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# Gaining Target Access for Deoxyribozymes

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Antisense oligonucleotides and ribozymes have been used widely to regulate gene expression by targeting mRNAs in a sequence-specific manner. Long RNAs, however, are highly structured molecules. Thus, up to 90% of putative cleavage sites have been shown to be inaccessible to classical RNA based ribozymes or DNAzymes. Here, we report the use of modified nucleotides to overcome barriers raised by internal structures of the target RNA. In our attempt to cleave a broad range of picornavirus RNAs, we generated a DNAzyme against a highly conserved sequence in the 5′ untranslated region (5′ UTR). While this DNAzyme was highly efficient against the 5′ UTR of the human rhinovirus 14, it failed to cleave the identical target sequence within the RNA of the related coxsackievirus A21 (CAV-21). After introduction of 2′-*O*-methyl RNA or locked nucleic acid (LNA) monomers into the substrate recognition arms, the DNAzyme degraded the previously inaccessible virus RNA at a high catalytic rate even to completion, indicating that nucleotides with high target affinity were able to compete successfully with internal structures. We then adopted this strategy to two DNAzymes that we had found to be inactive in our earlier experiments. The modified DNAzymes proved to be highly effective against their respective target structures. Our approach may be useful for other ribozyme strategies struggling with accessibility problems, especially when being restricted to unique target sites.



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*Keywords:* DNAzyme; deoxyribozyme; 2′-*O*-methyl; LNA; picornavirus

## Introduction

Antisense oligonucleotides and nucleic acids enzymes are valuable tools to regulate the expression of target genes in a sequence-specific manner.[1–3] These approaches employ oligonucleotides that bind to complementary mRNA molecules by Watson–Crick-type base-pairing. In theory, antisense oligonucleotides, ribozymes and deoxyribozymes can be designed to interact site-specifically with any RNA species. Despite this seemingly simple concept, one usually encounters numerous obstacles on the way to an efficient regulation of target gene expression. One of the major hurdles is the identification of accessible sites that allow the efficient interaction of ribozymes or antisense oligonucleotides with their specific target.

Numerous efforts have focused on solving the accessibility problem. Computer-based structural models of the target RNAs were employed to design efficient antisense oligonucleotides or ribozymes. Structure prediction, however, has severe limitations when focussing on complex targets like mRNAs. Therefore, a variety of experimental strategies have been developed to define accessible target sites. These approaches include the use of randomized or sequence-specific oligonucleotide libraries, ribozyme expression cassettes or, even more demanding, DNA arrays. For details the interested reader is referred to the reviews by Sohail & Southern[4] and Gautherot & Sodoyer.[5]

To identify accessible target sites on a complex and highly structured RNA by any of these methods is a time and labour consuming process that does not always meet with success. In general, only one out of eight antisense oligonucleotides is thought to be efficient for knockdown of target genes.[6] Likewise, up to 90% of the putative target

Abbreviations used: UTR, untranslated region; LNA, locked nucleic acid; HRV, human rhinovirus; VR1, vanilloid receptor subtype 1; CAV, coxsackievirus type A; CBV, coxsackievirus type B.

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sites on long RNAs (>700 bases) have been reported to be resistant to cleavage by deoxyribozymes.[7,8] In cases where the window of potential target sites within the sequence is narrow, this resistance may be prohibitive for antisense or ribozyme strategies.

In our present study, we report a strategy to design catalytically active oligonucleotides to cleave a seemingly uncleavable site. In the course of our attempt to design an effective antiviral strategy against picornaviruses, we employed the "10–23" DNAzyme, which has been obtained by *in vitro* selection.[9–12] As a target site we chose a region that is highly conserved between a large number of rhino- and coxsackieviruses.[13] While this DNAzyme proved to be effective against the RNA of the human rhinoviruses (HRV) 2, 4, 14 and 16, it showed virtually no detectable degradation of coxsackievirus mRNA. By introducing 2′-*O*-methyl RNA or locked nucleic acids (LNA) monomers into the binding arms of the DNAzyme, we succeeded in cleaving the apparently unsuitable target site. These findings were confirmed with the mRNA of the vanilloid receptor subtype 1 (VR1, TRPV1) as a second target system, suggesting that our strategy may be generally applicable.

