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Artificial hammerhead ribozymes: engineering and applications

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Abstract. The properties of hammerhead ribozymes are described. Various hammerhead ribozyme constructs for target RNA cleavage were considered. Approaches to enhancement of the stability of artificial ribozymes in biological media and to regulation of the catalytic activity of hammerhead ribozymes using effector molecules are described. The effect of ribozymes on extended structured natural RNAs is discussed. Applications of artificial hammerhead ribozymes as inhibitors of gene expression at matrix RNA level were considered. The bibliography includes 217 references.

I. Introduction

Selective inhibition of expression of particular genes at matrix RNA (mRNA) level is a topical problem in molecular biology, biotechnology and fundamental medicine. In recent decades, an intensive search has been carried out for agents that can interact with particular mRNA sequences with high specificity. Also, such agents are screened for applications in functional genomics and diagnosis and as pharmaceuticals for inhibition of expression of therapeutically important genes. The design of such agents, *viz*., antisense oligonucleotides, small interfering RNAs and catalytic nucleic acids, is based on the use of nucleic acid (NA) sequences complementary to particular fragments of a chosen target mRNA.[1 – 3] A promising avenue of research in this field is the design of catalytic nucleic acids (NA enzymes), *i.e*., oligoribonucleotides and oligodeoxyribonucleotides with a characteristic secondary structure, which can cleave RNA with high specificity.[4, 5] Among NA enzymes, of particular interest are hammerhead ribozymes.

Ribozymes, or catalytic RNAs, were discovered for the first time at the Prof. Cech laboratory (Colorado University) in 1982.[6] The authors showed that the precursor of ribosomal RNA from *Tetrahymena thermophila* contains an intron which can self-cleave *in vitro* involving no proteins or external sources of energy. Researchers headed by Altman [7] (Yale University) showed that processing of the transport RNA (tRNA) precursor by M1 RNA, a component of RNase P from *Escherichia coli*, occurs in the absence of protein cofactor. In 1989, Cech and Altman were awarded the Nobel prize in chemistry for their discovery of catalytic properties of RNA.

At present, there is a number of types of natural RNAs that exhibit the RNA-hydrolyzing activity (for more detail, see reviews [8, 9]), namely, Group I introns, Group II introns, RNase P RNA subunit, hepatitis delta virus RNA, Varcud satellite ribozyme, *glmS* ribozyme, hairpin ribozyme and hammerhead ribozyme. The discovery of catalytic properties of RNAs gave an impetus to investigations on the design of artificial ribozymes for target RNA cleavage based on natural sequences. From this point of view, most promising are short ribozymes at most 100 nucleotides long including hepatitis D virus ribozymes, the hairpin ribozyme and the hammerhead ribozyme. Initially, the hammerhead catalytic motif about 50 nucleotides long (Fig. 1) was found in RNA of some plant viruses [10] and then in amphibia, insects, flat worms and even in mammals; also it was obtained independently by *in vitro* selection.[11 – 13] This gives grounds to consider the hammerhead ribozyme as the most 'evolutionary ancient' catalytic RNA.

Having a relatively short oligonucleotide chain, the hammerhead ribozyme exhibits high catalytic activity. Its structure is such that the molecule can be separated to the conserved 'catalytic' and variable 'substrate' fragments. Combining these properties makes the hammerhead ribozyme a convenient object of study and serves a basis for the design of artificial ribozymes. This review is devoted to engineering of hammerhead ribozymes, to their properties

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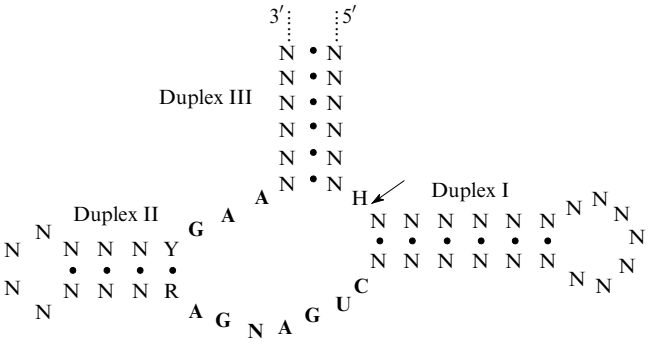


Figure 1. Secondary structure of hammerhead catalytic motif. Notations used from this point on are as follows: R is a purine ribonucleotide, Y is a pyrimidine ribonucleotide, N is a ribonucleotide, H is a ribonucleotide except G; conserved nucleotides are given in bold; the inter-nucleotide bond to be cleaved is asterisked.

and applications as selective agents for target RNA cleavage.

II. The structure and properties of *trans*-hammerhead ribozyme

It was Uhlenbeck who pioneered in the design of artificial hammerhead ribozymes for *trans*-cleavage of RNA molecules.[14] The hammerhead domain was separated into two partially complementary oligomers in such a manner that the ribozyme and substrate formed the duplexes I and II (the so-called I/II-format). However, in this case the substrate contained a conserved sequence, which made this approach of limited use.

A more appropriate separation is as follows:[15] the ribozyme and substrate form duplexes I and III (I/III-format), while duplex II and loop II are constituents of the ribozyme. The substrate contains a part of the natural sequence that comprises almost no conserved ribonucleotides (Fig. 2). This made it possible to go from a few unique examples of catalytic sequences to universal ribozymes for cleavage of a wide range of RNA sequences and gave an impetus to a vast majority of studies on the design of hammerhead ribozymes for target RNA cleavage.

It should be noted that if the hammerhead is separated into the ribozyme and substrate fragments as described above, the ribozyme 'loses' the loop protecting duplex I (*cf*. Figs 1 and 2). Recently, it was found that loop I plays a more important role than it was assumed earlier, *viz*., its interaction with loop II facilitates the formation of a cleavage-favouring conformation[17] and faster cleavage of the phosphodiester bond.[18–21] Recently, a number of studies on the design of *trans*-ribozymes with duplex I containing loop I or a bulge simulating it have been reported.[22,23] It was shown that such ribozymes can cleave RNAs at lower Mg^{2+} concentrations and can be used for cleavage of both short and extended RNA fragments.[23,24] However, the extended *trans*-ribozymes are still of limited use and the Haseloff–Gerlach model without loop I remains the most popular type of universal *trans*-ribozyme.

1. Substrate specificity of ribozyme

The hammerhead ribozyme can cleave RNA downstream the 5′-NUH-3′ triplet, where N is any of four ribonucleotides and H is any ribonucleotide except G.[25–28] The presence of guanosine residue in position 17 leads to the formation of a complementary pair $G_{17}C_3$ (Ref. 29) and to inactivation of the ribozyme. For the triplets 5′-NUH-3′ the efficiency of cleavage decreases in the order AUC, GUC > GUA, AUA, CUC > AUU, UUC, UUA > GUU, CUA > UUU, CUU and the rate constants for cleavage of the phosphodiester bond for the 'best' and 'worst' triplets differ by a factor of about 40. It was shown[30] that the mid-position of the triplet can also be occupied by cytosine and adenosine. In these cases, the rate constants for cleavage are about an order of magnitude lower than in the case of the GUC triplet. Replacement of adenosine in position 15.1 by inosine enables cleavage of the bond downstream the NCH triplet with almost the same efficiency as cleavage of the bond downstream the NUH triplet owing to the structural similarity between the A-U and I-C pairs in the ribozyme–substrate complex.[31] Based on these data, one can design hammerhead ribozymes for cleavage of various RNA sequences.

2. The molecular structure of ribozyme–substrate complex. The RNA cleavage mechanism

The structure of a hammerhead ribozyme in the complex with RNA substrate can be described as a three-way

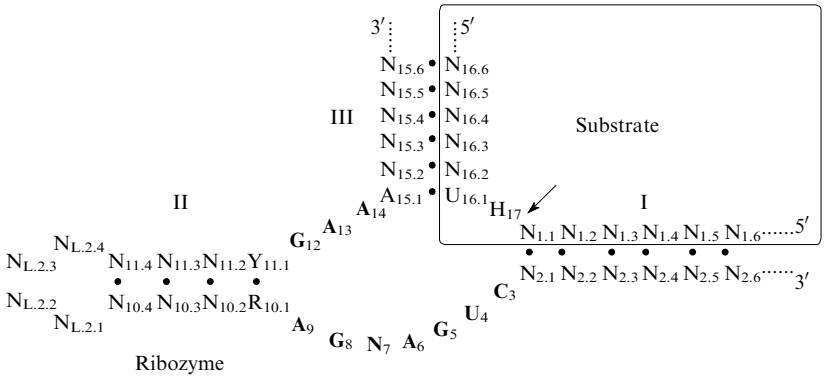


Figure 2. Separation of the hammerhead into ribozyme and substrate according to Haseloff and Gerlach.[15] The ribozyme and RNA substrate nucleotides are enumerated using the commonly accepted nomenclature.[16]

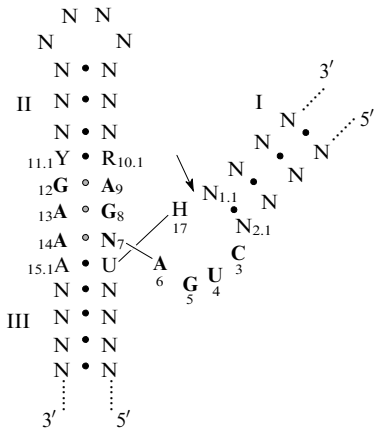


Figure 3. Y-Form of ribozyme–substrate complex.[32]

