Ribozyme- and Deoxyribozyme-Strategies for Medical Applications

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Abstract: Ribozymes are catalytically active nucleic acids capable of site-specific cleavage of target mRNAs. They have widely been employed as tools in functional studies and for therapeutic purposes. Different classes of ribozymes distinguished by size and mechanism of action have been discovered in natural systems or obtained by *in vitro* selection. After an introduction to different types of ribozymes with a special focus on the hammerhead and hairpin ribozyme, major challenges in the process of developing ribozymes for medical purposes will be described in the present review. Subsequently, examples of ribozyme applications in animal models for various diseases including cancer, viral infections, rheumatoid arthritis and cardiovascular diseases will be given. The course of phase I and II clinical trials with ribozymes designed to treat patients with virus infections or cancer will be outlined. Finally, the current significance of ribozymes will be discussed in the light of the emergence of new powerful anti-mRNA strategies, particularly RNA interference (RNAi).

Key Words: Ribozyme, Deoxyribozyme, DNAzyme, DNA enzyme, Hammerhead ribozyme, Group I intron, Hairpin ribozyme, catalytically active nucleic acids

INTRODUCTION

In the early 80s, Thomas Cech and his co-workers made the surprising discovery that RNA molecules are capable of catalysing reactions even in the absence of any protein component [1]. These ribonucleic acids with enzyme-like activity were named ribozymes. Since then, several classes of ribozymes have been discovered in natural systems, most of which catalyse intramolecular splicing or cleavage reactions. Furthermore, the three-dimensional crystal structure of the ribosome revealed that even one of the central cellular processes, the peptidyl transfer reaction of newly synthesised proteins, is catalysed by a ribozyme [2]: Only RNA, but no protein, is located in the vicinity of the catalytic centre of the ribosome.

These fundamental findings about the functional properties of RNA were not only of great academic interest, but also opened up new roads for functional investigations and therapeutic applications. Ribozymes were soon thought to be promising new tools to combat viral diseases, to control oncogenes and to inhibit the expression of deleterious genes. Since most of the naturally occurring ribozymes catalyse self-splicing or self-cleavage reactions, it was necessary to convert them into RNA enzymes which can cleave or modify target RNAs without becoming altered themselves (reactions '*in trans*'). In addition, *in vitro* selection techniques have extended the range of catalytically active nucleic acids even to DNA oligonucleotides with enzymatic properties, named DNA enzymes, deoxyribozymes or DNAzymes.

The current review will focus on the development of ribozymes for medical purposes. Ribozymes are widely used tools for functional studies. In addition, they can be employed to investigate the value of new targets in the process of drug development (target validation). Their properties also make them potential therapeutic agents themselves. First, the different types of ribozymes will be described with special emphasis on classes that have already been used in medically relevant applications. Then, major challenges in the process of ribozyme development will be outlined, and finally some examples of successful *in vivo* experiments with ribozymes will be given, leading to a discussion of clinical trials with respect to the potential of ribozymes as compared to alternative approaches.

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CLASSES OF RIBOZYMES

Catalytic RNAs 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 (VS) RNA as well as artificially selected nucleic acids. 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. Only a brief introduction into the different types of ribozymes can be given here; the interested reader is referred to the exhaustive review by Tanner [3] and to the more recent overview article by Doudna and Cech [4] for further details.

Group I Introns

A self-splicing intron in pre-ribosomal RNA of the ciliate *Tetrahymena thermophilia* was the first catalytic RNA molecule to be discovered in the early 80s [1]. Since then, further examples of group I introns have been found to interrupt genes for rRNAs, tRNAs and mRNAs in a wide

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range of organelles and organisms, including the nuclei of protozoa, mitochondria of fungi, chloroplasts of algae as well as bacteria and their phages. Group I introns perform a splicing reaction by a two-step transesterification mechanism (Fig. (**1A**)): The reaction is initiated by a nucleophilic attack of the 3'-hydroxyl group of an exogenous guanosine cofactor on the 5'-splice site. Subsequently, the free 3'-hydroxyl of the upstream exon performs a second nucleophilic attack on the 3'-splice site to ligate both exons and release the intron.

Substrate specificity of group I introns is achieved by an Internal Guide Sequence (IGS). A uridine at position -1 relative to the cleavage site was found to be highly conserved. It forms a functionally important G•U wobble base pair with the IGS. The catalytic centre of group I introns is built of two structural domains plus a specific binding site for the guanosine cofactor that initiates the reaction. A high resolution X-ray crystal structure of a complete group I bacterial intron together with both the 5'- and the 3'-exons and an organized active site with metal ions has been reported only recently [5].

The catalytically active site for the transesterification reaction resides in the intron which can be re-engineered to catalyse reactions *in trans.* It has been demonstrated that modified group I introns are suitable for the correction of deficient mRNAs (Fig. (**1B**)): A *trans*-active group I intron was used to repair mutant β-globin RNA in erythrocyte precursors from patients with sickle cell anaemia by replacing the mutated part of the β^s-globin RNA by the γ-globin-3'- exon. The resulting γ-globin was no longer susceptible to the pathological polymerisation of haemoglobin [6]. In a subsequent study, an expression cassette was developed for gene transfer of the ribozyme encoding sequence into mammalian cells to correct deficient β-globin RNA [7].

Additional attempts have been reported to address diseases caused by trinucleotide repeat expansions, including Huntington's disease and myotonic dystrophy. To this aim, a *trans*-active group I intron was designed to modify the 3'-end of the human myotonic dystrophy protein kinase mRNA [8]. The engineered ribozyme was shown to replace the large target transcript with twelve repeats by a smaller fragment with only five repeats *in vitro* and in mammalian cells. Taken together, these studies demonstrate that ribozymes are not only capable of cleaving deleterious RNAs, as will be outlined in detail below, but can also be employed to correct mutated RNAs which occur in the context of numerous genetic diseases.

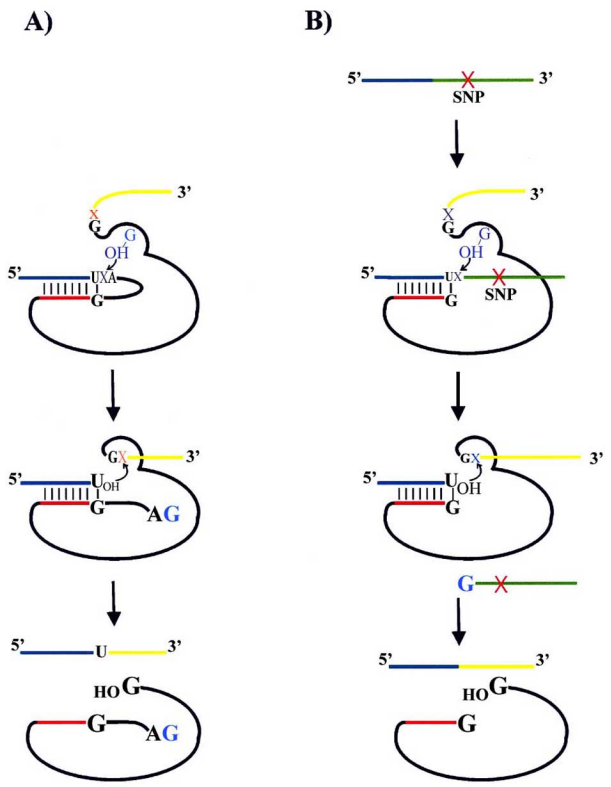

Fig. (1). Schematic representation of the catalytic action of group I introns. (A) *cis*-splicing reaction. Red: internal guide sequence; blue: 5'-exon; yellow: 3'-exon. The reaction is initiated by the attack of an external guanosine co-factor on the 5'-splice site (up), followed by joining of the exons by nucleophilic attack of the upstream exon on the 3'-splice site (middle), and dissociation of the joined exons from the intron (down). (B) Therapeutic application of a *trans*-splicing group I intron. A mutant exon (green) containing a single nucleotide polymorphism (SNP) is exchanged for a corrected form of the same exon (yellow).

