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Therapeutic applications of ribozymes and riboswitches

Jérôme Mulhbacher, Patrick St-Pierre and Daniel A Lafontaine

Therapeutic approaches employing RNA as a tool or as a drug target have recently emerged and have been employed for various applications—ranging from cancer treatment to virus infection. Despite the paucity of its molecular groups compared to proteins, RNA has nevertheless proved to be an excellent choice for researchers who have aspired to develop therapeutic tools. Ribozymes and riboswitches are RNA-based therapeutic tools that are most often employed to knockdown gene expression and to inhibit bacterial infections, respectively. The aim of this review is to summarize recent advances observed in ribozyme- and riboswitch-based therapeutic applications that, in some cases, have reached clinical trials.

Address
Groupe ARN/RNA Group, Département de biologie, Faculté des sciences, Université de Sherbrooke, Sherbrooke, Québec, Canada J1K 2R1

Corresponding author: Lafontaine, Daniel A (Daniel.Lafontaine@USherbrooke.ca)

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Introduction

Before the discovery that biologically relevant processes could rely on RNA-based mechanisms, RNA was only considered as a cellular messenger carrying out genetic information and proteins were only predicted to be catalytic molecular effectors involved in essential biological processes. The discovery of ribozymes, such as the group I intron and ribonuclease P [1,2], brought the idea that RNA could play a pivotal role in metabolic processing. RNA was also shown to be important in gene regulation mechanisms as exemplified by riboswitches [3], which are genetic regulatory elements that can bind small metabolites and regulate gene expression. Recently, by employing SELEX — (systematic evolution of ligands by exponential enrichment) — to manipulate at will the properties of ribozymes and riboswitches, a myriad of applications ranging from cellular detection to gene expression control were obtained, thus clearly illustrating the enormous potential of RNA as a genetic tool [4]. In this review, we plan to present the most recent advances on the field with a particular emphasis on the use of ribozymes and riboswitches for therapeutic purposes. We will build on previous reviews discussing ribozyme-based therapeutic applications [5–9] to provide a glimpse of future research activities dedicated to the tailoring of specific RNA therapeutic tools.