## Results

Recently, we reported the design of an optimized 10–23 DNAzyme with enhanced catalytic activity and stability against the 5′ non-coding region of human rhinovirus 14 (see Figure 1).[13] The deoxyribozyme, which we designated DH5, was directed towards a 19 nucleotide long target site that is highly conserved among a great number of rhino- and enteroviruses (Table 1) and has been shown to cleave the 5′ UTR of HRV14 (Figure 2(A) and (C)).[13] Although the 5′ UTR of CAV-21 contains the identical sequence element, it remained almost uncleaved by DH5 (Figure 2(B) and (C)). The observed weak degradation, however, is due to DH5, since virtually no degradation was observed when the viral RNA was incubated in the absence of the DNAzyme for 60 minutes (data not shown). These results suggest that the target sequence is well exposed in the three-dimensional structure of the rhinovirus 5′ UTR, while it is shielded in the 5′ UTR of CAV21.

To obtain the basic catalytic parameters of DH5 irrespective of folding problems due to higher-order structures, we determined $k_{cat}$ and $K_M$ values under multiple turnover conditions with a short synthetic RNA substrate of 19 nucleotides

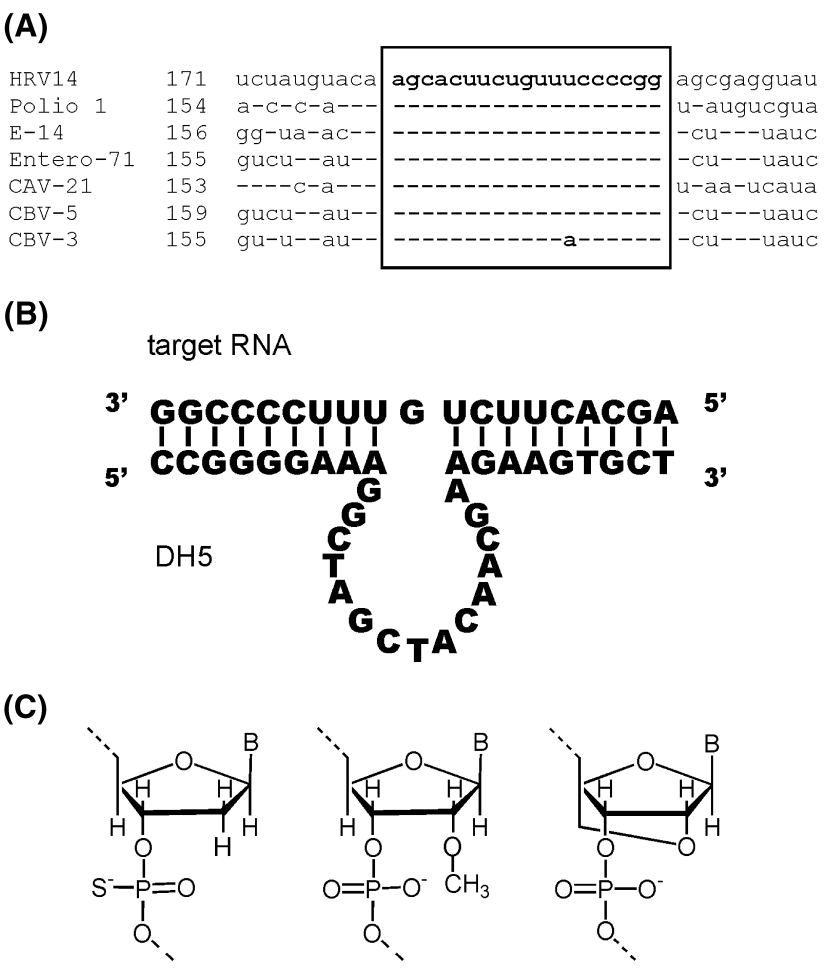


**Figure 1**. (A) Comparison of the primary sequence of different picornaviruses in the target region of the DNAzyme DH5. The target sequence is in bold. HRV14, human rhinovirus 14; Polio 1, human poliovirus 1; E14, human echovirus 14; Entero-71, human enterovirus 71; CAV21, coxsackievirus A21; CBV-3, coxsackievirus B3; CBV-5, coxsackievirus B5. (B) Cartoon of DH5 hybridized to the target sequence. (C) Nucleotide modifications used in this study: phosphorothioate, 2′-*O*-methyl RNA and locked nucleic acid.

