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In vitro selection of deoxyribozymes active with Cd^{2+} ions resulting in variants of DNAzyme 8-17

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Abstract

In vitro selection was performed to search for RNA-cleaving DNAzymes catalytically active with Cd^{2+} ions from the oligonucleotide combinatorial library with a 23-nucleotide random region. All the selected, catalytically active variants turned out to belong to the 8-17 type DNAzyme. Three DNAzymes were prepared in shortened, *cis*-acting versions which were subjected to a detailed study of the kinetic properties and metal ion preferences. Although the selection protocol was designed for Cd^{2+}-dependent DNAzymes, the variants showed broader metal ion specificity. They preferred Cd^{2+} but were also active with Mn^{2+} and Zn^{2+}, suggesting that binding of the catalytic ion does not require an extremely specific coordination pattern. The unexpected decrease of the catalytic activity of the variants along with the temperature increase suggested that some changes occurred in their structures or the rate-limiting step of the reaction was changed. Two elements of the catalytic core of DNAzyme 1/VIIWS, the nucleotide in position 12 and the three-base-pair hairpin motif, were mutated. The presence of a purine residue in position 12 was crucial for the catalytic activity but the changes at that position had a relatively small influence on the metal ion preferences of this variant. The middle base pair of the three-base-pair hairpin was changed from A-T to C-G interaction. The catalytic activity of the mutated variant was increased with Zn^{2+}, decreased with Mn^{2+}, and was not changed in the presence of Cd^{2+} ions. Clearly, this base pair was important for defining the metal ion preferences of the DNAzyme 1/VIIWS.

Introduction

For a long time, it has been believed that biological catalysis is dominated by protein enzymes. This view was changed in the 1980s when RNA molecules called ribozymes, capable of catalyzing reactions in the absence of any protein component, were discovered by Cech and Altman [1,2]. After the discovery of ribozymes, it became clear that also deoxyribonucleic acids of a particular sequence and a three-dimensional structure might be capable of carrying out specific chemical reactions [3]. In 1994, Breaker and Joyce used an *in vitro* selection procedure to isolate - from a random-sequence population of DNA - the first deoxyribozyme (DNAzyme) which was able to catalyze the cleavage of an RNA phosphodiester bond in the presence of Pb^{2+} ions [4,5]. Afterwards, many man-made DNAzymes have been reported to have catalytic activity towards specific substrates and catalyze many different reactions, such as the cleavage of DNA or RNA [6-9], phosphorylation and DNA ligation [10,11], porphyrin metallation [12] and carbon-carbon bond formation [13]. In this way, deoxyribozymes became the newest members of the enzyme family, joining protein enzymes and ribozymes.

The most studied deoxyribozymes include two DNAzymes known as 8-17 and 10-23, discovered by Santoro and Joyce in 1997 [14]. These DNazymes have the ability to carry out sequence-specific cleavage of all-RNA or chimeric RNA/DNA substrates in the presence of divalent metal ions. Metal ions are required to catalyze the attack of a specific RNA 2'-hydroxyl group on the adjacent phosphodiester linkage, forming a 2', 3'-cyclic phosphate and a 5'-hydroxyl group. The ions are believed to take part not only in the chemical step: they also play an important structural role, helping to organize the enzyme into its active conformation [15,16]. The 8-17 and 10-23 DNAzymes consist of a small 15-nucleotide catalytic core and two substrate-binding arms that may differ in terms of length and sequence. The 10-23 catalytic core is composed of an unstructured loop, in which no base-pairing elements have been defined while the 8-17 catalytic core is composed of a three-base-pair hairpin with the AGC apical loop, and four to five unpaired nucleotides [17,18]. The 8-17 DNAzyme variants are able to cleavage all 16 possible chimeric RNA/DNA dinucleotide junctions [19,20], whereas the 10-23 has the ability to cleavage any purine-pyrimidine junction [21].

Sequence variants of the 8-17 highly specific for metal cofactors such as Mg^{2+} [14], Ca^{2+} [22], Zn^{2+} [23], Mn^{2+} [24] and UO_2^{2+} ions [25,26] have been isolated by *in vitro* selection. That feature has made the 8-17 variants particularly attractive as biosensors for detection of

metal ions [27]. Besides, the 8-17 has been examined for numerous applications in nucleic acid research [28] and DNA computing [29]. On the other hand, the 10-23 has more often been used as a potential therapeutic agent to suppress the level of specific RNAs in the cell and to degrade viral RNAs [30,31]. Furthermore, deoxyribozymes seem to be attractive candidates for biochemical, nanotechnological and medical applications because of their high stability [27,32]. DNAzymes are more resistant to unspecific hydrolysis or nuclease degradation than ribozymes. They can be denatured and renatured many times without losing their catalytic activity.

Most deoxyribozymes discovered so far require divalent metal ions for their catalytic activity. The metal ions seem to be necessary either for structure formation and/or catalysis, however their exact role in the functioning of DNAzymes is still controversial. Selecting novel deoxyribozymes, active in the presence of particular metal ions, may help understand the functional specificity of DNAzymes, and in particular their different preferences towards metal ions that promote catalysis. Such information would also be useful in designing new deoxyribozymes for practical applications. Selection of DNAzymes active with Cd^{2+} ions seems to be particularly attractive. So far, no attempts have been made to select deoxyribozymes of such specificity, although several other divalent metal ions, like Mg^{2+}, Ca^{2+}, Zn^{2+} have been used in *in vitro* selection experiments. Moreover, Cd^{2+} is considered as one of the most extremely toxic metals for humans. The cytotoxic and metabolic effects of Cd^{2+} may be ascribed to its interference with the normal functions of some essential metal ions like Zn^{2+} and Ca^{2+} [33]. Therefore, it would be highly desirable to develop a simple method to detect trace amounts of Cd^{2+} in the environment. Nucleic-acid based biosensors could be applied towards this end.

Here, we describe the results of an *in vitro* selection experiment aimed to obtain RNA-cleaving DNAzymes catalytically active with Cd^{2+} ions. In the experiment, a DNA combinatorial library was used with a 23-nucleotide random region. Most probably, all the possible sequences were represented in the library; thus, we searched the entire oligonucleotide sequence space of that length for relatively small motifs capable of inducing cleavage of a single RNA bond. The selected DNAzymes were subjected to a detailed study of their kinetic properties and metal ion preferences in order to compare them with other known metal ion-specific deoxyribozymes.

Results and discussion

Construction of the oligonucleotide library and selection protocol

Following an analysis of three different strategies of construction of combinatorial libraries that have been used for selection of DNAzymes [34], we decided to use the procedure in which the random nucleotide region was localized in the predicted secondary structure of DNAzyme variants on the opposite side of a single ribonucleotide bond. The ribonucleotide was positioned between two double-stranded DNA segments, as shown in Figure 1. In the figure, the scheme of synthesis of the oligonucleotide library is also outlined. Chemically synthetized oligodeoxyribonucleotides led to double-stranded DNA in two PCR reactions. In the second reaction, the P4 primer was applied which contained a spacer of 18 units of ethylene glycol (18-spacer) and consequently, the two complementary DNA strands had different electrophoretic mobility. This enabled their separation by gel electrophoresis in denaturing conditions. The resulting library of single-stranded DNA oligomers contained a random region of 23-nucleotides in length, in which all the four basic nucleotides were present in every position.

The aim of our *in vitro* selection was to obtain Cd^{2+}-specific DNAzymes by searching a combinatorial library consisting of a full representation of all possible sequence variants. Therefore, in our experiment a DNA combinatorial library with a 23-nucleotide-long random region was used, which corresponds to approximately 7 x 10^{13} different sequences of that length. Most probably, all the theoretically possible sequence variants were present in the initial library and the entire sequence space was searched for variants that fulfill the selection criteria. From the experimental point of view, a 23-random region is basically the longest nucleotide stretch which allows obtaining a fully represented library in a standard experiment [35,36]. However, so short molecules can form relatively simple secondary and tertiary structural motifs. The probability of selecting variants with desirable but rare properties is therefore not high. Longer randomized regions, consisting of 40 – 50 nucleotides, have been used in several earlier performed *in vitro* selections of DNAzymes [14,23,25,37,38]. In spite of using long randomized regions, relatively simple structural motifs were usually identified to show catalytic properties.

