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A Discovery with Potential to Revitalize Hammerhead Ribozyme Therapeutics for Treatment of Inherited Retinal Degenerations

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Abstract

Hammerhead ribozymes (*hhRzs*), RNA enzymes capable of site-specific cleavage of arbitrary target mRNAs, have faced significant hurdles in development and optimization as gene therapeutics for clinical translation. Chemical and biological barriers must be overcome to realize an effective therapeutic. A new Facilitated ribozyme has been identified with greatly enhanced kinetic properties that lead new insight on the capacity of ribozymes to target mutant genes to treat inherited retinal degenerations.

Keywords

Gene therapy; Inherited retinal degenerations; Retinitis pigmentosa; Hammerhead ribozyme; RNA biologic therapies; Noncoding RNA

20.1 Hammerhead Ribozymes

20.1.1 The Beginning

Hammerhead ribozymes (*hhRzs*) are catalytically active noncoding RNA that facilitate cleavage of a target mRNA sequence, generating products that are degraded in the cell. Naturally occurring catalytically active RNAs were discovered by professors Sidney Altman and Thomas R. Cech (Altman 1990; Cech 1990), who received the Nobel Prize in Chemistry in 1989.

Ribozymes were first discovered as naturally occurring motifs in plant viroids (Forster and Symons 1987). They enhance replication in a “rolling-circle” fashion that allows for viroid RNA genome processing from long RNA concatemers to single circles. This provided evidence for the “RNA world” hypothesis in which early evolution of life was influenced first by catalytically active nucleic acids before proteins (Jaeger 1997). Further research discovered many structurally diverse, naturally occurring ribozymes such as the hairpin, hepatitis delta virus, and hammerhead. The *hhRz* has been the most extensively characterized and is the focus of this chapter.

20.1.2 Ribozyme Structure and Function

Study of these naturally occurring *cis*-acting (RNA target and *hhRz* on continuous nucleotide chain) *hhRzs* identified critical motifs driving the cleavage process (Hertel et al. 1994). The *hhRz* has two main motifs, a conserved catalytic core stabilized by a stem and capping loop, embraced by two antisense flanks which hybridize to target mRNA (Fig. 20.1 lower right). Once bound, a conformational change aligns nucleotides within the catalytic core with the cleavage site nucleotide. Deprotonation of the 2' hydroxyl of the ribose sugar allows it to act as a nucleophile in an $\text{S}_\text{N}2$ attack on the phosphodiester bond, which culminates in cleavage products, one with a 2',3'-cyclic phosphate and the other with a 5'-hydroxyl (Tanner 1999; Emilsson et al. 2003). The critical motifs of the *cis*-*hhRz* were morphed into a *trans*-cleaving molecule known as the “minimal” *hhRz* by removing the original *cis*-target and modifying the annealing antisense flanks to be able to recognize arbitrary targets (Bratty et al. 1993). *Cis*-acting *hhRzs* are capable of fast intramolecular cleavage of one molecule. *Trans*-acting ribozymes are capable of intermolecular cleavage of many target molecules in vitro. However, in vivo *trans*-acting ribozymes have demonstrated only single-turnover target cleavage and require that the enzyme concentration be in substantial molar excess over the target RNA (James and Gibson 1998).

Involvement of Magnesium— Mg^{2+} and monovalent ions provide structural stability for tertiary interactions that position the core nucleotides to better facilitate the acid-base chemistry of cleavage (Martick and Scott 2006). Naturally occurring *cis*-cleaving *hhRzs* are able to cleave at submillimolar Mg^{2+} concentrations; *trans*-cleaving *hhRzs* require >5 mM.

Physiologically relevant levels of Mg^{2+} are 0.1 mM–1 mM, so extensive work to optimize hhRz function at low Mg^{2+} is necessary for therapeutic development (Hean and Weinberg 2008).

