Chapter

Ribozymes as Therapeutic Agents against Infectious Diseases

Bao Chi Wong, Umama Shahid and Hock Siew Tan

Abstract

Ribozymes, also known as RNA enzymes, are catalytic RNA molecules capable of cleaving specific RNA sequences, leading to decreased expression of targeted genes. Recent studies suggest their role in cancer therapeutics, genetic diseases and retroviral infections. This book chapter will focus on ribozymes acting as therapeutic agents against infectious diseases caused by viral and bacterial pathogens. Firstly, we will introduce a brief history of ribozymes and a general overview of ribozymes and their characteristics. Next, different types of ribozymes will be explored regarding their targets and mechanisms of action. After that, ribozymes specific to viral and bacterial infections will be explored. We will briefly discuss the current status of ribozymes as therapeutic agents. Finally, the roadblock and challenges ribozymes face before being developed into therapeutic agents—such as their delivery and efficacy issues—will be discussed.

Keywords: ribozymes, therapeutic agent, antiviral, antibacterial, infectious diseases

1. Introduction

Proteins have always been the undefeated champions in most stories that any molecular biologist has to tell. A classic textbook elaborates extensively on these molecules, their structures, localisations and functions, followed by an essential section on enzymes. The Central Dogma of Molecular Biology states that deoxyribonucleic acid (DNA) precedes protein. DNA encodes important information, is converted into ribonucleic acid (RNA) and finally translated into the master molecule, protein [1]. So, in principle, proteins cannot exist without nucleic acids. However, the precursor here, i.e. DNA, is not even capable of replicating, much less forming a protein by itself, because it is found in a double-stranded form and hence is functionally inert. Therefore, DNA requires something capable of catalysing these reactions. Biologists have tried to explore the players involved in this phenomenon for years until a relatively recent discovery of catalytic RNAs by Thomas Cech and Sidney Altman proposed a possible explanation [2].

In 1978, Thomas Cech (University of Colorado) and his team decided to study RNA splicing, a considerably new field at the time. To explore RNA processing, they started working with a ciliated protozoan, *Tetrahymena thermophila*. Ribosomal RNA was chosen owing to its abundant amount in the selected system [3]. The 26S rRNA gene in *Tetrahymena* includes an intron of about 400 nucleotides, which must

be removed before the gene product can localise and function as required. However, they observed a 9S RNA fragment (approximately 400 nucleotides) in all their phenol-based nuclear extractions, leading them to assume the possibility of protein contamination in the nuclear extracts, which was responsible for the splicing of this intron. Extreme efforts were made to remove/denature the protein that was thought to be either contaminant or strongly attached to the RNA molecules. The samples were subjected to high salt concentrations and exposed to high temperatures (both being something that a protein does not like), but splicing was observed, nonetheless. This result hinted that no protein could be present/responsible for the processing. However, this was not enough evidence. Kruger et al. described the cloning of the *Tetrahymena* rRNA gene in the *Escherichia coli* plasmid, followed by its *in vitro* transcription in their 1982 article [4]. The rRNA thus transcribed was also capable of excising the intron itself, proving that it was, in fact, a self-splicing molecule and did not require any protein for the processing. In the same year, Cech and his team released an article explaining the actual working of rRNA self-splicing where they showed that a GTP was required as a co-factor [5]. A detailed mechanism of self-splicing will be explained later in this chapter.

The discovery of self-splicing RNA molecules raised consciousness in the molecular biology world. Where one set of researchers dismissed it by calling the finding 'not a big deal', others started investigating the possibility of more reactions that were catalysed by RNA. Sidney Altman, Norman Pace and their respective teams studied ribonuclease P, an enzyme responsible for tRNA processing. Ribonuclease P is an interesting molecule since 80% of its content is RNA, and only 10% is protein. Initially, the RNA part of ribonuclease P was considered leftover contamination from protein purification with no significance. However, both teams demonstrated that reactions could occur without the protein section of ribonuclease P, proving that the RNA component catalysed the cleavage [6]. In 1989, Cech and Altman shared a Nobel prize in chemistry for demonstrating the catalytic activity of RNA. Many terms were coined for these special RNA molecules, now named Ribozymes (Ribonucleic acids that act as enzymes). Though not as common in vertebrates, RNA catalysis is now known to be widely spread amongst bacteria, viruses, some lower eukaryotes and even plants. One is also found in humans [7]. The naturally occurring ribozymes are reported to aid in reactions such as Ribosyl 2'-O mediated cleavage [8], RNA cleavage and ligation [9], DNA cleavage and ligation [10], etc. In addition, researchers worldwide are generating artificial ribozymes through combinatorial screening of random RNA sequences, which has increased the catalytic repertoire to an even larger range, including phosphorylation [11], acyl transfer reaction [12] and an amazing RNA polymerase ribozyme capable of polymerising complex RNA structures such as aptamers, ribozymes and even tRNA, amongst others [13].

2. General characteristics of ribozymes

Catalytic RNAs, like proteins, form a 3-D structure to be functionally sound for catalysis. Metal ions such as K^+ or Mg^{2+} are required for the proper folding of ribozymes to recompense for the high negative charge of the oligonucleotides [14]. Ribozymes typically contribute to self-targeted reactions (such as self-cleavage, self-splicing, ligation and template-directed polymerisation) except for one, i.e. RNase P (involved in the processing of tRNA) [15]. RNA has a limited range of chemical functionalities with just four similar nucleotides as building blocks. Despite this,

RNA can catalyse phosphoryl transfer reactions by about a million-fold, if not more [16]. Generally, naturally occurring ribozymes catalyse these reactions by attacking sugar 2′ or 3′-hydroxyl on a phosphodiester linkage. This nucleophilic attack involves activation of the nucleophile, stabilisation of an electronegative transition state and stabilisation of the leaving group.