The *in vitro* selection protocol, which is schematically outlined in Figure 1, followed protocols used for selection of DNAzymes catalytically active in the presence of UO_2^{2+} [25] and Mg^{2+}/Mn^{2+} ions [39]. The crucial step of the procedure, separation of the active and inactive variants, was based on the different electrophoretic mobility of two pools of molecules of different length, cleaved and non-cleaved at the single adenosine residue. Then,

the active variants were subjected to amplification in two PCR steps. In the first step, the nucleotide stretch which had been cut off in catalysis was added, while in the second PCR step, the primer with 18-spacer was applied. Two DNA strands of unequal length were synthetized and following strand separation by gel electrophoresis in denaturing conditions the obtained new DNA pool was used in the next selection cycle.

Selection progress and identification of DNAzyme variants

Reaction conditions, which were applied during the selection experiment are specified in Table 1. In the first four cycles, the cleavage reaction was performed for 60 min, and the Cd^{2+} ions were used at the concentration of 1 mM in the first three cycles and 0.2 mM in the fourth cycle. The extent of the library cleavage ranged between 0.08 and 0.21% of the input DNA. Autoradiogram of the polyacrylamide gel from the fifth selection cycle after separation of cleavage products of DNA library in the presence of Cd^{2+} are shown in supplementary Figure S1. In order to discard variants catalytically active in other than Cd^{2+} divalent metals, an additional step called negative selection was included in the procedure after the fourth cycle. A mixture of 0.1 mM Mg^{2+}, and 0.05 mM Ca^{2+}, Co^{2+}, Ni^{2+}, Sr^{2+}, Mn^{2+} and Zn^{2+} ions was used instead of Cd^{2+}, and the cleavage reaction proceeded for 60 min. Subsequently, the fractions of the active and inactive variants were gel separated, the non-cleaved molecules were recovered from the gel and used in the next, fifth selection cycle.

Already after the five cycles of the selection-amplification process the library showed a measurable catalytic activity and ca. 6% of the input DNA was cleaved. Therefore, starting from the sixth cycle, the concentration of Cd^{2+} was decreased from 0.2 to 0.1 mM and the reaction time was reduced to 30 min, and in the following cycles to 10 and 5 minutes (Tab. 1). The highest cleavage extent of 8% was observed after the sixth cycle. Following ten selection-amplification cycles, two pools of DNAzyme variants were chosen, those obtained after the seventh and tenth cycles. The libraries were cloned using the pCR2.1-TOPO vector and *E. coli* cells and sequences of 28 and 27 individual clones from the first and the second library were determined.

The identified sequences were aligned taking into account the positions of the initially randomized region and adenosine residue at the catalytic cleavage site. Five groups of variants were distinguished based on the nucleotide composition of the initially randomized region (Fig. 2). In the first group consisting of 28 clones, 24 clones had identical sequences in the initially randomized region, and 25 clones had the A/G mutation in the same position of the downstream sequence. This mutation occurred in the constant region of the initial library

but it could be explained by its location beyond the hybridization site of the selection primer. In each of the following groups 2 – 5 the variants had identical sequences within the initially randomized region. Interestingly, one clone from group 3, three clones from group 4, and two clones from group 5 had the A/G mutation in the same position of the constant region as that one observed in almost all variants of group 1. Surprisingly, the A residue that had been present at this position of the initial library was deleted in all the variants of group 2. In these variants the T residue located on the opposite side of the initially randomized sequence was also deleted.

Catalytic activity of DNAzymes representing various selection groups

The catalytic activity of several DNAzyme variants representing selection groups 1 – 5 was assayed at 100 μM concentration of Cd^{2+}, and separately, using a mixture of 100 μM Mg^{2+}, and Ca^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Sr^{2+} ions, at the concentration of 50 μM each (Fig. 2). The cleavage extents were measured after 30 min incubation at 25 ºC. It turned out that in the presence of Cd^{2+} the following variants were catalytically active: 1/VII, 3/VII, 19/VII, 22/VII and 6/X from group 1, 2/X and 5/X from group 3, and 15/X from group 4. The cleavage extents were in the range of 10 – 55%. None of the variants exhibited a strict dependence on Cd^{2+} and the variants were also active in the presence of the divalent metal ions mixture. In most cases, the variants in the presence of the mixture of metals were cleaved slightly better, reaching 25 – 80% cleavage extents. Unexpectedly, two variants 7/VII and 13/X, representing selection groups 2 and 5, showed no measurable catalytic activity in both assay conditions. It is unclear whether these sequences were selected as having marginally low activity or they were inactive but survived in the selection process due to some unintended properties.

Two DNAzymes 1/VII and 22/VII from group 1 were chosen for a more detailed analysis. We wanted to confirm that the variants have a desired specificity towards Cd^{2+} and to establish their preferences towards some other divalent metal ions. The cleavage reactions were performed in the presence of Cd^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Ni^{2+} and Sr^{2+}, with the cleavage extents analyzed after 5 and 30 min (supplementary Figs S2 and S3). The comparison of the cleavage extents after these two time periods allowed us to qualitatively evaluate whether the observed differences were a consequence of different reaction rates or they reflected different final cleavage extents in the applied conditions. For example, the cleavage extents of both the 1/VII and 22/VII with Zn^{2+} and Cd^{2+} were similar after 30 min reaching approx. 10%, while after 5 min the 1/VII was cleaved to a 2-fold larger extent with Cd^{2+} than with Zn^{2+}, whereas the 22/VII was cleaved to a similar extent with both these metal

ions. This suggests a higher catalytic activity of the 1/VII compared to the 22/VII in the presence of Cd^{2+}. Moreover, this assay showed that both these variants were also active to some extent in the presence of Mn^{2+}, Co^{2+}, Ni^{2+} and Ca^{2+}. Importantly, in the presence of Mg^{2+} these DNAzymes showed essentially no catalytic activity (supplementary Fig. S3).

Secondary structure of DNAzyme 1/VII and the predicted structures of other DNAzymes obtained by *in vitro* selection

The computer analysis of the possible secondary structures of DNAzyme 1/VII with the use of the RNAstructure 5.4 program and constrains from S1 nuclease and DMS probing data yielded four structural models with a similar stabilization energy (Fig. 3A and supplementary Fig. S4). Three out of four models had a characteristic three-base-pair hairpin motif similar to that found in the catalytic center of the 8-17 DNAzyme variants [14,23]. Additionally, in two proposed structures a hairpin with a large 7-nucleotide loop was present at their 3' end. A similar hairpin motif at the 3' end of selected molecules has been noted by Liu and coworkers while selecting DNAzymes active in the presence of UO_2^{2+} [25]. All the proposed structures revealed a four-nucleotide stable loop of the GNRA type (N-any nucleotide, R-purine) which was present in the initial DNA library.

The secondary structure of the 1/VII was analyzed experimentally using enzymatic mapping with nuclease S1 and chemical modification with DMS (Fig. 3). Strong S1 cleavages occurred close to the 5' end of the molecule at A17.20 and A17.21, and in the loop regions at A2.11 and G16.16 confirming the single-stranded nature of these regions. Chemical modification with DMS in the presence of hydrazine was used to modify accessible cytosine residues at their N3 positions. No cytosine modification was observed in the catalytic region A2.4 –C16.5 while modification occurred at C16.22 and C17.17 close to the 5' and 3' ends, and at C1.12 and C2.10 in the apical loop region. (Fig. 3).

An analysis of the sequences of the clones obtained in the *in vitro* selection experiment showed that the three-base-pairs hairpin motif characteristic for the 8-17 DNAzyme might be formed in all the variants from groups 1, 3 and 4 (Fig. 2). Moreover, in all the variants a C13G14 dinucleotide conserved in the 8-17 DNAzyme was present in the expected positions. Consequently, we assumed that all the variants from selection groups 1, 3 and 4 fold into a secondary structure analogous to that known as the 8-17 type.

It is worth noting that all the catalytically active variants obtained in our selection experiment turned out to belong to the 8-17 type. Deoxyribozymes of that type have earlier been selected not only in the presence of Mg^{2+} and Ca^{2+} but also Zn^{2+} and Pb^{2+} [4,14,22,23].

However, in all those selections combinatorial libraries with randomized regions longer than those applied during our selection have been used, thus the libraries were highly underrepresented. In our experiment, all possible sequence variants were likely to be present in the initial DNA library. It seems that only variants of the 8-17 DNAzyme were able to meet the selection criteria. Although fully possible, no DNAzyme of the 10-23 type was selected. In the selected variants four initially randomized nucleotides replaced a part of the paired region in the initial library thus selection of the 8-17 variants seems to be strongly preferred.

