs and rRNAs in bacteria and many other organisms.[22, 29] They all have a common secondary structure and a common splicing pathway. The *Tetrahymena thermophila* precursor rRNA contains a group I intron capable of catalyzing its own excision. Self-splicing of the intron requires presence of a guanosine cofactor and a divalent cation, either Mg^{2+} or Mn^{2+}, and occurs via two sequential transesterification reactions.[30, 31]

Group I introns range in size from a few hundred nucleotides to around 3000. They are abundant in fungal and plant mitochondria, but they are also found in nuclear rRNA genes, chloroplast DNA (ctDNA), bacteriophage, and in the tRNA of ctDNA and eubacteria. But, they are not found in higher eukaryotes such as in vertebrates.[2] Group I intron ribozyme may prove to be an important tool for RNA-directed therapy. It can be specifically designed to repair abnormal mRNA molecules. [28] It has been demonstrated that modified group I introns are suitable for the correction of deficient mRNAs. For example, a trans-active group I intron was used to repair mutant β-globin RNA in erythrocyte precursors from patients with sickle cell anemia by replacing the mutated part of the β-globin RNA by the γ-globin-3'- exon. Additional attempts have been reported to address diseases caused by trinucleotide repeat expansions, including Huntington's disease and myotonic dystrophy.[25]

Group II introns

Group II introns have also been found in bacteria and in organellar genes of eukaryotic cells, mainly in mitochondrial and chloroplast RNAs of plants, fungi and yeast, but they are less widely distributed than group I introns. They are large catalytic RNA molecules that fold into a compact structure essential in RNA catalyzed self-splicing and intron mobility reactions. They are mainly present in mRNAs, but they also occur in tRNA and rRNA genes.[31, 32] They are multidomain RNA metalloenzymes that catalyze their own excision from their primary transcripts by a two step mechanism closely resembling that catalyzed by the spliceosome. Activity relies upon the presence of metal ions, which are involved in structural and catalytic roles, and formation of specific long range interactions.[33]

They catalyze a self-splicing reaction that is mechanistically distinct from the mode of action group I introns in that they do not require a guanosine cofactor. Much less is known about group II introns than about group I ribozymes, and their range of applications seems to be more limited. A subgroup of this class of ribozymes is able to insert itself into an intron less allele on the DNA level by reverse splicing and reverse transcription, a process called retro homing. It has been demonstrated that group II introns can be redirected to insert themselves into therapeutically relevant DNA target sites in human cells.[25]

Ribonuclease P

It is a ribonucleoprotein consisting of approximately 375-nucleotide RNA plus a small polypeptide. The RNA portion cleaves tRNA precursors to produce the mature tRNA.[27] It processes precursor tRNAs and other RNAs required for cellular metabolism.[34] It is a ribonucleo-protein complex whose catalytic activity lies in its RNA subunit. This enzyme is essential for the 5' processing of tRNAs, but a minimal model substrate contains only the acceptor stem and the T-stem of a precursor tRNA. Since the 3' part of the acceptor stem can be provided as a separate molecule, bound only through base pairing to the precursor RNA. Different studies have described the use of external guide sequences that enable the cleavage

of nearly any RNA using the endogenous RNase P.[35]

It is a ubiquitous enzyme that acts as an endonuclease to generate the mature 5'-end of tRNA precursors. In bacteria, it exists as a ribonucleo-protein complex, consisting of a long RNA, typically 300-400 nucleotides in length, and a small protein of approximately 14 kDa and the RNA portion cleaves tRNA precursors to produce the mature tRNA. It is the true naturally occurring trans-cleaving RNA enzyme. For full enzymatic activity under *in vivo* conditions, however, the protein component is essential. In human cells, RNase P contains multiple proteinaceous components and in the absence of protein the RNA moiety is thought to be catalytically inactive.[27, 31]

Hammerhead ribozyme

The hammerhead ribozyme was first discovered as a self cleaving domain in the RNA genome of different plant viroids and virusoids.[36] It is a small RNA molecule found in the satellite RNAs of some plant viroid pathogens and in the repetitive DNA of newts, cave crickets and schistosomes. [37] The smallest hammerhead ribozyme identified, is composed of approximately 30 nucleootides and is capable of site-specific cleavage of a phosphodiester bond.[28, 38] It was first discovered as a catalytic motif in different plant pathogen RNAs. It is the smallest, naturally occurring ribozyme, and the one most frequently employed for use in down-regulating cellular RNAs.[39] All of them are involved in the processing of long multimeric transcripts into monomer sized molecules. It is known, for at least for 14 of these RNAs, that this involves a self cleavage reaction catalyzed by the hammerhead domain. Ribozymes of the hammerhead type can catalyze the trans-cleavage of any RNA containing a trinucleotide amenable for cleavage.[40]