Ribozymes can be categorised into two categories based on their size and whether a ribozyme uses its sugar -OH group to target the 3′ phosphodiester bond or requires an exogenous nucleophile [15]. The first group is the small ribozymes (approximately 35–155 nucleotides) that utilise 2′-hydroxyl of an adjacent nucleotide for the nucleophilic attack. The second group is the large ribozymes (approximately 200–3000 nucleotides) that attack using exogenous groups such as water, hydroxyl group from a mononucleotide or even a distantly located nucleotide from the same stretch [17]. Ribozymes perform phosphoryl transfer reactions using two main mechanisms, which are acid-base catalysis (seen in hammerhead, hairpin and *glmS* ribozymes) and metal-ion-assisted catalysis (seen in RNase P, group I, group II introns, HDV ribozymes) [17].

2.1 Small self-cleaving ribozymes

In general, small self-cleaving ribozymes act on the same strand, i.e. act in *cis* and hence, have a single catalytic turnover. These classes work on general acid-base catalysis. They use adjacent nucleobases or external co-factors as the general base or acid. The base takes a proton from the 2′-hydroxyl group, thereby increasing oxygen's nucleophilicity, which can then attack the nearby phosphorous. As a result, a transition state is formed. On the other hand, 5′-oxygen gets protonated by a general acid leading to the release of leaving group and thus the formation of 2′,3′-cyclic phosphate and a free 5′-hydroxyl group (**Figure 1**) [18]. Below are the different classes of small self-cleaving ribozymes.

With a size of about 40–50 nucleotides, the **hammerhead ribozyme** is, by far, the most extensively studied. Originally found in plant viroids and satellites, they are a widely spread class of self-cleaving RNAs known to catalyse the conversion

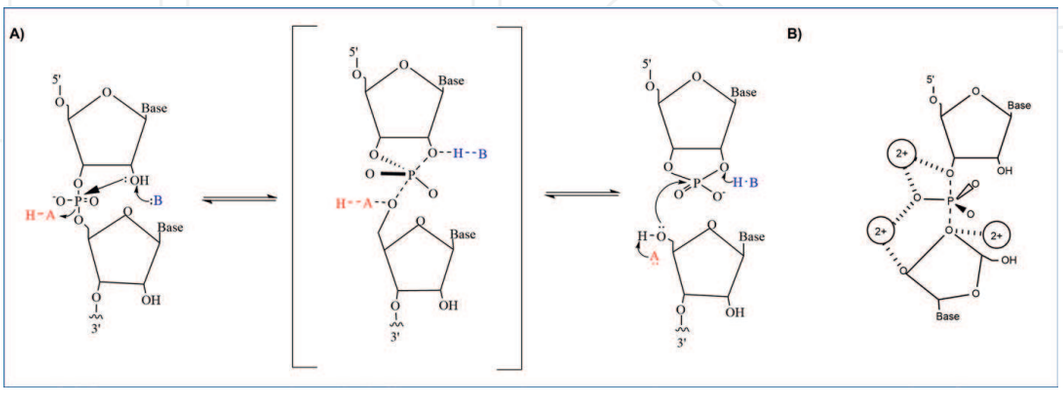

Figure 1.
Mechanism of catalysis in ribozymes: Ribozymes perform reversible nucleophilic reactions. (A) General Acid-Base catalysis. The general base (blue) deprotonates the 2′-hydroxyl in the cleavage reaction (or the 5′-hydroxyl in the reversed ligation reaction). The general acid (red) donates a proton to the 5′-oxyanion leaving group for cleavage (or the 2′oxyanion for ligation). A trigonal bipyramidal phosphorane is formed in the transition state (shown in the centre). B) RNA metalloenzymes. Large ribozymes, including RNase P and self-splicing introns, catalyse the phosphodiester bond breakdown via metal-ion catalysis. The figure is a representative group I intron where three metal ions bind to the transition state to bring about catalysis.

of their trimeric and dimeric forms into monomeric RNAs [19]. They are made up of three helical regions (Stem I, II, III), which are variable and a universally conserved junction sequence made up of three single strands (**Figure 2**) [20]. Hammerhead ribozymes cleave after an NUH [21] or NHH [22] triplet, where N can be any nucleotide, and H is any nucleotide except guanosine. They utilise N1 of G12 from stem II in their catalysis as a nucleophile. It forms a hydrogen bond with 2′ hydroxyl of C17 [23]. Some studies report that a divalent metal cation helps activate G12. Stabilisation of G8 occurs due to its base pairing with G3 [24]. Earlier, the 2′-hydroxyl group of G8 was thought to be the acid in this acid-base catalysis. However, a recent study reports that Mn^{2+}-bound water is the general acid during cleavage [25].

Hairpin ribozymes, like hammerhead ribozymes, are also found in plants' satellite viruses such as the tobacco ringspot (best studied), chicory yellow mottle and Arabis mosaic virus also catalyse the self-cleavage of multimeric RNA [26]. They comprise four stems that, when aligned, resemble a hairpin (**Figure 2**). A10 and G11 and A24 and C25 assemble as a ribose zipper and form a catalytic centre. The general base, in this case, is G8 (stem B), and A38 a (stem A) acts as a general acid, respectively. Rigorous *in vitro* selection of active mutants has shown that hairpin ribozymes prefer G at the +1 position of their cleavage site. N*GUY emerged as the best agreed-upon cleavage site, where N is any nucleotide, G is guanine, U is uracil and Y is any pyrimidine [27]. Later studies showed that substrates with G*GUN, G*GGR (R is any purine) and U*GUA could also be cleaved but with a considerably lower catalytic activity [28]. A crowded environment near the hairpin ribozymes increases their activity by stabilising the active conformation [29].