Group II Introns

Group II introns have also been found in bacteria and in organellar genes of eukaryotic cells, but they are less widely distributed than group I introns. They catalyse a self-splicing reaction that is mechanistically distinct from the mode of action described above in that group II introns do not require a guanosine cofactor. Instead, the 2'-hydroxyl of a specific adenosine at the so-called branch site of the intron initiates the reaction by a nucleophilic attack on the splice-site to form a lariat-type structure. 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 intronless allele on the DNA level by reverse splicing and reverse transcription, a process called retrohoming. It has been demonstrated that group II introns can be redirected to insert themselves into therapeutically relevant DNA target sites in human cells [9]. This emerging technology may ultimately be used to disrupt deleterious genes on the DNA level or to insert new genomic information specifically into target genes.

RNase P

RNase P is a ubiquitous enzyme that acts as an endonuclease to generate the mature 5'-end of tRNA precursors. In bacteria, RNase P exists as a ribonucleoprotein complex, consisting of a long RNA, typically 300-400 nucleotides in length, and a small protein of approximately 14 kDa. The discovery that the RNA component of the enzyme alone possesses catalytic activity *in vitro* provided the first example of an RNA-based catalyst that acts *in trans* on multiple substrates [10]. RNase P can be considered to be

the only true naturally occurring *trans*-cleaving RNA enzyme known to date. 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.

For gene targeting applications, cellular RNase P can be recruited to cleave a specific mRNA of choice. For this purpose, an External Guide Sequence (EGS) is exogenously introduced into cells or expressed intracellularly. EGSs are oligonucleotides designed to hybridise to the target region in such a way that the resulting double stranded RNA structurally resembles the top portion of the natural substrates of RNase P (for details of this method, see [11]). This technology has been successfully employed to down-regulate the expression of viral genes in cell culture and to interfere with the replication of herpes-, hepatitis-, human immunodeficiency-, influenza- and cytomegalovirus (see also [12] and references cited therein).

In a variation of this approach, a guide sequence has been covalently linked to the RNA component of *Escherichia coli* RNase P, referred to as the M1 ribozyme, to yield M1GS RNA [13], a sequence-specific ribozyme. M1GS RNAs have been employed to cleave various viral and oncogenic targets in cell culture. For details on the usage of RNase P ribozymes for therapeutic purposes, the reader is referred to recent reviews [14-15].

Since bacterial and eukaryotic RNase P differ fundamentally in structure and because the enzyme is essential for cell viability, RNase P has also been considered a target molecule for antisense-based approaches against multi-resistant bacterial pathogens. Oligonucleotides that efficiently block the RNA portion of RNase P from Escherichia coli have recently been identified [16].

Hammerhead Ribozyme

The hammerhead ribozyme is the most intensively studied and applied ribozyme to date. It has been found in several plant virus satellite RNAs, viroids and transcripts of a nuclear satellite DNA of newt. This ribozyme is 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. The development of hammerhead variants that cleave target RNA molecules *in trans* [17, 18] was a major advancement that made possible the use of ribozyme technology for practical applications. The hammerhead ribozyme motive that has widely been applied since then comprises three helical sections connected via a three-way helical junction (Fig. (**2A**)).

The crystal structure of the hammerhead ribozyme was the first example of an atomic structure of a catalytic RNA molecule to be determined. Interestingly, the three helices of the ribozyme are arranged in a Y shape rather than in a hammerhead-like structure [19, 20]. Further progress in unveiling details of the three dimensional structure of the hammerhead ribozyme and its relationship to folding and catalysis has exhaustively been described by Wedekind and McKay [21].

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. Usually, *in vitro* investigations of the hammerhead ribozyme are performed in the presence of 10 mM Mg^{2+} [22], whereas submilimolar Mg^{2+} concentrations exist in cells. In an attempt to overcome the need for high magnesium ion concentrations for optimal ribozyme activity, a minimised ribozyme was obtained by *in vitro* selection that maintained high catalytic activity at low magnesium ion concentrations [23].

Recently, it has been demonstrated that regions outside the catalytic core of the hammerhead ribozyme greatly enhance enzymatic activity under physiological conditions [24, 25]. In natural hammerhead ribozymes, an additional loop at stem I, which is lacking in the artificial variants, interacts with the loop at stem II. This loop-loop interaction drastically enhances catalytic activity at low magnesium ion concentrations and intracellular efficiency of the cleavage reaction.

The influence of the additional loop on magnesium ion-induced folding of the ribozyme has subsequently been analysed in more detail using Fluorescence Resonance Energy Transfer (FRET) [26]: The auxiliary elements enable folding of the ribozyme in a single step at µM concentrations of magnesium ions. Disruption of the loop-loop interaction, however, leads to a two-step folding mechanism dependent on high magnesium ion concentrations. It therefore seems as if researchers went too far in their attempt to reduce the size of the hammerhead ribozyme and removed elements that are vital for efficient folding under physiological conditions. Nevertheless, the truncated ribozyme motive depicted in Fig. (**2A**) has widely and successfully been applied for functional studies in cell culture and in animal models and has even entered the stage of clinical testing as will be outlined below.

Hairpin Ribozyme

Another catalytic RNA domain found in pathogenic plant virus satellite RNAs is the hairpin motive. 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. Depending on reaction conditions, the hairpin ribozyme may also favour RNA ligation over cleavage [27]. Three different hairpin ribozymes have so far been found in nature, of which the one from satellite RNA associated with tobacco ring spot virus (sTMSV) is the best characterised [28]; for a recent review see [29]. The other two hairpin motives, isolated from different satellite viruses, show sequence variations that preserve the overall structure of the molecule [30].

In naturally occurring hairpin ribozymes, the catalytic entity is part of a four-helix junction. A minimal catalytic motive containing approximately 50 nucleotides has been identified that can be used for metal-ion dependent cleavage reactions *in trans* (Fig. (**2B**)). It consists of two domains, each harbouring two helical regions separated by an internal loop, connected by a hinge region. One of these domains

results from the association of 14 nucleotides of a substrate RNA with the ribozyme *via* base-pairing.

In recent work, crystal structures of hairpin ribozymes bound to a modified, uncleavable substrate molecule, a transition state mimic, and a product complex have been obtained [31, 32]. These structures and related biochemical data show that the active site contains no tightly bound, well-ordered metal ions at the site of cleavage. Catalytic activity of the hairpin ribozyme thus results from distortion and precise orientation of the substrate RNA and general acid-base catalysis by neighbouring nucleotides without involvement of metal ions in catalysis [29]. The requirement for metal ions may be explained by a significant role in the folding process [33, 34]. It has also been shown that catalytic activity of the hairpin ribozyme can be supported by spermine, the major polyamine in eukaryotic cells [35].

Hairpin 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 cell culture experiments [36, 37]. Intracellularly expressed ribozymes conferred resistance to incoming HIV-1 [38]. 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 [39]. Additionally, it was shown that retrovirally expressed anti-HIV ribozymes confer a selective survival advantage on lymphocytes exposed to HIV-1 infection *in vitro* [40]. These promising results prompted researchers to conduct a phase I clinical trial to establish safety, feasibility and prospective efficacy of hairpin-ribozyme gene therapy against HIV-1 infection (*vide infra*).

An attempt has also been made to exploit the potential of hairpin ribozymes to act as sequence-specific ligases for RNA manipulation. In this approach, two hairpin motives were joined in such a way that a substrate molecule can be bound and cleaved at two predetermined sites. The excised fragment is less tightly bound than the rest of the substrate RNA and dissociates easily. Subsequently, an added "repair oligonucleotide" hybridises to the complex of ribozyme and cleaved substrate and is ligated into the RNA molecule in place of the excised fragment [41]. While this approach has until now been successfully employed only *in vitro*, such ribozymes may ultimately be of use in repairing defective RNAs.

Hepatitis Delta Virus and Varkud Satellite Ribozymes

Two more small catalytic ribozymes have been discovered, whose use in therapeutic approaches, however, is limited for the time being. Detailed reviews on the properties of the Hepatitis delta virus ribozyme [42] and the Varkud satellite ribozyme [43] have been published recently.

