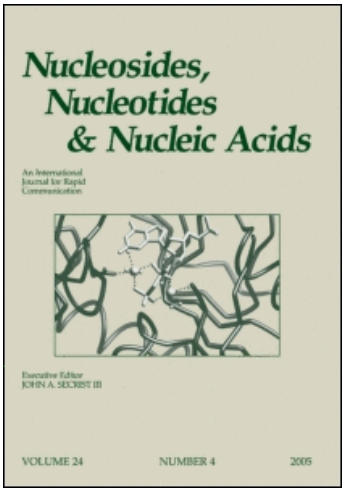

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Modified Binary Hammerhead Ribozymes with High Catalytic Activity

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MODIFIED BINARY HAMMERHEAD RIBOZYMES WITH HIGH CATALYTIC ACTIVITY

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▫ *A series of binary hammerhead ribozymes was designed and assessed in terms of cleavage activity and nuclease resistance. Enhanced nuclease resistance of binary ribozymes was achieved by incorporation of 2′-modified nucleotides at the selective positions along with addition of 3′-3′-linked thymidine cap. These modified binary ribozymes efficiently cleave 190-nucleotides long MDR1 mRNA fragment and display catalytic activity much higher then respective full-length analogs.*

Keywords RNA Cleavage, Hammerhead Ribozyme, Modified Binary Ribozyme, Nuclease Resistance, MDR1 mRNA

INTRODUCTION

Hammerhead ribozymes capable of site-selective RNA cleaving are promising tools for gene silencing. Recently we described binary ribozymes consisted of two oligoribonucleotides assembling into a hammerhead core structure upon binding to a target RNA. These binary ribozymes take advantages of improved catalytic properties provided by higher rate of products dissociation and simple synthetic procedures.[1,2] In this article, we would like to report on design of modified binary ribozymes that exhibit high catalytic activity and high nuclease resistance.

RESULTS AND DISCUSSION

Modified binary ribozymes targeting translation initiation region of a multiple drug resistance gene mdr1 mRNA were designed. To enhance nuclease resistance

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of binary ribozymes, all non-conservative ribonucleotides in substrate-binding domains, catalytic domain, and stem II were replaced by their 2′-O-methyl analogs with simultaneous modification of the 3′-end of each strand. 2′-O-Methyluridine or 2′-aminouridine residues were incorporated in positions 4 and 7 (see Figure 1A, **LRz-1 + RRz-1** and **LRz-2 + RRz-2**, respectively). According to data published in Beigelman et al.,[3] these modifications do not affect significantly cleavage efficiency of full-length hammerhead ribozymes. To estimate the effect of catalytic core modifications on binary ribozyme activity, ribozyme with non-modified catalytic core, **LRz-3 + RRz-3** was used as a control (Figure 1C).

Cleavage activity of the ribozymes was tested in experiments with 19-mer RNA substrate under single turnover reaction conditions (Table 1). Ribozyme **LRz-3 + RRz-3** in which non-conservative ribonucleotides in substrate-binding domains and stem II were replaced by 2′-OMe analogs exhibit similar cleavage activity (k_{cat}) as unmodified one **pLRz + RRzp**.[2] In case of modified binary constructions **LRz-1 + RRz-1** and **LRz-2 + RRz-2**, a decrease of cleavage rate was observed, probably because of partial distortion of ribozyme core structure.