Hepatitis delta virus-like ribozymes are self-cleaving ribozymes present in the genomic strand and the complementary/anti-genomic strand found in Hepatitis delta virus (HDV) (a single-stranded RNA virus that infects mammalian hepatocytes) [30]. These ribozymes also catalyse a transesterification reaction through a nucleophilic attack by a 2′ hydroxyl on the adjacent phosphate and result in the formation of a 2′–3′ cyclic phosphates and the release of 5′ hydroxyls. Their structure consists of five paired regions of helices, which, when coaxially aligned, are stacked over each other (P1 over P1.1 and P4; P2 over P3). Single-stranded joining strands link these helices. Crystallography reveals that they assume an extremely stable structure resembling a double pseudoknot. HDV-like ribozymes cleave at the first guanosine residue at the base of the P1 helix [31].

The **glucosamine-6-phosphate synthase (*glmS*) ribozyme** is found in several Gram-positive bacteria in the 5' UTR region of the *glmS* gene [32]. It regulates the expression of glutamine-fructose-6-phosphate transaminase and is the only known ribozyme which requires glucosamine-6-phosphate (GlcN6P) as a co-factor [33]. The *glmS* ribozyme comprises three parallel helices stacking each other (P1 on P3.1, P4 on P4.1 and P2.1). It also forms a core resembling a double pseudoknot. P3 and P4 are not essential for catalysis. However, they provide structural stability and enhance the activity of ribozymes. The P2.2 forms the binding site for GlcN6P, and the correct folding of P2.2 brings the ribozyme into active conformation [34]. A co-factor is required for the protonation of the 5′ oxygen leaving group, activation of the 2′-oxygen nucleophile and charge stabilisation [35].

Largest nucleolytic RNA with a length of ~150 nucleotides, the **Varkud Satellite (VS) ribozymes** are found in certain strains of Neurospora and help in the replication of single-stranded RNA [15]. VS ribozymes comprise seven helices (1–7), forming a three-way junction (2-3-6, 3-4- and 1-7-2). The inner loops of stem 1 act as their

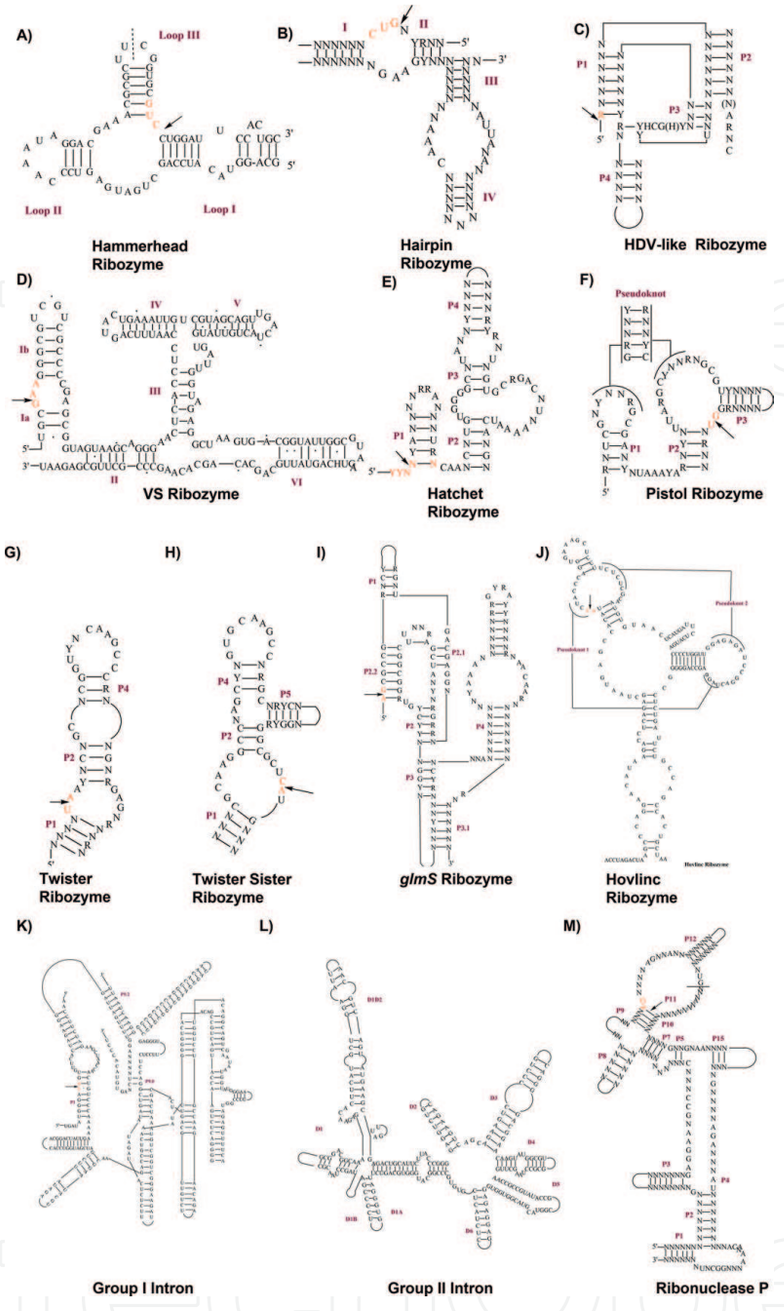

Figure 2.
Consensus secondary structures ribozymes. A, U, G and C represent adenine, uracil, guanine and cytosine. N represents any nucleotide. R stands for any purine and Y for any pyrimidine. The black arrows show the cleavage site, orange-coded nucleotides represent conserved bases near the cleavage sites, and the solid line shows a variable stretch of nucleotides.

cleavage site, while stems 6 and 1 harbour the catalytic centre [36]. A kissing loop forms between GUC in stem 1 and GAC in stem 5 to form an active site, bringing the cleavage site to the catalytic centre. The residues A756 and G638 act as the general acid and base, respectively. Additionally, Mg^{2+} is reported to interact with scissile phosphate and activate G638 [37].