The hepatitis delta virus ribozyme was found in a satellite virus of hepatitis B virus, a major human pathogen [44]. Both the genomic and the antigenomic strand express *cis*-cleaving ribozymes of ~85 nucleotides that differ in sequence but fold into similar secondary structures (Fig. (**2C**)). A crystal structure of the ribozyme has been determined [45], in which five helical regions are organised by two pseudoknot structures. There is strong evidence that the catalytic mechanism of the hepatitis delta virus 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. *Trans*-cleaving derivatives of this ribozyme have been developed [e.g. 46].

The Varkud Satellite (VS) ribozyme is a 154 nucleotides long catalytic entity that is transcribed from a plasmid discovered in the mitochondria of certain strains of Neurospora [47]. The VS ribozyme is the largest of the known nucleolytic ribozymes and the only one for which there is no crystal structure available to date. The global structure has been determined by solution methods, particularly Fluorescence Resonance Energy Transfer (FRET), which revealed a formal H shape of the five helical segments as depicted in Fig. (**2D**) [48]. Binding of the substrate is determined primarily by tertiary interactions.

Although all small nucleolytic ribozymes perform similar reactions, giving rise to the same reaction products, they differ considerably in structure and mechanism. They can therefore be viewed as different evolutionary solutions to the same problem [29].

DNAzymes

While ribozymes consisting of RNA are distributed widely in nature, no DNA molecules with catalytic activity seem to have evolved. This lack of DNAzymes in nature, however, is not indicative of a fundamental incapability of DNA to engage in catalysis. Using *in vitro* selection methods, various oligodeoxynucleotides have been obtained that catalyse chemical reactions (reviewed in [49]). One of the most active DNAzymes is the RNA-cleaving "10-23" deoxyribozyme shown in Fig. (**2E**) which was selected by Santoro and Joyce in 1997 [50]. Its name is derived from the fact that it was found as the 23^{rd} clone in the 10^{th} round of selection. The 10-23 DNAzyme cleaves its RNA substrate in a reaction dependent on divalent ions to yield a 2'-3'-cyclo phosphate and a free 5'-hydroxyl group. Neither the detailed mechanism of the cleavage reaction nor the crystal structure of the active conformation have yet been solved.

10-23 DNAzymes consist of a catalytic core of 15 nucleotides and two substrate binding arms of variable length and sequence. We have recently investigated into the sequence requirements of the catalytic centre and found an unexpectedly high degree of flexibility for base substitutions in the central part of the core, whereas the highly conserved boarders are likely to be directly involved in forming the catalytic site [51]. These findings were the basis of the development of a nuclease resistant DNAzyme with improved catalytic activity (see below). The kinetic properties of 10-23 DNAzymes compare favourably to those of RNA ribozymes [52, 53], and in a comparative study of 10-23 DNAzymes and hammerhead ribozymes directed against the same mRNA, the most active enzyme found was a DNAzyme [54].

Another advantage of ribozymes consisting of DNA is the relative ease of synthesis and handling compared to RNA ribozymes. Moreover, DNA molecules are less susceptible to

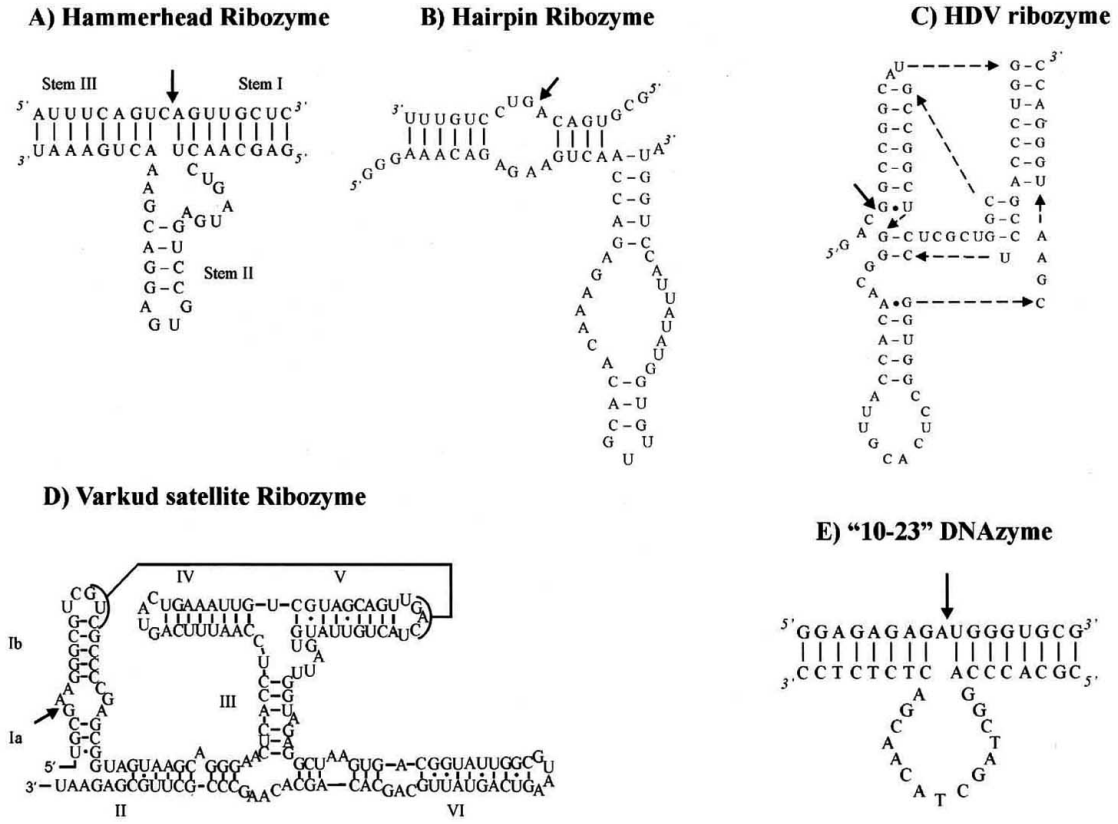

Fig. (2). Representation of the small nucleolytic ribozymes discussed in this review. Cleavage sites are indicated by solid arrows. Solid line in Varkud satellite ribozyme denotes tertiary interactions between loops. Of hammerhead and hairpin ribozymes, *trans*-cleaving forms are depicted. The DNAzyme (E) consists of deoxyribonucleotides.

degradation by nucleases in tissues and cell culture, making them considerably more stable for exogenous delivery in biological applications.

A variety of therapeutically relevant cellular and viral genes has been down-regulated by DNAzymes in cell culture experiments, an overview of which is given in [55]. For example, the 10-23 DNAzyme has been employed as an antiviral agent either directly targeting HIV-1 RNA [56] or preventing virus entry by downregulation of the CCR5 coreceptor [57]. Furthermore, DNAzymes have been developed to target cancer-related genes like the chimeric *BCR-ABL* oncogene [58, 59] or protein kinase C-α [60]. Their applicability for *in vivo* approaches has also been shown (see below).

DEVELOPMENT OF RIBOZYMES FOR THERAPEUTIC PURPOSES

Target Site Selection

It is fundamental to the success of therapeutic ribozyme approaches that a section of the substrate molecule is identified that allows for optimal catalytic efficiency. Each type of ribozyme described above has its own requirements concerning the nucleotide sequence at the cleavage site. In the following section, we will focus on the problem of target site selection for small nucleolytic ribozymes which have primarily been employed for therapeutic applications.

Cleavage by *hammerhead ribozymes* occurs after NUH triplets, where N is any nucleotide, U is conserved, and H can be any nucleotide except G. GUC and AUC are processed most efficiently, while other combinations yield lower reaction rates. In more recent work, the NUH rule has been somewhat relaxed as hammerhead ribozymes were found to also cleave after triplets containing A or C at the second position, albeit more slowly [61]. Moreover, Eckstein and colleagues have employed *in vitro* selection methods to obtain a purine-specific hammerhead ribozyme bearing nucleotide exchanges and two deletions compared to the consensus sequence. This modified enzyme cleaved 3' of both G and A and also tolerated the substitution of the central uridine by C or G [62]. *Hairpin ribozymes* cleave 5' of the guanosine in NGUC sequences. A systematic investigation of all possible substrate variants has revealed, however, that some other sequences of the form NGNN are also prone to cleavage at reasonable rates [63]. The *10-23 DNAzyme* cleaves its substrate RNA between a purine and a pyrimidine base, displaying the highest efficiency at AU and GU sites. The incorporation of inosine bases into the enzyme has been shown to improve cleavage at AC and GC sites [64].