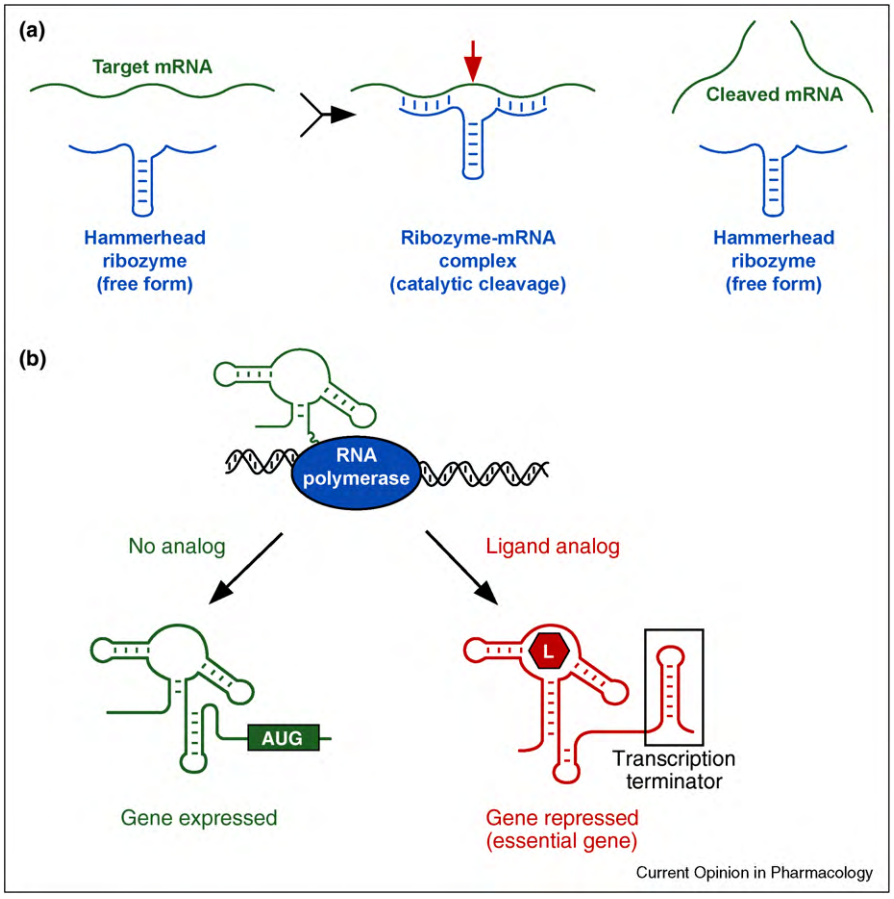
Therapeutic applications of ribozymes

Natural ribozymes perform various enzymatic reactions and are classified by their relative size [10–12]. Large ribozymes (>300 nt) are constituted by the group I and group II introns, and by the protein-assisted RNase P, while small nucleolytic ribozymes include the hammerhead, hairpin, hepatitis delta virus (HDV), Varkud satellite (VS), and glmS ribozymes. The hammerhead is the smallest and the best characterized small catalytic ribozyme [11], making it an interesting template for the development of ribozyme-based therapeutic agents. Recent advances have shown that a loop–loop interaction is important for the catalysis [13–16], but by using a minimal version in which the ribozyme arms are engineered to hybridize to a target mRNA, it is possible to direct the hammerhead cleavage toward a specific mRNA sequence as a way to knockdown the expression of a given gene (Figure 1a) [17]. A major aspect of the ribozyme design is the analysis of cleavage target sites and the identification of active ribozyme sequences. However, an important challenge consists in the ribozyme delivery. This can be achieved either by the transfection of expression vectors endogenously producing ribozymes or by the administration of exogenously synthesized ribozymes. Chemical modifications are usually employed to ensure longer half-lives of ribozyme molecules [18]. Interestingly, although hammerhead ribozymes appear slightly less effective than siRNAs, they exhibit less off-target effects as found in RNA interference mechanisms [19]. Ribozyme-inactivating mRNA strategies have been used by different research groups for therapeutic applications and several hammerhead ribozymes have been used in clinical trials [18]. In the following examples, we will survey the latest ribozyme therapeutic applications that strongly rely on hammerhead ribozymes.

Ribozyme-based anticancer strategy

The human epidermal growth factor receptor HER-2 is a member of the EGF receptor family. HER-2 encodes for a transmembrane receptor protein p185 that is overexpressed in 20–30% of adenocarcinoma, which are often associated with breast cancer. Lu and co-workers designed a hammerhead ribozyme targeting p185 mRNA as a way to reverse the malignant phenotype of breast

Figure 1



Schematic representation of action mechanisms of ribozymes and riboswitches used for therapeutic applications. **(a)** A hammerhead ribozyme targets an mRNA for cleavage. The ribozyme hybridizes to the mRNA target to perform enzymatic cleavage and dissociates to release the cleaved product. In principle, the ribozyme is free for a second round of reaction. **(b)** A guanine riboswitch is used as an antimicrobial target. Upon mRNA transcription by the RNA polymerase, the gene can be expressed in absence of the analog. However, in presence of a specific ligand analog (L) that binds to the aptamer, the expression of the gene is repressed. The gene repression is accomplished via premature transcription termination achieved by the formation of the transcription terminator (boxed).

cancer cell line MCF-7 [20]. The authors observed that the HER-2 mRNA, as well as the oncogene *k-ras*, produced at the end of the HER-2 pathway, were down-regulated in cells transfected with this ribozyme. Furthermore, the tumorigenicity of cells stably transfected with the ribozyme targeting HER-2 mRNA was significantly decreased in nude mice [20], suggesting that the use of anti-HER-2 ribozymes may help for gene therapy of breast cancer.

