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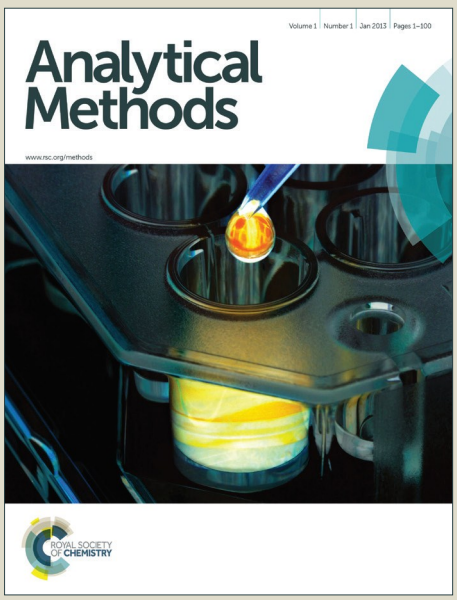

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Application of a fluorescently labeled *trans*-acting antigenomic HDV ribozyme to monitor antibiotic - RNA interactions

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Abstract

In order to determine if a fluorescently labeled *trans*-acting antigenomic HDV ribozyme can be used to monitor antibiotic-RNA interactions, several parameters that can influence the assay sensitivity and reproducibility were tested and optimized. Applicability of the assay for rapid screening of antibiotics targeting the HDV ribozyme was shown by using selected antibiotics and their complexes with copper(II) ions, also at various pH conditions. Potential antibiotics which are intrinsically fluorescent or intercalate can also be tested. The assay was very sensitive, since a single nucleotide mutation of the ribozyme target in the J4/2 region influencing the RNA-antibiotic interaction was detected. Consequently, the fluorophore-labeled HDV ribozyme is a good model for the rapid screening of antibiotics, and possibly, also other low-molecular-weight compounds which target RNA molecules.

Keywords: RNA, hepatitis delta virus ribozyme, HDV ribozyme, antibiotic, fluorescence

Introduction

RNA participates in important life processes such as replication, transcription and translation. Also many diseases are associated with disturbances in RNA pathways. Many low molecular compounds, including antibiotics, have been shown to be involved in the regulation of RNA function in the cell. Antibiotics are a large class of compounds that reduce the growth of pathogens by interacting with several cellular targets: proteins, cell wall components and nucleic acids.[1] Many antibiotics targeting RNA bind to ribosomal RNA, mRNA or tRNA, affecting the process of translation of the genetic message.[2,3] Recently resolved crystal structures of the bacterial ribosome in complex with several antibiotics have verified these hypotheses. It has been shown that different antibiotics bind to the A and P sites of bacterial ribosomes. The aminoglycoside antibiotics preferentially bind to the A site of the small 30S subunit.[4,5] On the other hand, antibiotics which belong to the oxazilidone and macrolide groups bind to the 50S large subunit, affecting the activity of the peptidyl transferase center which is crucial for peptide bond formation.[3]

Important information concerning the essential principles underlying the interactions of RNA with antibiotics can be obtained by analyzing their impact on RNA catalysis performed by ribozymes. The advantages offered by ribozymes as model systems include their complex structure and the possibility of testing ribozyme activity in relation to RNA structure and function.[6] The studies are facilitated by the known structure and function of several ribozymes, including large, self-excising introns of group I and II, as well as small ribozymes, such as hammerhead, hairpin, HDV (Hepatitis Delta Virus) and *glmS*.[7-9] Among these ribozymes, the HDV ribozymes are particularly attractive as a model system for studying RNA-antibiotic interactions.

The HDV ribozymes derive from the hepatitis delta virus which is a small circular RNA virus of 1.7 kb in length that replicates via a double rolling circle mechanism. During

replication, two viral strands are synthesized, genomic and antigenomic, which contain domains with the self-cleavage activity necessary for processing of both multimeric transcripts into unit-length linear RNAs.[10] The secondary structure of both genomic and antigenomic ribozymes has five pairings, P1–P4 and P1.1, which comprise two pseudoknots that provide a compact fold. Figure 1 shows the structure of an antigenomic variant designed in its *trans*-acting form which cleaves the phosphodiester bond between U1 and G1 to generate products with 2′,3′-cyclic phosphate and 5′-hydroxyl termini. This indicates that the ribozyme utilizes the transesterification reaction i.e. nucleophilic attack on the scissile phosphate by 2′ hydroxyl group of nearest nucleotide.