Hatchet ribozymes are one of the bioinformatically revealed ribozymes, and very little is known about them. They comprise four stems (P1–P4). P1 and P2 are linked with highly conserved residues, whereas internal loops (L2 and L3) connect the other three stems [38]. X-ray crystallography reveals that they appear as pseudo

symmetrical RNA and form long-range interactions of conserved residues near scissile phosphate. The cleavage site is located at the 5′ end of the P1 stem. N7 of G31 acts as the general base to deprotonate the 2′-OH of C (−1) for nucleophilic attack. In addition, Mg^{2+} is required for proper folding and catalysis [39].

Twister ribozymes are widely spread among many species of bacteria and eukaryotes [40]. They are made up of five stems (P1–P5) and internal loops held together by two pseudoknots. Twister ribozymes cleave folding-dependently, where central pseudoknot opens and closes at variable Mg^{2+} concentration [41]. These cations help position the phosphate oxygen at the U-A cleavage site and stabilise the transition stage to form an intermediate. Guanine is a conserved residue at the cleavage site. It acts as a general base in the general acid-base catalysis, whereas an adenine residue plays as the general acid. The active site comprises at least 10 conserved nucleotides, harbouring scissile phosphate between A and U joining P1 [42].

Twister sister ribozymes are highly similar to twister ribozymes in sequence and secondary structure. The only difference is that they do not have a double pseudoknot interaction. Long-range interactions that bring conserved nucleotides closer to the core are mediated by Mg^{2+} cations [43]. C62 and A63 flank the cleavage site on the internal loop between P1 and P2. Hydrogen bonds (N1H of G5, inner-sphere water of Mg^{2+} and phosphate oxygen) keep the scissile phosphate in its place [44]. The substrate specificity of these ribozymes has not been studied in detail yet.

Pistol ribozymes were discovered bioinformatically through comparative genomic analysis to search hammerhead and twister ribozymes-related sequences. Pistol ribozymes consist of three helical stems (P1–P3) connected by three loops (loops 1–3) and one pseudoknot. P1 and pseudoknot form a stacked structure [45]. N1 of G40 acts as a general base, and A32 acts as a general acid. Crystallography shows that Mg^{2+} cations have a significant role in catalysis. All information on this ribozyme is limited; some studies suggest that residues at positions 32 and 40 might affect the substrate specificity [46].

Hovlinc ribozymes are a recently discovered class of ribozymes that came up in a genome-wide search of human catalytic RNAs [47]. Although very little is known about hovlinc ribozymes, structure analysis shows that their catalytic core comprises two stem loops and two pseudoknots. They are pH-dependent and require divalent cations where their activity was shown to be highest in the presence of Mn^{2+} ($Mn^{2+} > Mg^{2+} > Ca^{2+}$) [48]. Further studies will be required to properly reveal its characteristic folding, cleavage site, catalytic centre and functioning.

2.2 Large ribozymes

These are often called ‘true catalysts’ because they can act on a substrate in a *trans* manner and thus have a catalytic turnover. In contrast to the small self-cleaving ribozymes, large catalytic RNAs act as metalloenzymes [7]. Metal ions are usually found in the active sites of these ribozymes and form inner-sphere complexes with oxygen atoms of the RNA (**Figure 1**).

Introns are intervening noncoding regions between a gene’s exon (coding regions). When a gene is transcribed, the pre-RNA thus formed undergoes removal, i.e. splicing all the introns to obtain mature RNA [49]. Naturally found in bacteria and bacteriophages, nuclear rRNA genes and chloroplast DNA, **Group I introns** are self-splicing in nature and can excise themselves without a protein enzyme [50]. These can migrate and insert themselves at different positions of the host genome, thus acting as mobile genetic elements [51]. Although widespread, the group I ribozymes have

very less sequence similarity. However, they all can fold into a conserved secondary structure with 10 paired segments (P1–P10). The catalytic core comprises P3, P4, P6 and P7 [9]. The intron is spliced from the pre-RNA by a two-step transesterification reaction. First, 3′ hydroxyl makes a nucleophilic attack on a guanosine co-factor at the 5′ splice site. The exon-intron phosphodiester bond is cleaved, and guanosine forms a 3′,5′-phosphodiester bond at the 5′ ends of the intron. Finally, a nucleophilic attack of now free 3′ hydroxyl of the 5′ at the 3′ splice site to form the ligated exons results in the release of an intron with the nonencoded guanosine. The intron is circularised by making a nucleophilic attack with its highly conserved 3′ terminal guanosine at a phosphodiester bond of C-15 or C-19. Each step is fully reversible and follows the SN2 reaction mechanism [52].

The self-splicing **group II introns** are widespread amongst the mRNA, tRNA and rRNA genes of plant and fungal mitochondria and chloroplasts (including algae and protists) [51]. The secondary structure of Group II introns was initially revealed via computational modelling and phylogenetic comparisons. They are composed of six helices (I–VI) radiating from a centrally located wheel [53]. Out of the six helical domains present, only I and V are crucial for their activity. Domain V, the most variable region, harbours the active site. A conserved Adenosine residue is present in the domain, which initiates the splicing reaction. 2′-Hydroxyl of adenosine performs the nucleophilic attack and forms a structure known as a lariat, which contains 3′–5′ and 2′–5′ phosphodiester bonds at the adenosine branch site. Following this, free 3′-hydroxyl of 5′ exon makes a nucleophilic attack at the 3′ splice site resulting in ligated exons and spliced out intron (as a lariat). Though not common, a nucleophilic attack may sometimes be initiated by water, resulting in a linear intron [54].