Defects in apoptosis are commonly found in malignant cells [21]. Survivin is a well-known anti-apoptotic factor which is overexpressed in all types of cancers and is therefore used as a biomarker for cancer diagnosis and prognosis. Survivin has also been selected as a target for cancer intervention [22]. In a recent study, four hammerhead ribozyme adenoviruses were developed to target single-strand regions of survivin mRNA [22]. The co-expression of three ribozymes was shown to be sufficient to significantly reduce survivin expression and produced the most potent anticancer effect. The suppression of survivin expression resulted in cell death via the caspase-3-dependent apoptotic pathway. The administration of ribozyme adenoviruses inhibited tumor growth in a hepatocellular carcinoma xenograft mouse model [22].

The *Tetrahymena* group I intron ribozyme is able to catalyze its auto-excision from mRNAs as well as perform splicing reactions in a *trans* fashion. This *trans*-splicing ribozyme could thus be used to reprogram a specific disease-associated transcript with transgene RNA exerting a therapeutic effect. The rational is that *trans*-splicing ribozyme targeting transcripts dominantly expressed in cancer cells would recognize its own substrates, switch them into suicidal gene transcripts and cause eventual cell death [23•]. Thus, because the human telomerase

reverse transcriptase, hTERT, is expressed only in stem cells and tumor cells, it is an excellent target for cancer therapy. Song *et al*. designed an adenoviral vector using a liver tissue-specific promoter controlling expression of a *trans*-splicing ribozyme targeting hTERT to replace it with either *lacZ* or herpes simplex virus thymidine kinase [24]. Intratumoral injection of a virus encoding for hTERT-targeting ribozyme into liver cancer cells in peritoneal carcinomatosis mice model produced selective and efficient regression of tumors [24]. This study demonstrates that the utilization of a hTERT-targeting *trans*-splicing ribozyme with a tissue-selective promoter represents a promising strategy for cancer gene therapy.

Ribozyme-based antiviral strategy

Several anti-HIV-1 strategies interfere with the functions of viral or cellular RNA and proteins [25]. Strategies that inhibit viral entry are of particular interest, as they would prevent healthy cells from being infected. It is known that HIV-1 recognizes CD4 as a primary receptor and uses CXCR4 or CCR5 as co-receptor to infect the cell [26]. CD4 and CXCR4 are essential for cells, but CCR5 is dispensable and it was already shown that individuals that do not express CCR5 are resistant to HIV-1 [27]. Nazari *et al*. attempted to downregulate CCR5 using a multimeric hammerhead ribozyme RZ_{1-7}, which targets seven unique sites within the human CCR5 mRNA [28]. This ribozyme proved to be more effective than a monomeric version to downregulate CCR5 mRNA. It almost completely inhibited the entry of HIV-1 in previously transducted cells after infection with R5-tropic HIV-1 (99–100% of inhibition). However, the treatment was not efficient against X4-tropic HIV-1 that appears to not rely on the CCR5 co-receptor to invade the cell [28]. The inhibition of virus production was observed for the duration of the experiments (2 months). The use of the multimeric hammerhead ribozyme RZ_{1-7} to cleave the CCR5 mRNA resulted in almost complete inhibition of the R5-tropic HIV-1 replication at the level of the entry and is thus a promising tool for a HIV-1 gene therapy.

HIV-1 infections have also been treated using highly active antiretroviral therapy (HAART), but have often been associated with toxicities, adverse interactions with other drugs and the emergence of viral resistance [29••]. Ribozyme-based therapies have been proposed as a long-lived alternative to small-molecule HAART [30]. Using a hammerhead ribozyme (OZ1) targeting the overlapping *vpr* and *tat* reading frames of HIV-1, it has been shown that the replication of laboratory and clinical isolates of HIV-1 could be inhibited *in vitro* and that resistance mutations in the ribozyme-targeted region were not observed in long-term cell culture. The OZ1 ribozyme was recently used in a phase 2 gene therapy trial where 74 HIV-1-infected adults were tested either using OZ1 or placebo delivered in autologous $CD34^+$ hematopoietic progenitor cells. The study showed that during the treatment (100 weeks), $CD4^+$ lymphocyte counts were higher in the OZ1 group [31]. This study suggests that ribozymes are promising therapeutic agents that can be employed against HIV-1 infections.