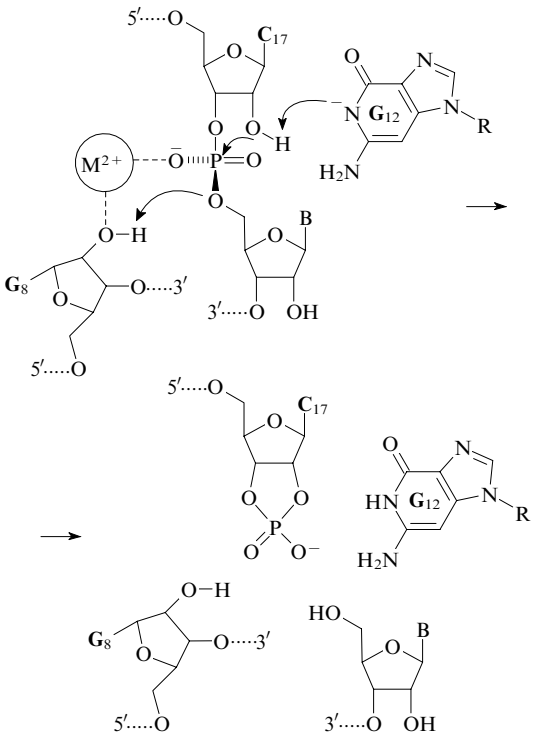
junction. Most conserved nucleotides are in the unpaired regions and are necessary for the formation of the characteristic structure of the catalytic core. Replacement of conserved nucleotides in positions 3–10 and 11–14 (Fig. 3) leads to almost complete inactivation of ribozyme; replacement of uridine in position 7 by any other nucleotide causes the cleavage constant to decrease 2–5-fold.[25]

According to X-ray diffraction data, in the crystal the complex of ribozyme with uncleaved RNA substrate containing deoxy- or 2′-*O*-methylribonucleotide in the cleavage site exists in the Y-form with the duplexes II and III arranged coaxially and form a pseudo-A helix, whereas duplex I makes an acute angle with duplex II (see reviews[32–34]) (see Fig. 3). Lilley and co-workers.[34–36] showed that the formation of the Y-form of the ribozyme–substrate complex involves two stages and requires the presence of divalent metal ions, usually, Mg^{2+}.

Hydrolysis of the phosphodiester bond in hammerhead ribozyme proceeds in the case of linear (*in-line*) orientation of 2′-O-, P- and 3′-O-atoms by the S_N2-mechanism with inversion of configuration of the substituents at the phosphorus atom. The mechanism involves an attack of the phosphorus atom on the 2′-OH group resulting in a pentacoordinate transition state and subsequent elimination of the 5′-OH group.[33,37] The cleavage products include the 2′,3′-cyclophosphate fragment and the 5′-OH group. The mechanism of RNA cleavage by the hammerhead ribozyme has been a subject of intensive research during almost two decades. The problem was that the results of X-ray diffraction analysis did not fit the biochemical data on particular functional groups of ribozyme necessary for RNA cleavage (see reviews[9,38,39]).

These contradictions were resolved in recent studies of 'extended' *cis*-ribozymes, in particular, X-ray studies.[17,40] The structures of 'extended' ribozymes in the crystal differ from those obtained for minizymes but are in good agreement with the biochemical data obtained previously. Based on these results, it was concluded that the structure of 'extended' ribozymes is similar to the transition-state geometry, whereas the minimal ribozyme–substrate complex whose structure was determined by X-ray diffraction analysis is inactive. To cleave RNAs, an additional conformational rearrangement resulting in a short-lived active form is required.[41,42] It was established that the guanosine residues G_8 and G_{12} are involved in catalysis. The N(1) atom of G_{12} acts as a base and favours deprotonation of the oxygen atom in position 2′,[17,43] and the 2′-OH group of G_8 participates in protonation of the leaving 5′-oxy anion.[44] The necessary acidity of the 2′-OH group of G_8 is attained through coordination with Mg^{2+} or other divalent cation.[44] In a general form, the mechanism of acid–base catalysis of cleavage of the phosphodiester bond by the hammerhead ribozyme is shown in Scheme 1.

Scheme 1



B is base.

3. The kinetic scheme of RNA cleavage by hammerhead ribozyme

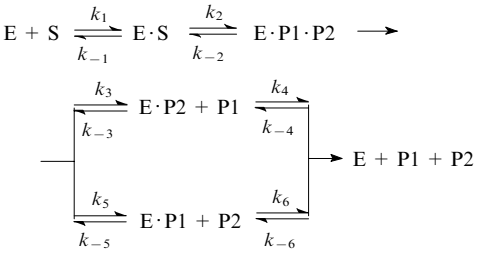
The minimum kinetic scheme of RNA cleavage by a hammerhead ribozyme involves the following stages (Scheme 2):[45]

1) formation of the ribozyme–substrate complex E·S;

2) cleavage of the phosphodiester bond to give a 5′-product P1 containing a 2′,3′-cyclophosphate moiety and the 3′-product P2 containing the 5′-OH group, which remain in the complex with the ribozyme E·P1·P2;

3) successive dissociation of products from ribozyme in different manner.

Scheme 2



E is ribozyme, S is RNA substrate, P1 is 5′-terminal product, P2 is 3′-terminal cleavage product.

All stages of the catalytic cycle are reversible; however, the forward reactions are much faster than the reverse ones. The rate constant (k_2) for cleavage of the phosphodiester bond ribozyme is on the average 1 min^{-1} under normal conditions (pH 7.5, 10 mM $MgCl_2$, 25 °C) while the rate constant for the reverse reaction (ligation) (k_{-2}) is 20–100 times lower. The other rate constants depend on the stability of duplexes with the substrate and cleavage products and can be either measured experimentally or estimated from the thermodynamic parameters of formation of RNA–RNA duplexes.[45] An approximate description of the ribozyme kinetics can be obtained using the Michaelis–Menten scheme. This allows one to compare the catalytic properties of ribozymes based on the K_M and k_{cat} values. The K_M values typical of hammerhead ribozymes lie in the range 0.01–1.00 μmol $litre^{-1}$ while the characteristic k_{cat} value is 1 min^{-1} (Ref. 45).

III. Design of artificial hammerhead ribozymes for target RNA cleavage

The design of artificial ribozymes should take into account some requirements, *e.g.*, the optimum length of the oligonucleotide chain, selective cleavage of target RNA, high catalytic activity and stability of the ribozyme under conditions of the intracell medium. Now we will consider the state of the art in the design of hammerhead ribozymes taking account of these requirements.

1. Minimization of the length of hammerhead ribozyme. Minizymes and maxizymes

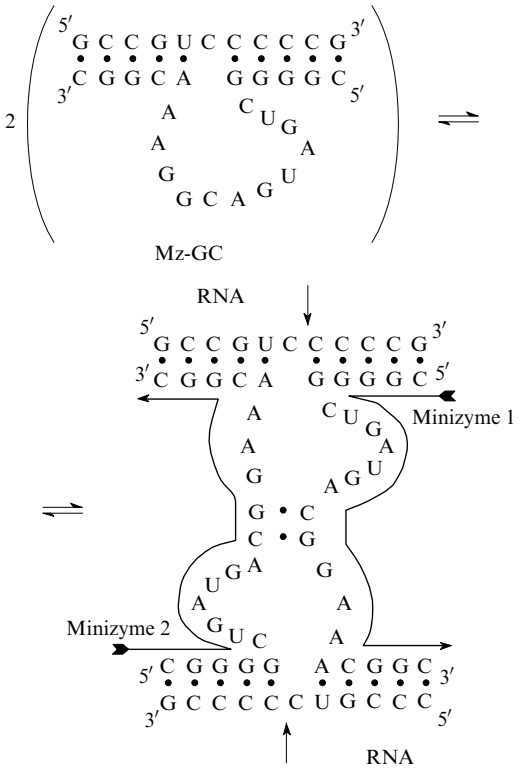
Carrying out NMR and X-ray diffraction studies of hammerhead ribozymes as well as clinical trials require large amounts of ribozymes. Therefore, the design of ribozymes should involve reduction of the oligonucleotide chain length. On the one hand, this makes the synthesis simpler and cheaper. On the other hand, this decreases the net negative charge and thus facilitates the delivery of ribozymes to cells. Shortening is possible only for the non-conserved regions of ribozymes, *viz.*, duplexes I and III, duplex II and the closing loop II. The optimal length of substrate-recognizing regions, which provides both efficient binding to RNA and fast elimination of cleavage products from ribozyme, can be different for different ribozymes and is 5–7 ribonucleotides for each fragment.[46,47]

The main method for shorteming of ribozymes is to decrease the number of non-conserved nucleotides in duplex II and in the teminal loop II. Ribozymes thus designed were called minizymes (see reviews[48,49] and references cited therein). The most appropriate systems are chimeric minizymes containing (i) a linker four deoxyribonucleotides long instead of duplex II and loop II and (ii) deoxyribonucleotides simultaneously introduced into the substrate-binding regions. Chimeric minizymes were more efficient in cleavage of extended RNA transcript than their full-length analogues.[50] A chimeric minizyme complementary to a fragment of human interleukine-2 mRNA inhibited expression of this protein in the cell culture with almost the same efficiency as the full-length ribozyme.[51] Minizymes are also constructed by *in vitro* selection. In this case, the catalytic core of the ribozyme remains unchanged while the composition of the shortened linker oligonucleotide sequence between $G_{10.1}$ and $C_{11.1}$ is varied. This approach was used to design the sequences of minizymes having high catalytic activity at reduced Mg^{2+} concentrations.[12,52,53]

Since most nucleotides in duplex II and loop II are non-conserved, it was proposed to replace this region by non-nucleotide fragments in order to decrease the number of synthesis stages (see review[49] and references cited therein). The highest efficiency was attained with ribozymes containing oligo(ethylene glycol) linkers at least thirteen atoms long. These constructs were almost as active as the unmodified ribozymes. In some cases, the modified ribozymes were more efficient than the unmodified ones; however, these studies got no further development.