Under physiological conditions, the HDV ribozymes require metal ions for catalytic activity, thus they belong to the metalloenzyme class. Divalent metal ions, like Mg^{2+}, Ca^{2+} and Mn^{2+}, are the most effective, but in the presence of monovalent ions like Na^{+} and K^{+}, a low catalytic activity of the ribozymes is also observed.[11,12] Metal ions can contribute to RNA catalysis through nonspecific and specific modes of action. Generally, they are bound specifically to the functional groups of nucleobases inside ribozyme spatial structures or non-specifically to sugar-phosphate backbones, neutralizing the negative charge of polynucleotide chains and stabilizing ribozyme tertiary structure.[13,14] In addition, a correctly positioned, strongly bound metal ion directly participates in the catalysis. According to a multichannel framework proposed for the genomic ribozyme, divalent metal ions contribute 3,000-fold to the reaction: ~125-fold to folding and ~25-fold to catalysis.[14] The genomic HDV ribozyme crystal structure and numerous biochemical experiments have shown that the ribozyme utilizes base–acid catalysis, involving nucleobases and divalent metal ions.[14-16] The catalytically important C75 nucleobase (C76 in the antigenomic variant), with a pK_a value shifted towards neutrality, lies at a hydrogen bonding distance from the 5'-oxygen leaving group and thus may play the role of a general acid and protonate the leaving group. Moreover,

the catalytic metal ion is positioned to interacting with the *pro*-R_P oxygen of the scissile phosphate and the phosphate group of U23 and acts as a Lewis acid, accepting the proton from the nucleophile and stabilizing the conformation of the cleavage site.

Although the HDV ribozymes are of viral origin, they are active in human cells. Moreover, the HDV-like ribozymes have been found in the second intron of human and other mammals' CPEB3 (Cytoplasmic Polyadenylation Element Binding protein 3) protein gene pointing to the existence of such types of ribozyme in the genomes of higher organisms.[17] Recently, HDV-like ribozyme sequences have also been revealed in LINE (Long interspersed Element) and SINE (Short Interspersed Element) retrotransposons.[18] In addition, minimal HDV-like ribozymes have been found in the human microbiome.[19] Therefore, by studying the impact of antibiotics on HDV ribozymes, we may obtain information on the possible interactions of antibiotics with other cellular RNAs.

Previous studies of the impact of various antibiotics on several ribozymes have shown that some of them inhibit RNA catalysis.[6] Basically, they can be divided into two groups: nonspecific antibiotics, which inhibit the catalytic activity of many ribozymes; and specific antibiotics, which inhibit individual ribozymes. A member of the first group is neomycin B, an aminoglycoside antibiotic that strongly inhibits the catalytic activity of small ribozymes, such as hammerhead, hairpin, and HDV of the genomic and antigenomic type, as well as large ribozymes, such as RNase P and ribozymes of group I intron.[6] Similar properties have been demonstrated in the case of 5-epi-sisomicin, another aminoglycoside antibiotic that effectively inhibits the catalytic activity of the hairpin, HDV and group I intron ribozymes. On the other hand, kanamycin acts very specifically, affecting the cleavage reaction only in the case of RNase P, and not inhibiting group I intron and HDV ribozymes.[6,20]

We recently characterized a set of antibiotics and their copper(II) complexes that interact with the HDV ribozymes in our laboratory.[21-24] We and others have found, however,

that a precise analysis of the impact of antibiotics on HDV ribozyme catalytic activity is usually difficult. The cleavage reaction is often very fast, which hampers precise determination of the kinetic parameters of the reaction using manual pipetting. Moreover, a commonly applied method of analysis of ribozyme activity involves the use of radioactively-labeled HDV ribozyme and quantification of the cleavage products after their separation by PAGE. This procedure is laborious and time-consuming. Here, we propose a new fluorescence-based assay for rapid screening of antibiotics targeting the HDV ribozyme. The applicability of the assay for studying antibiotic-ribozyme interactions was tested by using selected antibiotics which have earlier been shown to be able to modulate ribozyme activity.[21,23]

Experimental

Materials

All chemicals were from Serva or Fluka. Antibiotics were purchased from Sigma. Taq DNA polymerase, T_4 polynucleotide kinase, NTPs and dNTPs were from MBI Fermentas. [γ-^{32}P] ATP (5000 Ci/mmol) was from Hartmann Analytic. 5′-fluoroscein (3',6'-dihydroxyspiro[2-benzofuran-3,9'-xanthene]-1-one), 3′-dabcyl (4-{{4-(dimethylamino)phenyl} azo} benzoic acid) 13-mer RNA oligomer (R13FQ) was synthesized by Future Synthesis (Poznan, Poland).

DNA template construct

The dsDNA templates for the *in vitro* transcription of the *trans*-acting antigenomic HDV ribozyme were prepared as described.[22] Briefly, two DNA oligomers were synthesized: A, 5′-GAAAAGTGGCTCTCCCTTAGCCATCCGAGTGCTCGGATGCCC AGGTCGGACCGCGAGGAGGTGGAGATGCCC-3′ and B, 5′-*TAATACGACTCACTATA GG*GCATCTCCACC-3′ (the complementary sequences are underlined in both oligomers and letters in italics mark the T7 RNA polymerase promoter). Equimolar amounts of both oligomers A and B were annealed. The reaction mixture contained 1.5 μM of each oligomer,

10 mM Tris-HCl pH 8.8, 2 mM $MgCl_2$, 50 mM KCl, 200 μM each dNTP and 100 units/ml of DNA Taq polymerase. The reaction was performed on Biometra UNO II thermocycler for nine cycles, 30 s at 94 ºC, 30 s at 46 ºC and 2 min at 72 ºC. The double-stranded DNA was extracted with phenol/chloroform (1:1) and precipitated with ethanol at -20 ºC overnight. The HDV dsDNA template was recovered by centrifugation, dissolved in TE buffer and used in the transcription reactions.