Tertiary Accessory Elements (TAEs)—*Trans*-acting hhRzs have significantly lower cleavage rates than the naturally occurring *cis*-hhRzs, in part because of the second-order nature of the *trans* reaction, and that essential elements of the hhRz were removed during the initial enzyme engineering to achieve a small hhRz with site-specific cleavage capacity against an arbitrary target (Uhlenbeck 1987). In fact, upstream regions, now known as tertiary accessory elements (TAEs), were missing for over 20 years of research. This made for a schism between the initial structural biology as a means to fully explain the biochemistry.

De la Pena et al. demonstrated significant enhancement of cleavage rates of *trans*-acting hhRzs after adding upstream peripheral region nucleotides from the *cis*-forms they were derived from (De la Pena et al. 2003). Khvorova et al. discovered that adding loops to cap two helices in the minimal hhRzs to mimic *cis*-acting ribozymes increased cleavage rates at physiological levels of Mg^{2+} , exceeding rates observed by a wild-type enzyme (Khvorova et al. 2003). The loops form an RNA pseudoknot interaction that enhances the probability of achieving the catalytically active state (Martick and Scott 2006). Another enhancement found in nature is a 5' TAE that interacts with the variant loop capping a variant Stem II of the hhRz (Shepotinovskaya and Uhlenbeck 2010) producing a maximal observed rate (k_{obs}) of $<10 \text{ min}^{-1}$ (Breaker et al. 2003). The creation of these *extended* hhRzs precipitated engineering of hhRzs to enhance cleavage beyond their minimal forms.

Tetraloop receptors (TLRs) added 5' to the hhRz enhance cleavage efficacy by interacting with the tetraloop capping stem II stabilizing the core of the enzyme. TLR additions to minimal hhRzs enhanced rates by 4.5×, although still substantially less than *cis*-ribozymes at 1.4 min^{-1} (Fedoruk-Wyszomirska et al. 2009).

20.2 Therapeutics Development

20.2.1 Ribozymes Are an Ideal Posttranscriptional Gene Silencing Agent

HhRzs are an excellent candidate posttranscriptional gene silencing (PTGS) agent. “NUH↓” sites, where N = any nucleotide, U = uridine, and H = any nucleotide excluding guanosine, are the specific targets of cleavage. The GUC↓ motif was identified as the most cleavable site and is estimated to appear every 64 bases in the genome, indicating many potential genetic targets (Hean and Weinberg 2008).

The ability to reduce gene expression is a prime clinical tool. PTGS agents such as hhRzs, siRNA, and RNAi are being developed as potential therapeutics for disease caused by dominant gain of function mutations. By inhibiting toxic protein production, the disease state could be treated. HhRzs are optimal PTGS agents because they are capable of multiturnover reactions, do not rely on endogenous cell machinery, and have low potential for off-target effects due to high annealing specificity (Sullivan et al. 2011). Researchers

have developed hhRzs for many conditions including HIV, cancer, and ocular disease with successes in vitro (Birikh et al. 1997; Persidis 1997).

20.2.2 Hammerhead Ribozymes in Ophthalmology—adRP caused by mutations in the rhodopsin (*RHO*) gene has been the main therapeutic target of hhRz development for our group and others (Millington-Ward et al. 1997; O'Neill et al 2000; Sullivan et al. 2002; Gorbatyuk et al. 2005, 2007). Over 200 mutations have been reported in *RHO* that lead to adRP (Froebel et al. 2017). A knockdown-reconstitution strategy can be employed which targets both wild-type and mutant *RHO* with only one hhRz, creating a single therapeutic for the diverse set of mutations (Sullivan et al. 2011). *RHO* is then reconstituted, to avoid haploinsufficiency, in a form that creates WT protein but is immune to cleavage.

A critical factor of designing hhRzs is to model your mRNA target and to choose a NUH↓ site that is accessible in vivo. Computational secondary structure modelling is most commonly used, and our lab has developed the *multiparameter prediction of RNA accessibility* (mppRNA) to identify ideal sites. This method predicts that our lead candidate hhRz will be as effective against WT and ~90% of reported mutations (Froebel et al. 2017).