An interesting type of minizymes is represented by dimeric minizymes (maxizymes) described by Taira and co-workers,[54–56] who studied the properties of a series of minizymes in which duplex II and loop II were replaced by linker sequences 1–4 ribonucleotides long. Two minizymes with the 5′-GCGC-3′ (Mz-GCGC) and 5′-GC-3′ (Mz-GC) linkers showed high catalytic activity.[55] It was found that they form dimers with the common duplex II (Scheme 3) and that their catalytic activity enhances with the number of complementary pairs in duplex II.

Scheme 3



A dimer can be formed both by molecules of the same minizyme and by molecules with different linker sequences complementary to each other. Since maxizymes can cleave RNA at two sites simultaneously, they are particularly useful in the case of extended RNA molecules. For instance, the dimer was more efficient than the full-length ribozyme in cleavage of extended fragment of HIV-1 *tat* mRNA.[56–59] The ability to simultaneous binding to two RNA sequences was used in the design of allosterically regulated maxizymes, *i.e.*, heterodimers with one active site, which recognize two different RNA domains and cleave RNA in one of them.[60–65]

This strategy was efficiently used in the cleavage of *BCR/ABL* mRNA, a product of chimeric gene *BCR/ABL* which is expressed as a result of chromosome translocation and fusion of the genes *bcr* and *abl* (Philadelphia chromo-

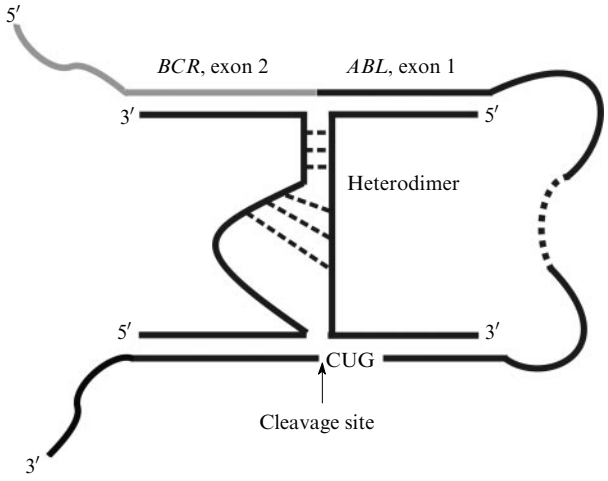


Figure 4. Design of a heterodimeric maxizyme targeted against chimeric *BCR*/*ABL* mRNA.[60]

some) (Fig. 4). The *BCR* and *ABL* gene fusion fragment has no 5′-NUH-3′ triplets in the corresponding mRNA, while the use of ribozymes in other regions of the *BCR*/*ABL* mRNA will inevitably lead to cleavage of 'normal' *BCR* mRNA or *ABL* mRNA. The heterodimeric maxizyme binds to the *BCR*/*ABL* fusion site and to the fragment containing the 5′-GUC-3′ triplet; cleavage occurs downstream this triplet. It was shown that such heterodimers selectively cleave *BCR*/*ABL* mRNA with high efficiency and selectively inhibit expression of *bcr*/*abl* gene. The dimer components were delivered to cells endogenously by introducing specially designed plasmids.

2. Binary ribozymes

Separation of a ribozyme into two individual, partially complementary oligonucleotides essentially simplifies the synthesis and isolation of ribozyme constructs. Ohtsuka and co-workers [66–69] reported the design and properties of ribozymes separated into two fragments in the region of loop II. Binary ribozymes efficiently cleaved both short and extended RNA substrates but showed a lower efficiency in the cleavage of full-length analogues. As a consequence, this interesting approach to the design of ribozymes for target RNA cleavage was not further developed. Venyaminova and co-workers [70–75] proposed yet another method for constructing binary hammerhead ribozymes. Such a ribozyme comprises two oligonucleotides that form a hammerhead structure with a common duplex II and no loop after assembling on the RNA substrate (Fig. 5).

The binary ribozyme taken in excess with respect to a 19-nucleotide RNA substrate cleaved it as efficiently as did the full-length analogue. The possibility of varying the concentration of each ribozyme component allows one to use the deficiency of one component with respect to RNA, while the second component is present in excess or in equivalent amount with respect to RNA. The concentration of the binary construct remains lower than the RNA concentration while cleavage occurs more efficiently than in the case of the full-length ribozyme. A study on the kinetics of RNA cleavage showed that separation of ribozyme into two oligomers does not decrease the association rate of the ribozyme–substrate complex and has almost no effect on the rate constant for cleavage of the phosphodiester bond, but significantly accelerates the elimination of reaction products from the ribozyme.[74–76]

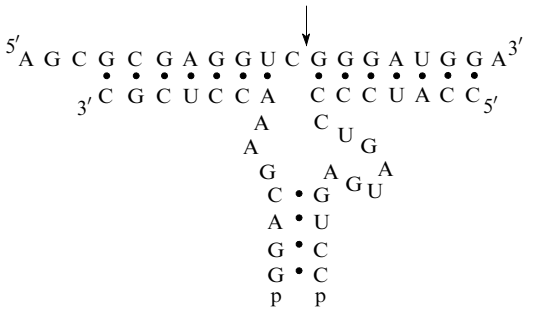


Figure 5. Binary ribozyme without loop II (hereafter, p is the 3′- or 5′-terminal phosphate).[70]

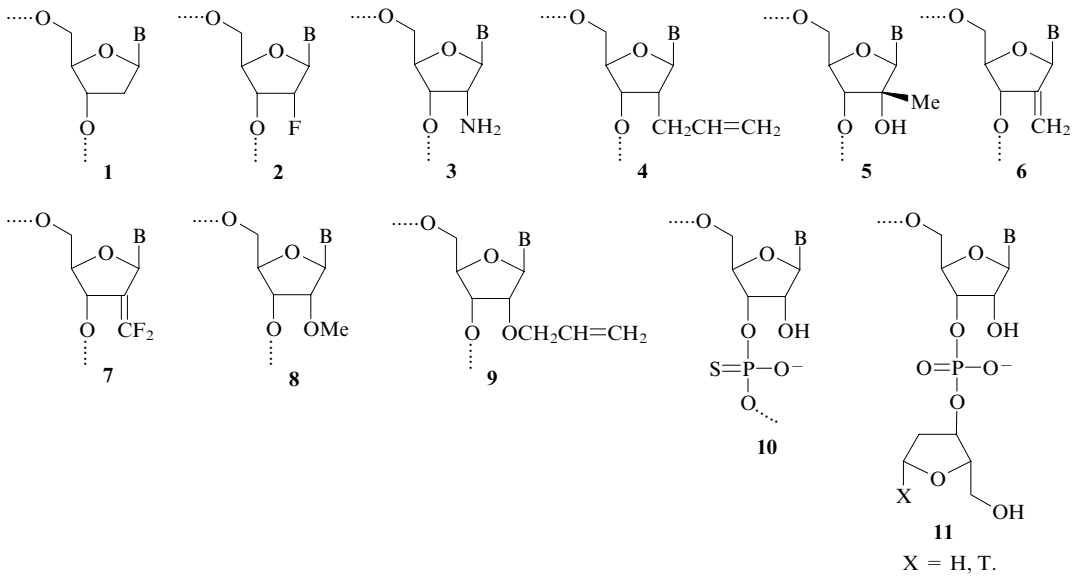
Taking a 190-nucleotide long mRNA fragment of human multidrug resistance gene *mdr*1 as an example, it was shown that binary ribozymes are more efficient in cleavage of extended structured RNA than their full-length analogues due to the possibility to use the excess amount of one component of the ribozyme.[72–75] It was assumed that this oligomer plays the role of an effector (see Section III.4) and unwinds the structured RNA, thus facilitaing the addition of the second component.

3. Enhancement of ribozyme stability in biological media

A severe problem in cell applications of hammerhead ribozymes is their fast degradation under the action of endo- and exo-nucleases. This gave researchers working in the field of design of artificial ribozymes for *in vivo* applications an impetus to search for methods for enhancement of their stability. The most obvious way is to replace most ribonucleotides by deoxyribonucleotides (**1**). Such chimeric ribozymes containing deoxyribonucleotides in the substrate-binding regions are not only more stable in biological media, but also exhibit higher catalytic activity (see reviews [48, 49]). To additionally enhance the stability of the chimeric ribozymes, most phosphodiester bonds beyond the conserved region, as well as the bonds in the catalytic core of the ribozyme that are the most sensitive against endoribonucleases are replaced by phosphorothioate bonds.

Another method of enhancement of the stability of ribozymes and, in particular, their protection from endoribonucleases is to introduce various group at position 2′ of the carbohydrate fragment of non-conserved ribonucleotides [2′-fluoro- (**2**), 2′-amino (**3**), 2′-*C*-allyl- (**4**), 2′-*C*-methyl- (**5**), 2′-methylidene- (**6**), 2′-difluoromethylidene- (**7**), 2′-*O*-methyl- (**8**) and 2′-*O*-allyl derivatives (**9**)], as well as the thiophosphate inter-nucleoside bond (**10**) or the 3′–3′-phosphodiester bond (**11**).