According to the substrate requirements described above, any mRNA can be expected to contain a number of possible reaction sites for sequence-specific cleavage by ribozymes. The vast majority of ribozymes designed to interact with their respective RNA substrates, however, is found to show

little or no activity in a cellular environment. This discrepancy is due to the fact that long RNAs are highly structured molecules that form complex secondary and tertiary structure motifs and engage in manifold interactions with cellular proteins. Such higher order target structures may interfere with ribozyme activity by precluding base pairing between substrate and ribozyme or by steric hindrance, impairing the formation of the catalytically active ribozyme structure at the reaction site. Thus, up to 90% of the putative cleavage sites have been shown to be inaccessible to antisense-based molecules like oligodeoxy-nucleotides, DNAzymes and ribozymes [54, 65, 66].

Numerous efforts have focused on identifying efficient cleavage sites for catalytically active oligonucleotides [67, 68]. An approximation of the overall topography of a folded substrate RNA can be obtained using computer-based modelling. When challenged with long RNAs, however, structure prediction has severe limitations. A variety of experimental procedures has thus been employed to define accessible target sites.

A comprehensive picture of the target RNA structure can be obtained using oligonucleotide libraries and RNase H. Cleavage of the substrate molecule is induced only upon binding of an oligonucleotide to an accessible region of the RNA. Subsequently, the length of the cleaved products is analysed using primer extension analysis [69, 70].

Instead of oligonucleotides, ribozyme libraries with randomised or semi-randomised substrate binding arms can be employed for mapping purposes. Such libraries have been generated for hammerhead and hairpin ribozymes by chemical synthesis or transcription from expression cassettes [71-73].

To identify appropriate external guide sequences for cleavage by RNase P, *in vitro* selection protocols have been developed [74]. For group I introns, a library containing randomised internal guide sequences can be used. Ribozymes directed against suitable sites perform a *trans*-tagging reaction, adding a short oligonucleotide tag to the 3'-end of the spliced substrate molecule [6].

To facilitate the laborious process of experimental screening of a target molecule for accessible target sites, Warashina *et al*. have developed a chimeric RNA molecule that brings together a hammerhead ribozyme and an RNA motif capable of recruiting cellular RNA helicases in order to melt down stable internal substrate structures. These RNA-protein hybrid ribozymes were shown to cleave target RNAs in cell culture experiments significantly better than customary hammerhead ribozymes [75].

In a recent study, we reported that the incorporation of 2'-O-methyl modified nucleotides or locked nucleic acids (LNA) into the binding arms of DNAzymes enhances their ability to compete with internal target structures. The modified nucleotides convey to the DNAzyme an increased affinity towards the target. Thus, we were able to cleave a number of seemingly uncleavable sites on highly structured RNA substrate molecules with high efficiency [76].

The affinity of a catalytically active nucleic acid towards its substrate can be adjusted by altering the length of the substrate binding region of the ribozyme. Although high affinity is usually desirable, an extended substrate binding region may cause problems regarding specificity and catalytic activity. Multiple turnover catalysis may be severely impaired if product release is slow due to strong binding to the ribozyme [22]. Ribozymes with short binding arms, however, may lack specificity. It has been estimated that a sequence of 15-17 nucleotides is sufficient to define a unique site in the human genome [77]. A database research is required anyway to exclude significant homology with other mRNAs. Thus, the length of the substrate binding region must strike a balance between specificity and unhindered product dissociation.

To increase ribozyme specificity, Taira and co-workers have employed allosterically controlled ribozymes, designated as maxizymes, which function as dimers and only cleave their target when they recognize an additional specific RNA sequence [78]. This approach was applied to target chimeric RNA for *PML-RAR* α that is found in patients with acute promyelocytic leukaemia (APL). Since there is no suitable target sequence near the junction site of the deleterious mRNA, ribozymes will cleave not only the chimeric RNA but also normal RNA. In contrast, the vector derived maxizyme was shown to specifically inhibit the expression of the chimeric gene *in vitro* and in cell culture.

Stability and Pharmakokinetics

The pharmacokinetic fate of a substance exogenously delivered into an organism results from characteristics like its stability in blood stream and tissues and its interaction with plasma proteins. These features are considered virtually independent of the nucleotide sequence, arising solely from backbone chemistry. Unprotected RNA molecules are almost instantaneously degraded by 3'-exonucleases and pyrimidine-specific endonucleases in human serum. A large number of nucleotide analogues more resistant to nucleolytic attack have been developed and evaluated for their usefulness in antisense technology applications (for a comprehensive review, see [79]). Some examples with relevance to ribozyme applications are depicted in Fig. (**3**) and will be discussed below.

To stabilise ribozymes, mainly modifications of the phosphodiester linkage and the 2'OH group of the ribose have been used. Substitution of one of the free oxygen atoms in the phosphodiester linkage by a sulfur atom yields phosphorothioate molecules [80]. For detailed reviews on pharmacokinetic properties and distribution of modified oligonucleotides, the reader is referred to excellent comprehensive reviews published recently [81, 82].

Phosphorothioates exhibit reduced target affinity and a high degree of non-specific binding to proteins in plasma and within tissues. This binding may be both advantageous and detrimental. On the one hand, plasma clearance of phosphorothioates and renal excretion have been found to proceed approximately 10fold slower than that of unmodified phosphodiester oligonucleotides [83]. Phosphorothioates are distributed widely to tissues after parenteral administration and are metabolised slowly with half-lives ranging from 20 to 120 hours in rats and monkeys, depending on the tissues and organs [81]. Adverse effects of

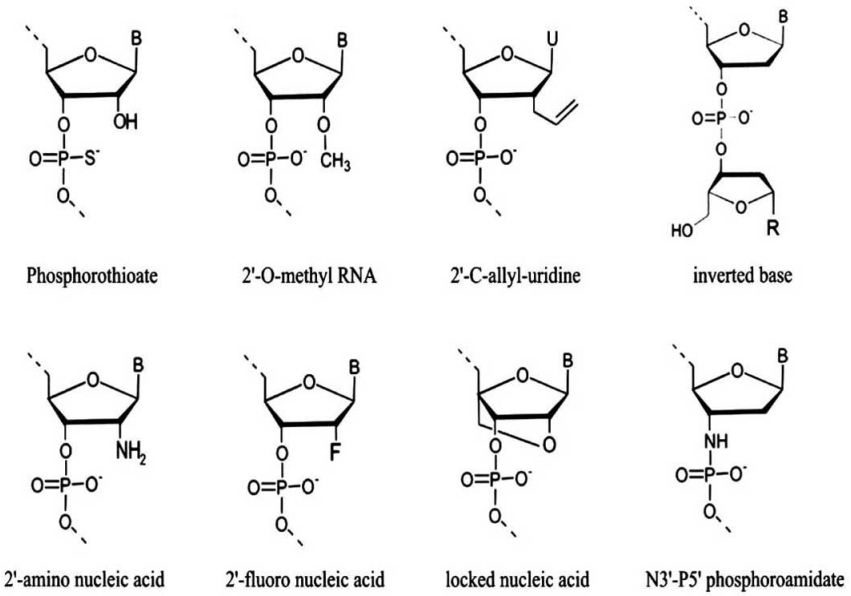

Fig. (3). Modified nucleotides used for the stabilisation of ribozymes. B denotes any nucleobase. In inverted bases, R can be any nucleobase, in an abasic sugar, it is OH.

non-specific protein binding, on the other hand, are unwanted side-effects and toxicity (summarized in [84]). Rapid infusion of phosphorothioate oligonucleotides into monkeys, for example, has caused negative cardiovascular effects, cumulating in cardiovascular collapse [85]. The doses used in clinical trials, however, were generally well tolerated, as will be outlined in a later section.