Catalytic properties of DNAzymes representing selection groups 1, 3 and 4 shortened at their 5' and 3' ends

In order to make the DNAzyme 1/VII shorter and to confirm its secondary structure two 19-nucleotide fragments from its 5' and 3' end were deleted. The catalytic activity of the truncated variant, DNAzyme 1/VIIWS, was analyzed in the presence of various divalent metal ions (Fig. 4A). It turned out that the cleavage extent after 5 min incubation in 50 μM Cd^{2+} solution was 8-times lower and in 100 μM Cd^{2+} - 36% lower compared to the cleavage extents of the full-length 1/VII (supplementary Fig. S3). After 30 min the shorter variant was cleaved to approx. 2% in the presence of 50 μM Cd^{2+} and to 9% in the presence of 100 μM Cd^{2+}, whereas the full-length DNAzyme was cleaved to 13% and 27% in these conditions. Other tested metal ions, including Sr^{2+}, Ni^{2+}, Ca^{2+} or Mg^{2+}, did not induce substantial cleavage of 1/VIIWS, either after 5 min or 30 min incubation. Thus, the removal of 19-nucleotide long fragments from the 5' and 3' ends resulted in the decrease of the catalytic properties of the 1/VII. These nucleotide stretches, however, were not required for catalysis.

Since the 19-nucleotide long terminal fragments of 1/VII were shown not to be required for catalysis we decided to also construct the truncated versions of two other DNAzymes, 5/X and 15/X, representing variants of selection groups 3 and 4. The catalytic activity of the 5/XWS and 15/XWS was assayed as in the case of 1/VIIWS (Fig. 4B, C). It turned out that the 5/XWS was less selective towards divalent metal ions required for catalysis than 1/VIIWS. The 5/XWS showed similar cleavage extents of approx. 3 – 5% in the presence of 50 μM Mn^{2+}, Cd^{2+} and Zn^{2+}, after 30 min incubation, but in relative terms, it was most active with Mn^{2+} (Figure 4B). The 15/XWS was substantially less active than the other two DNAzymes and besides, Cd^{2+} only Zn^{2+} supported catalysis to a reasonable extent (Fig. 4C).

Preferences of DNAzymes from selection groups 1, 3 and 4 toward Cd^{2+}, Zn^{2+} and Mn^{2+} ions

Since the semi-quantitative data suggested that the 1/VIIWS, 5/XWS and 15/XWS have different preferences towards catalytic divalent metal ions, the catalytic activity of these variants was analyzed in a greater detail. The variants were analyzed in their *cis*-acting forms in order to establish the catalytic properties in conditions reflecting those used during the selection process. This is contrary to some other studies of DNAzymes in which their *trans*-acting versions have been tested and a huge excess of the enzyme component over the substrate was applied.

The catalytic cleavage constants k_{obs} for 1/VIIWS, 5/XWS and 15/XWS were determined in the presence of three most active metal ions Cd^{2+}, Zn^{2+} and Mn^{2+}, and the reactions were performed at 25 ºC and 37 ºC (Fig. 5 and supplementary Fig. S5). It turns out that at 25 ºC the 1/VIIWS was most active of the three, but all the variants preferred Cd^{2+} in catalysis. The next preferred metal ions were Mn^{2+} ones, and the 5/XWS was especially active with them. On the other hand, the 15/XWS in the presence of Zn^{2+} was almost as active as with Cd^{2+}. Surprisingly, at 37 ºC all the three DNAzymes were less active in the presence of all the three metal ions by a factor of approximately 5 – 15. Bonaccio *et al.* [40] has reported that the rate of cleavage of 8-17 DNAzyme increases approx. 100-fold between 0 ºC and 37 ºC. The 8-17 construct was 2-times more active at 37 ºC in the presence of Mn^{2+}, Ca^{2+} or Mg^{2+} than at 25 ºC. In our studies, the largest decrease in activity was observed for 1/VIIWS, and with Cd^{2+} this DNAzyme was almost 15-times more active than at 37 ºC. Relative preferences of the three DNAzymes towards various metal ions were generally preserved, and only the 15XWS was much less active with Zn^{2+} at 37 ºC (Fig. 5). The unexpected effect of temperature on the catalytic activity of these DNAzymes might be connected with some rearrangements in their tertiary structure. Interestingly, it appears that optimal temperature of DNAzymes depends on the temperature which was used during their selection process. *In vitro* selection of Mg^{2+}-dependent DNAzymes carried out at 37 °C allowed to obtain two classes of 10-23 and 8-17 DNAzymes which are most active at 37 °C [14]. In contrast, selections performed at room temperature in search of DNAzymes with phosphoesterase activity [6] and of Cd^{2+}-dependent DNAzymes described in this study ended up with catalysts most active at room temperature.

Based on the selection outcome, a few conclusions can be formulated. First, although the selection process had been designed for Cd^{2+}-dependent DNAzymes it resulted in variants showing a broader metal ion specificity. The variants preferred Cd^{2+} but were also active with

Mn^{2+} and Zn^{2+} which suggests that binding of the catalytic ion does not require an extremely specific coordination mode. A broader metal ion specificity of the selected DNAzymes has also been confirmed by other *in vitro* selection experiments. For example, the Zn^{2+}-specific DNAzyme has been shown to be also active in other ions, in the following order: Zn^{2+}, Mn^{2+} ~ Co^{2+}, Cd^{2+}, Ni^{2+}, Mg^{2+} ~ Ca^{2+}, Sr^{2+} ~ Ba^{2+} [23]. The precursor 8-17 DNAzyme which has been selected with Mg^{2+} ions [14] shows higher activity with Ca^{2+} and Mn^{2+} [22,41]. Moreover, both these DNAzymes are much more active in the presence of Pb^{2+} than of any other metal ions tested [23]. Thus, the selection of DNAzymes of a strict metal ion specificity seems to be either very difficult or impossible. Although being most active with Cd^{2+}, the variants of 8-17 obtained in our studies differed in terms of their preferences towards other divalent metal ions. It is possible that the variants have different affinities towards metal ions or the catalytic ions are bound more or less correctly for inducing the cleavage.

Recent studies of the Zn^{2+}-dependent variant of the 8-17 DNAzyme using single-molecule FRET have suggested that in order to be active, the DNAzyme requires metal-dependent global folding [42]. The activity is dependent on metal ions with the following order of activity: Pb^{2+}, Zn^{2+}, Cd^{2+}, Mg^{2+} ~ Ca^{2+}, Ba^{2+} ~ Sr^{2+}, $NH4^{+}$ ~ Li^{+}, Na^{+}. A strong correlation between folding and activity has been found, suggesting that metal-ion dependent folding plays an important role in catalysis. The only exception are Pb^{2+} ions which despite being most active do not induce any changes in the global folding of the DNAzyme [42,43].

Finally, the unexpected dependence of the catalytic activity of the three DNAzymes obtained in our studies on the temperature suggests that some changes occur in their structures or the rate-limiting step of the reaction is changed. Some indications of temperature-induced transitions have been suggested by non-linear Arrhenius plots at temperatures between 20 ºC and 40 ºC [40]. It should also be noted that at higher temperatures not only was the activity of the three selected DNAzymes decreased but also the 15/XWS changed its metal ion preferences. Such a possibility has to be taken into account when using DNAzymes at a different temperature, and at different conditions than those applied during the selection experiment in which they were obtained.

Binding of Cd^{2+} ions to DNAzyme 1/VIIWS was also analyzed by circular dichroism technique. A non-cleavable version of 1/VIIWS was applied in these experiments in which ribonucleotide A at the cleavage site was replaced by its deoxy analogue. The 1/VIIWS at the concentration of 10 μM was incubated in the presence of 0 – 2000 μM Cd^{2+} ions. The CD spectra suggested the presence of DNA in its B form which was diagnosed by a positive Cotton effect at ~277 nm and a negative one at ~250 nm (Fig. 6A). Upon increasing the

concentration of Cd^{2+} the intensity of the band at 277 nm markedly decreased. This might be connected with the transition from form B to form Z of DNA, which has been considered important for DNAzyme catalytic activity [43]. However, no negative Cotton effect was observed at 294 nm which could indicate the formation of Z-DNA. The changes in the intensity of the CD band at 277 nm while increasing the concentration of Cd^{2+} were used for determination of the binding affinity of the metal ion to DNA molecule (Fig. 6B). The corresponding dissociation constant (K_d value) of 111±21 μM was calculated. This value is reasonable taking into account that for the Zn^{2+}-dependent DNAzyme the catalytic rate was half-maximal at 13.5 μM Pb^{2+}, 1 mM Zn^{2+}, or 10.5 mM Mg^{2+} suggesting different metal ion affinity of the studied DNAzyme in this range of metal ion concentrations [44].