An optimum variant is to combine several types of modifications within the same molecule. Systematic studies of the catalytic activity and stability against nucleases for a number of ribozymes containing various types of 2′-modifications (see structures **2**–**8**) were carried out.[77–80] The optimum activity/stability ratio was observed for the ribozyme in which the substrate-binding regions, the stem and loop II contained 2′-*O*-methylribonucleotides and the positions U_4/U_7 were occupied by 2′-aminouridine residues or by 2′-*C*-allyluridine/2′-*O*-methyluridine. An additional modification of the 3′-terminus by adding a thymidine or



1,2-dideoxyribose residue involved in the 3′–3′-phosphodiester ('inverted') bond had no effect on the catalytic activity; the ribozyme became stable against serum nucleases (Fig. 6).

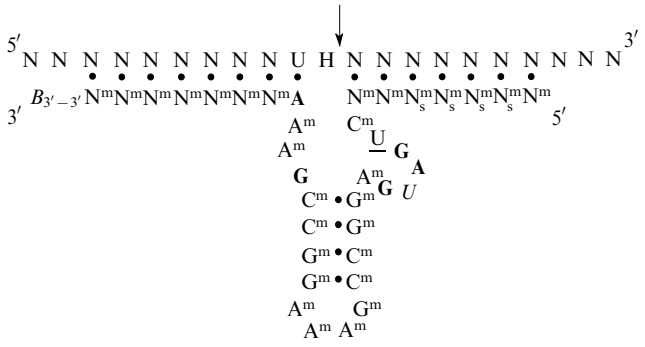


Figure 6. Design of maximally stabilized modified ribozymes.[81] Hereafter, N^m is 2′-*O*-methylribonucleotide, U is 2′-aminouridine or 2′-*C*-allyluridine, *U* is 2′-aminouridine or 2′-*O*-methyluridine, s is the phosphorothioate bond, $B_{3'-3'}$ is the hymidine or 1,2-dideoxyribose residue involved in the 3′–3′-phosphodiester bond.

Versatilty of this approach was confirmed in a number of subsequent studies. For instance, ribozymes constructed in this manner efficiently inhibited expression of *c-myb* and *N-ras* genes in the cell culture.[82,83] In 2010, it was proposed to introduce 2′-*C*-methyluridine at position 7 of 2′-O-methylated ribozymes.[84] The modified nucleoside retains the 2′-OH group, which allows it to adopt the C(3′)-*endo*-conformation and form hydrogen bonds and an additional methyl group in position 2′ protects it from nucleases. The ribozyme containing 2′-*O*-methylribonucleotides in all non-conserved positions and 2′-*C*-methyluridine in position 7 showed an efficient inhibition of expression of estrogen receptor in the cell culture.

Venyaminova and co-workers[72–75] used a combined modification strategy to design binary ribozymes stable against nucleases. The modified binary ribozymes contained 2′-*O*-methylribonucleotides in all non-conserved positions and 2′-aminouridine or 2′-*O*-methyluridine in positions 4 and 7 (Fig. 7). The 3′-termini of both components were protected with a thymidine residue involved in the 3′–3′-phosphodiester bond. This combination of modifiers enhanced the ribozyme stability in the serum culture medium from 1 min to 1 day. The catalytic activity of the constructs thus obtained in the cleavage of both short RNA substrate and extended 190-nucleotide long MDR1 mRNA fragments was the same or higher than that of the full-length ribozyme.

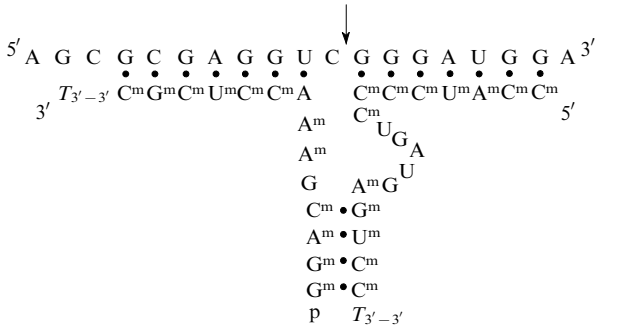
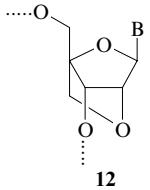


Figure 7. Modified binary ribozymes.[73] $T_{3'-3'}$ is the thymidine residue involved in the 3′–3′-phosphodiester bond.

To enhance the stability of the full-length and binary ribozymes in biological media and to improve their affinities to RNA, they were modified with 2′-*O*,4′-*C*-methylidenebicyclic nucleotides **12** (Locked Nucleic Acids, LNA).[85]



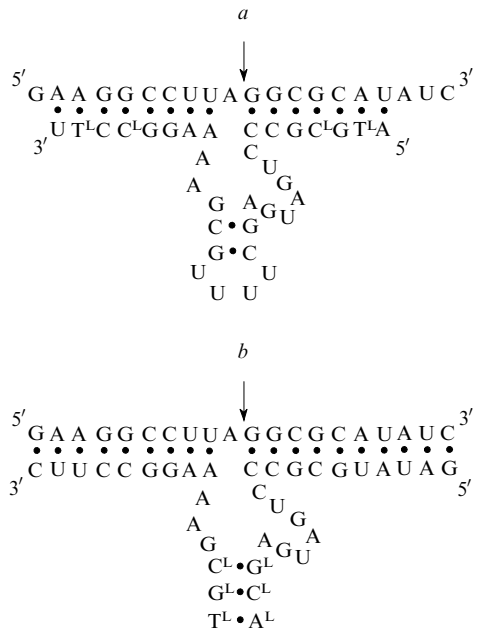


Figure 8. Modified full-length (*a*) and binary (*b*) ribozymes containing LNA-nucleotides (N^{L}).[85]

It should be noted that in all cases the modified binary ribozymes designed in that study (Fig. 8 *b*) cleaved RNAs with much lower efficiency than their full-length analogues (see Fig. 8 *a*). To search for optimum combinations of components of the binary ribozymes, the combinatorial approach was used.

4. Regulation of catalytic activity of ribozymes using effectors

To use ribozymes in complex multicomponent molecular biological systems, one should ensure their binding to highly structured target RNAs and have the possibility to regulate their catalytic activity depending on the local environment. Nowadys, a number of methods for regulation of ribozyme activity were proposed. They are based on the use of additional effector molecules ('facilitators', 'helpers', regulators). Effectors can be divided into three types with respect to the principle of action:

1) effector oligonucleotides complementary to RNA substrate;

2) allosteric effectors binding to ribozyme;

3) effector oligonucleotides capable of simultaneous binding to ribozyme and substrate.

a. Effector oligonucleotides complementary to RNA substrate

The first type of effectors includes oligonucleotides that complementary bind to RNA in the vicinity of the ribozyme binding site (Fig. 9). They were proposed to improve the catalytic activity of hammerhead ribozyme by Goodchild.[86] It was shown that in the presence of oligodeoxyribonucleotides that bind to RNA in the end-to-end fashion with the 3′-terminus of ribozyme, the cleavage becomes much more efficient owing to acceleration of the association of the ribozyme – substrate complex and its stabilization by stacking interactions between adjacent heterocyclic bases of the ribozyme and effector.[87,88]

Since an effector can also stabilize the complex with the cleavage product,[87] the length of the adjacent flanking fragment of ribozyme should be optimized. For instance, the optimum length of the flanking fragments is 5 – 6 nucleotides at an effector length of 13 nucleotides.[87,89] The influence of effectors on the catalytic activity depends on their positions, namely, the effectors which bind adjacent to the 3′-terminus of the ribozyme improve its activity, whereas the 5′-effectors inhibit the cleavage.[90] Probably, the binding of the 5′-effectors to the target RNA reduces the conformational mobility of duplex I and slows down the formation of the active ribozyme – substrate complex in the Y-form. The effectors used included not only oligodeoxyribonucleotides, but also oligoribonucleotides,[89–91] peptide nucleic acids (PNAs)[92] or oligo(2′-*O*-methylribonucleotides).[89] The PNAs, as effectors, improved the efficiency of cleavage to a lesser extent compared to oligoribo- and oligodeoxyribonucleotides; this was probably due to the reduced stability of the duplex with RNA.[92] It was shown[89] that ribo- and 2′-*O*-methylribo-oligomers improve the RNA cleavage by the ribozyme to almost the same extent and provide a more efficient cleavage than oligodeoxyribonucleotides.

The effector oligonucleotides appeared to be useful for cleavage of extended structured RNA,[89,91–95] namely, ribozymes efficiently cleaved RNA transcripts up to 900 nucleotides long and the positiive effect on the activity was reported for both 3′- and 5′-effectors. The efficiency of cleavage increased owing to unwinding of the structured

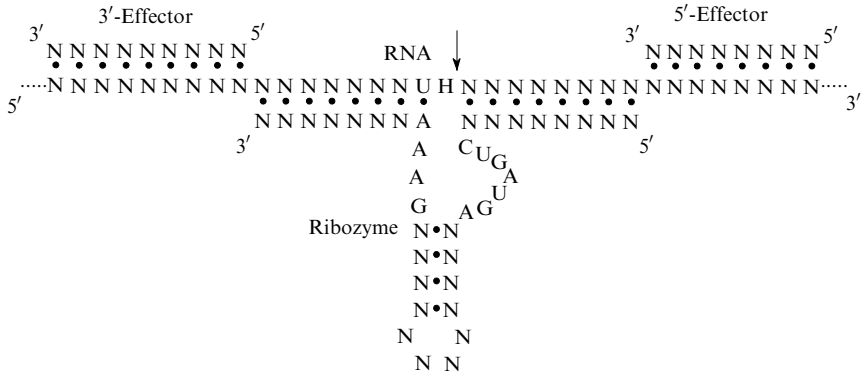


Figure 9. RNA – ribozyme – effector oligonucleotide system.