Ribozyme-based anti-prion strategy

Transmissible spongiform encephalopathies (TSE) are invariably fatal degenerative disorders of the central nervous system. The hallmark of these diseases is the accumulation of misfolded isoforms of a host-encoded protein, PrP^c or prion protein. TSE can be acquired by dietary exposure to infected tissues or by familial, spontaneous and iatrogenic means. According to the protein-only hypothesis, the disease is caused by the post-translational misfolding of the protein PrP^c of the host. The prion protein has been identified as the underlying causative agent as PrP knockout mice ($prnp^{0/0}$) are resistant to infection, suggesting that a significant reduction in the expression levels of PrP^c should interrupt disease progression. Hybrid hammerhead ribozymes including both a helicase recruitment signal and a $tRNA^{Val}$ promoter were used to target *prnp* mRNA. This strategy has been successfully used by allowing a 95% efficient knockdown of the *prnp* mRNA in neuroblastoma cells [32].

Therapeutic applications of riboswitches

Riboswitches are mostly found in untranslated regions of bacterial mRNA and are involved in gene expression regulation [33]. They are composed of two domains: an aptamer and an expression platform. The aptamer is involved in ligand recognition and the expression platform controls gene expression by adopting two mutually exclusive conformations depending on ligand binding. Metabolite-binding riboswitches represent a novel solution to multiple drug resistance (MDR) since they can be considered as antimicrobial targets when agonistic ligands are employed to knockdown the expression of the associated gene(s) (Figure 1b) [34]. Examples of such riboswitches used as antimicrobial targets are described below.

The TPP riboswitch

The thiamine pyrophosphate (TPP) riboswitch negatively regulates the expression of proteins involved in the biosynthesis and transport of thiamine in bacteria [35,36]. The TPP riboswitch binds its cognate ligand with a dissociation constant of 100 nM and discriminates by a factor of 100-fold against thiamine phosphate (TP), which only differs from TPP by one phosphate [37]. Pyrithiamine (PT) is an isosteric pyrimidine analog of thiamine that was shown to be toxic for fungi and bacteria [35,38,39]. PT, like thiamine, is phosphorylated in the cell to pyrithiamine pyrophosphate (PTPP) [40]. It is expected that PT and PTPP compete for thiamine and TPP binding sites on proteins to produce toxic effects. However, because bacteria and fungi that synthesize TPP *de novo* are susceptible to the effects of PT, the full understanding of PT toxic effects has remained elusive

for several decades, until recently [35]. Indeed, by using in-line probing assays, Breaker and co-workers have shown that PTPP is able to bind the TPP riboswitch with an affinity similar to TPP [35]. β-Galactosidase reporter assay showed that PT inhibits the expression of genes under the control of TPP riboswitch probably following its conversion in PTPP, highlighting its action mechanism as an antimicrobial compound. Bacteria selected for PT resistance show specific mutations disrupting ligand binding to TPP riboswitches and derepressing certain TPP metabolic genes, suggesting that the riboswitch was directly acting as an antimicrobial drug target [35].

The lysine riboswitch

Lysine riboswitches are involved in the control of biosynthesis and transport of lysine [41,42]. The importance of lysine and lysine intermediates in cellular metabolism has led to efforts to design new antibacterial compounds that bind to lysine riboswitches and repress the expression of important genes under the control of the riboswitch [43••]. Screening of lysine-related compounds identified lysine analogs exhibiting affinities similar to lysine for the riboswitch that were able to repress gene expression [43••]. Analogs put in culture in presence of *Bacillus subtilis*, as found for the TPP riboswitch, resulted in mutant strains obtained after serial passage that were resistant to lysine analogs. It was found that every resistant colony had a single mutation in the *lysC* riboswitch [43••], strongly suggesting that identified analogs repress bacterial growth via riboswitch binding.