This type of ribozyme is the most intensively studied and applied ribozyme [3] and the smallest of the naturally occurring ribozymes and processes the linear concatamers that are generated during the rolling circle replication of circular RNA plant pathogens. They have been used to cleave many RNA targets *in vitro*; however, there has been limited success in ribozyme-mediated gene inactivation *in vivo*.[41] A major problem that researchers face when aiming to apply hammerhead ribozymes intracellularly for therapeutic purposes is the requirement of elevated concentrations of divalent ions to obtain high catalytic activity.[25, 38]

Molecular modeling and kinetic analysis of the hammerhead cleavage reaction in the presence of monovalent or divalent salts support the idea that divalent metal ions are not essential for the catalytic step, although they do stabilize the structure of active ribozymes. The hammerhead ribozyme is probably the most widely studied ribozyme. It is also the most commonly used for gene inactivation assays due to its small size and catalytic efficiency.[3, 31]

Hairpin ribozyme

Another catalytic RNA domain found in pathogenic plant virus satellite RNAs is the hairpin motive. The hairpin ribozyme, like the hammerhead ribozyme, is found within RNA satellites of plant viruses, where it performs a reversible self-cleavage reaction to process the products of rolling circle genome replication [42] and similar to the other small ribozymes, hairpin catalysts cleave concatameric precursor molecules into mature satellite RNA during rolling-circle replication, giving rise to a 2'-3'-cyclo phosphate and a free 5'-OH terminus. Catalytic activity of the hairpin ribozyme results from distortion and precise orientation of the substrate RNA and general acid-base catalysis by neighboring nucleotides without involvement of metal ions in catalysis. The requirement for metal ions may be explained by a significant role in the folding process. It has also been shown that catalytic activity of the hairpin ribozyme can be supported by spermine, the major polyamine in eukaryotic cells.[25, 43]

These ribozymes have been developed mainly for antiviral applications. Two ribozymes targeted against different sites on the RNA of human

immunodeficiency virus type 1 (HIV-1) were shown to inhibit viral replication in some cell culture experiments. Intracellularly expressed ribozymes conferred resistance to incoming HIV-1. When $CD4^+$ lymphocytes from HIV-1 infected donors were transduced with an anti-HIV ribozyme vector and subsequently expanded, viral replication was delayed by two to three weeks.[25]

Hepatitis delta virus and varkud satellite ribozyme

The hepatitis delta virus (HDV) ribozyme is found in a satellite virus of hepatitis B virus, a major human pathogen.[27] It is the one which requires self-cleavage by closely related versions of a catalytic domain contained within the genomic and antigenomic RNAs. The two ribozymes are similar in sequence and structure, though the proposed common secondary structure differs from those of other, small, catalytic RNAs. It is an infectious agent that exits as a satellite RNA of the hepatitis B virus. Its genome is a single stranded circular RNA of 1700 nucleotides. The catalytic activity of HDV is more efficient in the presence of divalent cations.[28, 31]

Both the genomic and the antigenomic strand express cis cleaving ribozymes of ~85 nucleotides that differs in sequence but fold into secondary structures**.** There is strong evidence that the catalytic mechanism of the HDV ribozyme involves the action of a cytosine base within the catalytic centre as a general acid-base catalyst. The hepatitis delta ribozyme displays high resistance to denaturing agents like urea or formamide. The varkud satellite ribozyme is another important catalyst that is a 154 nucleotides long catalytic entity that is transcribed from a plasmid discovered in the mitochondria of certain strains of neurospora.[44] Table 1 shows some of the most common naturally occurring ribozymes with their respective catalytic activities and common organisms in which they are found.[28]

Table 1: The world of naturally occurring ribozymes [28]

Catalytic RNA motifs	Catalytic reactions	Organisms
Group I introns	RNA splicing	Tetrahymena
Group II introns	RNA splicing	Plants, fungi, bacteria
Hammerhead	RNA cleavage	Plants pathogen
Hairpin	RNA cleavage	Plants pathogen
M1subunit of RNaseP	RNA cleavage	Eubacteria
Hepatitis delta virus	RNA cleavage	Human pathogen
Varkud satellite	RNA cleavage	Neurospora