Some problems associated with phosphorothioate oligonucleotides can be overcome by using modifications at the 2'-position of the ribose. A large number 2'-modifications have been tested in antisense oligonucleotides, of which 2'-O-methyl and 2'-methoxyethyl RNA are the most widely used. Oligonucleotides bearing these modifications have been shown to be less toxic than phosphorothioates and display an enhanced affinity towards their target sequence. In addition, the bioavailability after oral and colorectal administration is significantly improved compared to phosphorothioates (summarised in [81]).

The introduction of modified nucleotides into catalytically active oligonucleotides is particularly challenging, since they might interfere with the three-dimensional structure and thereby reduce cleavage activity. Extensive work has been performed on the stabilisation of the hammerhead ribozyme without impeding catalytic activity. Beigelman and colleagues have used a combination of differently modified nucleotides to optimise the set-up of modified hammerhead ribozymes [86, 87]. The optimised molecule contains only five unmodified purine ribonucleotides in the catalytic centre. The 3'- and 5'-termini are protected by phosphorothioate linkages and an inverted 3'-3'-abasic sugar, respectively. Most other nucleotides are 2'-O-methyl RNA, one 2'-C-allyluridine is contained in the catalytic centre. The newly engineered molecule was found to be significantly more stable in human serum compared to the parent all-RNA ribozyme and was subsequently employed in clinical studies, as will be described below.

In an alternative approach, Zinnen *et al*. have used *in vitro* selection to generate an effective and stable nucleic acid catalyst [88]. The resulting ribozyme which was dubbed Zinzyme consists of 36 nucleotides and contains 2'-amino and 2'-fluoro modifications. Similar to the optimised hammerhead ribozyme, 2'-O-methyl nucleotides, phosphorothioate linkages and an abasic sugar were introduced in addition. The ribozyme displayed a serum half-life of >100 hours and reasonable *in vitro* catalytic activity. A Zinzyme directed against the oncogenic Her2 RNA has proceeded to clinical studies (*vide infra*).

DNAzymes are intrinsically more stable than ribozymes made of RNA. The half-life of an unmodified DNAzyme in human serum has been measured to be approximately two hours, compared to <1 minute for hammerhead ribozymes. For applications in cell culture and *in vivo*, however, additional stabilisation is indispensable. A significant improvement of nuclease resistance has been achieved by adding an inverted base to the 3'-terminus of the DNAzyme (e.g. [89]). Modifications in the binding arms include the use of 2'-O-methyl modifications [60] and locked nucleic acids (LNA) [90]. LNAs are nucleotide analogues that contain a methylene bridge between the 2'-oxygen and the 4'-carbon of the ribose [91]. Both modifications have the additional advantage of conferring higher substrate affinity to the DNAzyme, resulting in higher reaction rates [92, 93] and increased binding to less accessible target sites (see above, [76]). Most recently, phosphoroamidates have been used to stabilise DNAzymes directed against the translation initiation codon of hepatitis A virus [94].

We have recently established a design for a DNAzyme containing 2'-O-methyl RNA modifications both in the binding arms and the catalytic core. The optimised enzyme displayed an approximately tenfold higher catalytic activity compared to the unmodified molecule and a substantially increased resistance against exo- and endonucleases [93].

Delivery of Ribozymes

One of the most challenging parts in ribozyme applications is the delivery of catalytic oligonucleotides into the desired subcellular compartment of the targeted cell population within an organism. In a classic experiment, Sullenger and Cech exemplified the importance of colocalisation of ribozyme and target RNA. In this study, a ribozyme directed against the LacZ gene and its target RNA were expressed from separate retroviral vectors in a packaging cell line. Due to copackaging of ribozyme and target molecule into newly created virions, only genomic RNA was cleaved, whereas the ribozyme showed no activity against the cytoplasmic target LacZ mRNA [95].

Different cellular RNAs may be localised in different subcellular compartments as nucleus, nucleoli, or cytoplasm. The targeted delivery of ribozymes is thus a complex issue, and several methods have been described for various applications. In general, two ways of transfer can be discerned as depicted in Fig. (**4**): In exogenous delivery, pre-synthesized ribozymes are taken up by the target cells, whereas endogenous delivery denotes the enforced synthesis of catalytic RNAs directly within cells for gene-therapeutic approaches. Recent reviews of available methods for exogenous and endogenous distribution of ribozymes are found in more specialised overview articles [96-98].

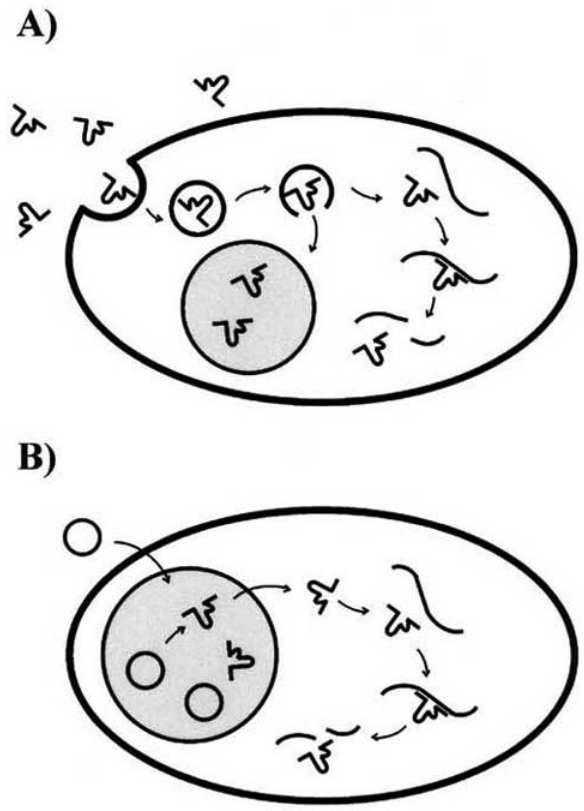

Fig. (4). Schematic representation of exogenous (A) and endogenous delivery of ribozymes (B). (A) Chemically presynthesised ribozymes are taken up by cells, released from endosomal particles and cleave their target RNA. Alternatively, plasmids may be introduced into the nucleus (B), where ribozymes are transcribed and transported into the cytoplasm.

A wealth of data has been collected for the exogenous delivery of antisense oligonucleotides which resemble ribozymes in that they are charged molecules like ribozymes that cannot be expected to cross hydrophobic membranes. The most frequently applied method to enhance uptake of antisense oligonucleotides and ribozymes by cells is the complexation with formulations of phospholipids containing positively charged headgroups. The cationic lipid reagents then assemble into oligonucleotide-containing liposomes that adsorb to cell membranes and are taken up by endocytosis. To facilitate the subsequent release of the nucleic acid from endosomes or lysosomes, helper lipids are often added that interfere with endosomal membranes. A number of transfection reagents working in this way are commercially available [97], and most trials in cell culture have been conducted using one of these formulations. *In vivo*, liposomes are often toxic and immunostimulatory. Furthermore, they are usually quickly removed from the circulation by cells of the reticuloendothelial system. Numerous methods for improved *in vivo* delivery have therefore been developed [96]: A sustained circulation can be achieved by reducing phagocytosis using liposomes modified with polyethylenglycol (PEG). Other macromolecular systems for exogenous delivery include highly branched carbohydrates referred to as dendrimers, and biodegradable polymer matrices like polylactides and lactic acid/glycolic acid copolymers. Delivery by means of biodegradable polymers has the advantage of displaying sustained release of the ribozyme over a prolonged period of time, sometimes exceeding one month. Pluronic gels have successfully been used for the sustained delivery of DNAzymes in *in vivo* studies (see below, [99]).

Specific targeting of defined cells can be achieved by coupling the ribozyme to antibodies or ligands that are recognised by cellular receptors [100]. Endocytotic pathways may be avoided altogether by coupling ribozymes to short carrier-peptides. The tat-protein from human immuno-deficiency virus type 1 (HIV-1), transcription factor VP-22 from herpes simplex virus and the transcription factor ANTP from *Drosophila melanogaster* are examples of protein transduction domains that promote transport of oligonucleotides across biological membranes independent of receptor- or transporter proteins. Alternatively, influenza-virus hemagglutinine or amphipatic helix-peptides can be used that permeabilise endosomal membranes [97].