The compound *S*-(2-aminoethyl)-L-cysteine (AEC) was also previously shown to exhibit antibacterial activity by binding to the lysine riboswitch [41]. Moreover, it was previously reported that strains exhibiting mutations in the *lysC* riboswitch were resistant to AEC [41]. Thus, as for the other lysine analog compounds, it was conceived that AEC exhibited antimicrobial effects by inhibiting *lysC* expression due to its binding to the lysine riboswitch. However, it was recently shown that the lysine riboswitch is not the primary cellular target of AEC [44••]. Indeed, it was found that lysyl-tRNA synthetase (LysRS) can load AEC on its cognate tRNA and thus perturb downstream protein synthesis and inhibit bacterial growth. Bacteria containing LysRS mutants and wild-type lysine riboswitches were obtained that allowed growth in the presence of AEC, indicating that LysRS is the primary AEC target. It is expected that riboswitch mutations are generated to increase the cellular pool of lysine to compete with AEC for aminoacylation and protein synthesis. This study demonstrates that antimicrobial compounds can exhibit complex mechanisms of action.

The FMN riboswitch

Roseoflavin, which is a natural pigment isolated from *Streptomyces davawenensis*, exhibits an antimicrobial activity involved in the inhibition of riboflavin biosynthesis [45]. Roseoflavin might potentially target the FMN riboswitch because of its structural similarity to flavin mononucleotide (FMN) [46,47]. It was found that roseoflavin binds FMN riboswitch *in vitro* [34] and downregulates the expression of a *LacZ* reporter gene under the control of FMN riboswitch [48,49]. Growing bacteria in presence of roseoflavin also allowed selection of resistant mutants bearing mutations in the FMN riboswitch, suggesting that roseoflavin exerts its antimicrobial activity via riboswitch binding and gene inactivation.

The guanine riboswitch

The guanine riboswitch controls the expression of genes involved in purine biosynthesis and transport [50]. This riboswitch is one of the smallest and the most structurally characterized [50–56], making it an excellent choice to rationally design drugs targeting riboswitches. Kim *et al.* used rational design to identify several riboswitch-compatible guanine analogs that could potentially bind guanine riboswitches and repress bacterial growth [57]. Several compounds were found to bind the riboswitch *in vitro* and to inhibit *B. subtilis* growth. Serial inoculation of media was performed to generate resistant mutant strains, and of all strains sequenced, no mutations were found in guanine riboswitches. Several mutations were found in the terminator stem of the expression platform of the *pbuE* adenine riboswitch, suggesting that mutant strains export guanine analogs. Guanine-related antimicrobial compounds were also explored in a second study where pyrimidines were employed to cause bacterial growth inhibition [58••]. In this study, Mulhbacher *et al.* used a pyrimidine compound mimicking guanine interactions with the riboswitch that showed bacterial activity. By screening various bacterial strains, it was observed that the bacterial inhibition was only obtained when the guanine riboswitch was upstream of *guaA*, a GMP synthetase, which led to guanine auxotrophy when inactivated in *Escherichia coli* [59]. Several clinical strains exhibiting MDR were inhibited, including *Clostridium difficile* and *Staphylococcus aureus*, suggesting that the pyrimidine compound targets a different metabolic pathway. The efficiency of the analog to inhibit bacterial growth was also shown in a murine infection model [58••].

Conclusions

Ribozymes and riboswitches offer new therapeutic approaches to address human diseases. The use of ribozymes appears to be a promising alternative to RNAi technology as no off-target hits have yet been observed [19,60]. For the hammerhead ribozyme, at least 12 complementary nucleotides are needed for the ribozyme hybridization/formation with a gap of one nucleotide between the two hybridization sites to allow cleavage [61], which is likely important for the specificity of the reaction. In addition, newly-discovered riboswitches are interesting antibiotic targets as they offer new opportu-

nities to fight MDR. The first demonstration of their efficiency in an infection model should promote attempts to use them as antibiotic targets. In addition, riboswitches could be employed to control enzyme production in genetic disorders by using SELEX-designed RNA genetic elements.

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