Ribozyme-Based Therapeutic Approaches for Autosomal Dominant Retinitis Pigmentosa

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PURPOSE. To design, generate, and compare in vitro a range of hammerhead ribozymes targeting retinal transcripts implicated in autosomal dominant retinitis pigmentosa (adRP) and thereby identify ribozymes that may be valuable as therapeutic agents for adRP. To address mutational heterogeneity in rhodopsin and peripherin-linked adRP using mutation-independent ribozyme-based therapeutic approaches.

METHODS. Ribozyme and cDNAs constructs were cloned into pcDNA3 and expressed in vitro from the T7 promoter. Cleavage reactions were separated on polyacrylamide gels, visualized by autoradiography, and quantified using an instant imager. Ribozymes targeting rhodopsin and peripherin transcripts in a mutation-independent manner (Rz9, Rz10, and Rz40) and a multimeric ribozyme (RzMM) targeting rhodopsin transcripts were evaluated for in vitro activity. Parameters such as $V_{\text{max}}$, $K_{\text{m}}$, $k_2$ and $k_{-1}$ were established for each ribozyme.

RESULTS. Four ribozymes targeting retinal transcripts were evaluated. Mutation-independent ribozymes targeting degenerate sites or untranslated regions in retinal transcripts resulted in cleavage products of predicted size, whereas transcripts from modified replacement genes remained intact. Detailed kinetic evaluation of ribozymes revealed substantial differences in cleavage rates between ribozymes.

CONCLUSIONS. Mutation-independent hammerhead ribozymes targeting rhodopsin and peripherin have been screened in vitro, and a number of extremely efficient ribozymes identified subsequent to detailed kinetic analyses, suggesting that these ribozymes may provide mutation-independent methods of treating adRP. These are the first ribozymes reported that potentially will provide benefit for inherited retinopathies. (Invest Ophthalmol Vis Sci. 2000;41:2865–2869)
(RzMM) that simultaneously targets four sites in human rhodopsin RNA have been tested in vitro, and detailed kinetic profiles have been generated for these ribozymes. Kinetic profiles of ribozymes in vitro can be used as broad predictors of potential efficiencies in vivo.\textsuperscript{2,8} Ribozymes follow Michaelis–Menten kinetics\textsuperscript{2,8}; parameters such as \(V_{\text{max}}\) (maximum velocity of the reaction), \(K_m\) (Michaelis–Menten constant), \(k_2\) (the rate of the cleavage step), \(k_{-1}\) (the dissociation rate), and \(t_{1/2}\) (the half-life of substrate RNA) can be determined. Kinetic values obtained in the study indicate that some ribozymes tested are extremely efficient in vitro and may be valuable in the development of mutation-independent therapeutic approaches for adRP.

### MATERIALS AND METHODS

**cDNA and Ribozyme Constructs**

Human peripherin (M62958) and human rhodopsin (K02281) constructs were linearized with BseFI and BglII, respectively, and transcribed from a T7 promoter using Ribomax kits (Promega, Eugene, OR) incorporating \(\alpha^{32}\text{P}\) uridine triphosphate.\textsuperscript{8} Resultant transcripts were gel purified. Hammerhead ribozymes were designed to target accessible regions in target mRNAs, as predicted by the computer program RNAPlotFold (Wisconsin Package, Madison, WI).\textsuperscript{9} The integrity of open loops was evaluated from the 15 most probable conformations. Rz10 and Rz40 were designed to cleave a GUC motif at positions 475-477 of the 5' human rhodopsin sequence. Both ribozymes were cloned into the dIII, XbaI or Xho sites of pcDNA3 or pcDNA3.1(−) (Invitrogen, San Diego, CA). Constructs were linearized with Xbal, transcribed, and purified as described earlier. Ribozyme sequences with antisense arms are italic in print: Rz9: 5'-GAAACCUUCUGAGGUGGAGGACGAAACGAGCC3'; Rz10: 5'-GGUCGCGUCUGAGUAGUCGGAGAGCAAGUAGG3'; Rz40: 5'-GGACGGUAGUGAGCUAGGAGGGAGG3'; and RzMM: 5'-GGACGGUAGUGAGCGAGGAGG3'.

