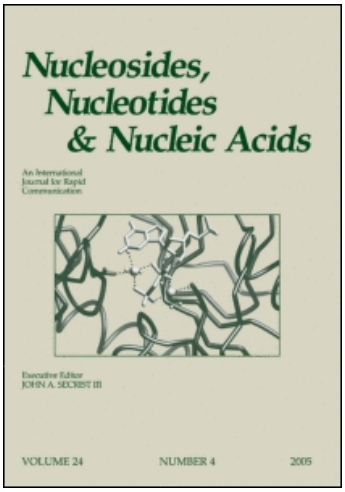

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Binary Hammerhead Ribozymes with High Cleavage Activity

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Online publication date: 10 July 2004

To cite this Article Kuznetsova, M. , Novopashina, D. , Repkova, M. , Venyaminova, A. and Vlassov, V.(2004) 'Binary Hammerhead Ribozymes with High Cleavage Activity', Nucleosides, Nucleotides and Nucleic Acids, 23: 6, 1037 — 1042
To link to this Article: DOI: 10.1081/NCN-200026063
URL: http://dx.doi.org/10.1081/NCN-200026063

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 23, Nos. 6 & 7, pp. 1037–1042, 2004

Binary Hammerhead Ribozymes with High Cleavage Activity

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ABSTRACT

Binary hammerhead ribozymes consisted of two oligoribonucleotides capable of assembling into hammerhead structure (without loop II) on the RNA target were engineered. Catalytic activities of such ribozymes were investigated in comparison with their full-length analog and ribozyme where two strands were jointed by non-nucleotidic linker. Binary constructs were shown to be significantly more active than the parent full-length hammerhead ribozyme.

Key Words: RNA cleavage; Hammerhead ribozyme; Binary ribozyme; Jointed ribozyme; Oligoethylene glycol linker.

INTRODUCTION

One of the most extensively progressing approaches to the selective action on particular mRNAs is the use of small catalytic nucleic acids which possess the properties of RNA sequence-specific recognition and site-specific cleavage.[1] The goal

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DOI: 10.1081/NCN-200026063
1525-7770 (Print); 1532-2335 (Online)

of the present study was to design hammerhead ribozymes with improved catalytic activity. The site in the translation initiation region of a multiple drug resistance gene MDR1 mRNA was chosen as a cleavage target.[2]

RESULTS AND DISCUSSION

For optimal design of ribozyme constructions the influence of the length of substrate-binding arms on ribozyme catalytic activity was investigated. Three hammerhead ribozymes have been synthesized and tested for their catalytic activity (Fig. 1). The 19-mer synthetic fragment of MDR1 mRNA (nucleotides 127–145) was used as a model substrate in this study. In a single turnover mode all three ribozymes effectively cleaved RNA substrate (data not shown). In a multiple turnover mode the limiting extent of cleavage for **HHRz 7/7** was markedly higher than for ribozymes with 8- and 9-nucleotide binding arms (Fig. 1).

The probable reason for such difference was the stability of duplexes between ribozyme arms and RNA substrate. Using burst kinetics method,[3] we have shown that for ribozymes **HHRz 8/8** and **HHRz 9/8** the rate-limiting step was the products release. For **HHRz 7/7** the cleavage rate was almost the same as for **HHRz 9/8**, but the limiting extent of cleavage was higher because the chemical step became rate-limiting due to less stable duplexes of ribozyme arms with the cleavage products. Thus for the subsequent design of new ribozyme constructs we have chosen **HHRz 7/7** as a basis.

Binary ribozymes consisted of two short oligoribonucleotides (18-mer and 14-mer) capable of assembling into hammerhead structure (without loop II) on the RNA target

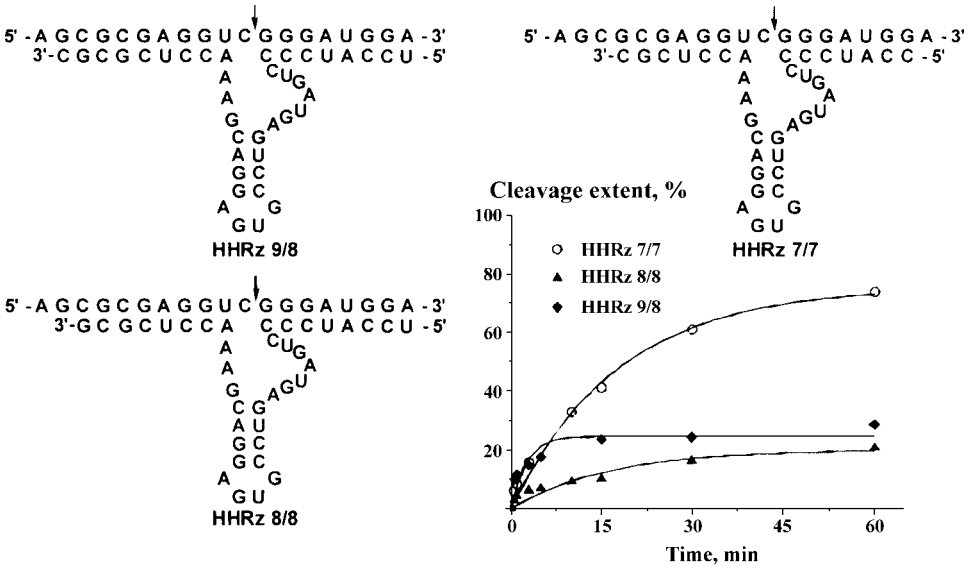

Figure 1. The cleavage of RNA by full-length hammerhead ribozymes **HHRz 9/8**, **HHRz 8/8**, and **HHRz 7/7**. Cleavage conditions: 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 37°C; initial RNA concentration 1.0×10^{-7} M. Concentration of ribozymes was 1.0×10^{-8} M.