RNA by the effector oligonucleotide and to acceleration of the association of the ribozyme and RNA.[91,94] The addition of effector oligonucleotide allows one to cleave extended RNAs when ribozyme is in deficiency.[94] A successful use of the ribozyme + effector system was reported.[96] The ribozyme with the 20-unit effector oligodeoxyribonucleotide targeted against mRNA of hepatitis B virus X protein not only cleaved the RNA transcript *in vitro*, but also considerably reduced the level of this RNA in the cell culture.

b. Allosteric effectors

At present, allosteric regulation of ribozyme activity becomes increasingly more popular. Allosteric regulators were described for the first time for enzymes. They interact with an enzyme beyond the active site and induce conversion of the enzyme to the active conformation. RNAs can form various 3D structures and, like proteins, recognize certain molecules with high specificity. Such RNA sequences are called aptamer sequences. Recently, the possibility of introducing aptamer sequences into the catalytic RNA has been the subject of intensive research and led to allosteric ribozymes. Interest in the design of such ribozymes is due to the possibility of using them for analytical purposes (for detection of various compounds) and for control of gene expression at mRNA level.[97,98]

Allosteric ribozymes are constructed using various molecular design strategies including rational design and *in vitro* selection. Rational design is based on the combination of the hammerhead catalytic motif with one or more preset aptamer RNA sequences. *In vitro* selection involves successive screening of ribozyme libraries for specified properties; therefore, this method offers greater prospects. The best results are obtained by combining both approaches.[98]

Allosteric activation occurs through stabilization of the structure of the active conformation of the ribozyme–substrate complex. In particular, ribozymes containing ATP-, FMN- (FMN is flavine mononucleotide) and theophylline-recognizing aptamers in duplex II were obtained.[99–106] The stability of duplex II was artificially reduced by the presence of non-canonical pairs (see, *e.g.*, Fig. 10). In the absence of effector, the activity of these ribozymes was very low while binding of the effector led to stabilization of duplex II and induced transition of the ribozyme to the active state. The combined approach was used[107] to design the ribozymes capable of cleaving RNA in the presence of coffein or aspartame; these ribozymes serve a basis for fluorescence sensors for these molecules in solution. An interesting type of photo-activated allosteric ribozyme was reported;[108] a

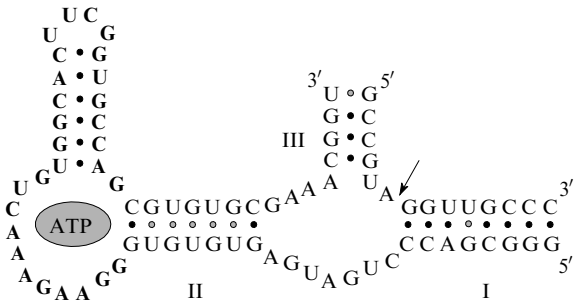


Figure 10. ATP-dependent allosteric ribozyme.[99]
The ATP-binding aptamer sequence is given in bold.

ribozyme constructed by rational design is activated in the presence of an isomer of dihydropyrene. Irradiation with visible light ($\lambda > 400$ nm) leads to reversible isomerization of the activator and the ribozyme is switched off; exposure to UV light ($\lambda < 300$ nm) again switches the ribozyme on.

Oligonucleotides can also be used for allosteric regulation of the catalytic activity of ribozymes. Nucleotides $G_{10.1}$ and $C_{11.1}$ were linked[109–111] by an oligonucleotide sequence that could not form the duplex II and loop II and contained a fragment complementary to the so-called regulator oligonucleotide, or effector (Fig. 11). In the absence of effector, the ribozyme showed no catalytic activity; however, complementary binding to the effector was followed by the formation of a pseudo-junction replacing duplex II, and the ribozyme could cleave RNA. The effectors used were oligoribonucleotides and oligo(2′-*O*-methylribonucleotides) and the ribozyme activity was higher in the latter case.

Another route to allosteric ribozymes activated by oligonucleotide is the targeted ribozyme-attenuated probe (TRAP) design.[112,113] In this case, the ribozyme contains an auxiliary sequence (attenuator) bound to the catalytic core by a linker. In the absence of effector, the attenuator binds to the catalytic core and inactivates the ribozyme. The effector oligonucleotide binds to the linker sequence, the duplex of the attenuator with the catalytic core becomes broken, and the ribozyme goes to the active state. The ability of ribozymes to cleave RNAs only in the presence of effector oligonucleotide of a particular sequence can be used for the determination of (i) RNA in diagnostic purposes or (ii) alien RNAs in the cells. For instance, the ribozyme bound to an attenuator (a specified viral RNA sequence or

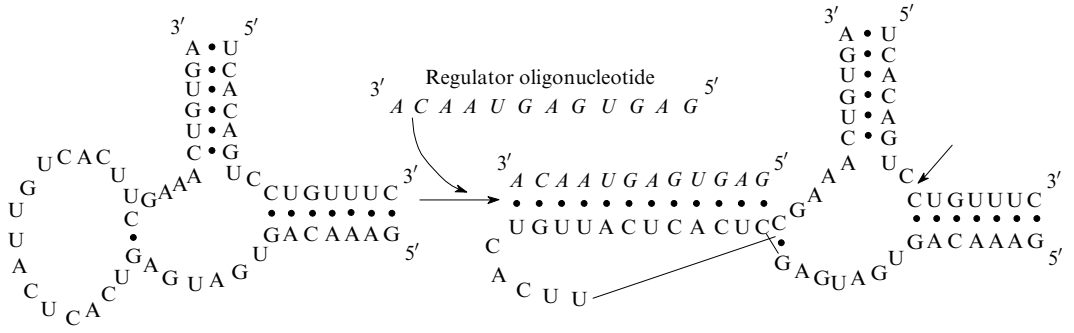


Figure 11. Allosterically regulated ribozyme.[109–111] Regulator oligonucleotide is given in italic.

oncogene RNA sequence) can cleave the reporter gene mRNA.[112]

Thus, the possibility of allosteric regulation of hammerhead ribozymes by effectors of different nature, which bind to the ribozyme beyond the active site, offers prospects for the design of biosensor ribozymes with specific response to particular molecules present in the medium.

c. Effector oligonucleotides capable of simultaneous binding to NA enzyme and substrate

The third class of effectors includes oligonucleotides capable of simultaneous binding to both ribozyme and RNA. This approach was first proposed[114] for the DNAzyme '10–23' and then adapted for regulation of activity of the hammerhead ribozyme.[115] The ribozymes contained octa- and trinucleotide substrate-bindng domains and an effector oligodeoxyribonucleotide which forms six complementary pairs with the 3′-terminal domain of the ribozyme and six pairs with the RNA substrate (Fig. 12 *a*).

The next step in the evolution of this approach was the design of a novel, FMN-responsive ribozyme (Fig. 12 *b*).[116] The binary construct comprising the ribozyme and the effector was replaced by a single FMN-binding aptamer sequence introduced into the 3′-substrate-binding domain; the addition of FMN improved the ribozyme activity by an order of magnitude. The constructs thus designed are similar to the allosteric ribozymes described above, but in this case the effector influences the step of association of the ribozyme–substrate complex rather than the formation of the active conformation. The extent of activation in the presence of effector was lower than for conventional allosteric ribozymes, although it is believed that further geometry optimization should lead to the design of efficient ribozymes with controlled catalytic activity. It should be noted that this approach is still in the early stage of development.

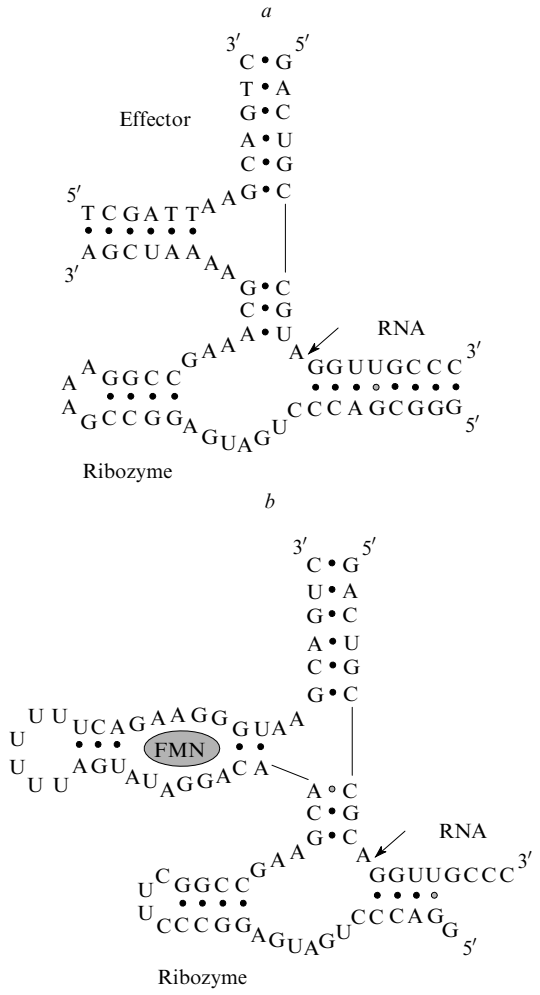


Figure 12. Design of expansively regulated hammerhead ribozyme[115] (*a*) and a FMN-responsive ribozyme construct[116] derived from it (*b*).