Changing the metal ion preferences of selected DNAzymes by introducing mutations in their catalytic core

We were curious to know to what extent changes of some nucleotides in DNAzyme 1/VIIWS which are important for the formation of the secondary structure of and/or which take part in the reaction mechanism could influence its catalytic properties. In 8-17 DNAzymes 4 out of the 15 nucleotides that comprise the catalytic core are absolutely conserved: A6, G7, C13 and G14. We focused our attention on the nucleotide present in position 12 and the stem of the characteristic three-base-pair hairpin. First, three mutants of 1/VIIWS were synthesized with single nucleotide substitution of A12 (Fig. 7A). The replacement of adenosine by guanosine resulted in only 1.7-fold lower activity with Cd^{2+} and 2-fold lower activity with Zn^{2+} and Mn^{2+}. Strikingly, the change to cytosine or thymidine resulted in reduced catalytic activity: at least 10-fold with Cd^{2+}, 7-fold with Zn^{2+} and 17-fold with Mn^{2+}. Thus, the presence of a purine residue (adenosine or guanosine) in position 12 is crucial for the catalytic activity of the 1/VIIWS. The level of protonation of these purines and/or their involvement in the formation of intramolecular interactions are likely to determine the folding of the molecule into a catalytically active structure. On the other hand, the change of the nucleotide in position 12 had a relatively small influence on the metal ion preferences of the 1/VIIWS.

Earlier studies of 8-17 DNAzyme have shown that the replacement of T12 by any other nucleotide decreases its catalytic activity in the presence of Mg^{2+}, Ca^{2+} and Mn^{2+} ions. However, cytosine is slightly preferred for the Mg^{2+}- and Ca^{2+}-induced catalysis while guanosine or adenosine for Mn^{2+}-induced reactions [41]. In another study, the activity of Zn^{2+}-dependent DNAzyme with Zn^{2+} was decreased 8-fold when T12 was changed to adenosine [44]. Interestingly, when at the same time all the three base pairs between

nucleotides in positions 3-5 and 9-11 were changed, the DNAzyme was less active by only 25%. This clearly shows that both elements, the nucleotide in position 12 and the stem of the three-base-pair hairpin, may act together in defining the DNAzyme activity and its metal ion preferences.

Wang *et al.* [45] have suggested that the nucleotide in position 12 plays two functions important for the catalytic activity of 8-17 DNAzyme. It may act as a stabilizing element which ensures the correct position of conservative C13. Deletion of the nucleotide from position 12 may substantially influence the system of hydrogen and stacking interactions, which results in changes in the arrangement of C13 in the catalytic center, thereby decreasing the activity or making the DNAzyme inactive. Moreover, the nucleotide in position 12 may have a chaperon function being important for correct folding of the DNAzyme into a catalytically active structure [45].

Another structural element of the catalytic core of 1/VIIWS that we changed was the middle base pair of the three-base-pair hairpin (Fig. 7A). The A-T interaction was replaced by the C-G base pair. The catalytic activity of the mutated variant was increased with Zn^{2+}, decreased with Mn^{2+}, and it was not changed with Cd^{2+}. Clearly, this base pair is important for defining the metal ion preferences of the 1/VIIWS. Interestingly, when the mutated variant of 8-17 DNAzyme had T12 and the same composition of the three-base pair hairpin as those present in 1/VII but with C5-G9 reversed to G5-C9, the activity with Mn^{2+} was reduced more than 10^2-fold [41]. It is also worth noting that in the 1/VIIWS the three-base-pair hairpin contains only one GC pair. Earlier results have suggested that the stem should contain at least two GC interactions [14,23]. Later observations, however, have shown that stems containing one or even no GC pair could be compatible with catalytic activity [24]. It has also been observed that if the middle base pair of the stem is different from the canonical C4-G10, the activity of DNAzymes increases [41]. This could be connected with the fact that the interactions of transition metals with DNAzyme are complicated. Transition metal ions usually bind to the oxygen atom of the phosphate group and to the N7 atom of the base. This may indicate that the N7 group of G10 imposes a specific geometry to the stem [41,46].

Finally, in the 1/VIIWS, the ribonucleic bond at the cleavage site was replaced by its thio analog with a phosphorus atom at the non-bonding position of the phosphate residue (PS modification). The largest effect, a 10-fold decrease of the catalytic activity, was observed in the presence of Mn^{2+}, a smaller 3-fold decrease with Zn^{2+}, and almost no effect occurred in the presence of Cd^{2+} (Fig. 7A). This is contrary to the very recent observation where in

another RNA-cleaving DNAzyme active with trivalent lanthanides PS modification at the cleavage site shifted the activity to soft tiophilic metals Cd^{2+}, Hg^{2+} and Pb^{2+} [47].

Another DNAzyme from our *in vitro* selection experiment that was mutated, was DNAzyme 5/X chosen from selection group 3. After shortening at its 5'and 3' ends by 19-nucleotide long stretches, the middle base pair in the three-base-pair hairpin was changed from T-A to C-G interaction (Fig. 7B). This resulted in an almost equal, 5-fold decrease of the catalytic activity in the presence of the three metal ions studied, Cd^{2+}, Zn^{2+} and Mn^{2+}. Consequently, the change resulted in lowering the activity of the 5/XWS without changing its metal ion preferences.

The DNAzyme 1/VIIWS was also constructed in *trans*-acting versions. In the first two variants the molecule was cut between C1.12 and G2.12, and in the second two variants between G1.10 and C2.10 (see Fig. 7A for the numbering system used to describe the construction of the variants). The catalytic activity of these variants was tested in the presence of 0.1, 1 and 10 mM Cd^{2+} ions in 50 mM HEPES-NaCl pH 7.0. The DNAzyme components were used in a 100, 1000 and 10000-fold excess over the oligonucleotide substrate. Both the *trans*-acting variants did not show measurable activity in the applied conditions (data not shown).

In other *trans*-acting variants of DNAzyme 1/VII, the catalytic center (nucleotides 3 – 15.0) was embedded between two double-stranded segments derived from 8-17 DNAzyme. Two oligonucleotide substrates were synthetized with adenosine and guanosine residues at the catalytic cleavage site. The *trans*-acting variants were inactive in the presence of 100 μM Cd^{2+} and in 100 μM Mg^{2+} ions. The DNAzyme was able to cleave the oligonucleotide substrate with adenosine to a small extent after incubation with 10 mM Mg^{2+} ions for 3 hours. When the substrate with guanosine residue at the cleavage site was used, slightly stronger cleavage occurred (data not shown).

It has been shown that the presence of 2'-O-methyl and LNA modifications in the substrate binding arms of 10-23 DNAzyme significantly increase the catalytic activity [48]. It seems that a similar approach could be used to increase the activity of our Cd^{2+}-dependent DNAzymes. Moreover, a catalytic cleavage could be performed in the presence of low-molecular weight compounds like histidine used in the *in vitro* selection of some DNAzymes [49] or imidazole applied to rescue the activity of a mutant of HDV ribozyme [50].

Conclusions

We described the results of an *in vitro* selection experiment that aimed to obtain RNA-cleaving DNAzymes catalytically active with Cd^{2+} ions. All the variants turned out to belong to the type of 8-17 DNAzyme. The variants preferred Cd^{2+} but were also active with Mn^{2+} and Zn^{2+} suggesting that binding of the catalytic ion does not require an extremely specific coordination mode. The broader metal ion specificity of the selected DNAzymes has also been observed in other *in vitro* selection experiments [22,23,41]. Consequently, selection of DNAzymes strictly dependent on a particular divalent metal ion seems to be either very difficult or impossible. Although being most active with Cd^{2+}, the variants of 8-17 obtained in our study showed differentiated preferences towards other metal ions. Taking into account the variants' catalytic activity and selectivity, DNAzyme 1/VIIWS seems to show the best performance. However, we are aware that for some specific purposes other variants might be better suited.

The unexpected decrease of the catalytic activity of three selected DNAzymes along with the temperature increase of the reaction environment, suggests that some changes occur in their structures or the rate-limiting step of the reaction is changed. Moreover, at the higher temperature not only was the activity decreased but the 15/XWS DNAzyme changed its metal ion preferences. Such a possibility has to be taken into account when using DNAzymes at a different temperature, and/or in other conditions, different than those applied during the selection experiment in which they were obtained.