**Table 1.** Picornaviruses containing the target site for DNAzyme DH5

| | Number of serotypes | Serotypes known to contain the target sequence |
|---|---|---|
| A. *Rhinoviruses* | | |
| Human rhinovirus | 102 | 1A, 1B, 2, 7,14, 16, 21, 29, 37, 58, 62, 72 |
| B. *Enteroviruses* | | |
| Poliovirus | 3 | 1, 2, 3 |
| Echovirus | 30 | 3, 5, 6, 13–15, 18–20, 24, 27, 29–31 |
| Enterovirus | | Porcine enterovirus 9, enterovirus 71 |
| Coxsackievirus A | 23 | A1–22, A24 |
| Coxsackievirus B | 6 | B5 |

(Figure 3). We found DH5 to follow Michaelis–Menten kinetics, showing a $k_{cat}$ value of 3 ($\pm$0.1) $min^{-1}$ and a $K_M$ value of 18.1 ($\pm$3.5) nM. Both values are in good agreement with those found in the literature for the 10–23 DNAzyme, where $k_{cat}$ values of around 1 $min^{-1}$ and $K_M$ values in the low nanomolar range have been most frequently reported.[10] According to this finding DH5 does not display any unusual properties.

We thus analyzed the influence of target structure on the cleavage activity in more detail. As can be seen in Figure 4, a 19mer oligodeoxynucleotide complementary to the target region of DH5 induced efficient RNase H cleavage of both the 5′ UTRs of HRV14 and CAV-21, respectively. This finding indicates that the conserved sequence motif is accessible to complementary oligonucleotides in both viruses. The question remains, why the 5′ UTR of CAV-21 is resistant to cleavage by DH5.

Since binding of complementary oligonucleotides occurred efficiently to both RNAs, while cleavage was restricted to one RNA only, it was necessary to investigate the hybridization of the DNAzyme more closely. In an effort to separate substrate binding of the DNAzyme from its cleavage activity, DH5 was rendered inactive by substituting the sixth nucleotide in the catalytic core (guanosine) with a cytidine residue, according to previous findings.[14] The accessibility of the target site on both viral RNAs was investigated by incubating inactivated DNAzyme and target RNA together with RNase H. It was found that DH5 induced RNase H cleavage of HRV14 mRNA to a significant extent. CAV-21 mRNA, however, remained virtually uncleaved (<10% degradation) after 30 minutes of incubation (Figure 4). This