HhRz screening against multiple targets throughout *RHO* has proven successful in vitro, with varied but suboptimal success in vivo (Gorbatyuk et al. 2007). A recent study demonstrated a *RHO* hhRz that was highly effective at cleavage in vivo, but the high titer of AAV virus needed to transduce the retina was toxic (Cideciyan et al. 2018).

20.2.3 Failure of Ribozyme In Vitro Results to Translate In Vivo—The pervasive problem in hhRz development is that hhRzs that prove successful in vitro do not have the same cleavage efficacy in cell culture or animal screening stages. With clinical goals in mind, optimization of hhRz function in the cell requires input from biology. Ribozymes designed to target HIV were the first in clinical trial. Two trials with different HIV targets and ribozyme designs both ended with very low to no expression of ribozyme detected in patients' cells, and the antiviral efficacy was not evaluated (Scarborough and Gatignol 2015). There is another HIV targeting ribozyme in clinical trial to be evaluated for both safety and efficacy as a treatment for AIDS-related non-Hodgkin lymphoma to be completed in 2031 (<https://clinicaltrials.gov/ct2/show/study/NCT01961063>). Currently, there has been no hhRz developed for any condition to enter the market.

These problems with hhRzs led many researchers to abandon them and focus on newer techniques such as RNAi and CRISPR. While these techniques have merit, many of the original problems with in vivo hhRz optimization remain. Critical factors of enhancing therapeutic RNA expression, cellular trafficking, and half-life enhancement are influential in all of these techniques and the abandonment of hhRz technology for therapeutics indeed may have been premature.

20.3 Facilitated Ribozyme Provides New Insight on Enhancing Enzymatic Activity

Our Facilitated-hhRz (*F-hhRz*) was first identified in attempts to merge hhRz and miRNA functionality (Myers et al. 2018). We discovered an RNA element that when attached to a hhRz allows Michaelis-Menten enzymatic performance, meaning functionality under substrate excess conditions. The *F-hhRz* also operates at cellular free levels of Mg^{2+} for minimal and full-length target substrates. The Facilitator was identified as an element placed 3' to the hhRz, not the natural position of a 5' upstream TAE/TLR of hhRzs *in cis*. Minimal hhRzs generally have a cleavage rate around 1–2/min either under single or multiturnover conditions for a variety of substrates, with larger substrate rates orders of magnitude lower. Here, we have identified rates up to 150/min for *F-hhRzs* against short *RHO* substrates (Fig. 20.1 *Graph*).

Emilsson et al. (2003) found enzymes (RNA and protein) that catalyze RNA cleavage in a hhRz-like manner generally follow four rate-enhancing mechanisms. RNA enzymes only use two of these accelerating mechanisms to get to a ceiling of 1–2/min, (Stage-Zimmermann and Uhlenbeck 1998), whereas RNaseA appears to use all four mechanisms and operates on the scale of enhancement of 10^4 /min.

At 1–2 log order enhanced rates with *F-hhRzs*, we realize that these RNA catalysts have a novel functionality. Obviously, this enhanced performance of the *F-hhRz* could potentially revitalize hhRz therapeutics in gene therapy. *F-hhRzs* stabilized for long lifetime in cells could promote strong knockdown of target mRNAs with much lower levels of expression than the historical minimal and enhanced hhRzs. This chemical advantage may allow for a lower titer of AAV to be transduced into the cell to reduce reported toxic effects. Recent studies have demonstrated RNA structure-functional correlates that begin to explain these high rates of functional activity (Myers and Sullivan 2019; Sullivan et al. 2019).

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Fig. 20.1.

Cleavage kinetics of minimal (hhRz266), F-hhRz266, F2-hhRz266, and F3-hhRz266. Data are from fluorescent cleavage assays against a short substrate in low magnesium and substrate in 10× excess to enzyme conditions. Cleavage of substrate alleviates FAM quenching causing a fluorescent signal. Right minimal hhRz, left F-hhRz. Green represents target with arrow site of cleavage