**Cleavage Reactions**

Transcripts were quantified by scintillation counting, and molar ratios were determined.\textsuperscript{5} Cleavage reactions were performed in 0 to 15 mM MgCl₂, 50 mM Tris-HCl (pH 8.0) at 37°C for up to 3 hours. Time points were performed using optimal MgCl₂ concentrations. Labeled transcripts were separated on polyacrylamide gels (4%–8%) and analyzed by autoradiography and instant imaging.\textsuperscript{8} Percentage cleavage was determined as follows: \(\%\) cleavage = (cpm P1 + cpm P2)/(cpm target + cpm P1 + cpm P2) where cpm is counts per minute, and P1 and P2 are cleavage products 1 and 2, respectively.

### Determination of Steady State Intervals and Ribozyme Saturation

Target and ribozyme RNAs were combined in molar ratios of 1:0.2 to 1:100. RNAs were heated at 90°C for 3 minutes, incubated at 37°C and cleavage reactions initiated with 10 mM MgCl₂. Linear time frames of reactions and ribozyme saturation were determined from graphs of percentage cleavage versus time.

### Single- and Multiple-Turnover Kinetics: Determining \(t_{1/2}\), \(k_2\), \(k_{-1}\), \(V_{\text{max}}\) and \(K_m\)

Ribozyme and substrate RNAs were preannealed at 37°C for 5 minutes in saturating conditions of ribozyme excess, thereby enabling determination of single-turnover parameters including the \(t_{1/2}\) of substrate RNA, \(k_2\), and substrate \(k_{-1}\).

**Kinetic Pathway for a Hammerhead Ribozyme**

\[
Rz + S \rightleftharpoons RzS \rightleftharpoons RzP1P2 \leftrightarrow Rz + P1 + P2
\]

where \(Rz\) is ribozyme; \(P1\) and \(P2\) are cleavage products 1 and 2, \(RzS\) is the ribozyme–substrate complex, and \(S\) is the substrate.

The \(t_{1/2}\) was determined over two \(t_{1/2s}\) from linear profiles of a graph of the fraction of uncleaved target RNA versus time, \(k_2\) was calculated from \(k_2 = \ln(2)/t_{1/2}\),\textsuperscript{8} and \(k_{-1}\) was determined using a pulse–chase experiment with saturating molar excesses of ribozyme and a trace of labeled substrate and chased with a 100-fold excess of unlabeled substrate.\textsuperscript{6,8} Multiple-turnover parameters such as \(V_{\text{max}}\) and \(K_m\) (affinity of ribozyme for target RNA) were determined within steady state intervals under conditions of substrate

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932909/ on 09/06/2018)

**Figure 1.** Structure of hammerhead ribozyme Rz40. The two antisense arms (steps 1 and 3) flanking the conserved catalytic core and the double-stranded region (step 2) of the ribozyme are shown. The GUC target site 475-477 is italicized with an arrow indicating the exact position of cleavage in human rhodopsin mRNA.
excess. \( V_{\text{max}} \) and \( K_m \) were calculated using the following equations:\textsuperscript{10}

\[
V_{\text{max}} = \frac{\sum v^2/a^2 \Sigma v^2 - (\Sigma v^2/a^2)^2}{\sum v^2/a^2 \Sigma v - \Sigma v^2/a \Sigma v/a^2}
\]

\[
K_m = \frac{\Sigma v^2/a^2 \Sigma v - \Sigma v^2/a \Sigma v/a}{\sum v^2/a^2 \Sigma v - \Sigma v^2/a \Sigma v/a}
\]

where \( a \) is substrate concentration, and \( v \) is the rate of reaction.

Reactions were also performed under multiple-turnover conditions in the presence and absence of a 250-fold excess of COS-7 cellular RNA to ribozyme RNA. Molar ratios of target to ribozyme RNA ranged between 1.25:1 and 5:1.

**Size Marker**

MspI–cut pBR322 DNA was labeled with [\( \alpha^{32}\text{P} \)] dCTP. Fragment sizes were 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, and 76 bases.