5. Methods for improvement of the efficiency of cleavage of structured natural RNAs by ribozymes

The main field of application of artificial hammerhead ribozymes is selective cleavage of particular natural mRNAs for regulation of the expression of corresponding genes. In this connection, the accessibility of a chosen RNA sequence to cleavage becomes a severe problem. Since mRNAs have complex secondary and tertiary structures, the number of regions accessible to binding to the ribozymes is limited. There are two approaches to the design of ribozymes capable of cleaving natural RNAs with high efficiency. One of them involves the determination of the sites accessible to hybridization and containing the 5′-NUH-3′ triplet. This is done by probing mRNA-transcripts with RNAase H using a set of oligodeoxyribonucleotides,[117,118] by choosing the regions accessible to binding based on analysis of the secondary structure of the target mRNA[119,120] or by analyzing the cleavage of the mRNA transcript by a ribozyme library with random sequences of substrate-binding domains.[121–123] It should be noted that these methods do not state that these regions will also be accessible in the cell because the secondary and tertiary structures of a full-length mRNA in the cell can differ from those of the transcript. In addition, a mRNA exists in the cell in the form of complexes with proteins that may prevent it from binding to ribozymes.

The second strategy implies the design of ribozyme constructs that can interact with a chosen RNA sequence irrespective of its secondary and tertiary structures. Taira and co-workers[124,125] proposed a strategy for the design of ribozymes for cleavage of highly structured extended RNAs, which is based on fusion of the hammerhead ribozyme and RNA helicase (this protein can non-specifically bind to RNA, migrate along the molecule and unwind its secondary structure). A system was developed for intracell expression of hybrid ribozymes, which allows one to produce $tRNA^{Val}$ transcript with extended 3′-terminus including the ribozyme sequence.[126] The attached tRNA sequence did not inhibit the catalytic activity of the ribozyme and enhanced its stability against cell nucleases. To activate RNA helicase, a RNA CTE-sequence (CTE is constitutive transport element) was introduced into the construct. The principle of the interaction of hybrid ribozymes with RNA is shown in Fig. 13. The CTE sequence interacts with the adapter protein that forms a complex with RNA helicase. This complex scans mRNA and unwinds its secondary structure. Ribozyme binding to the target sequence is followed by RNA cleavage. It was shown that hybrid ribozymes are efficient in mRNA cleavage in the regions that are inaccessible to binding to conventional

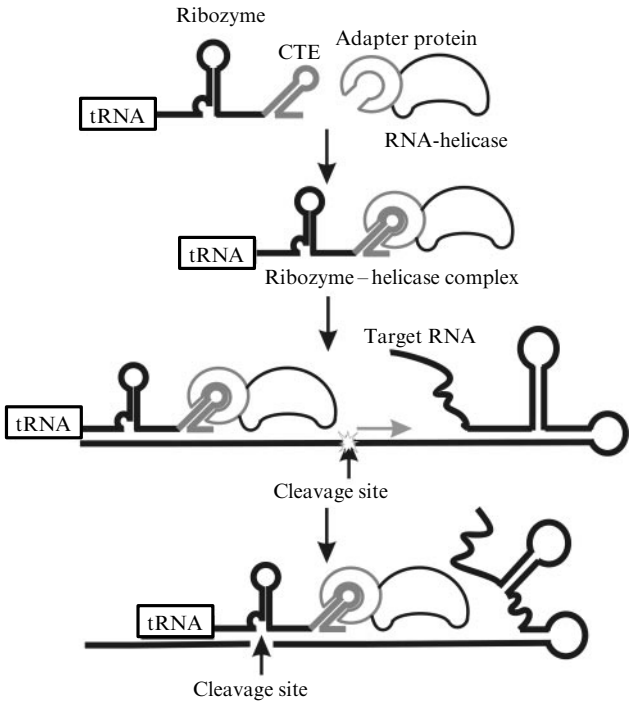


Figure 13. Schematic view of mRNA cleavage with a hybrid ribozyme capable of activating RNA-helicase.[127]

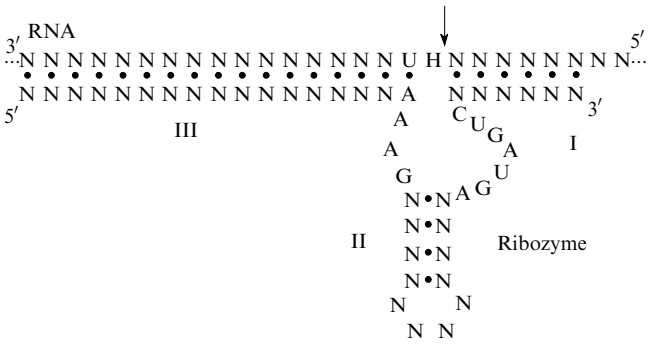


Figure 14. Asymmetric hammerhead ribozyme.

ribozymes.[124, 125, 128] Successful use of libraries of hybrid ribozymes with randomized RNA-recognizing sites for determination of functional genes was reported.[129–134]

Yet another way of improving the efficiency of cleavage of structured RNA is to elongate a substrate-binding ribozyme fragment.[135–139] Such constructs were called asymmetric ribozymes (Fig. 14).

An asymmetric ribozyme can conditionally be separated into two functional domains, *viz*., a long flanking fragment which unwinds the target RNA and a domain responsible for cleavage (catalytic core with adjacent short flank). Asymmetric ribozymes resemble the ribozyme + effector oligonucleotide system, namely, one component of the construct is responsible for unwinding of RNA while the other component is responsible for cleavage. Extension of the 3′-flank has a positive effect on the activity of the asymmetric ribozyme, in this case the minimum length of the 5′-domain is three nucleotides.[140–142] Contrary to this, elongation of the 5′-flank makes cleavage less efficient. It is assumed [142] that the extended stable duplex I formed by the 5′-flank and RNA slows down the conversion of the ribozyme to the active conformation. Interestingly, this regularity is also characteritic of the effector oligonucleotides that bind to one ribozyme terminus in the end-to-end fashion (see above), namely, the effectors stabilized by duplex I, *i.e*., those 'extending' the 5′-flanking arm of ribozyme, also had a negative effect on its catalytic activity. Asymmetric ribozymes can efficiently cleave extended structured fragments of AML1/MTG8 mRNA [140] and HIV-1 RNA;[135, 136] transduction of plasmids expressing these ribozymes to cells led to noticeable inhibition of expression of the AML1/MTG8 chimeric gene or to inhibition of HIV-1 replication. A common drawback of asymmetric ribozymes is the formation of stable complexes of the extended domain (to a few hundreds of nucleotides long) with the cleavage products; as a result, each ribozyme molecule cleaves only one RNA molecule.

Venyaminova and co-workers [143, 144] proposed a strategy for the design of asymmetric binary ribozymes, which is based on physical separation of the unwinding and cleaving domains. Three asymmetric binary ribozymes with heptanucleotide cleaving 5′-domains differing in length of the unwinding 3′-terminal domain (10 to 20 nucleotides long) (Fig. 15) were studied. To enhance the biological stability of the ribozymes, they were modified with 2′-*O*-methylribonu-

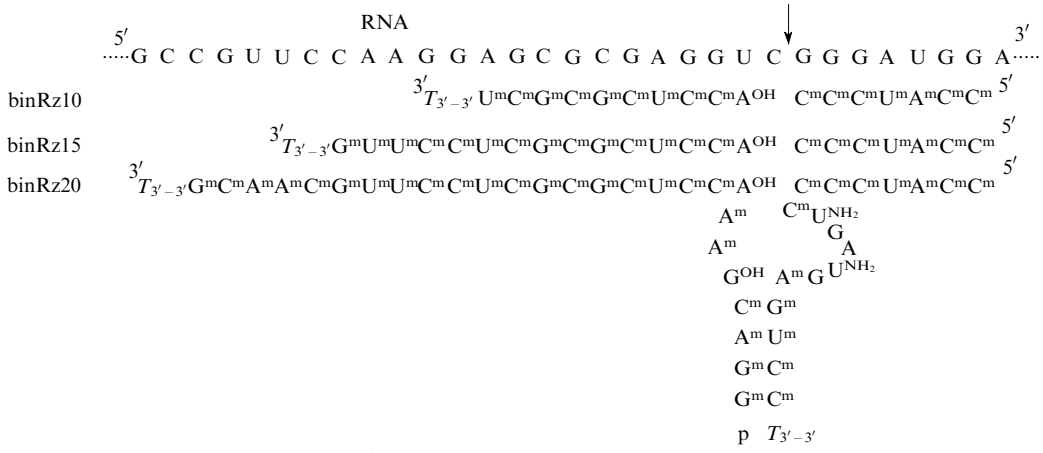


Figure 15. Asymmetric binary ribozymes.[143] U^{NH_2} is 2′-aminouridine.

cleotides, 2′-aminouridine and 'inversion' of the 3′-terminus (see above). It was shown that the unwinding components of the ribozymes rapidly and efficiently form complexes with the structured 190-nucleotide long MDR1 mRNA fragment. Because of this, the asymmetric binary ribozymes were much more efficient in cleavage of this RNA fragment than their symmetric analogue. As the length of the 'unwinding' domain increases, the cleavage efficiency also increased; asymmetric binary ribozymes cleaved RNA not only when taken in excess, but also when the ribozyme was in deficiency with respect to RNA.