Other ribozymes

Apart from the aforementioned ribozymes, there are different types of ribozymes that have been studied and characterized more recently. For instance, the glmS ribozyme is a recently discovered ribozyme that is unique in the world of naturally occurring ribozymes in two respects. First, it is a ribozyme that is also a riboswitch. Second, the regulatory effector of the ribozyme, glucosamine-6-phosphate, is actually a functional group that binds to the ribozyme active site and participates in the acid-base catalysis of RNA self-cleavage.[45] The glmS ribozyme is derived from a self-cleaving RNA sequence found in the 50-untranslated region of the glmS message; it cleaves itself, inactivating the message, when the cofactor glucosamine-6-phosphate binds. Glucosamine-6-phosphate production is thus regulated in many gram-positive bacteria via this ribozyme-mediated negative-feedback mechanism. The structure of the glmS ribozyme is thus of particular interest both as a riboswitch and as an unusual catalytic RNA. As it is known

to occur only in gram-positive bacteria, it is also a potential antibiotic target.[34, 45, 46]

Catalytic Activities of Ribozymes

Ribozymes are RNA molecules endowed with catalytic activity and capable of cleaving mRNA molecules in a sequence specific, catalytic manner. They contain sequences for selective ligation with target mRNAs which confers upon them high specificity. They also contain sequences that perform cleavage reactions with the target mRNA. By modifying the substrate recognizing sequences, ribozymes can be specifically tailored for the suppression of particular genes. A large variety of gene products have been targeted successfully using this strategy.[13, 47] The predominant activity found in naturally-occurring ribozymes is the ability to splice or cleave RNA molecules in a sequence-specific manner. Sequence specificity results from the base pairing of ribozyme sequences with nucleotides near the cleavage site of the target RNA. Ribozymes function in an intramolecular (cis) reaction to splice or cleave their own RNA sequence, and they can also function in trans to cleave another RNA molecule. Because of their sequence specificity, ribozymes show promise as therapeutic agents to down-regulate a given RNA species in the background of cellular RNAs. Specifically, the mRNA coding for a protein associated with a disease state may be selectively cleaved as shown in Figure 2. This cleavage event renders the mRNA untranslatable and attenuates the protein's expression.[23, 48]

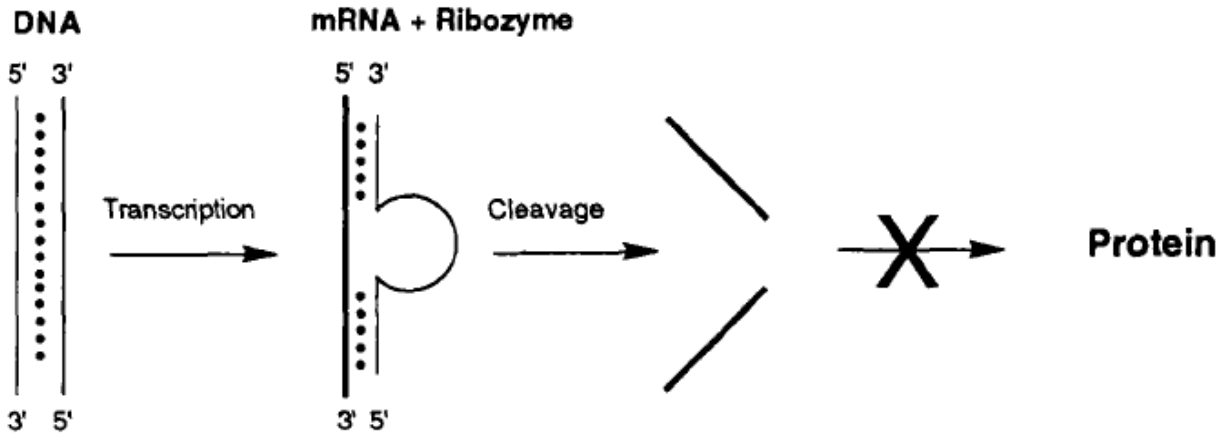

Figure 2: Messenger RNA cleavage by a ribozyme [48]