Taken together, there is no simple rule to uptake and subcellular localisation of ribozymes in cells. Many parameters, including the delivery system and cell cycle, are involved in the outcome of a ribozyme targeting approach [97]. Surprisingly, simple injection of chemically stabilized ribozymes in a saline solution has led to efficient uptake and biological effects *in vivo* (see below). This finding points to yet unknown uptake mechanisms inside organisms that are not encountered in cell culture.

A different strategy to deliver ribozymes to their areas of action is their expression from vectors inside cells. Two challenges have to be met: efficient transfection of target cells and expression from suitable promoters. A variety of viral transduction methods has been employed to introduce ribozyme-coding genes into cells. Murine-based retroviral

vectors are well characterised and have already been employed in clinical studies (see below, summarised in [101]). They randomly integrate the ribozyme-coding sequence into the host genome leading to sustained expression, but holding the risk of insertional mutagenesis. Adenoviral vectors have also been engineered to deliver ribozyme genes. Subsequent expression, however, is only transient since their genetic material is not integrated into the host genome. In addition, adenoviruses are known to be immunogenic. An alternative may be the usage of adeno-associated viral vectors. Adeno-associated viruses are non-pathogenic, integration of the ribozyme gene into the host genome occurs at defined sites, and the transduction of non-dividing cells is also possible.

Promoter choice is crucial to subcellular localization. Ribozymes can either be transcribed from polymerase II or polymerase III promoters. Transcription by Pol II leads to the addition of a 5'-cap and a poly (A)-tail, resulting in enhanced stability and cytoplasmic localisation. In addition, Pol II promoters can be used for tissue-specific transcription. Ribozymes may also be inserted into RNAs with defined subcellular localisations including tRNAs, small nuclear RNAs like U1 and small nucleolar RNAs (for an overview, see [98]).

Several mRNAs contain signals in their 3'-untranslated regions that lead to transport and anchoring in specific subregions. These signals are referred to as zip-codes. Lee *et al*. made use of the zip-codes of different actin mRNAs to colocalise ribozymes and their lacZ-target mRNAs. Subcellular co-localization was enhanced threefold if localization signals of ribozyme and target matched. The inhibitory activity of the ribozymes was found to correlate with the correct localization [102].

Finally, interference of a ribozyme with its target gene may lead to growth advantages or disadvantages of the organism or cell population. For this reason, means of inducible expression have been sought. An example of this is the employment of the tetracycline transactivator protein tTA, consisting of a transcriptional activation domain from herpes simplex virus and the tetracycline repressor from *E. coli*. If tetracycline is present, it binds to the repressor domain and thus inhibits transactivation of transcription. Reverse repressors have also been developed that inhibit transcription only in the absence of tetracycline (see [103] for a review).

RIBOZYMES FOR TARGET IDENTIFICATION

A first step in the development of new therapeutic approaches is the identification and validation of suitable target molecules. Ribozyme libraries with randomised substrate recognition arms have been employed for this purpose. In one study, an inverse genomic approach based on a randomised hairpin ribozyme library was applied to identify genes regulating the expression of *BRCA 1* which is down-regulated in many cases of breast and ovarian cancer [104]. The ribozyme gene library was introduced into human ovarian cancer-derived cells that stably expressed a reporter gene under control of the *BRCA 1* promoter. Treated cells with an increased reporter gene expression were selected and the dominant negative transcriptional regulator *Id4* was identified as a modulator of *BRCA 1* expression. The identification of genes involved in regulation of tumour relevant genes might facilitate the development of new therapeutic options.

In an independent approach, a library of helicase-coupled randomised hammerhead ribozymes was employed to identify genes involved in tumour invasion [105]. Cells expressing ribozymes that enhance the invasive properties of NIH 3T3 fibroblasts were selected in a filter-based invasion assay. Genes that were likely to enhance the metastatic properties of tumour cells were then identified by sequence analysis of the ribozyme genes. This approach has subsequently been transferred to an *in vivo* model [106]: Weakly metastatic melanoma cells that had been treated with a ribozyme library were injected intravenously into mice. Ribozymes were isolated from cells converted into strongly metastatic cells and sequenced to identify the targeted genes relevant to metastasis. Again, several candidates were found which are involved in the complex mechanism of tumour metastasis and might be new targets for cancer therapy.

These examples and several further studies demonstrate that ribozymes are valuable tools to identify new targets for therapeutic approaches. The following sections will focus on the *in vivo* application of ribozymes and their potential as therapeutic agents in clinical trials.

APPLICATIONS OF RIBOZYMES AND DEOXYRIBOZYMES IN ANIMAL MODELS

Before testing a new therapeutic strategy in humans, a proof-of principle must be offered in animals as a basis for clinical studies. For numerous diseases, animal models have been established that may be employed for target validation and functional genomics, also in cases in which development of an effective agent as a drug is not intended. It is therefore not surprising that different approaches with ribozymes in animal models have been followed early on.

In one of the first reports showing the specific elimination of gene expression in animals, a synthetic hammerhead ribozyme that targets amelogenin, a protein which guides the formation of tooth enamel, was injected into the mandibular molar area of newborn mice [107]. As a consequence, abnormal tooth development was observed in the tooth buds next to the injection site. In another *in vivo* study, ribozymes targeting the matrix metalloproteinase stromelysin which is a key mediator of arthritic diseases were employed [108]. Following intraarticular administration, radioactively labelled ribozymes were found to be taken up by cells in the synovial lining. The chemically modified ribozymes were stable in the synovium and reduced interleukin 1α-induced stromelysin expression.

An interesting aspect of these studies was the finding that ribozymes were taken up into the cells without the use of any delivery system. As detailed above, nucleic acids are highly charged biomacromolecules that are unlikely to cross a hydrophobic cell membrane. *In vivo*, however, spontaneous cellular uptake by a yet unknown mechanism has been achieved in many cases for antisense oligonucleotides and ribozymes. Nevertheless, improvement of delivery systems is one of the major tasks for the successful application of these techniques [96, 97].

To achieve long-term inhibition of target gene expression, ribozymes have been endogenously expressed *in vivo*. A ribozyme-expression cassette was delivered into transgenic mice that produced human growth hormone by adenovirus-mediated gene-transfer [109]. An up to 96% reduction of hepatic human growth hormone was achieved over a period of several weeks. In another publication, researchers reported the generation of transgenic mice carrying a gene for a ribozyme specific for bovine α-lactalbumin [110]. These animals were cross-bred with transgenic mice expressing the target gene. As a consequence, the levels of target mRNA and protein were reduced demonstrating the potential of the ribozyme strategy to down-regulate target transcript in transgenic animals.

After the general proof-of-concept that ribozymes can be employed to knock down target genes in a sequence-specific manner *in vivo*, numerous studies have been performed using ribozymes for functional studies or as lead candidates for drug development. Work has been performed in different fields of high medical relevance, in particular cardiovascular diseases, cancer, viral infections and arthritis. Due to space restraints, only a few examples from different areas can be discussed and some very recent reports will be mentioned. The interested reader will be referred to comprehensive specialized reviews for the various topics.

The potential of the ribozyme strategy to treat cardiovascular diseases has been demonstrated with a hammerhead ribozyme targeting the transforming growth factor β (TGF-β) in rats [111]. The ribozyme was delivered by a sophisticated transfer agent consisting of the hemagglutinating virus of Japan combined with liposomes. The selective decrease of TGF-β resulted in significant inhibition of neointimal formation after vascular balloon-injury in a rat carotid artery model. In another study, a ribozyme approach was used to investigate the reason for intimal thickening during cardiac allograft vasculopathy [112]. A ribozyme targeting the matrix metalloprotenase 2 which was delivered into donor hearts just before transplantation significantly decreased luminal occlusion compared to non-treated allografts. For further nucleic acids based strategies to modulate gene expression for the treatment of cardiac diseases, see the recent review by Poller and colleagues [113].