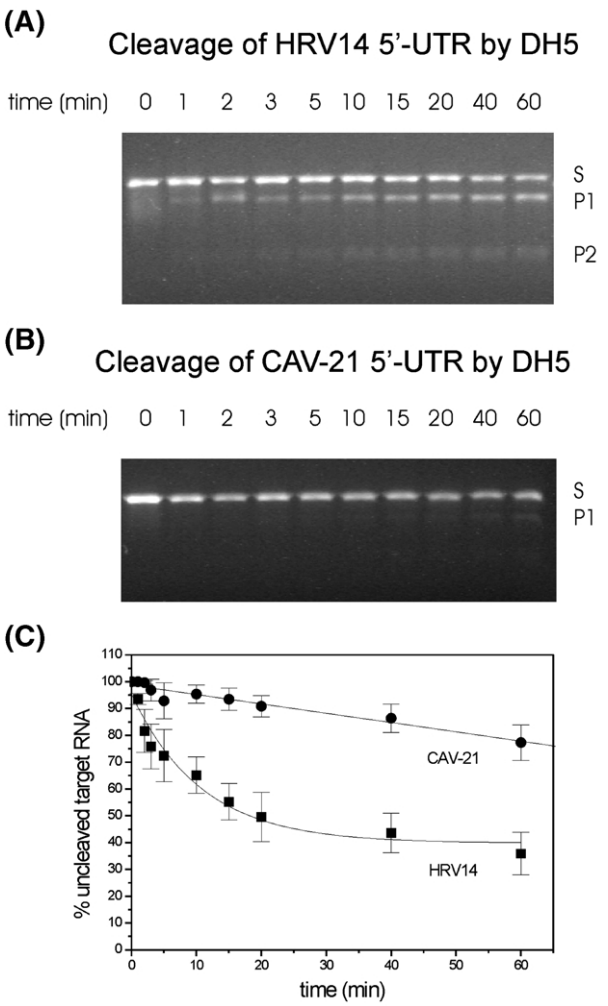


**Figure 2**. Time-course of the cleavage of *in vitro* transcribed 5′ UTRs of HRV14 (A) and CAV-21 (B) by unmodified DNAzyme DH5 under single turnover conditions. S, uncleaved substrate; P1 and P2, product bands. (C) Comparison of the cleavage of the 5′ UTRs of HRV14 (squares) and CAV-21 (circles) by unmodified DH5. Exponential fit of the mean of three independent experiments.

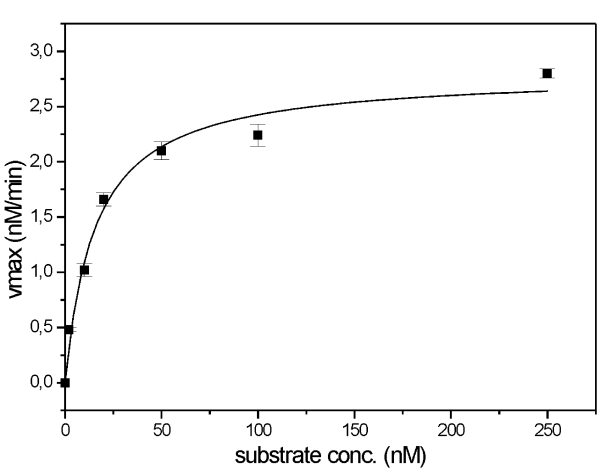


**Figure 3**. Kinetic measurements on DNAzyme DH5 with the 19mer target sequence under multiple turnover conditions.

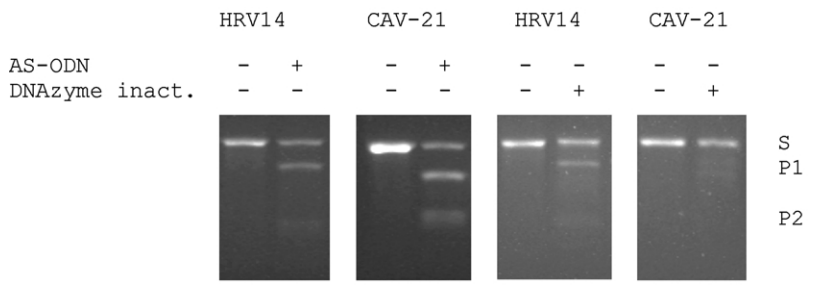


**Figure 4**. RNase H cleavage of transcripts of the 5′ UTRs of HRV14 and CAV-21 induced by a 19mer antisense oligodeoxynucleotide (AS-ODN) and inactive DH5, respectively. S, uncleaved substrate; P1 and P2, product bands.

result confirms our previous assumption that the lack of coxsackievirus-RNA cleavage has its cause in impaired accessibility of the target nucleotides for DH5.

It was therefore tempting to investigate whether a DNAzyme with increased affinity to the binding region is able to compete with internal interactions of the target RNA. In a preceding study, we reported that modification of arm design, i.e. adjusting arm length and content of modified nucleotides can improve the catalytic activity of DNAzymes.[13] We wanted to test whether this optimization strategy could be employed to cleave the apparently unsuitable target site of CAV-21 RNA. Thus, we introduced modified nucleotides into the substrate binding arms to increase the affinity of the DNAzyme towards its target RNA. Binding arms were designed according to the optimal set-up described previously: the arm length was shortened to seven nucleotides on each side and five 2′-*O*-methyl RNA or three LNA monomers were introduced at both ends (Figure 1(C)). The respective DNAzymes were designated DH5 OMe 7/5 and DH5 LNA 7/3, accordingly.