RNase P is a widespread enzyme processing tRNA precursors [55]. It is known to exist in a ribonucleoprotein complex consisting of about 350–400 nucleotide long RNA stretch and about 14kDa of small protein subunit [56]. Though protein moiety is important for catalysis *in vivo*, the RNA component is enough *in vitro*. The reaction occurs at a high salt concentration, and protein was assumed to promote RNA enzyme and substrate interaction. However, studies have shown that the protein component of RNase P plays a significant role in site specificity and turnover [57]. No high sequence conservation is observed in RNase P across different organisms. However, they all can fold into a similar secondary structure [56]. RNase P from *E. coli* M1 RNA consists of 18 paired helices, but RNase P from *Bacillus subtilis* lacks P6, P13, P14, P16 and P17 but contains a few extra helices (P5.1, P10.1, P15.1 and P19). Despite these differences, comparative analyses of RNase P secondary structure have deduced a catalytic core composed of P1–P5, P7–12 and P15. This ribozyme uses water to make a nucleophilic attack [58].

In eukaryotic cells, intron removal occurs through a ribonucleoprotein complex called **spliceosomes**. These complexes are not preformed; five RNAs and about 100 proteins assemble directly into a spliceosome on their substrate [59]. Splicing events can be divided into four main reaction steps: assembly, activation, catalysis and disassembly [60, 61]. The catalytic centre of spliceosomes highly resembles Group II introns, and even the splicing mechanism is quite like the latter [62].

The **ribosome** is a protein translating machinery formed by 30S and 50S subunits in bacteria and 40S and 60S subunits in eukaryotes, respectively [63]. The larger subunit contains the peptidyl transferase centre (PTC), which forms peptides by joining amino acids. X-ray crystallography and electron microscopy have elucidated two main reactions involved in protein synthesis: aminolysis to form peptide bonds and

peptidyl hydrolysis to release protein after synthesis. The catalysis does not occur via nucleobase-mediated catalysis, rather is mediated by 2′-hydroxyl of tRNA [64]. Both these reactions occur in the PTC, known to be made completely of RNA [65].

3. Ribozymes as antiviral and antibacterial infection alternatives

The potential of ribozymes as therapeutic agents has been explored from other perspectives, including cancer and inherited diseases. Ribozymes downregulate the expression of the target gene(s) through the cleavage of mRNA transcripts. If the expression of a gene could lead to pathogenesis, then the downregulation of that gene expression via ribozymes can be performed as a therapeutic option. Previous studies have selected a few important genes responsible for viral replication as targets. By decreasing the viral replication, the application of ribozymes will inevitably treat the viral infection.

Multiple viruses have been used as targets in antiviral ribozyme research, including the human immunodeficiency virus (HIV), herpes simplex virus (HSV) and human cytomegalovirus. Different types of ribozymes were used, demonstrating their potential to be used as therapeutic agents in both *in vitro* and *in vivo* conditions (**Table 1**). There are different strategies for studying the efficiency of antiviral ribozymes. If a target gene is shortlisted and the cleavage site is determined, the ribozyme can be designed rationally. If the cleavage site is undetermined, the potential target cleavage site can be screened to discover any region exposed to the ribozyme for easy binding. Another method is to use a library of ribozymes to find any ribozymes with

Target	Ribozyme	Design	Delivery	References
Herpes simplex virus (HSV)				
Thymidine kinase	RNase P	*In vitro* selection	Endogenous—Retrovirus	[66]
Infected-cell polypeptide 4 (ICP4)	RNase P (M1GS)	*In vitro* selection	Endogenous—Retrovirus	[67]
Latency-associated transcript (LAT)	Hammerhead	Rational design	Endogenous—Adenovirus	[68]
Human cytomegalovirus				
Capsid assembly protein (AP) and protease (PR)	RNase P (M1GS)	Rational design	Endogenous—Retrovirus	[69]
Assembly protein (mAP) and M80	RNase P (M1GS)	Rational design	Endogenous—Retrovirus (*in vitro*); hydrodynamic transfection (murine)	[70]
M80.5 and protease	RNase P (M1GS)	Rational design	Endogenous—Salmonella	[71]
Immediate early proteins 1 and 2	RNase P	Screening of target sites	Endogenous—Retrovirus	[72]
Assemblin (AS)	RNase P (M1GS)	*In vitro* selection	Endogenous—Retrovirus (*in vitro*); hydrodynamic transfection (murine)	[73]

Target	Ribozyme	Design	Delivery	References
Human immunodeficiency virus 1 (HIV-1)				
Vpr and tat region	Hammerhead	Rational design	Endogenous—Retrovirus	[74]
Glycoprotein (gp41)	Hammerhead	Rational design	Exogenous	[75]
Tat region	RNase P	*In vitro* selection	Endogenous—Retrovirus	[76]
Glycoprotein (gp41)	Hammerhead	Rational design	Endogenous—Plasmid	[77]
Influenza A virus				
Conserved regions of Influenza A virus mRNA	Hepatitis delta virus ribozyme	Rational design	Endogenous—Plasmid	[78]
Conserved RNA secondary structure motifs	Hammerhead	Rational design	Endogenous—Plasmid	[79]
Sindbis virus				
Within the 26S subgenomic RNA	Hairpin	Rational design	Endogenous—Plasmid	[80]
Genomic RNA	Hairpin	Screening of target sites	Endogenous—Plasmid	[81]
Chikungunya virus				
Conserved genomic sequences among 100 strains	Hammerhead	Rational design	Endogenous—Retrovirus (*in vitro*); *piggyBac* vector (mosquito)	[82]
Hepatitis C virus				
5′ UTR of HCV genome	M1GS ribozyme	Rational design	Exogenous	[83]
SARS virus and mouse hepatitis virus (MHV)				
SARS and MHV consensus sequences	Chimeric DNA-RNA hammerhead	Rational design	Exogenous	[84]