IV. Applications of hammerhead ribozymes

Based on the hammerhead model, one can design various ribozyme constructs for particular purposes. Because of this, artificial ribozymes have found wide use as regulators of gene expression, as tools for functional genomics and as agents affecting mRNAs of therapeutically important genes. The main avenue of applied research on ribozymes is the inhibition of expression of the genes responsible for the development of various pathologies, *viz*., viral infections, oncological and hereditary diseases. In this Section, we will consider the most important results obtained in this field.

An important problem concerning the use of ribozymes for inhibition of gene expression is how they are delivered to cells. This can be done in two ways, endogenously and exogenously (see, *e.g*., a review [145]). The former way implies the incorporation of a ribozyme-encoding DNA sequence into the plasmid or retroviral vector. After transfection into the cell, the encoding construct is incorporated into the host genome or expressed independently, thus ensuring the expression of ribozyme, which then interacts with the target mRNA. The advantage of this approach is that the construct delivered to the cell resides there permanently. As a result, one deals with expression of new portions of ribozyme, so its concentration in the cell remains constant. When constructing the expressing vector, the ribozyme can be modified with RNA sequences (tRNA, small nuclear RNAs, *etc*.) which provide localization of the ribozyme within the same compartment as the target mRNA.[146–149] A drawback of the endogenous strategy is the delivery of alien genetic material to cells, which may be dangerous owing to possible mutagenesis.

In the exogenous route, ribozyme is synthesized chemically or enzymatically and then introduced into cells. This eliminates the problem of vector choice, but imposes new restrictions due to penetration of ribozymes through the cell membrane and to their degradation under the action of cell nucleases. To enhance the stability of ribozymes against nucleases, various types of chemical modification were developed, which allow one to significantly enhance the ribozyme stability and to retain high catalytic activity (see above). To ensure penetration of the ribozyme through the cell membrane, carriers are used in most cases, *i.e*., cationic lipids that can form micelles whose inner surfaces bear positive charges that interact with the negative charges of oligonucleotides; these micelles are then transported to cells by endocytosis.[150] The addition of helper lipids (*e.g*., cholesterol) to the cationic lipids favours the release of ribozymes from endosomes or lysosomes.[4] Cationic lipids proved themselves when using in cell cultures, but their applications at the level of living organisms are precluded by toxicity and immunostimulating properties. Recently, alternative biodegradable carriers, namely, dendrimers (branched carbohydrates) and polymers based on lactic and glycolic acids were developed.[151] Specific action on particular types of cells is also attained using conjugates of ribozymes with antibodies or cell receptor ligands.[152] Interestingly, in some cases ribozymes with hydrophobic groups (*O*-methyl and *O*-allyl) in position 2′ were quite efficiently delivered to cells without lipid carriers;[153, 154] however, this was observed only at the level of living organisms rather than in cell cultures.

The use of hammerhead ribozymes appeared to be a highly efficient tool for inhibition of viral gene expression. Ribozymes affect with high specificity only the infected viral cells and inhibit expression of viral genes and have no effect on the host cell RNA. Ribozymes were successfully used for inhibition of expression of human immunodeficiency virus HIV-1 (see reviews [155, 156]). In most cases, this was done involving endogenous ribozymes which were targeted against the *env*, *tat*, *pro* and *rev* genes and against the long 5′-terminal repeats (5′-LTR) of viral RNA. To additionally improve the efficiency of the effect on HIV-1, it was proposed to use 'multimeric' ribozymes, *viz*., a plasmid or vector simultaneously encodes a number of ribozymes targeted against different regions of the target mRNA. The ribozyme transcript thus obtained can then act as a polyribozyme molecule [155, 157–159] or undergo self-splicing to give a number of individual ribozymes.[160, 161] Endogenous anti-HIV ribozymes chosen based on the results of cell culture studies successfully passed phase I clinical trials.[162–164] Also, an endogenously expressed ribozyme OZ1 (Johnson and Johnson) targeted against highly conserved region of overlapping reading frames of *vpr* and *tat* genes [165] (Fig. 16) and a multimeric ribozyme [Ribozyme Pharmaceuticals, Inc. (USA)] targeted against *tat* and *rev* genes [167] have passed phase II clinical trials.

A combined approach proposed to suppress HIV infection involves the delivery to cells of vectors for encoding various RNA-based constructs [hammerhead ribozymes, small hairpin RNA (shRNA), RNA decoys] targeted

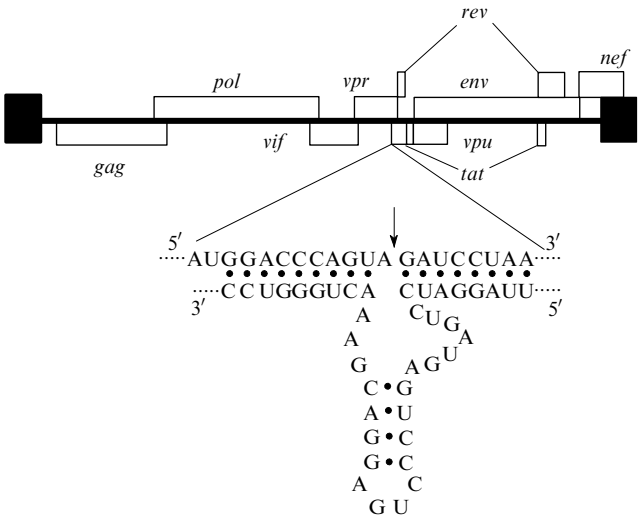


Figure 16. Schematic view of HIV-1 genome and the nucleotide sequence against which the OZ1 ribozyme is targeted.[165, 166]

against various stages of the lifecycle of the virus.[168] For instance, a lentiviral vector was designed which encodes the three constructs mentioned above; introduction of this vector into cells makes them stable against immunodeficiency virus.[169, 170] Moreover, stem cells transfected by such a vector and injected into HIV-infected mice developed into normal T-cells which were also virus-resistant.[170] This strategy was also employed in another study: [171] a joint use of hammerhead ribozymes and DNAzymes with a '10–23' catalytic motif exogenously delivered to cells led to more efficient inhibition of viral gene expression compared to the use of individual agents.

The possibility of using hammerhead ribozymes to inhibit hepatitis C viral gene expression was studied. Both endogenous [172] and exogenous ribozymes were investigated, but better results were obtained with exogenous chemically synthesized ribozymes modified with 'inversion' of the 3′-terminus, replacement of most ribonucleotides by their 2′-*O*-methyl analogues and replacement of some phosphodiester bonds by phosphorothioate bonds. In the cell culture, the extent of inhibition of viral gene expression by ribozymes targeted against the 5′-untranslatable region of viral RNA was 50% to 90%.[173] Based on the results obtained using these ribozymes, the most efficient among them, HEPTAZYME™ [Ribozyme Pharmaceuticals Inc., (USA)], was chosen as a therapeutic agent and passed phase I and II clinical trials.[81] However, the clinical trials were stopped in 2002 because high activity of HEPTAZYME was observed only in 10% of patients.[174] *In vitro* selection was used to obtain chimeric constructs comprising a ribozyme targeting the IRES site of hepatitis C RNA and an aptamer directed towards an essential domain in the IRES region.[175] The ribozyme and aptamer, as components of the chimeric construct, retained their functions and inhibition of IRES-dependent translation *in vitro* was observed.

Ribozymes were used to inhibit hepatitis B virus expression. Endogenous ribozyme constructs efficiently inhibited virus expression in cell extracts being, however, inactive in cell culture.[176] Chimeric minizymes exogenously introduced into a cell culture appeared to be efficient inhibitors of virus expression.[177] It was also shown that artificial 2′-modified ribozymes can inhibit hepatitis B virus not only in cell culture, but also at the level of organisms.[178] The most efficient of them, HepBzyme [siRNA Therapeitics Inc., (USA)] (Fig. 17 *a*), is under clinical trials.[167, 180]

Banerjea and co-workers [181] proposed to use chimeric constructs comprising a siRNA and ribozyme (Fig. 18) to inhibit expression of hepatitis B virus X gene. These constructs were cleaved in cell extracts into individual

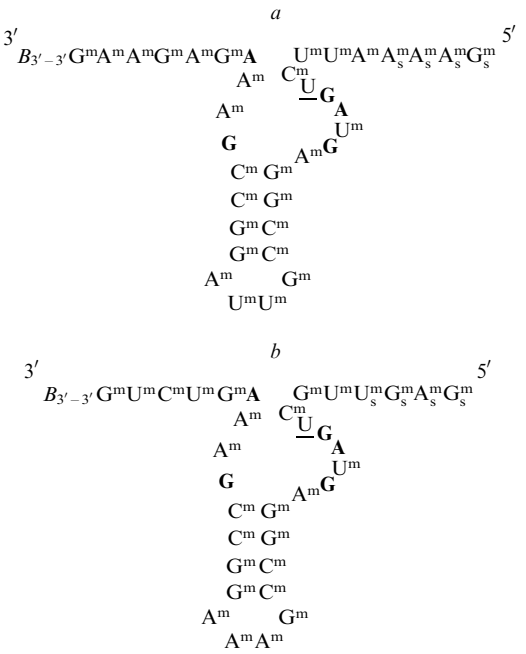


Figure 17. Artificial modified ribozymes HepBzyme [178] (*a*) and ANGIOZYME [179] (*b*).
U is 2′-*C*-allyluridine, $B_{3'-3'}$ is 1,2-dideoxyribose residue involved in the 3′–3′-phosphodiester bond.

ribozyme and siRNA molecules that inhibited the expression of X gene on both mRNA and HBx protein level.