**RESULTS**

The computer program RNAPlotFOLD was used to identify single-stranded areas in rhodopsin and peripherin transcripts that may be accessible to hammerhead ribozymes.\textsuperscript{5,6,9} Three

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**Figure 2.** Cleavage time points of (A) human peripherin RNA and Rz9 and (B) human rhodopsin RNA and Rz10. Graphs were used to determine ribozyme saturation and also to identify the linear component of the cleavage reaction. Cleavage times were 0, 5, 7.5, 10, 20, 30, 60, and 180 minutes for Rz9 and Rz10. All reactions were performed with 10 mM MgCl\(_2\) at 37°C. Right: Molar ratios of substrate to ribozyme RNA.
monomeric and one multimeric ribozyme were designed to target large predicted open-loop structures in these two transcripts. Rz10, Rz40, and RzMM target degenerate sites in human rhodopsin transcripts and Rz9 targets the 5′ UTR of the human peripherin transcript. The hammerhead structure of Rz40, including the consensus sequence of the catalytic core,\textsuperscript{11} GUC cleavage motif, and flanking antisense sequences that provide specificity of the ribozyme for human rhodopsin mRNA, is presented in Figure 1. Kinetic profiles have been generated for all ribozymes in vitro.

\textbf{Figure 3.} Cleavage gels of human rhodopsin RNA by Rz10 in the absence (A) and presence (B) of a 250-fold excess of total cellular RNA. Lanes 1 through 8: Molar ratios of substrate RNA to ribozyme of 1:0.2, 1:0.4, 1:0.5, 1:0.6, 1:0.8, 1:2, 1:5, and 1:10, respectively. Rates of each multiple-turnover reaction were used to determine $V_{\text{max}}$ and $K_{\text{m}}$ parameters. \textit{Arrowheads:} Human rhodopsin RNA and 5′ and 3′ cleavage products (584 and 277 bases). (C) Lineweaver–Burk plot of human rhodopsin RNA cleavage by Rz10 under multiple- and single-turnover conditions, in both the presence and absence of total cellular RNA.

\textbf{Figure 4.} Cleavage gels of human rhodopsin RNA by a 10-fold molar excess of Rz10 alone (A) and in the presence of a 100-fold excess of nonradioactively labeled chase rhodopsin RNA (B). Lanes 1 through 7: Reaction time frames of 0.5, 1, 3, 5, 10, 15, and 20 minutes, respectively. \textit{Arrowbeads:} Target rhodopsin RNA, 5′ and 3′ cleavage products. (C, D) Percentage of cleavage of human rhodopsin RNA by (C) Rz10 and (D) Rz40 in the absence and presence of chase RNA. Control reactions are also indicated. Results were used to determine $k_2$ and $k_{-1}$ parameters. (E) Cleavage gel of human peripherin RNA by a 50-fold molar excess of Rz9. Lanes 1 through 8: Cleavage times of 10 seconds and 5, 10, 20, 30, 60, 120, and 180 minutes, respectively. \textit{Arrowbeads:} Intact peripherin target RNA (545 bases) and 5′ and 3′ cleavage products (194 and 295 bases). Notably, cleavage of the highly structured target RNA almost reached completion. (F) Cleavage profile of human rhodopsin RNA by the multimeric ribozyme construct. Lane 1: DNA ladder. Lanes 2 through 8: A 10-fold molar excess of RzMM and rhodopsin RNA for 0, 5, 10, 20, 40, 60, 90, and 120 minutes. Reactions were performed in the presence of 10 mM MgCl\textsubscript{2} at 37°C. \textit{Filled arrowbeads:} Target rhodopsin RNA and labeled ribozyme. \textit{Open arrows:} Spectrum of cleavage products. Because any one or more of the monomeric ribozymes within the multimeric construct can function at any given time, it was not possible to determine which cleavage products were the result of a specific ribozyme or combination of ribozymes. However, cleavage of the target RNA almost reached completion.
Steady State Intervals and Ribozyme Saturation

Saturating levels of ribozyme over target transcripts were identified using graphs of percentage cleavage versus time (Figs. 2A, 2B). Saturation was deemed to have been achieved when no additional substrate cleavage was observed regardless of the molar excesses of ribozyme used. Notably, molar excesses of ribozyme to substrate as low as 8:1 (Rz9 to human peripherin RNA) and 4:1 (Rz10 to human rhodopsin RNA) were sufficient to achieve saturation (Figs. 2A, 2B). Linear time frames for Rz9, Rz10, and Rz40 cleavage reactions were 30, 6, and 6 minutes, respectively, as determined using time points with varying ratios of substrate RNA to ribozyme (Fig. 2).