Both the 2′-*O*-methyl set-up or the LNA modifications led to fast and efficient cleavage of the target RNA (Figure 5(A)). After only 20 minutes of reaction, virtually the complete CAV-21 RNA had been degraded by DH5 OMe 7/5 and DH5 LNA 7/3. In comparison, the unmodified DNAzyme cleaved approximately 15% of the target RNA within this period. Fitting the data to a single exponential decay function predicts that the reaction of the unmodified DH5 enzyme will not run to completion. According to the fit, about 60% of the target molecule will remain inaccessible to the enzyme.

These data support an interpretation that DNAzymes with higher affinity towards the target region are capable of competing with internal RNA structures. This explanation implies that molecules with a lower target affinity should be even less active than the unmodified DNAzyme. Phosphorothioates are known to weaken binding of an oligonucleotide to its complementary sequence.[15] We have previously shown that the introduction of phophorothioate monomers into the binding arms of DH5 significantly reduces cleavage of the rhinoviral RNA.[13] As can be seen in Figure 5(A), this substitution completely abolished enzymatic activity against CAV-21 UTR.

To test whether our strategy to direct modified DNAzymes against the consensus site can be employed to develop antiviral tools against an even broader range of viruses, we extended our investigation to an additional picornavirus. CBV-3 is an enterovirus of high clinical relevance as it is one of the major pathogens causing severe heart diseases. Unmodified DH5 revealed only weak cleavage activity when targeted against CBV-3 RNA (Figure 5(B)). Again, introduction of 2′-*O*-methyl RNA monomers improved the efficiency of the reaction, indicating a similar competition of the binding arms with internal RNA structures as found for CAV-21. The cleavage rate can be further improved by a single base substitution in the binding arm of DH5, since the CBV-3 5′ UTR contains A rather than U in the 13th position of the target region (Figure 1(A)). The resulting DNAzyme was named DCo5 OMe 7/5. It cleaves CBV-3 5′ UTR with high efficiency (Figure 5(B)).

Subsequently, we wanted to investigate whether our strategy is transferable to an unrelated DNAzyme. We therefore employed a DNAzyme directed against a target site on the mRNA of the vanilloid receptor subtype I (VR1). Earlier, we reported DNAzyme DV28 to show no cleavage activity when challenged with the approximately 2600 nucleotide long target RNA.[7] The results obtained with DV28 and VR1 mRNA were similar to those seen with DH5 and CAV-21 5′ UTR. A 19mer antisense oligodeoxynucleotide induced efficient cleavage in an RNase H assay, indicating that the target region is accessible to oligonucleotides (Figure 6(A)). The unmodified DNAzyme targeted against the same region, however, showed virtually no cleavage in a single turnover experiment after a two hour period of incubation. To validate the potency of our strategy, we designed a DNAzyme following the modification scheme of DH5. The resulting DNAzyme DV28 OMe 7/5 contains five 2′-*O*-methyl RNA monomers at each terminus of the seven nucleotide long binding arms. Again, the modified DNAzyme showed substantial cleavage of the target molecule (Figure 6(B)). Analysis of the data predicted the reaction to almost reach completion. These findings indicate that enhancing the affinity of catalytically active nucleic acids is a suitable strategy to cleave a target RNA at otherwise inaccessible sites.