***In vitro* transcription**

In vitro transcription proceeded with the AmpliScribe T7-Flash kit according to the manufacturer's procedure using 2 μg HDV dsDNA template. The mixture was incubated for 1 hour at 37 ºC. The reaction products were purified on an 8% polyacrylamide gel under denaturing conditions. The band corresponding to the ribozyme was localized by UV shadowing, cut out and RNA was eluted from the gel with 0.3 M sodium acetate (pH 5.2), 1 mM EDTA, ethanol precipitated and dissolved in sterile water containing 0.1 mM EDTA. RNA was divided into small aliquots and stored at -20 ºC.

Catalytic cleavage reaction

The *trans*-acting antigenomic HDV ribozyme was prepared by mixing 0.01 μM fluorescently labeled RNA (R13FQ) substrate with 1 μM HDV ribozyme in the buffer 50 mM Tris-HCl pH 7.5 to obtain a 100:1 ribozyme – substrate ratio. The mixture was subjected to a denaturation-renaturation procedure by incubating for 1 min at 100 ºC and chilling on ice for 5 min. The antibiotics or their complexes with Cu^{2+} ions were then added to the appropriate concentrations and the mixture was incubated for an additional 5 min at 37 ºC. Subsequently, the appropriate volume of the sample was transferred to a well of a 96-well polystyrene microplate for fluorescence measurement. The ribozyme cleavage reaction was initiated by adding magnesium chloride to a final concentration of 10 mM and the reaction proceeded at 37 ºC.

Fluorescence measurements

Fluorescence intensity was measured using a multimode reader X4 Victor™ (Perkin Elmer) according to pre-programmed protocols. Fluorescein was excited at 485 nm and fluorescence emission was measured at 535 nm. The measurement was performed once with the control sample at 37 ºC, after 1s mixing, with 1s wave excitation, before the addition of Mg^{2+} ions. The analysis of the kinetics of cleavage reactions was performed by measuring fluorescence after a repeated specific time period at 37 ºC, with 1s wave excitation, after addition of 10 mM $MgCl_2$ and stirring for 1 s. Reaction rate constants were calculated by fitting the experimental data to a single exponential equation: $[P]_t=[EP](1-e^{-kobs \times t})$, where k_{obs} is the first order constant and $[P]_t$ and [EP] are the fraction cleaved at time t and at the reaction end point, respectively. These values were calculated using Microcal Origin Pro 8.5 software. The values presented in the figures are the means of 2–3 independent experiments.

Results and Discussion

Optimization of the fluorescence-based assay for an analysis of ribozyme activity

The HDV ribozymes of the genomic and antigenomic type seem to be particularly attractive model systems for studying the interactions of RNA with antibiotics. Both ribozymes have similar structures and cleavage mechanisms, which have been studied in detail.[10] Moreover, the structure of the genomic variant has been determined with atomic resolution.[15,16] In our studies, two *trans*-acting antigenomic HDV ribozymes, wild type and containing G80U mutation[25], were used (Fig. 1). In the crystal structure of the genomic variant, nucleotide G80 is located in the single-stranded region J4/2. In this region nucleotides G74, C75, and G76 are present, which form the trefoil turn motif ensuring the correct positioning of the catalytic C75 in the active center.[15,16] The G80U mutant retains full catalytic activity[25,26], and even a C80 deletion mutant is still active in RNA catalysis.[27] Interestingly, despite the identical cleavage rates for wild type and G80U mutant ribozymes, when the cleavage site was modified with a

phosphorothioate, in the presence of Mg^{2+} the S_P/R_P ratio of the cleavage rate was 25 for wild type ribozyme and decreased to 6.4 for the G80U mutant.[28] Thus, there are subtle differences in functioning of these two ribozymes and both variants were used in evaluating the sensitivity of our fluorescence-based assay.

Two oligonucleotide substrates, 13-mer RNAs with fluorescein on the 5′ end and with fluorophore-quencher system (FRET, Forster resonance energy transfer) on the 5′ and 3′ ends, were tested (Fig. 1). Dabcyl was used as a fluorescent quencher and the cleavage progress was followed by fluorescence increase. Fluorescein was excited with 494 nm light and fluorescence intensity was measured at 521 nm. The oligonucleotide substrate containing a fluorophore-quencher system was found to be more precise in tracking fluorescence changes, thus this substrate was applied in all subsequent experiments.

The ribozyme/substrate ratio (20, 50 and 100-fold excesses of the ribozyme), type of reaction buffer (Tris, MES and HEPES), reaction temperature (25, 37 and 50 ºC), final reaction volume (10, 30 and 60 µl), and frequency of fluorescence measurements (2, 15 and 60 s) were tested and optimized for their influence on the assay's sensitivity and reproducibility. This resulted in the following optimized assay conditions: 30 pmol of HDV ribozyme was mixed with a 100-fold lower amount of fluorophore-quencher 13-mer RNA substrate in a total volume of 30 µl. Cleavage reactions were carried out in appropriate buffers, usually 50 mM Tris-HCl pH 7.5. The reaction was initiated by adding $MgCl_2$ to the final concentration of 10 mM. After stirring the sample for 1 s fluorescence intensity was measured after repeated 2 s time periods with 1 s wave excitation. Checking two types of 96-well plates, black and white, better sensitivity was obtained using black plates.