Multiple-Turnover Kinetics

$V_{\text{max}}$ and $K_m$ for Rz9, Rz10, and Rz40 were determined within linear time-frames under conditions of substrate excess. Rz10, despite its long target (861 bases), was found to be extremely active, achieving a $V_{\text{max}}$ of 0.71 min$^{-1}$, in the same range as that observed for ribozymes targeting short, unstructured transcripts. The $K_m$ value for Rz10 was 8.5 nM. Similar $V_{\text{max}}$ and $K_m$ values of 0.55 min$^{-1}$ and 6.75 nM respectively were obtained with Rz40. The $K_m$ value for Rz40 suggests that this ribozyme may have a slightly higher affinity for rhodopsin RNA than Rz10. This may be due to the single-base mismatch present in one antisense arm of Rz10 that is absent in Rz40. Given the lower $V_{\text{max}}$ value obtained with Rz40, it may be that the inclusion of such a mismatch should be given consideration in the future design of therapeutic ribozymes to increase their catalytic efficiencies. Data indicate that Rz9 ($V_{\text{max}} = 0.23$ min$^{-1}$, $K_m = 13.1$ nM) is approximately three times less active than Rz10 or Rz40 and has a reduced affinity for its target. Kinetic profiles suggest that these ribozymes are extremely efficient in vitro and thus may be valuable as potential mutation-independent therapeutic agents for adRP.

Inhibition experiments were performed as described earlier but with a 250-fold excess of cellular RNA to Rz10 (Figs. 3A, 3B, 3C). Cleavage of the target by Rz10, although reduced by approximately 25%, remained specific. $V_{\text{max}}$ in the absence and presence of total RNA was similar: 0.71 min$^{-1}$ and 0.8 min$^{-1}$, respectively (Fig. 3C), whereas $K_m$ values increased from 8.3 nM to 26.55 nM, suggesting a reduction in binding of Rz10 to human rhodopsin RNA. Typically, competitive inhibition results in an increased $K_m$, and $V_{\text{max}}$ remains unaltered, whereas uncompetitive inhibition causes reduced $V_{\text{max}}$ values with unaltered $K_m$. The data suggest that addition of cellular RNA to cleavage reactions with Rz10 causes competitive inhibition.

Single-Turnover Kinetics

The $k_2$, substrate $k_{-1}$, and $t_{1/2}$ of substrate RNA were determined for Rz10 and Rz40 under single-turnover conditions. The $t_{1/2}$ for human rhodopsin RNA, cleaved by Rz10 and Rz40, were 28 and 50 seconds, respectively (Figs. 4A through 4D). Similar $k_2$ of 1.47 min$^{-1}$ and 1.38 min$^{-1}$ for Rz10 and Rz40, respectively, were generated from the $t_{1/2}$ (Figs. 4A through 4D). Rz10 and Rz40 were notably efficient. More than 50% of the 861-base rhodopsin target was cleaved in less than 30 seconds (Fig. 4A). The single-base mismatch in Rz10 would be predicted to increase the $k_{(\text{intermax})}$ because of possible destabilization of substrate and ribozyme binding. As predicted, $k_{-1}$ for Rz10 and Rz40 showed that the $k_{-1}$ for Rz10 (0.55 min$^{-1}$) was slightly greater than that for Rz40 (0.4 min$^{-1}$). In contrast Rz9 was found to be significantly less efficient than either Rz10 or Rz40 under both single- and multiple-turnover conditions (see earlier description). However, cleavage of human peripherin RNA by Rz9 over time almost reached completion under conditions of ribozyme excess (Fig. 4E).

Cleavage Profile of Multimeric Ribozyme RzMM

A multimeric ribozyme (RzMM) consisting of four hammerhead ribozymes directed to degenerate sites in human rhodopsin RNA was generated and tested in vitro. Figure 4F illustrates the cleavage profile of RzMM. Because any single ribozyme or multiple ribozymes within the multimeric cassette may potentially cleave the transcript, a number of cleavage products were observed. Using a 10:1 molar ratio of RzMM to target RNA, approximately 85% of target transcripts were cleaved (Fig. 4F). Because of the large number of potential cleavage products generated by RzMM, kinetic parameters could not be accurately determined. However, RzMM does not appear, at least in vitro, to be more efficient than Rz10 or Rz40.