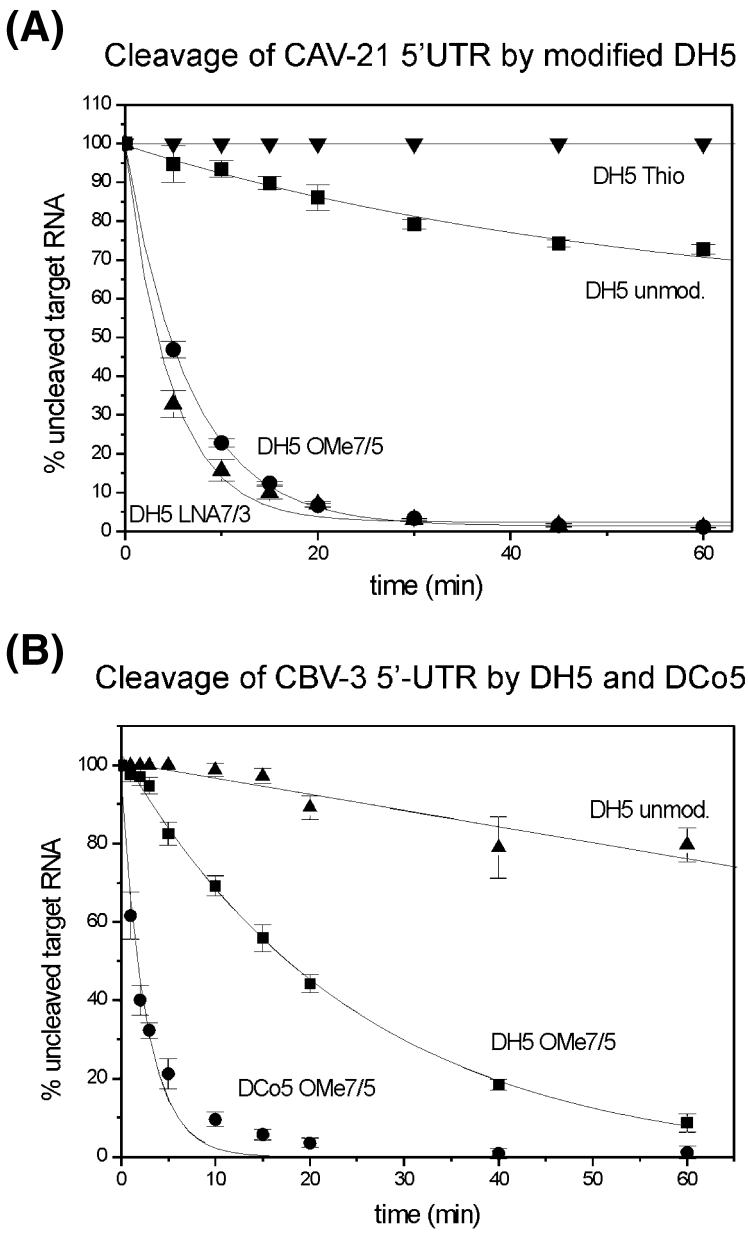


**Figure 5**. (A) Enhanced cleavage of CAV-21 RNA by DH5 due to the introduction of modified nucleotides into the binding arms. Squares, unmodified DH5; circles, DH5 OMe7/5; up triangles, DH5 LNA7/3; down triangles, DH5 Thio. (B) Cleavage of *in vitro* transcribed CBV-3 mRNA by optimized DNAzymes. Up triangles, unmodified DH5; squares, DH5 OMe7/5; circles, DCo5 OMe7/5. Exponential fit of the mean of three independent experiments.

## Discussion

The design of efficient antisense oligonucleotides or ribozymes is usually guided by the accessibility of the target site. For many applications, it may be an easy and obvious strategy to comply with the requirements of the target structure and to exclude those sites that are found inactive of further considerations. In some cases, however, one may be committed to a particular target sequence.

As it was our aim to develop a single oligonucleotide-based antiviral agent to target numerous picornaviruses, we were focussing on a highly conserved region of the viral 5′ UTR. In our initial experiments to target the consensus site, we obtained promising results with a DNAzyme designated as DH5 that efficiently cleaved the RNA of HRV14. Surprisingly, the identical sequence element of CAV-21 remained almost uncleaved. Coxsackie- and rhinoviruses are related members of the picornavirus family (reviewed by Zeichhardt & Grunert[16]) and the overall secondary structure of both viral RNAs has been predicted to be similar.[17,18] The inability of DH5 to cleave the CAV-21 RNA, however, points to different local structures of the related virus 5′ UTRs.