Table 1.
Examples of antiviral ribozymes.

high binding or cleavage activity towards the target virus. Finally, two main delivery methods exist for introducing ribozymes into the system. While some studies propose the potential of ribozymes as therapeutic agents for viral infections, there is still a distinct lack of ribozymes that successfully passed their pre-clinical or clinical trials.

To our best knowledge, there are currently no studies on using ribozymes to cleave specific target genes in bacteria to treat bacterial infections. Instead, Felletti et al. [85] successfully cleaved the bacterial 3′-untranslated region (UTR) using twister ribozymes, affecting the expression of the gene downstream. By designing the ribozymes specific to the 3′-UTR of essential bacterial genes, these ribozymes have potential as antibacterial agents.

4. Current status of ribozymes

As of 2022, only four clinical trials are registered on ClinicalTrials.gov for using ribozymes as therapeutic agents (**Table 2**). Among these four, three clinical trials are targeted towards human immunodeficiency virus (HIV), while the other ribozyme is targeted towards kidney cancer.

Two clinical trials were conducted for OZ1, a ribozyme designed to target the overlapping region between two essential genes. The multifunctional viral protein R (vpr) is involved in host infection, immune system evasion and infection persistence [86]. The tat protein is also involved in viral replication, enhancing the efficiency of viral expression [87]. The ribozyme OZ1 is a hammerhead ribozyme encoded within a Moloney murine leukaemia gammaretroviral vector LNL6 [74]. By cleaving the overlapping region in the *vpr* and *tat* gene, the ribozyme could inhibit the replication of HIV-1. A phase I clinical trial was conducted by delivering the OZ1 ribozyme through a retroviral vector to the mature CD34+ hematopoietic cells [74]. It was determined that the gene expression of ribozyme was detected within the patients, demonstrating that the ribozyme OZ1 can be maintained. Another Phase I study was done using a similar delivery vector to CD4+ T lymphocytes, demonstrating similar results whereby the cells can express the ribozyme long term [88]. A Phase II clinical trial (NCT00074997) was conducted with OZ1 ribozymes targeting the CD34+ hematopoietic cells. They did not achieve their primary efficacy endpoint as the mean

Title of clinical trial	Ribozyme	Target gene	Disease	NCT number	Time
An Efficacy and Safety Study of Autologous Cluster of Differentiation 34 (CD34+) Hematopoietic Progenitor Cells Transduced With Placebo or an Anti-Human Immunodeficiency Virus Type 1 (HIV-1) Ribozyme (OZ1) in Participants With HIV-1 Infection	OZ1	vpr/tat	HIV-1	NCT00074997	2002–2008
Long Term Follow-Up Study of Human Immunodeficiency Virus Type 1 (HIV-1) Positive Patients Who Have Received OZ1 Gene Therapy as Part of a Clinical Trial	OZ1	vpr/tat	HIV-1	NCT01177059	2004–2017
Gene Therapy in HIV-Positive Patients With Non-Hodgkin's Lymphoma	L-TR / Tat-neo	Tat, Rev mRNA	Non-Hodgkin lymphoma, HIV infections	NCT00002221	2001 – N/A
RPI.4610 in Treating Patients With Metastatic Kidney Cancer	ANGIOZYME	VEGF-1	Kidney cancer	NCT00021021	2001–2004

Table 2.
Clinical trials of ribozymes registered on ClinicalTrials.gov. All trials were completed in phase 2 trials.

plasma HIV-1 viral load difference was lower but not significantly different from the placebo. However, no serious adverse events were linked to OZ1 gene transfer, indicating that using the retroviral vector to perform this gene therapy is safe, albeit with low efficacy. A second Phase II clinical trial (NCT01177059) was performed with the same group of patients from the previous trials to investigate the long-term effect of the ribozymes. There was no serious adverse effect on the participants due to the treatment. The OZ1 and the retroviral vector LNL6 marking analysis showed that they were only detected in a few participants. Unfortunately, there are no further studies on this ribozyme, perhaps due to its low efficiency in the human system.

Another Phase II clinical trial (NCT00002221) also investigated the usage of ribozymes against HIV. In this trial, a retrovirus containing two ribozyme sequences named L-TR/Tat-neo that target the tat and rev region of the virus RNA was used. Like the tat protein, the rev protein is also essential for viral replication [89]. The ribozymes were delivered to the participants of the clinical trials through *ex vivo* retroviral modified CD34+ stem cells. However, no results have been provided for this clinical trial.

Finally, RPI.4610 (ANGIOZYME), a ribozyme that targets vascular endothelial growth factor receptor 1 (VEGF1) was used to treat patients with metastatic kidney cancer. VEGF is an angiogenesis-promoting molecule, and when its preRNA is cleaved, it can inhibit angiogenesis and tumour growth [90–92]. Clinical trials with ANGIOZYME have demonstrated that it is well tolerated. However, due to its lack of efficacy, this drug could not proceed with further development [93].

5. The roadblock to commercialisation

While ribozymes have the potential to be one of the alternatives to treat infectious diseases, it cannot be denied that there are still multiple roadblocks before they can be developed as marketable drugs. Like other nucleic-acid therapeutics, ribozymes' challenges include selecting the appropriate ribozyme type and target mRNA sequence, delivery to the target site, efficiency *in vivo* and potential side effects as therapeutic drugs.