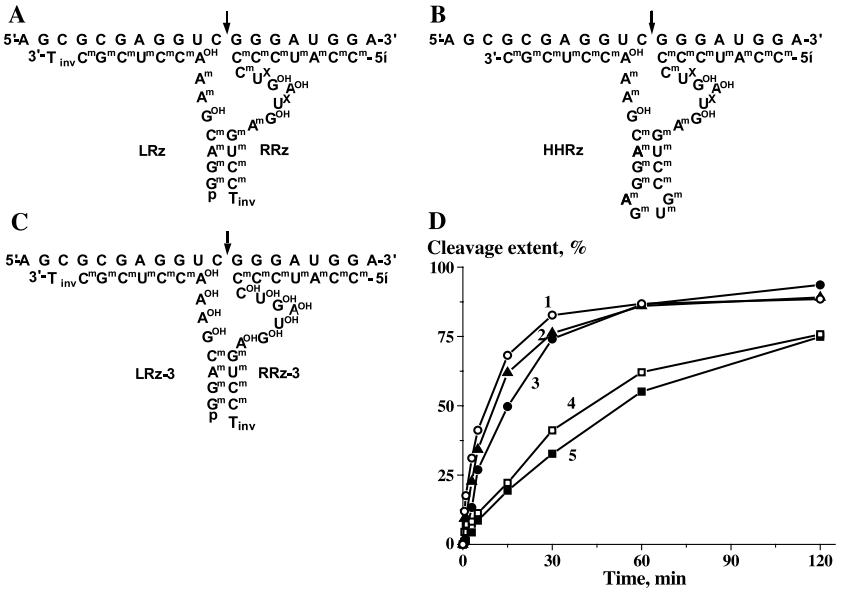

FIGURE 1 Binary and full-length ribozymes cleavage activity in reaction with 19-mer synthetic RNA. Abbreviations: LRz, "left" strand of binary ribozyme; RRz, "right" strand of binary ribozyme; HHRz, full-length hammerhead ribozyme; N^m, 2′-O-methylribonucleotide; T_{inv}, thymidine residue linked by 3′-3′ phosphodiester bond. Cleavage site is shown by an arrow. **A**. Structure of modified binary ribozymes **LRz-1 + RRz-1** (X = 2′-OCH_3) and **LRz-2 + RRz-2** (X = 2′-NH_2). **B**. Structure of modified full-length ribozymes **HHRz-1** (X = 2′-OCH_3) and **HHRz-2** (X = 2′-NH_2). **C**. Structure of binary ribozyme **LRz-3 + RRz-3** with unmodified catalytic core. **D**. Kinetics of 19-mer RNA cleavage. Cleavage conditions: 37°C; 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, initial RNA concentration 0.1 μM. [RRz] = 0.1 μM, [LRz] = 0.02 μM, [HHRz] = 0.02 μM. Kinetic curves are numbered as follows: 1, **HHRz-2 (-○-)**; 2, **LRz-3 + RRz-3 (-▲-)**; 3, **LRz-2 + RRz-2 (-●-)**; 4, **HHRz-1 (-□-)**; 5, **LRz-1 + RRz-1 (-■-)**.

TABLE 1 Kinetic Parameters of 19-mer mRNA Fragment Cleavage Under Single Turnover Reaction Conditions[a]

Ribozyme	K_M (nM)	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1} \times mM^{-1}$)
LRz-1 + RRz-1	449 ± 168	0.8 ± 0.1	1.8
LRz-2 + RRz-2	296 ± 73	4.2 ± 0.3	14
LRz-3 + RRz-3	830 ± 237	22.2 ± 2.5	27
pLRz + RRzp[b]	893 ± 169	21.5 ± 1.7	24

[a]Determined according to Hendry et al.[4]
[b]Unmodified binary ribozyme.[2]

However, ribozyme **LRz-2 + RRz-2** containing 2′-NH_2 uridine at positions 4 and 7 was at least five times more active than **LRz-1 + RRz-1** containing 2′-OMe uridine at the same positions. This difference can be attributed to the ability of 2′-NH_2 group of hydrogen bonding necessary to support the active structure of catalytic core.[3]

It was shown that modified binary ribozymes efficiently hydrolyze RNA under multiple turnover conditions (5-fold excess of RNA substrate), and the rate of cleavage depends on the type of catalytic core modification (Figure 1D). We compared the efficiency of RNA cleavage in multiple turnover mode by modified binary ribozymes **LRz-1 + RRz-1** and **LRz-2 + RRz-2** and full-length ribozymes **HHRz-1** and **HHRz-2**. Binary ribozymes with modified catalytic core displayed the rate and the maximal extent of RNA cleavage similar to that of full-length ribozymes. It is worth noting that the same modifications introduced in the core structure similarly affect activity of both full-length and binary ribozymes: compare curves 1 and 3 (2′-NH_2 U in positions 4 and 7) and curves 4 and 5 (2′-OMe U in positions 4 and 7) (Figure 1D).

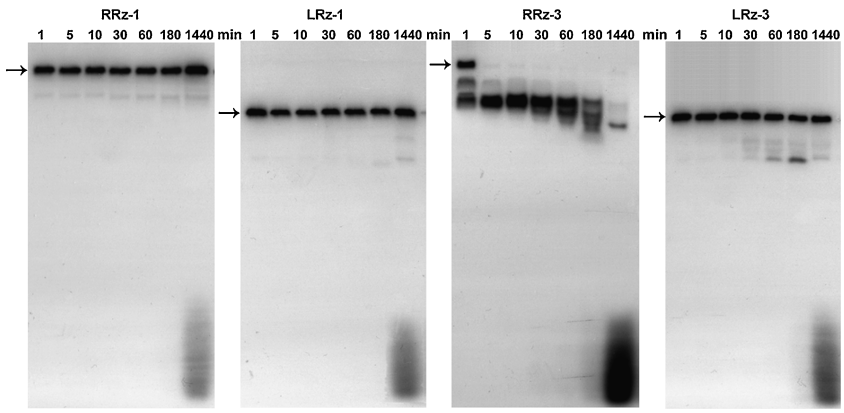

FIGURE 2 Nuclease resistance of modified binary ribozymes. 5′-^{32}P-Labeled ribozymes were incubated in cultural medium IMDM supplemented with 10% FBS at 37°C. Aliquots taken at the indicated times were fractionated by electrophoresis in a 20% denaturing polyacrylamide gel. Positions of initial oligomers are shown by arrows. Incubating times are shown at the top.