Impact of selected antibiotics and their complexes with Cu^{2+} on ribozyme activity

In order to check the reproducibility and sensitivity of the fluorescence-based assay the impact of selected cyclic peptide antibiotics, capreomycin, gramicidin S and viomycin, as

well as of actinomycin D, which belongs to the chromopeptide antibiotic group, on HDV ribozyme activity was studied. We have previously found that the inhibitory properties of some antibiotics significantly increase when complexed with Cu^{2+} so the impact of Cu^{2+}-antibiotic complexes on ribozyme activity was also analyzed.[21-24] We were curious to know whether Cu^{2+} ions might influence the application of the fluorescence-based assay.

Figure 2 shows the results of determination of the ribozyme catalytic activity at different concentrations of capreomycin and its complex with Cu^{2+}. In the control reaction with no antibiotic, the cleavage rate constant k_{obs} was 0.35 s^{-1}. In the presence of capreomycin used at the concentrations of 0.2 and 0.6 mM an approx. 60% increase of k_{obs} was observed. At higher concentrations of the antibiotic of 0.8 and 1.0 mM, 2-fold higher k_{obs} values were determined in comparison with the control reaction. Complete inhibition of the HDV ribozyme activity was observed at peptide antibiotic-Cu^{2+} complex concentrations over 0.5 mM (data not shown), therefore, lower concentrations of the complex were used. First, we tested the cleavage reaction environment containing Cu^{2+} ions based on the yield of the fluorophore-quencher system. Thus, the control ribozyme cleavage reaction was performed in the presence of only 0.2 mM Cu^{2+} ions. There was no difference between the two k_{obs} values, which were determined in the presence and absence of Cu^{2+} (Fig. 2A, B). For the capreomycin-Cu^{2+} complex, the dependence of the cleavage rate on the complex concentration revealed a bell-shaped curve. For the 0.05 mM capreomycin-Cu^{2+} complex the k_{obs} value was 0.54 s^{-1} and in the presence of 0.1 mM complex, an almost twice as high k_{obs} was determined compared with the control reaction. An increased complex concentration (to 0.2 mM) slightly inhibited the reaction, while at the concentration of 0.5 mM, k_{obs} was 3-fold lower than the value for the uninhibited reaction. It must be emphasized that during our previous study concerning an analysis of ribozyme activity with [^{32}P]-labeled oligonucleotide

substrate and PAGE analysis[23] we have observed an essentially identical impact of capreomycin and its Cu^{2+}-complex on HDV ribozyme as in this study.

Previously, it has been determined that capreomycin and its complex with Cu^{2+} ions bind to the antigenomic HDV ribozyme at pH 7.5 with K_d values of 50 μM and 11 μM, respectively.[23] Such K_d values, which are in μM range, are typical for drug – RNA interactions. Antibacterial lysine derivatives L-4 oxalysine and L-aminoethylcysteine AEC bind to *B subtilis* riboswitch with the corresponding K_d values of 13 μM and 30 μM. Also netilmicin and 5-(N,N)-dimethylamiloride drugs show affinity to HIV-1 TAR structure with K_d values of 1.35 μM and 121.35 μM, respectively.[29] Several factors have been suggested which might be responsible for an impact of antibiotics on the HDV ribozyme cleavage reaction, such as the displacement of the catalytic Mg^{2+}, or an impact on the cleavage reaction trajectory by changing the ribozyme structure.[22] Recently, we have mapped the interactions of selected antibiotics and their Cu(II) complexes with the antigenomic HDV ribozyme. It turned out that they bind to its different regions or bind in different ways. Importantly, their high binding affinity seem to be of primary importance for effective inhibition of ribozyme catalysis.[6,20,22]

In the next step we checked the sensitivity of the assay in monitoring the RNA–antibiotic interactions when small structural changes had been introduced to the HDV ribozyme target. To this end, in addition to the wild type ribozyme, the G80U mutant was used. Irrespective of a changed nucleotide in the J4/2 region, this mutant retains full catalytic activity.[25-27] A different cleavage rate of the G80U mutant in comparison with the wild-type ribozyme was observed at the highest 1 mM concentration of capreomycin (Fig. 2C). Moreover, in the case of wild type ribozyme, stimulation of the cleavage reaction was observed with 0.2 mM capreomycin-Cu^{2+} complex, whereas an inhibitory effect was observed for the G80U mutant under these conditions. Thus, our fluorescence-based assay allowed

monitoring changes in the antibiotic-RNA interactions upon subtle structural changes introduced in the targeted RNA.

Impact of pH on HDV ribozyme cleavage rate

It has been noted that some antibiotics act in a pH-dependent manner, being able to inhibit or stimulate ribozyme activity.[20] The concentration of neomycin required for 50% inhibition of HDV ribozyme increases from 0.5 μM at pH 5.5 to 1.5 mM at pH 9.0. It has been suggested that this aminoglycoside antibiotic requires its protonated amino groups to effectively bind to the ribozyme.[6,20] The corresponding pH-dependent change is only four-fold for viomycin (unpublished results), a cyclic peptide antibiotic structurally related to capreomycin.