The most fascinating property of RNA is the potential to both carry information and perform catalysis within the same molecule. Despite their relative chemical instability, RNA molecules remain the most suited biopolymers for building self-sustained biochemical systems. Thus, the real challenge is the development of ribozymes that have processive ligation or polymerization activities or that have the capability of coupling a catalytic function to a molecular switch. Building such molecules will certainly open the way to the development of artificial RNA-based networking systems. More over the development of ribonucleoproteins RNA associated with small peptides appears to be the more promising route for catalysis of a broader range of chemical reactions.[21, 49, 50]

Production of Ribozymes

Design of ribozymes

Successful use of ribozymes to knockdown target gene expression is dependent on a number of factors, including target site selection as well as ribozymes gene delivery, expression, stability and intracellular localization.[8] The two types of ribozymes that have been used most extensively are hairpin and hammerhead ribozymes.[12, 51] Each has its own minimal target sequence requirements, the hairpin ribozyme requiring a GUC at the site of cleavage, whereas the hammerhead ribozymes requires NUH (where

N denotes any base and H denotes A, C,or U). Not all target sites, however, are accessible for cleavage; secondary structures, binding of proteins and nucleic acids and additional esoteric factors will influence ribozymes activity. To ensure success, one should plan to analyze several different ribozyme sites within the same mRNA to identify the sites that are the most susceptible to *in vivo* cleavage. One strategy for enhancing activity is to design 'facilitator' molecules: short RNA or DNA fragments complementary to the sequences flanking the ribozyme site. Binding of the facilitators can open up target secondary structure to allow ribozyme binding. Some have taken site selection one step further by using random ribozyme libraries to select for the most available sites in a target.[11, 51]

For designing a ribozyme, one first selects a region in the target RNA containing NUX, where N stands for any nucleotide, U represents uridine and X is any nucleotide except guanosine. Two stretches of antisense nucleotides 6-8 nucleotides long that flank the 21 nucleotide sequence forming the catalytic hammerhead between them are then designed based on the target sequence surrounding the third nucleotide (X) of the target triplet. Hairpin ribozyme design employs the same approach except that the catalytic core is larger (34 nucleotides) and the ribozyme targeting domains require more specificity. Hairpins recognize the sequence NNYNGUCNNNNNN, where N is any nucleotide and Y is a pyrimidine. The underlined target bases (NGUC) are not base-paired with the ribozyme.[27, 52] The ability to design ribozymes against selected RNA targets has expanded their therapeutic potential.[51] Hammerhead ribozymes can be modified in their binding arms to be complementary to any target RNA which contains a UH (where H is any nucleotide except guanosine). The optimum length of the binding arms appears to be 7/7 nucleotides on the 3'- and 5`- ends respectively.[27, 53, 54]

Modification of ribozymes

The discovery of cis-cleaving RNA by Czech *et al*. in the early 1980's and the subsequent research leading to trans-cleavage made possible the development of ribozymes as a new class of therapeutics and biological tools. Synthetic ribozymes comprised of all ribonucleotides are susceptible to endo- and exonuclease breakdown. To overcome the stability, substrate specificity and cell permeability limitations imposed by all-ribonucleotide ribozymes, much effort has been devoted to the chemical modification of ribozymes while maintaining their ability to cleave substrate mRNA.[55, 56] The appropriate modifications of the backbones ribonucleotides and the cumulative research over the years have delineated a number of properties essential for ribozymes to demonstrate better catalytic activity.[55] Some of the most common modified ribozymes are discussed as follows;

Catalytic Antisense RNAs

The extension of ribozyme substrate recognition arms was the first modification aimed at achieving optimal association between ribozymes and their substrates. This resulted in a new class of ribozymes called catalytic antisense RNAs. These extended arms help ribozymes gain access to target sequences in long RNA substrates. A similar strategy, based on the use of facilitators, pursues the same aim without having to modify ribozymes. This method makes use of external oligonucleotides specifically designed to bind the sequences flanking the target site in the substrate RNA.[31]

Retroviral Nucleocapsid Proteins

The interaction or association of RNA molecules with cellular factors such as proteins is an important difference between *in vitro* and *ex vivo* assays. Moreover, essential biological processes are mediated by retroviral nucleocapsid protein complexes. These associations are important for achieving RNA stability and RNA catalysis. Several proteins have been characterized that bind to hammerhead ribozymes and act as RNA chaperones, promoting the unwinding of the RNA substrate, strand-exchange and annealing, and co-localization of the ribozyme with its specific target. This methodology has been shown to be

useful to overcome limitations in specificity and turnover.[31]