**DISCUSSION**

Therapies for diseases such as RP could be targeted to specific mutations that give rise to pathologic changes in disease. For example, the Gly51Val rhodopsin mutation known to cause adRP creates a hammerhead ribozyme target NUX site that fortuitously occurs in an accessible region of the human rhodopsin RNA (determined by RNAPlotFold; data not shown). However, most mutations do not create NUX sites and are not situated in accessible regions of target transcripts. In addition, given that more than 150 different rhodopsin and peripherin mutations can give rise to adRP and various photoreceptor degenerations, it is apparent that mutation-independent approaches for therapy that circumvents genetic heterogeneity would be highly preferable. Such approaches may be valuable not solely for RP but for many other inherited diseases with similar high levels of genetic heterogeneity. Two mutation-independent approaches were adopted in the present study that exploit inherent features of the genome, either the degeneracy of the genetic code or UTRs of a transcript. For example, Rz10, Rz40, and RzMM target human rhodopsin transcripts at various wobble sites, thereby exploiting the degeneracy of the code and enabling the introduction of a replacement gene with modified sequences around wobble sites. For all mutation-independent ribozymes tested replacement genes coding for wild-type protein but with marginally altered sequences around the ribozyme target site have been shown to escape ribozyme cleavage in vitro. A connected multimeric ribozyme (RzMM) has also been evaluated for cleavage of human rhodopsin transcripts. RzMM simultaneously targets four degenerate sites in open-loop structures of human rhodopsin RNA. Studies have indicated that it may be advantageous to use multimeric ribozymes to achieve efficient cleavage of target RNAs. Populations of alternative RNA conformations may be cleaved at one or multiple sites by different ribozymes in the multimeric cassette. RzMM cleaved human rhodopsin RNA into a range of cleavage products as expected for a multimeric
hammerhead ribozyme and represents the first such multimeric ribozyme designed to target a retinal transcript (Fig. 4F).

Kinetic profiles of ribozymes in vitro can be used as broad predictors of activity in vivo. All ribozymes developed in the study elicited sequence-specific cleavage of retinal target transcripts, yielding cleavage products of predicted sizes. Detailed kinetic analyses showed that Rz10 and Rz40 were extremely active under all conditions tested. For example, the $V_{\text{max}}$ obtained for Rz10 targeting a degenerative site in an 861-base rhodopsin transcript was 0.71 min$^{-1}$ thereby achieving a $V_{\text{max}}$ in the range obtained with ribozymes that target short, unstructured RNAs (approximately 20 bases). The efficiency of Rz10 is highlighted by the observation that typically reductions in $V_{\text{max}}$ of 200- to 1000-fold have been observed for ribozymes directed to long-structured RNAs. Although Rz9 was three times less active than Rz10 and had a slightly higher $K_m$ value, the ribozyme nevertheless was more active in vitro than many other ribozymes (Fig. 4E). In addition, single-turnover kinetic parameters were determined for Rz10 and Rz40. Values for $k_2$ may vary considerably; however, it has been suggested that $k_2$ typically ranges from 0.4 to 2.5 min$^{-1}$ for ribozymes that target unstructured RNAs. It is of note that $k_2$'s for Rz10 and Rz40 (1.47 min$^{-1}$ and 1.38 min$^{-1}$, respectively) were significantly greater than their corresponding $k_1$'s (0.55 min$^{-1}$ and 0.4 min$^{-1}$, respectively) contributing to the efficiency of both ribozymes.

Efficiencies of ribozymes in vitro only broadly predict potential efficiencies in vivo. In this regard, a number of mouse models of rhodopsin-linked retinal degenerations that could be used to test the functionality of these ribozymes—for example, mice carrying human rhodopsin transgenes with Pro23His Val20Gly, Gln344ter, Pro296Glu, or Pro347Ser mutations—are available. Moreover, there are now many reports demonstrating that hammerhead ribozymes can be functional in both cell and animal systems. One such study used a ribozyme targeting a mutant human rhodopsin transcript in a rat model of a retinal degeneration. Given the efficiency of the ribozymes evaluated in the present study they should be valuable in the development of mutation-independent therapeutic approaches for inherited photoreceptor degenerations such as adRP.

In summary, hammerhead ribozymes Rz10, Rz40, and RzMM, which target human rhodopsin transcripts, and Rz9, which targets human peripherin transcripts, have been found to be extremely efficient in vitro after detailed kinetic analyses. Comparative analyses with previously reported ribozymes suggest that, despite their longer and more structured targets, these retinal ribozymes achieve cleavage rates similar to many ribozymes that target short, unstructured RNAs of 20 to 40 bases. Moreover, the design of these ribozymes is such that they are the first therapeutically relevant ribozymes that will enable mutation-independent gene silencing of retinal genes implicated in dominantly inherited retinopathies.

References