The finding that both target sites are accessible to antisense oligonucleotides, while the DNAzyme is unable to bind to the CAV-21 RNA as demonstrated by the mutated inactive control indicates that hybridization of DNAzymes is more complex than annealing of antisense oligonucleotides. The bulky catalytic center of DNAzymes can be expected to be an additional barrier to accessing target sites. Furthermore, the enzyme must be able to fold into its active structure and perform the cleavage reaction.

This interpretation suggests that incorporation of

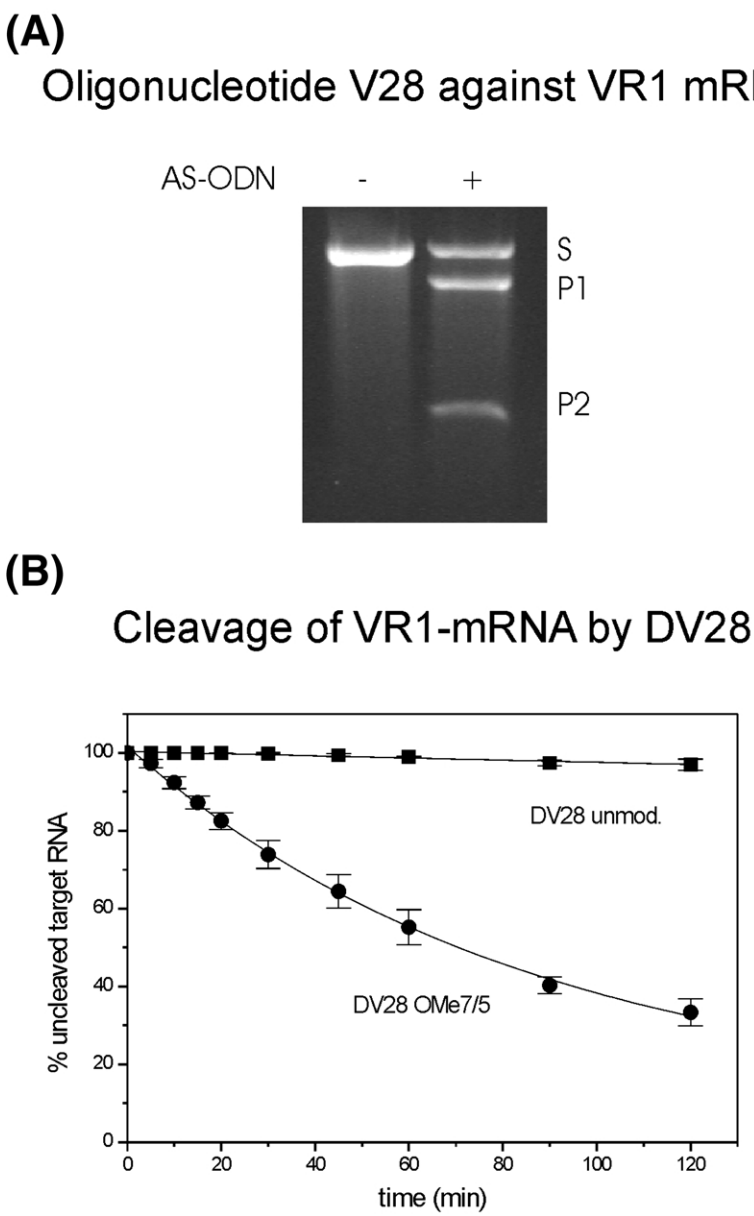


**Figure 6**. Transferability of the optimized arm-design to a DNAzyme with different specificity. (A) RNase H cleavage of VR1 mRNA induced by a 19mer antisense oligodeoxynucleotide (AS-ODN). (B) Cleavage of VR1 mRNA by unmodified DV28 and DV28 OMe7/5, respectively. An exponential fit of the mean of three independent experiments is given. Squares, DV28 unmodified; circles, DV28 OMe7/5.

modified nucleotides with enhanced affinity towards complementary sequences might help to overcome unfavorable target structures and to allow tight binding to form the active conformation. We therefore employed modified nucleotides that are known to increase binding affinity of an oligonucleotide substantially[3,19,20] and to enhance its resistance against nuclease degradation.[21–24]