Allosteric Ribozymes

Allosteric ribozymes are a modified type of catalytic RNA whose activity can be regulated by external factors. Control of their cleavage activity is achieved by binding an effector molecule to an allosteric binding site. This strategy can be exploited in situations in which a regulation of RNA-cleavage activity by an external factor is desirable, e.g., cleavage of mRNAs or viral RNAs in infected cells. Maxizymes are allosteric ribozymes whose activity is regulated by a specific sequence in the target mRNA. This sequence, called the sensor sequence, is physically different from the cleavage site. They were first developed as ribozymes to cleave target RNA at two different sites. They are composed of two minizymes (deleted hammerhead ribozymes) which are active only when they form a dimer.[31, 57, 58] The maxizyme is an allosterically controllable ribozyme with powerful biosensor capacity that appears to function even in mice. Its biosensor functions allow specific inhibition of expression of a gene of interest only, without any effect on the normal mRNA. By modulating the sequences of sensor arms, it is possible to adjust the activity of the maxizyme.[59, 60]

Delivery of Ribozymes to Cells

In order to make the therapeutic applications of these enzymes a real, the development of safe, effective and tissue specific formulations and delivery systems is one of the central issues.[13, 61] More recently, both the viral and the non-viral vectors have been developed for delivery of ribozymes. Among these, the viral based strategies offer a promising potential for cell specific delivery of ribozymes as a result of the naturally evolved ability of viruses to deliver genes to cells.[10, 13, 61]

Viral vectors

Viral-based vectors are continuously reviewed in the context of gene therapy. The same considerations apply for the use of such vectors for delivery of ribozymes. Delivery of genes encoding ribozymes (i.e. endogenous delivery of ribozymes) using viral gene therapy vectors is the principal alternative to direct delivery of ribozymes (i.e. exogenous delivery of ribozymes) to tissues for inhibition of gene expression.[9, 54, 61] The most widely used viral vectors are the retroviral vectors, [10, 61] adenovirus vectors [11, 62] and adeno-associated virus vectors.[9, 11, 35, 61] The viral vectors have been modified to eliminate their toxicity and maintain their high gene transfer capability. Especially the replication-competent or non-replicating viruses with viral coding sequence partially or completely replaced by therapeutic genes have been the focus of many basic research and clinical studies. Due to their safety compared to replicating viral vectors, these vectors have been widely investigated in vaccine development as well as in gene replacement therapy.[63, 64] Despite the high transfection efficiencies attainable using viral vectors, they are limited in the size of plasmid they can transport and are hampered by manufacturing difficulties, limited targeting ability and safety concerns.[65, 66] Thus a lot has to be done to develop vectors for delivery of ribozymes to cells that feature simplicity, reproducibility, reduced immunogenicity and improved targeting ability.

Non-viral vectors

The problems associated with the toxicity and immunogenicity of viral vectors has encouraged researchers to increasingly focus on non-viral vectors as alternative delivery systems to the use of viral vectors.[67, 68] Non-viral vectors are mainly used for exogenous delivery of ribozymes.[54] Although their low efficiency remains a major drawback, non-viral vectors have many advantages over viral vectors, including ease of production, lower toxicity and immunogenicity and lower cost.[68] Moreover, non-viral vectors provide various means of physical protection of the ribozymes for safe delivery into the cells. Protection from the renal filtration system, systemic enzymatic degradation and immunological processes is necessary for the ribozymes to reach the targeted cell and exert their ultimate effect.[67, 69] Most of the non-viral

vectors that have been developed so far are generally cationic in nature.[70] Cationic lipids,[71, 72] cationic polymers,[73] dendrimers, cationic peptides[74] and cationic liposomes [62, 74, 75] are by far the most widely used vectors for exogenous delivery ribozymes in to cells. Besides the cationic non-viral vectors, there is wide interest in the potential of physical such as electroporation[76] or mechanical means like microinjection [60, 62] for enhancing the efficiency of ribozymes delivery in to cells. In particular, microinjection is a simple mechanical means used to direct the ribozyme to a specific intracellular compartment using a needle roughly 0.5 to 5 micrometers in diameter and that can penetrate the cell membrane and/or the nuclear envelope of cells.[62]