Ribozyme approaches have also widely been used in cancer research. Telomerase is an interesting target for the development of new anti-cancer drugs. In most normal cells, telomeric DNA shortens progressively with each cell division, ultimately leading to cell death after reaching a critical short length. Cancer cells, however, escape senescence through activation of telomerase, a riboprotein complex that maintains the termini of eukaryotic chromosomes. Recently, a systemically administered, plasmid-expressed ribozyme targeting the RNA component of telomerase was shown to significantly reduce the metastatic progression of murine melanoma *in vivo* [114].

In several studies, oncogenic targets were silenced by ribozymes to prevent tumour growth. For example, down-regulation of the receptor tyrosine kinase c-Met inhibited metastatic growth of human colorectal carcinoma cells in the liver of athymic nude mice [115]. The maxizyme technology described above was employed to target the chimeric *BCR-ABL* gene which is the major cause of chronic myelogenous leukaemia (CML). Expression of the allosterically controlled ribozyme inhibited tumour-cell infiltration in mice and drastically improved survival rates [116].

An alternative approach is to reverse the resistance of cancer cells to chemotherapy. A major target in these attempts is the multiple drug resistance-1 gene. Recently, a hammerhead ribozyme targeting survivin was stably expressed in human melanoma cells to enhance tumour sensitivity to chemotherapeutic agents [117]. Xenograft tumours in athymic nude mice grown from these cells were significantly more susceptible to the topoisomerase-I inhibitor topotecan than control tumours. A detailed list of genes associated with malignancies that have been targeted by ribozymes is given in the review by Wright and Kearney [118]. Several ribozymes against cancer genes have been developed to the stage of clinical trials and will be described in the following section.

Worldwide, more than 40 million people are infected with the human immunodeficiency virus HIV-1 and even the latest highly active anti-retroviral therapy (HAART) is still unsatisfactory. HIV can escape many therapies and severe side effects occur upon antiviral treatment. Therefore, the improvement of therapies for HIV infections still remains a major goal for virologists. RNA-based strategies have also been employed to this end. For example, HIV-1 *tat-rev* or *env* ribozymes as well as Rev aptamers have been introduced into $CD34^+$ hematopoietic progenitor cells [119]. In a SCID-hu mouse model, these cells retained their capacity to reconstitute human fetal thymus and liver tissue grafts. Differentiated thymocytes derived from these grafts expressed the ribozymes and aptamers and showed significant resistance to HIV-1 infection upon challenge. Ribozymes targeting HIV-1 were among the first to enter clinical tests in the mid 90s (see below).

The use of ribozymes as antiviral agents, however, is not restricted to HIV-1. For example, hepatitis B virus has been targeted by a ribozyme generated intracellularly from a self-processing triple-ribozyme cassette [120]. In a transgenic mouse model, these constructs reduced viral DNA in the liver by >80% after two weeks. A nuclease-resistant ribozyme against hepatitis C virus has been tested in a clinical phase II trial as will be outlined in the following section. For a comprehensive overview of the development of antiviral ribozymes and deoxyribozymes, the reader is referred to the recommendable recent review by Peracchi [121].

Furthermore, early attempts to treat arthritic diseases with ribozymes have continued and led to some successful approaches reported recently. Cathepsin L is a matrix degrading enzyme whose mRNA has been targeted by a retrovirus-encoded ribozyme [122]. Rheumatoid arthritis synovial fibroblasts transduced with the ribozyme vector were coimplanted with human cartilage into SCID mice. The ribozyme decreased target protein synthesis and led to a significantly reduced cartilage destruction compared to mock-transduced cells. In a similar approach, a ribozyme against the matrix metalloproteinase 1 reduced cartilage invasion by rheumatoid arthritis synovial fibroblast [123].

These studies demonstrate that intracellular expression of ribozymes constitutes a feasible tool to inhibit the production of matrix-degrading enzymes that are involved in arthritic degeneration.

In addition to ribozymes made of RNA, the 10-23 DNAzyme has successfully been applied in animal models. In the first *in vivo* study published in 1999, a DNAzyme directed against the early growth response factor-1 (Egr-1) was shown to inhibit neointima formation after balloon injury to the carotid artery wall of rats [99]. Further successful *in vivo* applications of DNAzymes employed for cardiovascular research reported since include the improvement of hemodynamic performance in rats with postinfarction heart failure by targeting TNF-α [124] and the impairment of neointima formation in rodents and swine by cleaving Egr-1 mRNA [125, 126] or c-Jun [127, 128]. Furthermore, the DNAzyme has been used in cancer research. Growth of breast carcinoma solid tumours was inhibited by a deoxyribozyme targeting Egr-1 [129] and interference with raf-1 inhibited juvenile myelomonocytic leukaemia cell growth [89]; a reduction of tumour growth was also achieved by cleaving the vascular endothelial growth factor receptor 2 in mouse models [130].

CLINICAL TRIALS WITH RIBOZYMES

Several clinical studies with *trans*-cleaving ribozymes have been carried out to treat infectious diseases or cancer. Both strategies described above, i. e. endogenous expression of ribozymes or exogenous delivery of chemically presynthesized ribozymes, were employed in these studies (summarized in [101]). Gene therapeutic studies have focused on the treatment of patients infected with the human immunodeficiency virus (HIV).

In the mid 90s the first Phase I clinical trials were initiated, in which retroviral vectors were employed to deliver anti-HIV ribozymes. Lymphocytes or hematopoietic precursor cells were isolated from HIV infected individuals or from a healthy identical twin. After transduction with the ribozyme-vector *ex vivo* the cells were infused in the patient (Fig. (**5**)).

In one of the studies, peripheral blood T-lymophocytes were collected from asymptomatic HIV-1 seropositive individuals [131]. These cells were transduced with a murine retroviral vector encoding a hairpin ribozyme against HIV-1 and transferred back into the infected patient. Despite preliminary evidence that infusion of genetically modified cells was safe and that ribozyme-transduced cells may possess a transient survival advantage compared to cells transduced with a control vector, the utility of the hairpin-ribozyme approach for clinical applications has recently been questioned [132].

For two further studies, a retroviral vector was used which contained a hammerhead ribozyme directed against the regulatory HIV-1 *tat* gene. The first trial involved identical twins, discordant for infection with HIV-1 [133]. $CD4^+$ lymphocytes were collected from the uninfected twin and transduced with the retroviral vector encoding the ribozyme gene. After culturing and expansion *ex vivo*, the transduced cells were transfused into the bloodstream of the HIV-positive twin. Preliminary data indicate that the