We tested the possibility of applying a fluorescence-based assay to investigate the impact of viomycin and a viomycin-Cu^{2+} complex on the HDV ribozyme activity at different pH conditions (Fig. 3). Approximately 3-fold stimulation of cleavage was observed with 0.2 mM concentration of viomycin at pH 7.5. However, in the presence of 0.05 mM viomycin-Cu^{2+} complex, only 2-fold increase of k_{obs} value was observed. On the other hand, at a lower pH of 5.5 the concentration of 0.2 mM viomycin completely inhibited the cleavage reaction, while its Cu^{2+}-complex at 0.05 mM stimulated the reaction 2-fold. Such an impact of viomycin and the complex on the catalytic properties of HDV ribozyme resembles that observed previously for capreomycin.[23] This is not surprising, as both these cyclic peptide antibiotics are structurally related and bind to an identical site on bacterial 70S ribosome i.e. in a cleft between the small and large ribosomal subunits, interacting with helix 44 of 16S rRNA and helix 69 of 23S rRNA.[30]

Analysis of HDV ribozyme activity in the presence of antibiotics with fluorescent and intercalating properties

It was important to test some possible limitations of the fluorescence-based assay: first of all, to determine whether selected groups of antibiotics or reaction conditions might disturb the

measurements of fluorescence intensity of the system. To this end, we extended our studies with two antibiotics not tested earlier, gramicidin S and actinomycin D, which have different chemical properties than capreomycin and viomycin. Gramicidin S appeared to have a limited influence on ribozyme cleavage. In the concentration range of 0.01 – 0.05 mM the cleavage rate was almost identical to that observed for the control reaction, with no antibiotic added (Fig. 4A). However, the impact of actinomycin D on HDV ribozyme was more complex. This antibiotic at a low concentration of 0.1 mM caused a 2-fold inhibition of ribozyme activity while at a higher concentration of 0.5 and 1 mM, moderate stimulation of the reaction was observed (Fig. 4B).

Unlike viomycin and capreomycin, gramicidin S which also belongs to the cyclic peptide antibiotics group, contains a tryptophan residue and is thus inherently fluorescent.[31] Fluorescence excitation of this antibiotic requires 295 nm light and fluorescence emission occurs at 334 nm. In our studies the *trans*-acting HDV ribozyme contained a fluorescein-labeled oligonucleotide substrate and Victor filters were used to provide fluorescence excitation at 485±15 nm and fluorescence emission at 535 ± 25 nm. Therefore, proper selection of filters allowed the use of the fluorescence assay to follow the binding of gramicidin S to the HDV ribozyme, despite the inherent fluorescence of the antibiotic. On the other hand, actinomycin D with a phenoxazone ring intercalates into the DNA and RNA helix, preferentially between the GC base pair, and the cyclic penta-peptide moiety is located in the major groove.[32] Actinomycin D represents a large group of antibiotics with intercalating properties and we showed that it was possible to follow the binding of this antibiotic to RNA using a fluorescence-based assay.

An approach to screening antibiotics that bind to fluorophore-labeled RNA similar to that described in our communication has previously been investigated.[33-37] A fragment of 16S RNA containing an antibiotic binding site or fluorescein-labeled hammerhead ribozyme was

used as a research model.[33-35] In addition, Jenne *et al*. constructed a hammerhead ribozyme model containing 3′ TAMRA (carboxytetramethylrhodamine) and 5′ FAM (6-carboxyfluorescein) dyes allowing FRET assay to search for antibiotics which affect the cleavage reaction.[36] Blout & Tor prepared TAR (regulatory element of HIV virus) constructs labeled with a pyrene fluorophore.[37] Changes of fluorescence intensity of the pyrene as a function of aminoglycoside concentration revealed that among seven different aminoglycosides, neomycin binds to the TAR element most effectively. In our assay, the HDV ribozyme was applied, which is active in human cells, thus importantly, in the same cellular environment in which the antibiotics are active.

Conclusions

We have developed a new fluorescence-based assay for rapid screening of antibiotics targeting the HDV ribozyme. The applicability of the assay for studying antibiotic – ribozyme interactions was confirmed by using selected antibiotics able to modulate the activity of the HDV ribozyme and their complexes with copper(II) ions, also in various pH conditions. Other selected antibiotics having intrinsic fluorescence properties or showing intercalating abilities were also suitable to be used in the assay.

The fluorophore-labeled HDV ribozyme model for studying the RNA – antibiotic interactions has several advantages: 1) HDV ribozyme is an exceptional ribozyme which is present in human, other eukaryotic as well as bacterial cells, in contrast to most other ribozymes occurring in plants, 2) the ribozyme has a complex structure facilitating binding of a wide spectrum of antibiotics, 3) the ribozyme is relatively stable and active in various conditions.

The fluorophore-labeled HDV ribozyme is a good and universal model for rapid screening of antibiotics which interact with RNA molecules as well as for revealing the details of RNA – antibiotic interactions.