Nuclease resistance of modified binary ribozymes was assayed in cultural medium supplemented with 10% FBS (Figure 2). It is seen that modified binary ribozymes **LRz-1 + RRz-1** and **LRz-2 + RRz-2** are entirely resistant toward serum nucleases during incubation times. On the contrary, components of binary ribozyme containing an unmodified catalytic core, namely **RRz-3** and at much low extent **LRz-3** degraded in culture medium. These differences in nuclease resistance of "right" and "left" strands resulted from sequences of corresponding parts of catalytic core.

The ability of binary hammerhead ribozymes to cleave native folded RNA was examined in experiments with 190-nt 5′-terminal fragment of multiple drug resistance gene mdr1 mRNA[5] (Figure 3A). Modified binary ribozymes specifically and efficiently cleaved this RNA. As in the case of short RNA substrate, ribozyme **LRz-1 + RRz-1** was found to be more active than ribozyme **LRz-2 + RRz-2** containing 2′-OMe uridine at positions 4 and 7 (Figure 3, curves 1 and 3).

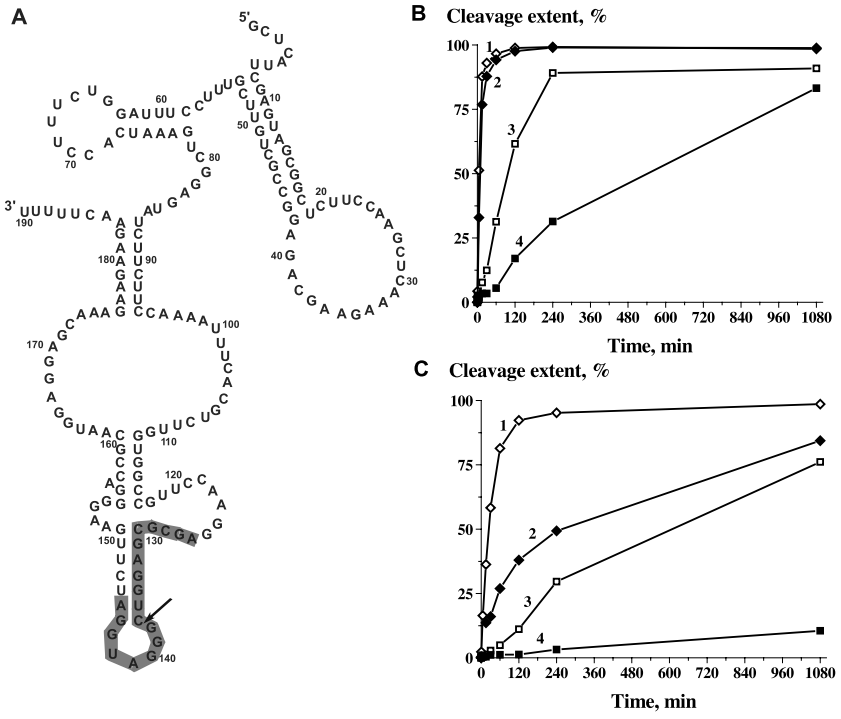

FIGURE 3 Cleavage of 5′-terminal fragment of MDR1 mRNA by modified binary and full-length ribozymes. **A**. Secondary structure of the 190-nucleotide long fragment of MDR1 mRNA.[5] The sequence complementary to the ribozymes is shown by gray filling. Cleavage site is indicated by an arrow. **B**. Kinetics of RNA cleavage in a single turnover mode: [RRz] = [LRz] = [HHRz] = 1 μM. **C**. Kinetics of RNA cleavage in multiple turnover mode: [RRz] = 0.5 μM, [LRz] = 0.1 μM, [HHRz] = 0.1 μM. Cleavage conditions: 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 37°C, initial RNA concentration 0.5 μM. Kinetic curves are numbered as follows: 1, **LRz-2 + RRz-2 (-◇-)**; 2, **HHRz-2(-◆-)**; 3, **LRz-1 + RRz-1 (-□-)**; 4, **HHRz-1 (-■-)**.

Under the conditions of single turnover reaction (Figure 3B) binary ribozyme **LRz-1 + RRz-1** was significantly more active than full-length ribozyme **HHRz-1** while binary ribozyme **LRz-2 + RRz-2** and corresponding full-length one **HHRz-2** displayed similar kinetics. In contrast to 19-nt RNA cleavage, sharp differences between binary and full-length ribozymes were observed under multiple turnover reaction conditions (Figure 3C). Catalytic activity of both modified binary ribozymes **LRz-1 + RRz-1** and **LRz-2 + RRz-2** greatly exceeded under these conditions the activity of full-length ones **HHRz-1** and **HHRz-2**. We suppose that binary ribozymes are favorable for extended RNA cleavage due to improved rate of association/dissociation upon ribozyme cleavage cycle. Thus, obtained results show evidence for the advantages of newly designed binary ribozymes and allow one to consider them as effective tools for gene silencing.

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