Exogenous chimeric ribozymes containing deoxyribonucleotides in non-conserved positions efficiently inhibited expression of severe acute respiratory syndrome (SARS) virus in cell culture.[182] It was also shown that ribozymes endogenously delivered to cells using a retroviral vector inhibited the expression of Dengue fever virus.[183] An adenoviral vector for endogenous expression of ribozyme targeted against herpes simplex virus type I RNA was reported.[184] It efficiently protects mice from herpes.

Yet another rapidly progressing field of application of hammerhead ribozymes is inhibition of growth of malignant tumours. In this case, the ribozymes are targeted against mRNAs of various gene families for which defects in gene expression lead to malignification of cells. Among them, mention may be made of the following:

— mRNAs of oncogenes involved in regulation of cell growth and proliferation. Popular oncogene targets are *ras*

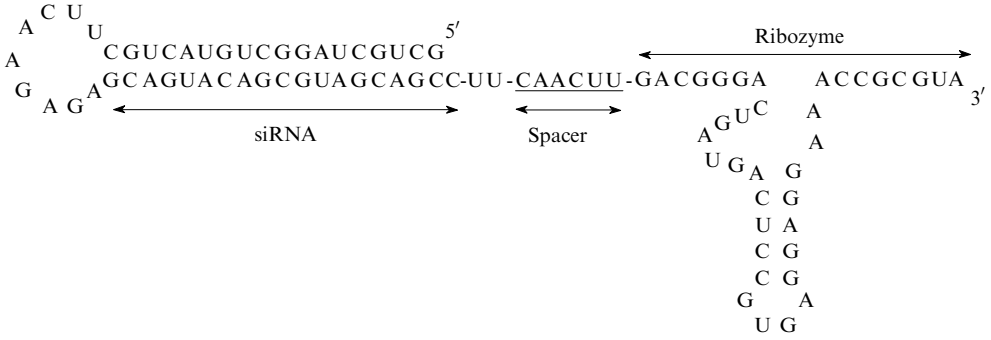


Figure 18. 'Hybrid' ribozyme + siRNA construct targeted against hepatitis B virus X gene mRNA.[181]

genes characterized by high level of mutations in the coding region. The design of ribozymes targeted against mutant RNA enables specific inhibition of expression of *ras* genes in malignant cells without changing the level of their expression in normal cells. Ribozymes targeted against *N*-ras,[185] *H*-ras[186] and *K*-ras[187] gene mRNAs were used for reversal of malignant phenotype in cell cultures. Oncogene targets also include tyrosine kinase *c-erb*, *c-met* and *RET* genes and the *c-myc* transcription factor.[166,188] An endogenously expressed ribozyme targeted against the NF-κB transcription factor inhibitor mRNA inhibits melanoma invasion and metastasis in mice.[189]

— *pml*/*rar* and *bcr*/*abl* chimeric gene mRNAs appeared as a result of chromosome translocations. Selective cleavage of chimeric mRNA is attained with endogenously expressed ribozymes targeted against the gene fusion region (see Section III.1); this type of ribozymes inhibits expression of chimeric genes and leukemia in animals.[61,65,166]

— mRNA of genes involved in regulation of apoptosis (*bcl*-2, survivin protein gene). Ribozyme-mediated inhibition of expression of these genes induces apoptosis of tumour cells and enhances their sensitivity to chemotherapeutic agents and radiotherapy.[166,188,190–192] An interesting method of delivery of a ribozyme targeted against survivin mRNA to cells using a φ29 bacteriophage RNA vector was reported.[193] The ribozyme incorporated into the bacteriophage RNA retained its ability to cleave the target mRNA while the highly structured vector protected the ribozyme from cell nucleases. Such constructs efficiently inhibited the expression of survivin in cancer cells, which led to their apoptosis. An adenoviral vector encoding four ribozymes targeted against different regions of survivin mRNA was designed and it was shown that it induces apoptosis in the cell culture and inhibits the growth of xenograft mice model for human hepatocellular carcinoma.[194]

— telomere repeat RNA (hTR) and telomerase reverse transcriptase (hTERT) mRNA whose expression level is increased in tumour cells. Synthetic 2′-*O*-methyl-containing ribozymes targeted against hTR inhibit telomerase activity in tumour cell culture.[195] Endogenously expressed ribozymes targeted against the same RNA inhibited melanoma growth in mice and prevented metastasis.[195,196] Ribozymes targeted against hTERT gene were efficient in reducing the telomerase activity in cell cultures, inhibiting tumour cell growth and reducing resistance of tumour cells to chemotherapeutic agents.[188,197]

— multidrug resistance gene *mdr*1 mRNA. The product of *mdr*1 gene, the transmembrane protein P-glycoprotein, eliminates pharmaceuticals from cells, thus complicating tumour chemotherapy. Exogenously delivered (chemically or enzymatically synthesized)[198–201] and endogenous[202–207] ribozymes reduce the level of *mdr*1 gene expression in tumour cell cultures, recover their sentivity towards pharmaceuticals and inhibit expression of *mdr*1 gene in animals.[188] The design of endogenous 'complex' polyribozyme targeted against mRNAs of some multidrug resistance genes (MDR1, BCRP, MRP2) was reported.[208] In the cell models, the vector encoding this polyribozyme inhibits expression of corresponding genes and recovers the cell resistance to daunorubicin.

— mRNAs of blood vessel growth factor genes and their membrane receptors (VEGF, Flt-1, KDR). Inhibition of expression of these genes blocks the growth of blood vessels in the tumour and thus stops the growth of the tumour. The most efficient was the use of ribozymes to inhibit expression of the gene encoding the VEGF receptor. The VEGF receptor mRNA was affected by a number of synthetic ribozymes containing the same chemical modifiers as those chosen for HEPTAZYME™ (see above). The most efficient of these ribozymes called ANGIOZYME™ [Ribozyme Pharmaceuticals Inc. (USA)] (see Fig. 17*b*) successfully passed phases I and II clinical trials.[5,81,179,188,209] Investigations revealed that a combination of ANGIOZYME with conventional chemotherapeutic agents enhances their action by reducing the level of the VEGF receptor.

Ribozymes can also be used for treatment of hereditary diseases. It was shown that endogenous ribozymes targeted against mRNA of the rhodopsin mutant gene responsible for the development of *retinitis pigmentosa* and blindness, efficiently inhibit expression of this gene and can induce elimination of clinical indicators of disease in animals.[210–212] Chemically synthesized ribozymes targeted against fibrillin-1 mutant gene mRNA responsible for imperfect osteogenesis can selectively cleave only the mutant RNA which differs from the wild mRNA in only one nucleotide.[213] Based on these data, endogenous ribozymes capable of reducing the expression level of mutant fibrillin in cell culture were designed.[214,215]

Ribozymes were also proposed as a tool for treatment of restenosis following surgical procedures (angioplasty, bypass) due to proliferation of smooth muscle cells vessels. To inhibit such a proliferation, a number of exogenous, synthetic modified ribozymes containing deoxyribonucleotides and phosphorothioate bonds and endogenously delivered ribozymes (see review[188]) was used. The ribozymes were targeted against mRNA of the proteins responsible for regulation of cell cycle and cell mobility. For instance, 'chimeric' exogenously delivered ribozymes targeted against mRNA growth factor PDGF-A and endogenously expressed ribozymes targeted against TGF-β growth factor mRNA inhibited the growth of smooth muscle tissue in rats. A combination of ribozymes targeted against the E2F1 transcription factor mRNA and cyclin E mRNA completely inhibited proliferation in a smooth muscle cell culture.[188,216] Chemotaxis of smooth muscle cells was affected by exogenously delivered 'chimeric' ribozymes targeted against 12-lipoxygenase mRNA;[217] it was found that they significantly inhibit arterial restenosis in rats.

Thus, at present there are convincing proofs that hammerhead ribozymes can not only cleave extended structured mRNAs *in vitro*, but also inhibit the expression of various genes in cell cultures and in some cases even at the level of an organism. It should be noted that the extent of inhibition depends on particular mRNA, cell line, modifiers introduced into the ribozyme, carrier chosen to deliver the ribozyme, ribozyme concentration and the lifetimes of the target mRNA and the corresponding protein. All these factors complicate a comparison of the results obtained by different research groups even for the same gene.

V. Conclusion

Among all known ribozymes, the hammerhead ribozyme has been best studied to date. Its molecular structure and the mechanism of cleavage of phosphodiester bonds in RNA are determined. Owing to their ability to site-specific cleavage of RNA with high efficiency, hammerhead ribo-

zymes obtained both chemically and enzymatically can be used for inhibition of gene expression at mRNA level. Hammerhead ribozymes offer some unique advantages over other agents (antisense oligonucleotides, siRNA):

— RNA cleavage involving no additional enzymes;

— very high specificity; *viz*., RNA cleavage requires hybridization of both flanking regions of the ribozyme with the target and the presence of the 5′-NUH-3′ triplet;

— possibility of regulation of the catalytic activity depending on the local environment of the ribozyme;

— no interferon response in mammalian cells after delivery of ribozymes.

Researchers developed methods for enhancement of the resistance of ribozymes to cell nucleases, for exogenous delivery of ribozymes to cells and to construction of vectors for endogenous expression of ribozymes. Approaches to regulation of the catalytic activity of ribozymes and improvement of the efficiency of cleavage of natural structured RNAs were proposed. Hammerhead ribozymes are successfully used to inhibit the expression of a broad spectrum of genes in cell cultures. In some cases, ribozymes selected after cell experiments and in animal models have passed phase I and II clinical trials as therapeutical agents for treatment of malignant tumours and viral infections.

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