5.1 Selection of target and ribozymes

There is a wide variety of genes to choose from within the target pathogen, be it virus or bacteria, which can be used as a ribozyme target. The selection of these targets would thus depend on the aim of the ribozyme. An antiviral ribozyme may target the mRNA of genes important for viral replication, while an antibacterial ribozyme to decrease antibiotic resistance may target antimicrobial resistance genes (AMR) instead. More importantly, the cleavage site within the mRNA transcript must be carefully determined for the best cleavage efficiency. Designing sequence-specific ribozymes can be done through rational design or by *in vitro* selection.

To design a ribozyme that targets a specific gene, it needs a target-specific sequence that leads the ribozyme to the target mRNA transcript and cleaves it. Different ribozymes have different target cleavage sites due to their structural variety. For instance, hammerhead ribozymes have an NUH or NHH sequence specificity. In comparison, hairpin ribozymes catalyse site-specific reversible cleavage on the 5' side of a GUC triplet [94]. Another criterion to consider is the accessibility of the cleavage site to the ribozymes. RNAs can fold to specific three-dimensional structures; multiple methods exist to study these structures [95]. One of them is the usage of dimethyl sulfate (DMS), a chemical that can covalently modify both purines and pyrimidines

that are accessible [96, 97]. Through DMS probing and footprinting, it is possible to detect the RNA secondary and even tertiary structure, determine the potential region most accessible to DMS modification and presumably ribozyme binding.

On the other hand, Zhang et al. used a random pool of ribozymes to find accessible target sites [81]. As we progress into the post-genomic era, some may look towards in-silico analysis and bioinformatics to determine the best cleavage site, shortlisting a few for wet lab validation. RiboSoft [98] and RiboSubstrates [99] are some web applications that allow a comprehensive ribozyme design. Unfortunately, these two websites are not maintained. RNAiFold is another web server used to design a hammerhead ribozyme through computational design with experimental validation, showing that this method can be used for synthetic ribozymes [100].

Other than rational design, another method to obtain specific and efficient ribozymes is through an *in vitro* selection process using a ribozyme library [101]. Multiple studies have used this process to identify ribozymes with high cleavage efficiency. A putative self-cleaving hairpin ribozyme library was used whereby ribozymes that successfully bind and cleave a target sequence were identified [102]. Not only does this method allows the identification of effective target sites within the target mRNA, but it can also identify the most efficient ribozyme for a particular target site. The in vitro selection was used by Maghami et al. [103] to identify efficient trans-acting adenylyl transferase ribozymes that can label specific RNA sites. The ribozymes developed can be modified to target other RNA sequences by changing the sequence-specific region of the ribozymes. This method can be modified to different types of ribozymes and towards different targets.

Finally, it is worth noting that while the discovery of ribozymes is not recent, there is still undiscovered land in this field. Firstly, ribozyme variants may provide higher efficiency in their catalytic activity, which can be discovered through *in vitro* selection from a random pool of ribozymes. Deep sequencing of a ribozyme library [104] or a high-throughput analysis [105] can help elucidate novel ribozymes and their properties. Secondly, new types of ribozymes are continually being discovered and studied. A new RNA polymerase ribozyme discovered can also act as a reverse transcriptase enzyme [106]. In contrast, a type of novel ribozyme called hatchet ribozyme was reported in 2019 [38], while a pseudoknot-type hammerhead ribozyme was studied in 2020 [19]. These discoveries demonstrate that new ribozymes with improved potential still continuously emerge in recent times.

5.2 Stability and delivery of ribozymes

Like most nucleic acids, Ribozymes are vulnerable to nuclease attacks by the host cells. An unmodified ribozyme would be rapidly degraded and would not be effective when exposed to nuclease-rich fluids and tissues. Additionally, some ribozymes require co-enzymes or a certain concentration of metal ions for sufficient stability and efficiency. For example, the *glmS* ribozyme-riboswitch requires the presence of the intracellular small molecule co-enzyme GlcN6P for effective catalysis [107]. On the other hand, divalent metal ions, such as magnesium ions, are generally required by ribozymes to form a tertiary structure or catalytic activity [108]. Certain modifications or delivery vectors are needed to ensure their efficiency *in vitro* and *in vivo*.

Ribozymes can be modified to improve their stability and resistance towards nucleases. Some modifications include using locked nucleic acids (LNAs) [109], cholesterol [83], nanoparticles [110], or low-molecular-weight polyethyleneimine [111]. Modifications to the ribozyme tertiary structure or interactions can improve their stability. For instance, a tertiary interaction between a GAAA tetraloop and a

tetraloop receptor within a hammerhead ribozyme showed higher activity even under low magnesium conditions [75]. Another method of modification is to simply conduct an *in vitro* selection to determine which variants of ribozymes can remain effective. An RNase P ribozyme from *in vitro* selection showed a higher cleavage efficiency than the wild-type ribozyme. This variant was used towards the thymine kinase [66] and major transcription activator ICP4 [67] or the herpes simplex virus, as well as the assembly (AS) of murine cytomegalovirus [73]. A coenzyme-independent variant of *glmS* ribozyme was also successfully isolated through *in vitro* selection [112]. This variant contains the wild-type structure that can catalyse the cleaving reactions effectively with the presence of divalent cations alone. These studies demonstrate that it is feasible to develop variants of known ribozymes and modify their requirements for co-enzymes or increase their efficiency.