Applications of Ribozymes

Therapeutic applications

Ribozymes are RNA molecules endowed with catalytic activity and capable of cleaving mRNA molecules in a sequence specific, catalytic manner. They contain sequences for selective ligation with target mRNAs which confers upon them high specificity. They also contain sequences that perform cleavage reactions with the target mRNA. By modifying the substrate recognizing sequences, ribozymes can be specifically tailored for the suppression of particular genes. A large variety of gene products that are responsible for different pathological conditions have been targeted successfully using this strategy.[13, 77]

Like other antisense therapeutic agents, ribozymes offer promising potential in the treatment of cancer, infectious diseases and genetic disorders.[13, 67, 78-80] Currently, there are few examples of ribozymes that are under clinical trails for application to human diseases such as for AIDS and cancer. The first clinical trial using a ribozyme targeted human immunodeficiency virus 1(HIV-1) had shown that an anti-HIV-l gag ribozyme that was delivered intracellularly can interfere with both pre-integration and post-integration events of the HIV replication cycle, by cleaving incoming viral RNA and transcribed mRNAs.[14, 15]

The recently defined mechanisms of cellular proliferation, as well as the recent explosion of the biology of human cancer at molecular level have helped scientists to identify different molecular targets for anticancer ribozymes discovery and development. Some of the targets that have attracted attention are those genes involved in signal transduction cascades, such as genes for expression of growth factors and their corresponding receptors, genes for the induction or progression of tumors (i.e. different oncogenes), genes important in tumor angiogenesis and the genes important in cancer therapy (such as MDR-1-multidrug resistance).[8, 31] Only two clinical trials are in progress to evaluate the potential of chemically modified hammerhead ribozymes to fight cancer. The first, ANGIOZYME (Sirna Therapeutics, Inc.), a hammerhead ribozyme that targets mRNA that encodes VEGF, is being examined in a phase II trial for treatment of metastatic colorectal cancer.[12, 25, 31, 81] The other ribozyme in cancer clinical trials, HERZYME, is of a class of modified ribozymes (so called Zinzymes) that has high catalytic activity under physiological Mg^{2+} conditions and targets the mRNA that encodes human epidermal growth factor-2, and is in phase I clinical trials to determine toxicity and efficacy in breast and ovarian cancer patients.[12, 82]

Ribozymes as tools to study gene function and in target validation

Ribozymes can be used to study the function, regulation and expression of genes.[4] They provide a unique tool for understanding gene function because they allow one to assess cellular responses to a rapid ablation of target gene expression.[83] They are also unique in that they can inactivate specific gene expression, and thereby can be used to help identify the function of a protein or the role of a gene in a functional cascade. The use of ribozymes for target validation is critical for both basic biological research and drug discovery and development. Compared to other means of target validation such as use of transgenic animals, ribozymes offer specificity and ease of design and usage.[12, 84]

As researchers adopt the recent improvements in ribozyme technology, the effectiveness of ribozymes in target validation will be certainly improved. However, application of more conventional ribozyme technology has already yielded important information regarding proposed drug targets for the treatment of cancers, arthritis, viral infections and other diseases. For example, several groups have utilized ribozymes to increase sensitivity to agents that induce apoptosis. Other groups have used ribozymes to decrease both the rate of cell proliferation and the growth of tumors in nude mice. Especially, validation of other cellular genes as potential targets for treatment of viral infection remains an active area of research.[85, 86]

SUMMARY

Ribozymes are nucleic acid enzymes that are discovered by Thomas Czech and Sidney Altman who were awarded the Nobel Prize in Chemistry in 1989. The different classes of ribozymes may play an important role as therapeutic agents, and for applications in functional genomics and gene discovery. Although they are among the newly emerging groups of biopharmaceuticals, a lot of interesting future perspectives had been generated. As a new class of pharmaceutically important antisense compounds, ribozymes offer promising potential in the treatment of cancer, infectious diseases and genetic disorders. Currently, there are few examples of ribozymes that are under clinical trails for application to human diseases such as AIDS and cancer.

In order to make the therapeutic applications of these enzymes a real, the development of safe, effective and tissue specific delivery systems is one of the central issues. More recently, both the viral and the non-viral vectors have been developed for delivery of ribozymes. Among these, the viral based strategies offer a promising potential for cell specific delivery of ribozymes. There is hope that these approaches, together with more precisely targeted expression systems will make ribozymes novel pharmaceutical agents for the treatment of cancer and viral infections in the coming years.

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