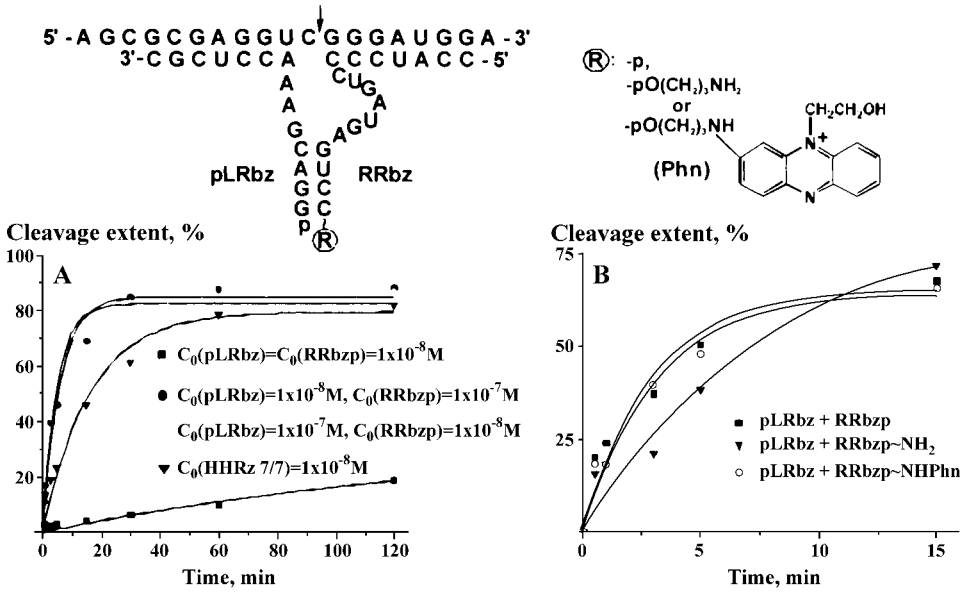

Figure 2. The cleavage of RNA by the binary and full-length ribozymes in multiple turnover mode. Cleavage conditions: 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 37°C; initial RNA concentration 1.0×10^{-7} M; (B) concentrations of (L) and (R) ribozyme strands were 1.0×10^{-8} M and 1.0×10^{-7} M, respectively.

were engineered (Fig. 2). We suggest that this strategy would improve the activity of ribozyme due to the faster rate of products dissociation and will also simplify the synthesis and purification of ribozyme.

Two-strand ribozymes corresponding to hammerhead ribozyme with the scission in loop II were described earlier by Ohtsuka,[4] but these constructs were not investigated further probably due to reduced activity comparing with parent ribozymes.

The catalytic activity of our binary ribozymes was evaluated in comparison with the full-length hammerhead ribozyme **HHRz 7/7**. In a single turnover mode the binary ribozyme hydrolyzes RNA with the same efficiency as its full-length analog (the limiting extent of cleavage was about 85% after 3 min of incubation). In a multiple turnover mode, at the concentration of both ribozyme strands 1×10^{-8} M, the efficiency of cleavage was dramatically reduced, and the limiting extent of cleavage did not exceed 20%. However, as it is seen from Fig. 2A, it was enough to raise the concentration of only one of the ribozyme strands to increase the cleavage efficiency by several times, thus the limiting extent and the rate of cleavage becomes higher than in the case of the parent full-length hammerhead ribozyme.

Kinetic parameters of RNA cleavage by the binary ribozymes were obtained. It was shown that in a multiple turnover mode RNA cleavage kinetics did not follow Michaelis equation, so the estimation of kinetic parameters of RNA cleavage was carried out in a single turnover conditions (Table 1).

In the case of binary ribozymes we observed a considerable increase of K_M value comparing with the full-length ribozyme. We propose that such rise of K_M reflects the

Table 1. Kinetic parameters of RNA cleavage by the designed hammerhead ribozymes at 25°C in single turnover mode (determined according to Ref. [5]).