There are two ways to deliver the ribozymes into the cells: exogenous delivery (as preformed ribozymes) or endogenous delivery (as ribozyme genes). The preformed ribozymes can be delivered through electroporation or lipofection for exogenous delivery. A ribozyme stabilised by GAAA tetraloop and its receptor motif was transfected into human HeLa cells using Lipofectamine 2000 and showed effective target gene silencing [75]. A chimeric DNA-RNA hammerhead ribozyme was transfected using a polyethylenimine reagent into the cells [84]. Due to the vulnerability of ribozymes within the biological system, exogenous delivery relies on modifications that improve the stability of ribozymes. Other studies utilise endogenous delivery. In endogenous delivery, the ribozymes are introduced through ribozyme genes carried within plasmids or expression vectors. These plasmids can then be introduced through transfection to the cells, allowing the cells to express the ribozyme within the cytoplasm. The ribozymes can then catalyse the intended cleavage reaction within the cells [80, 81]. Besides plasmids, the ribozyme genes can be inserted in retroviral-derived or adeno-associated viral-derived vectors (refer to **Table 1**: Delivery). While unsuccessful, the clinical trials of multiple ribozymes using Moloney murine leukaemia virus retroviral vector LNL6 demonstrated its feasibility as delivery agents of ribozymes [113]. Endogenous delivery also benefits from modifications aiming to improve ribozyme stability. Peng et al. used a novel scaffold RNA to stabilise the ribozyme structure, improving its catalytic activities [114]. However, modifications performed on the ribozymes require further investigation. Czapik et al. showed that modifications such as adding a hairpin motif to the hammerhead ribozyme decreased their catalytic activity compared with the unmodified ribozymes [79].

The delivery methods of ribozymes are not limited to these traditional methods. Rouge *et al.* successfully transfected ribozymes into cancer cells without auxiliary transfection agents using the spherical nucleic acid (SNA) architecture to stabilise the ribozymes [110]. The ribozyme, targeted towards a gene involved in chemotherapeutic resistance of solid tumours, increased the sensitisation of the cancer cells towards therapy-mediated apoptosis. On the other hand, an attenuated strain of Salmonella that contains the expression vector encoding the ribozymes was used to deliver these ribozymes to mice [71]. The success of Salmonella-mediated oral delivery of the ribozymes introduced an alternative delivery method other than those mentioned before.

5.3 The efficiency of ribozymes under in vivo conditions

It is easily shown that they can cleave their target mRNA transcripts *in vitro* through the direct cleavage of RNAs or *in vitro* studies. However, it is not as simple to translate these data from *in vitro* conditions to *in vivo*. Multiple studies have used

animal models to prove the potential therapeutic use of ribozymes, and they have successfully demonstrated that in models such as rats and rabbits. Nevertheless, there are still some challenges before the ribozymes can be used in the human body.

Ribozymes, like all enzymes, also require co-factors for their optimal function. One crucial co-factor is the divalent ions, such as magnesium ions. Mainly, these ions are required for the ribozymes to achieve the correct folding of the active site and their tertiary structures [108]. However, the requirements differ between ribozymes. For instance, magnesium is essential for the catalysis activity of hammerhead ribozymes, but hairpin ribozymes do not require magnesium [77, 115].

Further research into the effects of ion concentration on the catalytic core or structure of the ribozymes allowed specific modifications to be made. A section of the ribozyme responsible for substrate-binding and tertiary stabilisation functions can be separated into discrete structural segments to ensure that trans-cleaving hammerhead ribozymes can be used in intracellular applications [116]. This separation provided the resulting ribozymes with an efficient catalytic activity at lower magnesium ion concentration. Additionally, with careful selection, ribozymes may be evolved to require a lower concentration of metal ions for their efficient activity *in vitro* and *in vivo* [117].

Finally, the efficiency of the ribozymes to cleave their targets within the *in vivo* system is also a key to the success of ribozymes as antiviral or antibacterial therapeutic agents. As mentioned previously, the ribozyme ANGIOZYME, while showing promising results in pre-clinical trial studies, did not manage to proceed further than Phase 2 clinical trials due to their lack of efficiency in the patients [93]. Other studies have also highlighted the difficulty in translating the efficiency of ribozymes from *in vitro* to *in vivo*. Due to their rapid degradation during *in vivo* conditions decreasing their concentration within the system, it was proposed that ribozymes are more suitable for acute diseases and not chronic diseases [84]. There were also significant differences in the ribozyme efficiency in recognising and cleaving the target sequences when comparing *in vitro* and *in vivo* cells [77]. Due to these challenges, ribozymes' development as therapeutic agents, in general, has slowed down in the past years. More research must be conducted to improve the feasibility of ribozymes in the *in vivo* system by focusing on their stability and efficiency to bring ribozymes back to the table.

6. Conclusion

Ribozymes are catalytic RNAs that can catalyse reactions similarly to protein enzymes. There is a wide variety of ribozymes classes with different characteristics and structures, and even now, novel ribozymes are being discovered through research. Ribozymes have the potential to be used as therapeutic agents for infectious diseases. While there is a lack of actual ribozymes for antibacterial purposes, multiple ribozymes are tested to successfully target viruses such as human immunodeficiency virus (HIV), human cytomegalovirus and herpes simplex virus. Unfortunately, their uses have not been translated into real-world applications, mostly due to their vulnerability to nucleases in the biological system and the difficulty in translating their efficiency from the *in vitro* system to the *in vivo* system. However, progress has been made in improving their stability and delivery, and it is hoped that with more research, ribozymes can be the next therapeutic agent used for infectious diseases.

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Conflict of interest

The authors do not have any conflict of interest.

Author details

Bao Chi Wong, Umama Shahid and Hock Siew Tan*
Monash University Malaysia, Selangor, Malaysia

*Address all correspondence to: tan.hocksiew@monash.edu

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