We were curious to know to what extent the changes of the selected nucleotides in DNAzyme 1/VIIWS could influence its catalytic properties. We focused our attention on two elements of the catalytic core, the nucleotide in position 12 and the three-base-pair hairpin motif characteristic of type 8-17 DNAzymes. The presence of a purine residue in position 12 was crucial for the catalytic activity of the 1/VIIWS but had a relatively small influence on its metal ion preferences. The middle base pair of the three-base-pair hairpin was changed from A-T to C-G interaction. The catalytic activity of the mutated variant was increased with Zn^{2+}, decreased with Mn^{2+}, and it was not changed with Cd^{2+} ions. Clearly, this base pair is important for defining the metal ion preferences of the 1/VIIWS. Earlier studies have suggested that the three-base-pair hairpin of 8-17 DNAzyme should contain at least two GC interactions [14,23]. Later data have shown that stems containing one or even no GC pair could be compatible with catalytic activity [24]. This is consistent with our observation that in the 1/VIIWS the three-base-pair hairpin contains only one GC pair.

Experimental

Oligonucleotides and reagents

Oligonucleotides were prepared by automated DNA synthesis (FutureSynthesis, Poznan, Poland or Integrated DNA Technologies, Leuven, Belgium). Random-sequence DNA library was synthesized using an equimolar mixture of the four standard phosphoramidities. All DNA oligonucleotides were purified using denaturing polyacrylamide gel electrophoresis. The other materials used in this study were from the following sources: [γ-^{32}P]ATP and [α-^{32}P]dCTP with specific activity of 5000 Ci/mmol were from Hartmann Analytic, T4 polynucleotide kinase, S1 nuclease, Taq polymerase and dNTPs were from MBI Fermentas. All other chemicals were purchased from Sigma-Aldrich and Serva.

In vitro selection procedure

Preparation of a random DNA pool and the *in vitro* selection procedure are schematically outlined in Figure 1. The initial DNA pool contained a 23-nucleotide random region (N23) flanked by two constant primer-binding domains. In the middle of this DNA strand, a ribo-adenosine (rA) was introduced which was the cleavage site for *in vitro* selection.

Step I: The starting pool of DNA was generated by extending 400 pmoles of primer P3 (5'-GGGCAGAATTCTAATACGACTCACTATrA-3') in the presence of 200 pmoles of random DNA template (5'-GTGCCAAGCTTACCGTCAC-N23-GAGATGTCGCCATCTCTTCC TATAGTGAGTCGTATTAG-3') in a 300 µl reaction mixture containing 0.05 U/µl Taq polymerase, 2 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl (pH 8.8 at 25 ºC), 0,01% Tween 20, 100 µCi of [α-^{32}P]dCTP and 0.2 mM of each dNTP, for seven thermal-cycles (1 min each at 92 ºC, 52 ºC and 72 ºC).

Step II: 1 nmol each of P3 (5'-GGGCAGAATTCTAATACGACTCACTATrA-3') and P4 (5'-CAACAACAACAA(Spacer18)GTGCCAAGCTTACCG-3') were added to the reaction mixture for seven additional cycles of PCR amplification. The ribo-adenosine (rA) was introduced through P3. P4 contained a PEG spacer (Spacer18) that was incorporated into the strand without rA and led to stop the PCR extension.

Step III: PCR products were precipitated with ethanol, dissolved in loading buffer with bromophenol blue dye and purified by electrophoresis on a 12% polyacrylamide gel containing 1 mM EDTA and 8.3 M urea. The band corresponding to the random DNA pool was visualized by autoradiography, cut out and eluted from the gel with 50 mM HEPES (pH 7.0), 100 mM NaCl, 1 mM EDTA and twice ethanol precipitated.

Step IV and V: The cleavage reaction was carried out by incubating single-stranded DNA containing rA with 50 mM HEPES (pH 7.0), 100 mM NaCl and 1 mM $CdCl_2$. The reaction proceeded at 25 ºC for a designated period time, quenched with addition of EDTA and precipitated with ethanol. The cleavage product was purified on a 12% denaturing polyacrylamide gel, excised from the gel and ethanol precipitated. To increase the selection stringency, the reaction time was gradually decreased from 60 min to 5 min during the 10 rounds of the selection. The concentration of cadmium(II) chloride for the following rounds of selection was reduced from 1 mM for round 1 to 100 µM for round 10.

Step VI and VII: Two rounds of PCR were used to amplify the recovered 62-nt cleavage product. The reaction mixtures contained 150 pmoles each of primers P1 (5'-GTGCCAAGCTTACCG-3') and P2 (5'-CTGCAGAATTCTAATACGACTCACTATAGGAAGAGATGGCGAC-3') over 25 thermal cycles. One-third of the double-stranded DNA product from the first reaction was used as the template in the next PCR. The second reaction mixture containing 1nmol each of primers P3 and P4, 0.05 U/µl Taq polymerase, 2 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl (pH 8.8 at 25 ºC), 0.01% Tween 20, 100 µCi of [α-^{32}P]dCTP and 0.2 mM of each dNTP was carried out for seven thermal-cycles (1 min each at 92 ºC, 52 ºC and 72 ºC).

Step VIII: PCR products were precipitated with ethanol and purified by electrophoresis on a 12% denaturing polyacrylamide gel. The band corresponding to the random DNA pool (90 nt) was cut out, eluted, twice ethanol precipitated and finally used to initiate the next round of selection. Steps IV – VIII were repeated for ten rounds.

Cloning and sequencing of selected DNA populations

DNA sequences from the seventh and tenth rounds of selection were amplified by PCR and cloned using the TA-TOPO Cloning Kit (Invitrogen). The plasmids containing individual clones were prepared using a BIO BASIC INC. MiniPreps Kit. Concentrations of the 55 randomly selected clones were determined. All the clones were diluted to a concentration of 50 ng/µl and were submitted to the Oligo.pl service, IBB PAN Warsaw, for sequencing.

Synthetic DNAzyme variants

Each, full length c*is*-acting DNAzyme was prepared as follows: 150 pmol each of primers P3 and P4, 60 ng of plasmid containing individual DNAzyme template were annealed in the total volume of 300 µl. The reaction mixture also contained 0.05 U/µl Taq polymerase, 2 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl (pH 8.8 at 25 ºC), 0.01% Tween 20, 10 µl of [α-32^P]dCTP and 0.2 mM of each dNTP. The reaction was carried out for 25 thermal-cycles (92 ºC/30 sec, 60 ºC/30 sec and 72 ºC/1 min). The dsDNA template was precipitated with ethanol

and purified by electrophoresis on a 12% denaturing polyacrylamide gel. The band corresponding to the c*is*-acting DNAzyme (90 nt) was cut out, eluted, and twice ethanol precipitated. Shortened variants of DNAzymes (19 nucleotides were truncated from their 3' and 5' ends) were prepared by automated DNA synthesis (FutureSynthesis, Poznan). All shortened DNAzymes were also purified using polyacrylamide gel electrophoresis denaturing conditions.

Structural probing

The 5'-^{32}P-end-labelled non-cleavable DNAzymes (with single ribo-A replaced by deoxy-A) were supplemented with tRNA carrier to a final concentration of 2 A_{260}/ml and renatured in 40 mM NaCl, 10 mM Tris-HCl pH 7.5 and 10 mM $MgCl_2$ by heating at 100 ºC for two minutes and cooling at 0 ºC for five minutes. Reactions with nuclease S1 were performed at 25 ºC for different time intervals with a 1:100 dilution of the enzyme. The reactions were terminated by adding a 7 M urea/ dyes/ 20 mM EDTA solution and freezing on dry ice. The reaction products were analyzed by polyacrylamide gel electrophoresis.

Chemical modification of cytosine residues with DMS was carried out as follow: the 5'-^{32}P-end-labelled non-cleavable DNAzymes in 80 mM HEPES (pH 7.0), 40 mM NaCl, 10 mM $MgCl_2$ were renatured by heating at 65 ºC for five minutes and cooling to room temperature and then incubated with 1/20 volume of DMS in ethanol (1:12 v/v) at 25 ºC for 10 minutes. The reaction was terminated by standard ethanol precipitation (twice). The pellet was dissolved in a 10 µl ice-cold solution of hydrazine in water (1:1 v/v) and incubated for ten minutes at 0 ºC. After precipitation with ethanol (twice), the pellet was dissolved in a 20 µl 1/10 diluted piperidine and incubated at 60 ºC for 15 minutes. After precipitation with ethanol (twice) the DNA was dissolved in 7 M urea/ dyes/ 20 mM EDTA and loaded on a 12% polyacrylamide gel.