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Figure legends

Figure 1. The sequence and secondary structure of the *trans*-acting antigenomic HDV ribozyme. A filled triangle marks the cleavage site in oligonucleotide substrate R13FQ. On the left, the structures of fluorophore (F) and quencher (Q) are shown. The numbering of the nucleotides corresponds to the wild-type ribozyme sequence. Base-paired segments are denoted as P1–P4, and single stranded regions as J4/2, L3, and L4. The site of the replacement of guanine by uracil in G80U mutant ribozyme is marked.

Figure 2. Dependence of the cleavage rate constant (k_{obs}) for the HDV ribozyme wild type (A, B) and G80U mutant (C, D) on the concentration of capreomycin and the capreomycin-Cu^{2+} complex. Cleavage reactions were performed in the presence of capreomycin at a concentration in the range of 0 - 1 mM (A, C) and in the presence of capreomycin-Cu^{2+} complex at a concentration in the range of 0 - 0.5 mM (B, D).

Figure 3. Effect of pH on the cleavage rate constants. A) Kinetic curves of fluorescein-dabcyl labeled antigenomic HDV ribozyme cleavage reactions at pH 5.5 (left) and 7.5 (right); control (blue triangles), in the presence of 0.2 mM viomycin (red squares), and 0.05 mM viomycin-Cu^{2+} complex (green circles). The cleavage reactions were induced by 10 mM Mg^{2+} ions and fluorescence intensity was measured after repeated 15 s time periods with 1 s wave excitation. B) The k_{obs} values determined for the HDV ribozyme with no antibiotic added (control), 0.2 mM capreomycin and with 0.05 mM capreomycin-Cu^{2+} complex at pH 5.5 and 7.5.

Figure 4. Histogram showing cleavage efficiency of fluorescein-dabcyl-labeled antigenomic HDV ribozyme in the presence of gramicidin S (A) and actinomycin D (B).