Ribozyme	K_M, nM	k_{cat}, min^{-1}	k_{cat}/K_M, $mM^{-1} \times min^{-1}$
HHRz 7/7	208 ± 45	15.9 ± 1.0	76
pLRbz + RRbzp	890 ± 170	21.5 ± 1.7	24
pLRbz + RRbzp ~ NH_2	1150 ± 440	19.4 ± 2.7	17
pLRbz + RRbzp ~ NHPhn	700 ± 350	23.2 ± 4.6	33
HHRz 7/7 HEG	234 ± 78	11.0 ± 1.1	47

downturn of stability of RNA:binary ribozyme complex due to less stable helix II in the absence of tetraloop. Thus, to provide the further increase of binary ribozyme activity, stabilization of helix II is necessary. To enhance the stability of this duplex, an intercalator N-(2-hydroxyethyl)phenazinium residue was attached to the 3′-end of the ''right'' ribozyme strand through aliphatic amino linker. As it is seen from kinetic curves (Fig. 2B) and kinetic constants (Table 1), binary ribozymes bearing phosphate group or N-(2-hydroxyethyl)phenazinium residue at the 3′-terminus of the ''right'' ribozyme strand were more active than ribozyme containing aliphatic amino linker in the same position.

To evaluate the effect of loop II deletion, we have also designed hammerhead ribozyme which strands were jointed with flexible non-nucleotidic linker based on ethylene glycol units[6] (Fig. 3). The catalytic activity of such ribozyme construct was investigated comparing with the binary ribozyme and the parent full-length hammerhead ribozyme.

In a single turnover mode K_M value for the jointed ribozyme **HHRz 7/7 HEG** was several times smaller than for the binary ribozyme (Table 1), while k_{cat} was higher for

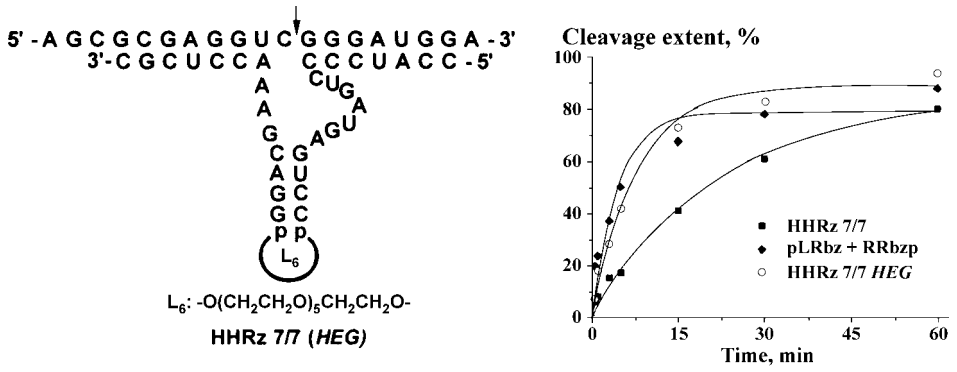

Figure 3. The cleavage of RNA by full-length, binary and linker-containing ribozymes in multiple turnover mode. Cleavage conditions: 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 37°C; initial RNA concentration 1.0×10^{-7} M; concentration of ribozymes was 1.0×10^{-8} M; in the case of binary ribozyme, concentrations of ''left'' (L) and ''right'' (R) ribozyme strands were 1.0×10^{-8} M and 1.0×10^{-7} M, respectively.

Table 2. Kinetic parameters of RNA cleavage by linker-containing and full-length ribozyme at 37°C in a multiple turnover mode (determined according to Ref. [5]).

Ribozyme	K_M, nM	k_{cat}, min^{-1}	k_{cat}/K_M, $mM^{-1} \times min^{-1}$
HHRz 7/7	97	0.4	4.1
HHRz 7/7 HEG	273	1.4	5.1

the binary construct. This leads to the conclusion that in our case removing of loop II does not cause any adverse changes of the catalytic core structure, and even accelerates the chemical step of the reaction, but complicates the assembly of reactive complex. Comparing the RNA cleavage by **HHRz 7/7** and **HHRz 7/7 HEG** we observed that kinetic parameters of cleavage in a single turnover mode were almost the same, but in a multiple turnover mode k_{cat} value and the overall catalytic efficiency (k_{cat}/K_M) was higher in the case of HEG-containing ribozyme (Table 2).

Such unexpected increase of catalytic activity may be tentatively explained by dimerization of ribozyme in analogy with Refs. [6,7]. The indirect evidence of this hypothesis is the increase of K_M value in a multiple turnover mode, which characterizes the formation of reaction complex. Further experiments will be fulfilled to prove the formation of ribozyme dimers.

To summarize, we have compared catalytic activities of different types of hammerhead ribozymes described above (Fig. 3). It was shown that in a catalytic mode the parent ribozyme was less efficient than newly designed constructs.

The details of the synthesis and properties of ribozyme constructs will be published elsewhere. The results obtained allow one to consider constructed ribozymes and the proposed strategy of their design as promising for the inhibition of expression of genes, including MDR1 gene.

ACKNOWLEDGMENTS

This work was supported by the Russian State Program ''Gene Targeted Biologically Active Compounds as Antiviral and Anticancer Drugs,'' SB RAS Integration Program (grant No. 50), CRDF (grant No. REC-008-X1), and RFBR (grants No. 02-04-48586 and 03-04-06089-mas).

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Received December 2, 2003
Accepted April 23, 2004