Catalytic cleavage reaction

Full length c*is*-acting DNAzymes internally ^{32}P-labeled or their 5'-^{32}P-end-labeled shortened variants, were subjected to a denaturation-renaturation procedure in the standard reaction buffer, 50 mM HEPES (pH 7.0), 100 mM NaCl by incubating at 65 ºC for 1 min, slow cooling to 35 ºC and then incubating at 25 ºC (or at 37 ºC) for 10 min. The cleavage reactions were initiated by adding divalent metal chloride solution to the desired concentrations shown in the plots. The reactions proceeded at 25 ºC or at 37 ºC and were quenched at various time points with a stop-buffer containing 7 M urea, 20 mM EDTA and dyes for electrophoresis. Products of the catalytic cleavage were analyzed on a 12% polyacrylamide, 0.75% bisacrylamide, 8.3 M urea gels. For quantitative analysis, gels were exposed to

phosphorimaging screens and quantified using the FLA 5100 image analyzer (Fuji) and MultiGauge software (Fuji). The rate constant for the cleavage reaction k_{obs} was determined by plotting the natural logarithm of the fraction of DNA that remained unreacted versus the reaction time.

Spectroscopic measurements

To perform the CD spectroscopic studies, a non-cleavable version of DNAzyme 1/VIIWS was used in which the single ribo-A was replaced by a deoxy-A. DNAzyme(dA) in 50 mM HEPES (pH 7.0), 100 mM NaCl was renaturated by heating at 65 ºC for one minute and slow cooling to 35 ºC and then to 25 ºC for ten minutes. The final concentration of DNA was 10 μM in all the experiments. The CD spectra were recorded on JASCO-715 spectropolarimeter using 1 mm path length cuvette and 250 μl sample volume. The spectra are the averages of five sequential accumulations for each sample between 220 and 400 nm. Cadmium(II) ions were titrated by incremental addition of a concentrated metal solution. The sample with cadmium ions was incubated at room temperature for 10 minutes before measurements.

Acknowledgements

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Notes and references

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Table 1. Reaction conditions for selection of DNAs that catalyze the cadmium(II)-dependent cleavage of an RNA phosphoester bond.

selection cycle	incubation time [min]	Cd^{2+} [mM]	cleavage extent [%]
1	60	1	0.08
2	60	1	0.12
3	60	1	0.16
4[a]	60	0.2	0.21
5	30	0.2	6.20
6	30	0.1	7.70
7	10	0.1	4.80
8	5	0.1	3.20
9	5	0.1	2.90
10	5	0.1	3.60

[a] negative selection with 100 μM Mg^{2+} and 50 μM Ca^{2+}, Co^{2+}, Ni^{2+}, Sr^{2+}, Mn^{2+} and Zn^{2+} was incorporated after round 4; incubation time was 60 min.

Figure legends

Figure 1. Synthesis of the combinatory library of *cis*-acting DNAzymes (steps I – III) and the scheme of the selection-amplification procedure used to search for their metal-dependent RNA-cleaving variants (steps IV – VIII). The random sequence domain is marked as N23. The catalytic cleavage site is denoted as rA.

Figure 2. Nucleotide sequences and cleavage activities of the metal-dependent DNAzymes obtained by the *in vitro* selection. Regions that were randomized in the initial DNAzyme library are marked in green and single nucleotide changes within each selection group in that region are marked by orange fonts. In the constant regions all nucleotides that differ from those found at the corresponding position in the initial library are also denoted in orange. The catalytic cleavage site is marked as rA in red. In the parenthesis the numbers of clones with identical sequences are shown. Cleavage extents (in percentages) were determined for some selected DNAzymes after 30 min in the presence of 100 μM Cd^{2+} (in the figure – Cd^{2+}) and mixed metal ions solution: 100 μM Mg^{2+} and 50 μM Sr^{2+}, Ni^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} (in the figure – Me^{2+}).

Figure 3. Secondary structure arrangement of cadmium-dependent DNAzyme 1/VII. (A) Secondary structure model of DNAzyme 1/VII and the result of its structural enzymatic and chemical probing. The DMS-modified cytosine residues are marked by dark grey circles. S1 nuclease digestion sites are denoted by arrows. Italic black letters mark the catalytic core of DNAzyme 1/VII. The sequence truncation sites are denoted by black triangles. (B) Autoradiograms showing the probing of cytosine residues with 0.4% DMS (C) Autoradiograms of S1 nuclease digestion. All reactions were performed with 5'-^{32}P-end-labeled DNAzyme 1/VII at 25 °C. Lanes: C, control reaction, AC and GA, sequencing lanes. Selected nucleotide residues are numbered on the autoradiograms on the right. Those at which nuclease S1 cleavages or DMS modifications were detected are shown in bold.

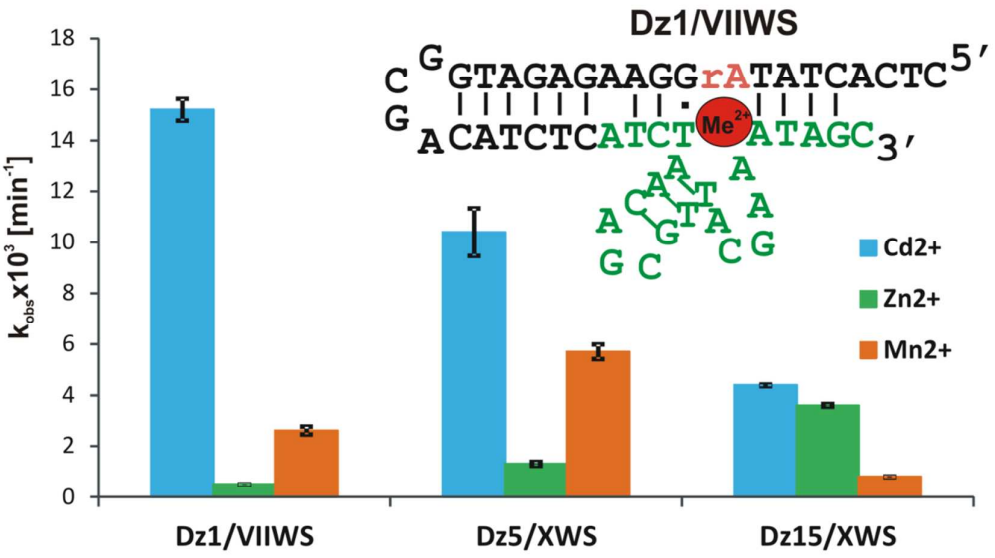
Figure 4. Metal-ions specificities of the shortened *cis*-acting DNAzymes. Cleavage extents (in percentages) were determined for DNAzyme (A) Dz1/VIIWS (B) Dz5/XWS (C) Dz15/XWS after 5 min (grey bars) and 30 min (bars with diagonal lines) in the presence of different divalent metal ions.