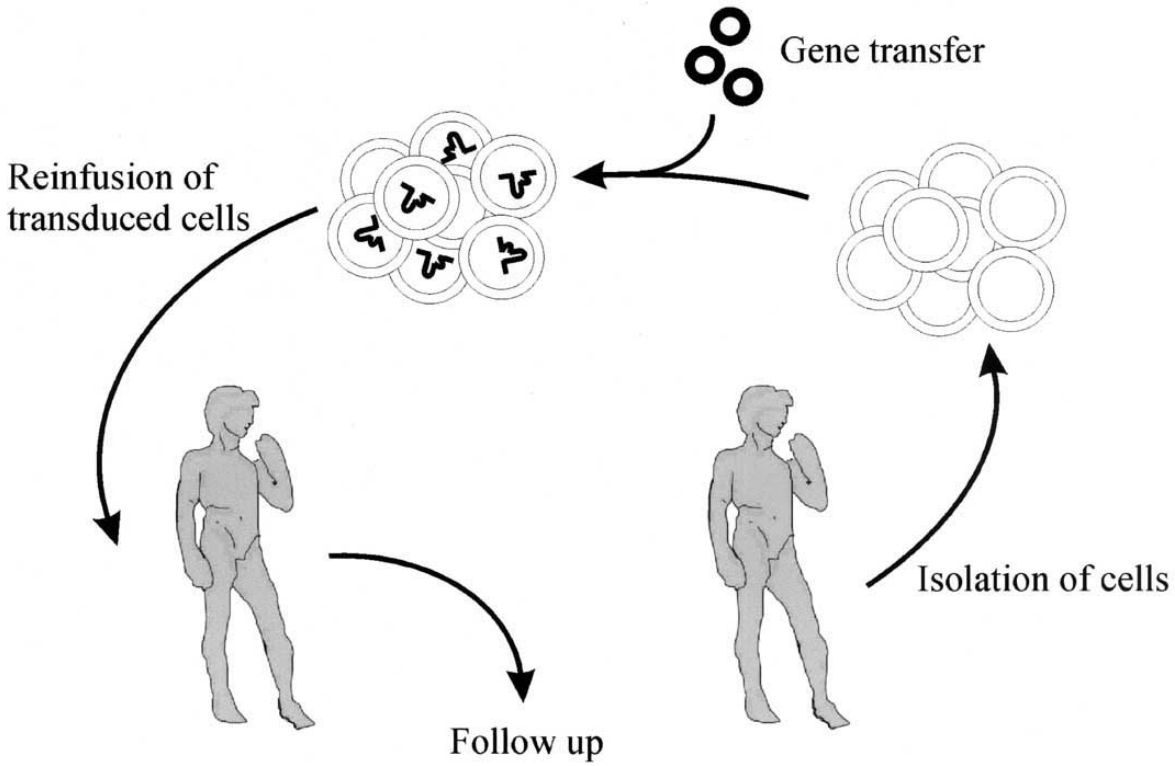

Fig. (5). Gene therapeutic approach using anti-HIV ribozymes. Blood cells are isolated from a patient or a healthy twin, transduced *ex vivo* with vectors carrying therapeutic ribozyme genes and (re-)infused into the patient.

procedure is safe and that transduced cells can be detected in all patients [134]. The other trial involves *ex vivo* retroviral transduction of the same ribozyme vector into purified $CD34^+$ hematopoietic progenitor cells [135]. These stem cells are expected to differentiate and give rise to a variety of lineages that express the ribozyme. After re-infusion of transduced autologous $CD34^+$ cells in HIV-infected patients no serious adverse events were reported. In the follow-up, the presence of the transduced gene and its transcript were detected by PCR after 2.5 years [136]. The authors of the study conclude that *de novo* T-cell development can occur from genetically engineered hematopoietic progenitors in HIV-infected patients. Details of further phase I trials in which hematopoietic progenitor cells were transduced with anti-HIV tat or tat-rev ribozymes to increase resistance to viral infections are outlined in Michienzi *et al*. [137]. Here, persistance of genetically modified $CD34^+$ cells ceased after one year. These results demonstrate that efficient transduction of pluripotent hematopoietic stem cells is a major topic for long term protection against HIV.

In addition to gene therapy based studies, several nuclease resistant, chemically synthesized ribozymes have been tested in clinical trials. Ribozyme Pharmaceticals Inc. (RPI) was the leading company to explore the therapeutic potential of exogenously delivered, catalytically active RNA molecules. The stabilized ribozymes were employed to treat cancer and hepatitis C virus (HCV) infections.

Tumours are rapidly growing tissues with a high demand for oxygen and nutrition. Therefore, the formation of new blood vessels, a process called angiogenesis, is required for sustained tumour growth. The furthest developed chemically synthesized ribozyme 'ANGIOZYME' was designed to inhibit tumour angiogenesis. It is directed against the receptor tyrosine kinase Flt-1 (VEGF-R1), the high-affinity receptor for the vascular endothelial growth factor VEGF. ANGIOZYME is a hammerhead ribozyme stabilized according to the modification scheme that has been described in a previous section [86].

A phase I clinical trial to test ANGIOZYME's safety, tolerability and pharmacokinetics in healthy volunteers began in late 1998 [138]. The ribozyme was found to be maintained in the plasma for several hours following single s. c. or i. v. infusion. Furthermore, it was well tolerated at the concentration under investigation. The ribozyme was then tested in a multidose phase I/II trial [87]. Again, it was well tolerated by all subjects and has subsequently been investigated in phase II trials in patients with breast cancer as well as in combination with chemotherapy in humans with metastatic colorectal carcinoma. According to the company's information, the effect of ANGIOZYME could not be separated from the chemotherapeutic drug, but it may result in decreased level of the VEGF receptor and improved clinical outcome.

Although any kind of virus can be treated with ribozymes by cleaving essential RNAs, RNA viruses are particularly suited to be addressed by this class of therapeutics. The hepatitis C virus (HCV), a plus strand RNA virus, causes substantial human disease worldwide, since a chronic infection can lead to liver cirrhosis, liver failure and hepatocellular carcinoma. RPI developed a ribozyme (HEPTAZYME) against a target site in the viral 5'-untranslated region that is highly conserved in numerous clinical isolates. HEPTAZYME inhibited replication of HCV/poliovirus chimera that are better suited than native HCV for propagation in cell culture by up to 90% [139]. A combination of HEPTAZYME and type 1 interferon was found to have a more potent antiviral activity than either compound individually [87].

These encouraging results led to the initiation of a clinical phase II trial of HEPTAZYME alone and in combination with interferon to evaluate safety and efficiency in treating chronic hepatitis C. Initial results showed a 10% reduction of HCV RNA levels in the serum of patients that had been treated with the ribozyme. Their results together with the outcome of a toxicological study led to the decision not to proceed with clinical investigations of HEPTAZYME [121].

HERZYME is the third ribozyme that was studied in a clinical trial by RPI. It is directed against the mRNA of the human epidermal growth factor receptor-2 (HER-2), which is overexpressed in aggressive breast cancer. This ribozyme was based on the chemistry developed by Zinnen *et al*. [88] as described above. In a phase I trial it was found to be well tolerated with no significant systemic adverse events observed according to the company's information.

Taken together, these clinical studies demonstrated that the ribozymes were well tolerated and that their application in humans is safe. They did, however, not fulfil early expectations to become general tools that can easily be employed to treat any disease caused by the expression of a deleterious gene. The efficacy or ribozymes to prevent tumour growth or virus replication has not yet been proven convincingly. Consequently, the above mentioned company RPI as a major player in clinical applications of ribozymes now focuses on RNA interference approaches under the name Sirna Therapeutics.

CONCLUSION

Ribozymes are a fascinating class of biomacromolecules which have now been in the focus of RNA research for more than 20 years. Their natural properties and catalytic mechanisms have intensively been studied, and crystal structures have been obtained for most of the known ribozymes. The generation of ribozymes which can cleave target molecules in a multiple turnover process opened the road to using ribozymes as tools for regulating the expression of intended genes. Consequently, *trans*-active ribozymes have widely been applied for functional studies and target validation. Several studies have been described above in which the role of certain genes in various diseases was analysed with ribozyme-based approaches in cell culture or in animal models. The long-awaited breakthrough in clinical trials, however, has not yet been achieved.

The question therefore arises whether ribozyme strategies will be able to compete with the more recently developed RNA interference (RNAi) technology. RNAi utilises an endogenous cellular mechanism to inhibit gene expression and is characterised by an extraordinarily high efficiency. Several warnings, however, have been reported in recent

years which indicate that the application of small interfering RNAs (siRNA) might cause unwanted side effects by off-target regulation, the induction of the interferon response or by inhibiting translation in a micro RNA-like manner (summarized in [140]). It will therefore be important to clarify whether small interfering RNAs can be applied as therapeutic agents with an acceptable risk of adverse effects. Ribozyme- and antisense-strategies can be seen as complementary approaches that provide mechanistically distinct ways to prove the outcome of RNAi experiments with an independent method.

Furthermore, the combination of ribozymes and siRNAs may be a promising attempt to treat virus infections like HIV-1 that are characterized by the emergence of escape mutants upon long-term treatment of cells with siRNAs. It has been suggested to incorporate several different anti-HIV RNA transcriptional units expressing ribozymes, siRNAs and decoys within a single vector in order to target various components of the virus with different antiviral agents [137]. Finally, experiences made in the ribozyme-field will help to expedite clinical applications of siRNAs. Interestingly, several of the companies and physicians that announced clinical trials with siRNAs to be initiated in the near future have also been involved in testing ribozymes in human patients.

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