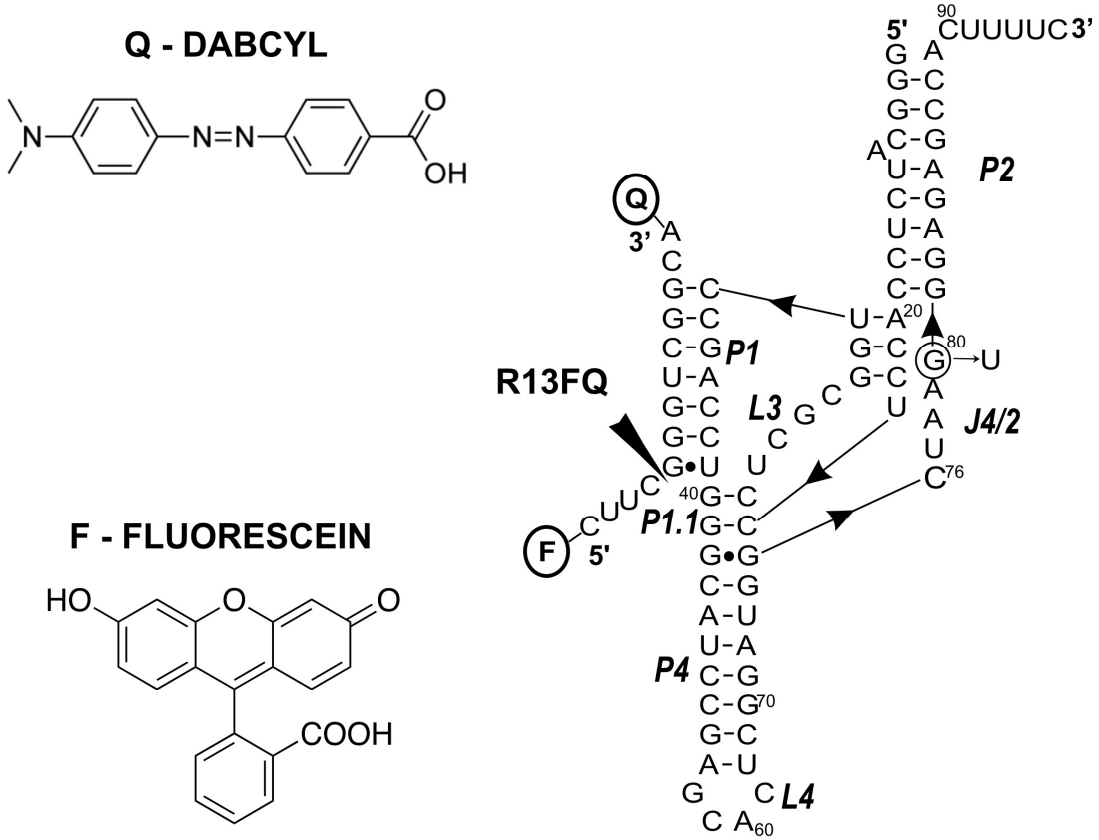

Fig. 1

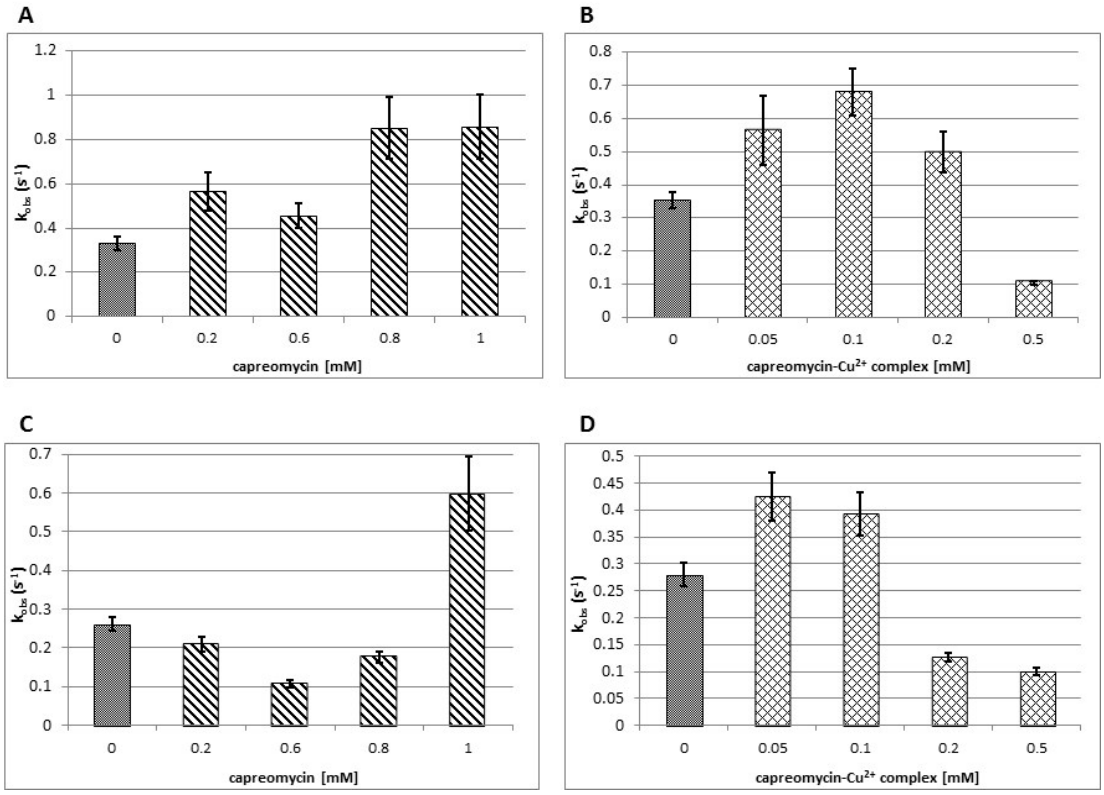

Fig. 2