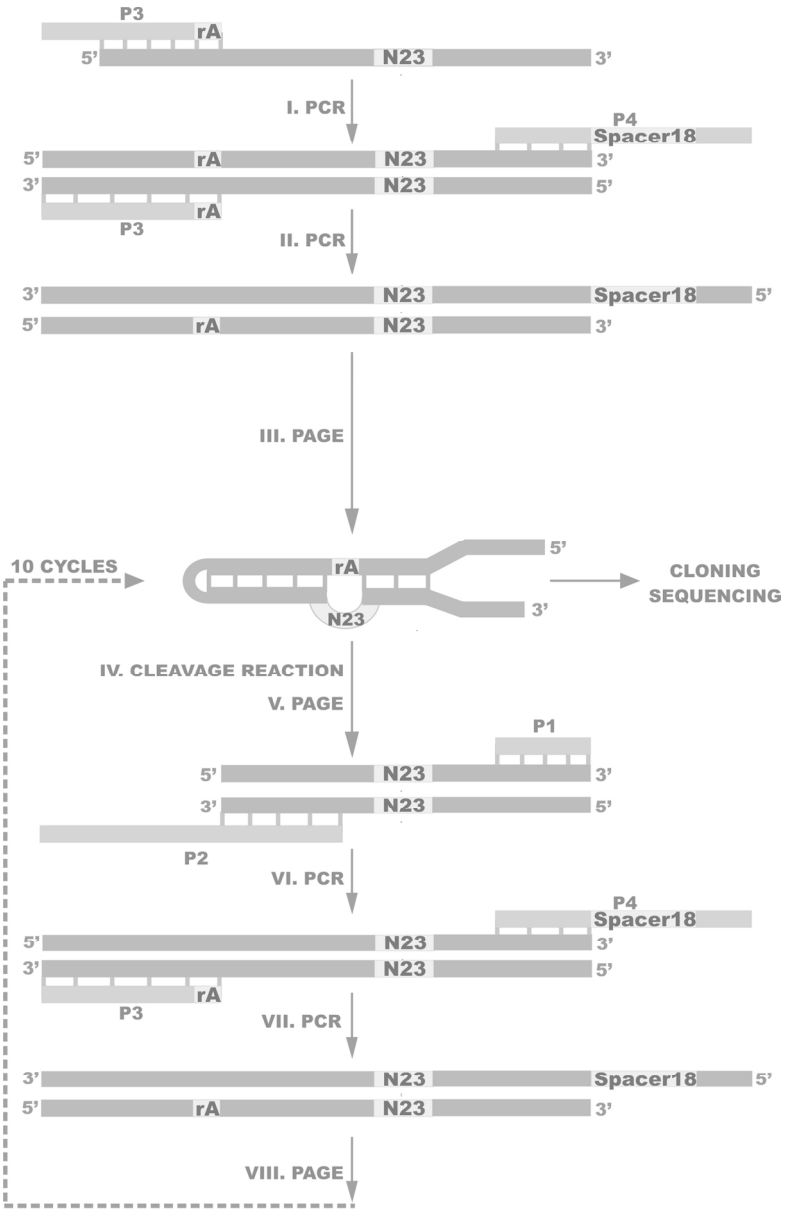
Figure 5. The cleavage rate constant, k_{obs} of the shortened *cis*-acting DNAzymes – Dz1/VIIWS, Dz5/XWS and Dz15/XWS in the presence of Cd^{2+} (grey bars), Zn^{2+} (bars with diagonal lines) and Mn^{2+} ions (bars with caro pattern). The assays were carried out with 50 μM metal ions at pH 7.0. (A) The reactions were performed at 25 °C. (B) The reactions were performed at 37 °C.

Figure 6. (A) CD spectra of DNAzyme 1/VIIWS at different concentrations of Cd^{2+} ions. A non-cleavable version of 1/VIIWS was applied in which ribonucleotide A at the cleavage site was replaced by its dideoxy analogue. The spectra were recorded in 50 mM HEPES, pH 7.0 and 100 mM NaCl (B) Relative CD signal at 277 nm as a function of cadmium(II) concentration.

Figure 7. Dependency of the DNAzymes activity on sequence variations. Secondary structure and residues numbering of the shortened: (A) Dz1/VIIWS, (B) Dz5/XWS. Italic black letters mark the catalytic cores of DNAzymes. The site of cleavage is denoted by underlining. The encircled letter indicates the mutated nucleotide. In the tables the relative maximal cleavage yields (Y_m) are shown which were determined for selected DNAzymes and their variants in the presence of Cd^{2+}, Zn^{2+} and Mn^{2+} ions after 30 min incubation at 25 °C. The Y_m values for the wild type Dz1/VIIWS and Dz5/XWS with Cd^{2+} were taken as 1 and they were compared to the Y_m values obtained with Zn^{2+} and Mn^{2+}. The Y_m value for a given mutant in the presence of a particular metal ion was calculated as the ratio $(Y_m)_{mutant}/(Y_m)_{wild\ type}$.

In vitro selection was performed to search for RNA-cleaving DNAzymes active with Cd^{2+} ions. All the selected, catalytically active variants turned out to belong to the 8-17 type DNAzyme.





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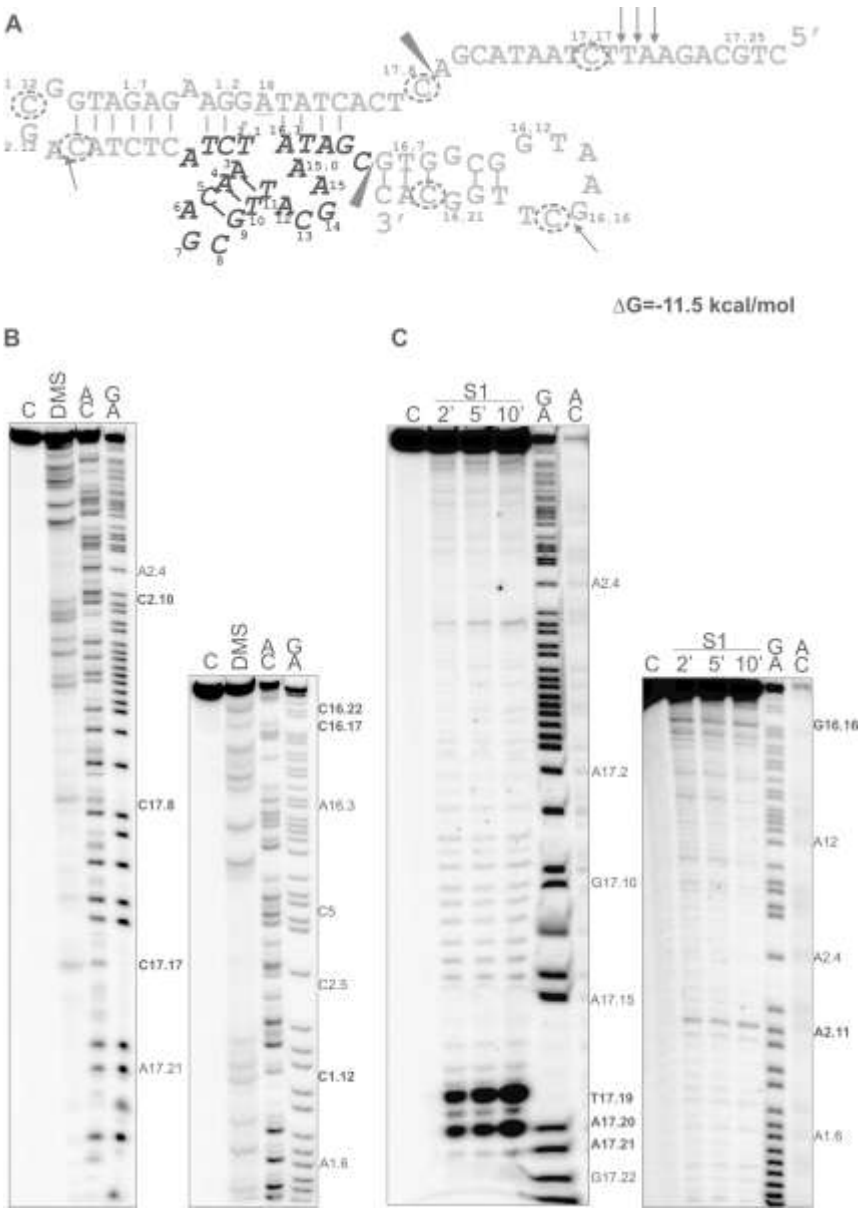

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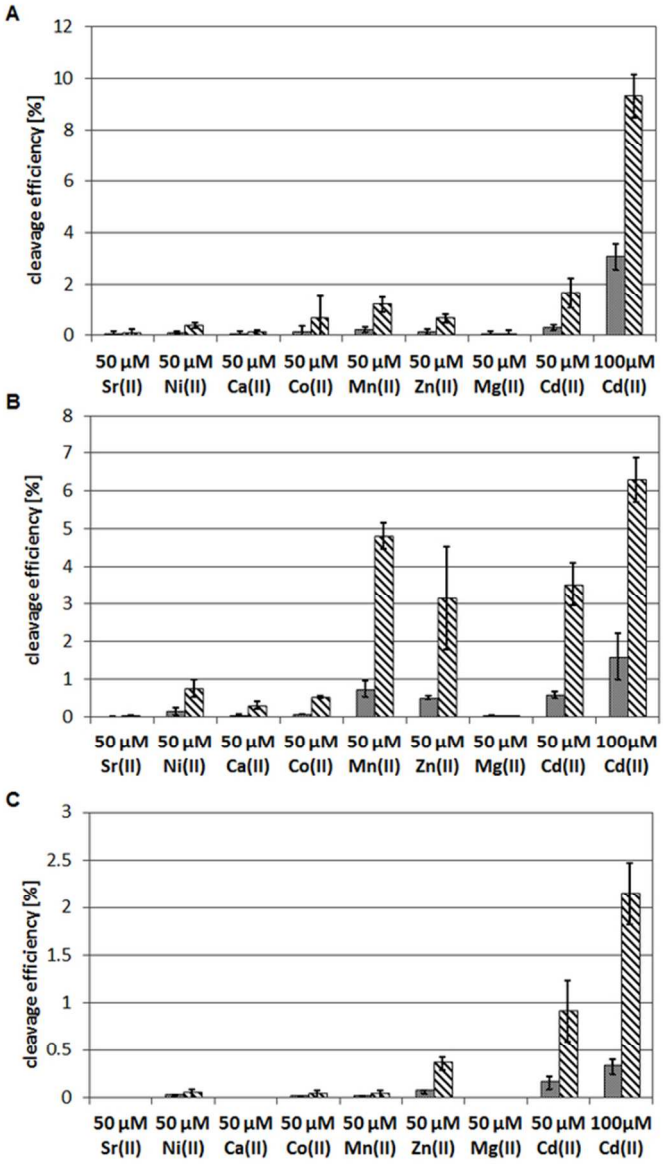

Figure 4
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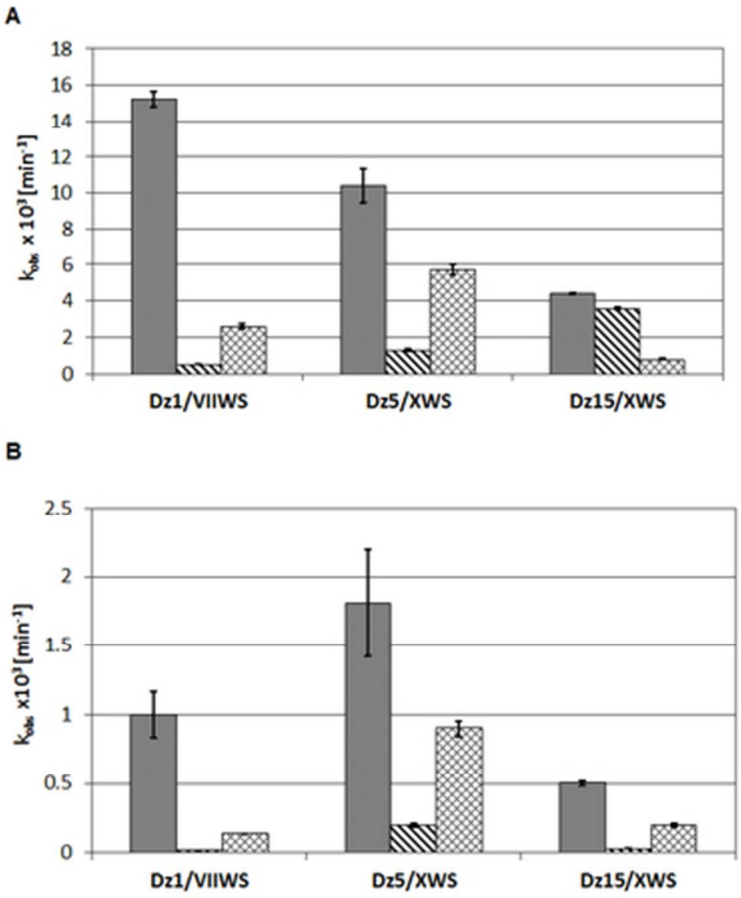

Figure 5
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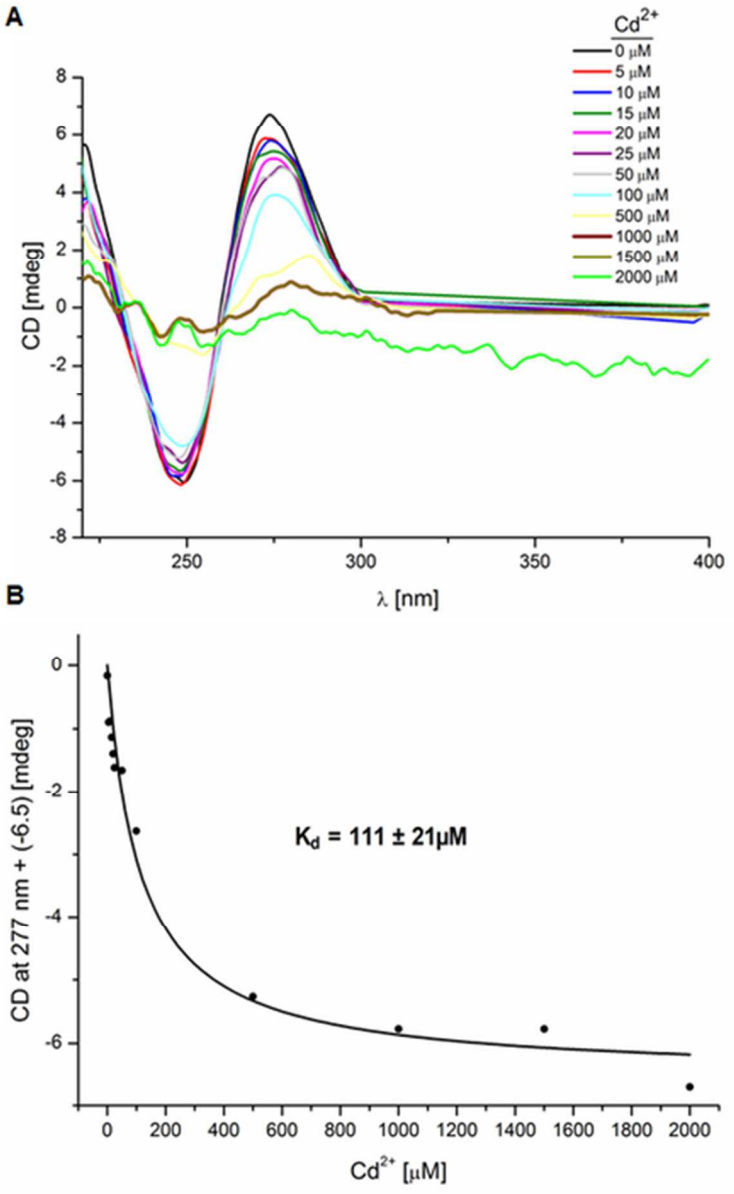

Figure 6
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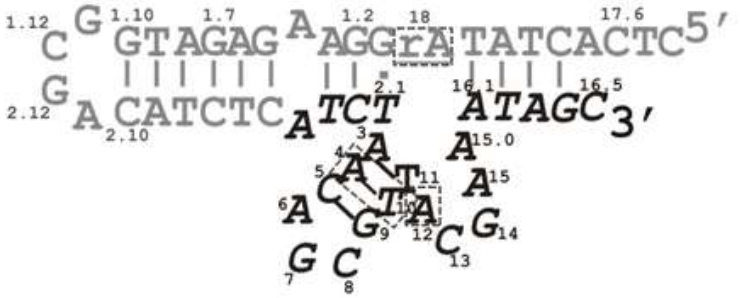
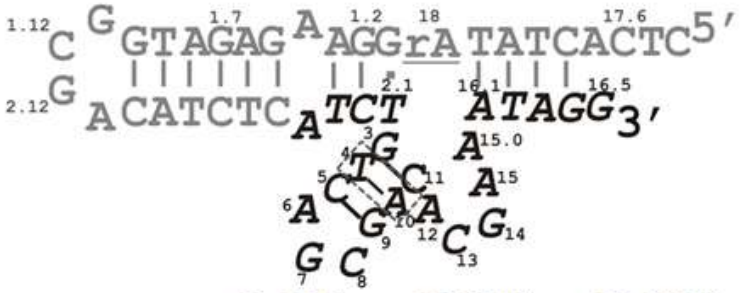
A



	50 μM Cd^{2+}	50 μM Zn^{2+}	50 μM Mn^{2+}
Dz1/VIIWS_WT	1.00	0.40	0.70
A4C/T10G	0.97	0.70	0.20
rA_PTO	0.80	0.13	0.07
A12C	0.10	0.03	0.04
A12T	0.07	0.06	0.03
A12G	0.60	0.20	0.30

B



	50 μM Cd^{2+}	50 μM Zn^{2+}	50 μM Mn^{2+}
Dz5/XWS_WT	1.00	0.90	1.36
T4C/A10G	0.20	0.17	0.30

Figure 7
38x59mm (300 x 300 DPI)