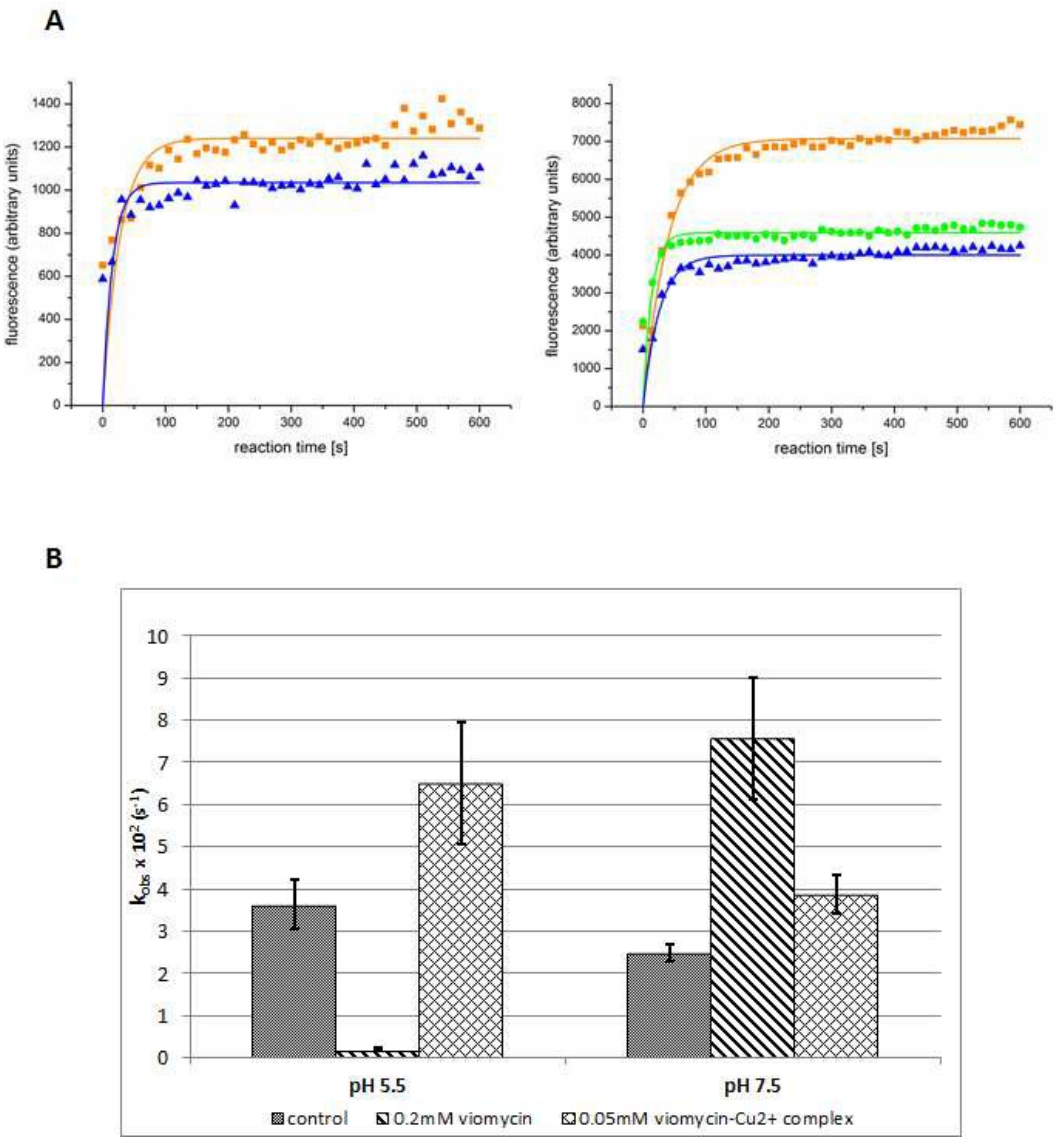

Fig. 3

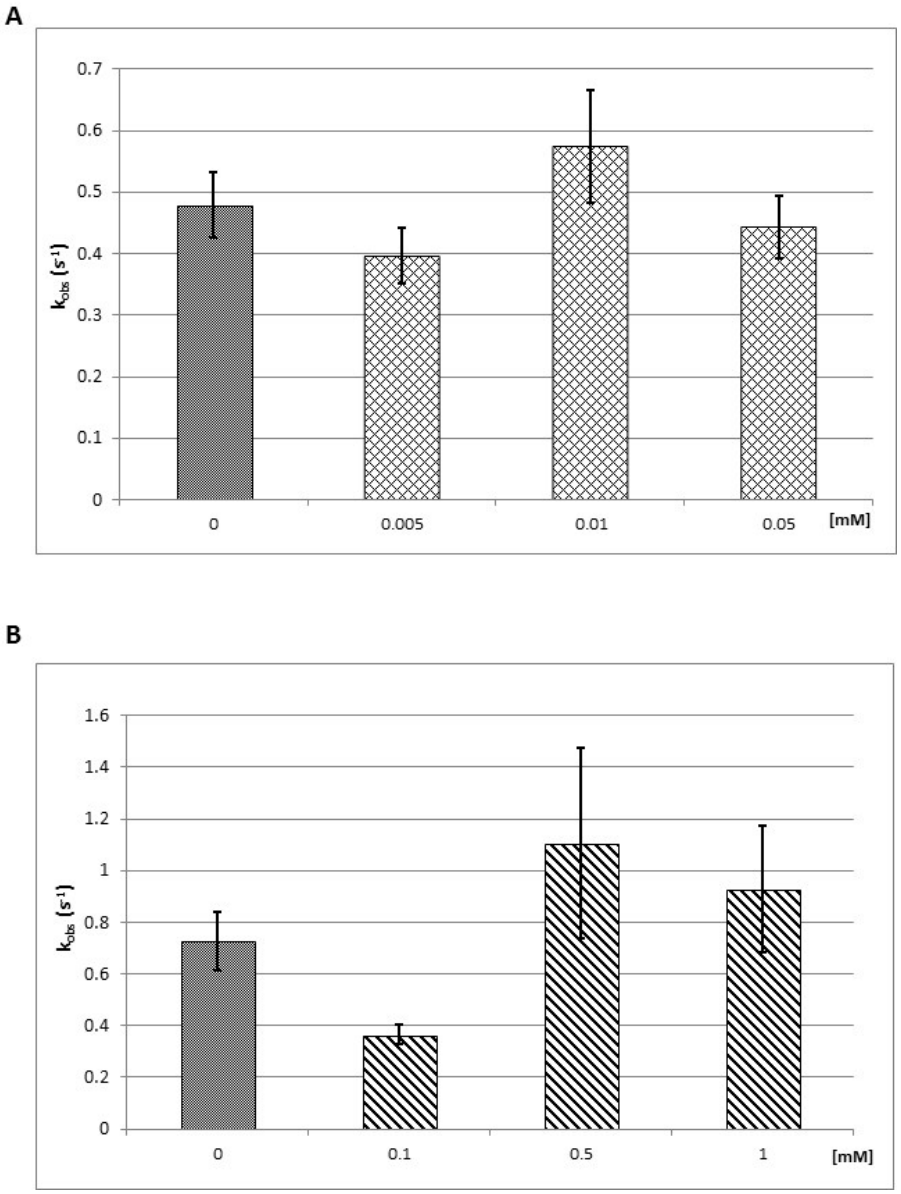

Fig. 4