Vickers *et al*. demonstrated that incorporation of 2′-*O*-methyl RNA into antisense oligonucleotides can improve their potency to inhibit the expression of a reporter gene.[25] Furthermore, a DNAzyme containing LNA nucleotides in the binding arms has been shown to cleave the highly structured 23S RNA more efficiently than its unmodified counterpart.[26,27]

Here, we demonstrate that a DNAzyme with 2′-*O*-methyl RNA monomers in the binding arms is capable of cleaving the seemingly inaccessible target site of CAV-21. This finding was confirmed with a DNAzyme containing LNA as a second modified nucleotide with high target affinity. It has been observed frequently that catalytically active nucleic acids leave a substantial amount of the target RNA uncleaved.[28] Besides displaying a high catalytic activity, the modified DNAzymes presented here have the ability to cut the target RNA to completion. These findings are in agreement with the assumption that the inability of a DNAzyme to cleave a certain site reflects its failure to compete with internal target structures.

To prove the applicability of our strategy further and to broaden the range of virus RNAs cleaved by our DNAzyme, we employed the 5′ UTR of CBV-3, one of the major myocardial pathogens. As observed with CAV-21, the CBV-3 RNA remained almost uncleaved by unmodified DH5. A substantial cleavage was achieved already by the introduction of 2′-*O*-methyl RNA monomers

into the substrate binding arms, even though CBV-3 contains a single base substitution within the consensus region. Since DNAzymes are highly sequence-specific,[29] cleavage was further improved by employing a DNAzyme with perfect homology to the target site of CBV-3.

Finally, a DNAzyme targeting the VR1 mRNA was used as an independent control for our approach. VR1 is an important pain receptor that is activated by heat, protons and capsaicin, the hot component of chili peppers.[30] The results obtained for VR1 reflect our earlier observation with the coxsackievirus RNA: the DNAzyme was inactive although the target region was found to be accessible to antisense oligonucleotides in the RNase H assay. Again, the target RNA was efficiently cleaved after introduction of modified monomers into the binding arms of the DNAzyme. These findings imply that our strategy to overcome unfavorable target structures by improving the target affinity of nucleic acids enzymes may be generally applicable.

We expect this strategy to be particularly valuable in applications in which the choice of target sequences is limited. For example when targeting chimeric RNAs, a major cause for various diseases including leukemia, one is necessarily confined to the junction sequence.[31] Furthermore, nucleic acid enzymes can be used as a tool to process heterogeneous termini of RNA molecules.[32,33] Here again, the experimental design will necessitate the cleavage of unique sequences that can be inaccessible to unmodified DNAzymes.

The approach to cleave highly structured RNA molecules by modifying the binding arms may be applicable to other types of *in vitro* selected DNAzymes like the 8–17 DNAzyme[9] or the bipartite DNAzyme,[34] as well as to RNA ribozymes like the hammerhead or the hairpin ribozyme.[2] The results reported here may thus be incentive to revisit ribozyme approaches against structured targets that looked hopeless at first sight.

## Materials and Methods

### Oligonucleotides

Unmodified oligodeoxynucleotides and phosphorothioates were obtained from MWG-Biotech AG, Ebersberg, Germany. 2′-*O*-methyl-containing oligonucleotides and RNA oligonucleotides were purchased from IBA GmbH (Göttingen, Germany). Oligonucleotides containing LNA were obtained from Proligo, Boulder, CO, USA. The sequences of the unmodified oligonucleotides and DNAzymes used in this study are:

| | |
|---|---|
| H5 | CCG GGG AAA CAG AAG TGC T |
| DH5 | CCG GGG AAA GGC TAG CTA CAA CGA AGA AGT GCT |
| Target sequence DH5 | AGC ACU UCU GUU UCC CCG G |
| DH5 inactive | CCG GGG AAA GGC TAC CTA CAA CGA AGA AGT GCT |
| DCo5 | CCG GGG TAA GGC TAG CTA CAA CGA AGA AGT GCT |
| V28 | AGC TCC AGA CAT GTG GAA T |
| DV28 | AGC TCC AGA GGC TAG CTA CAA CGA ATG TGG AAT |

Modified nucleotides were introduced as described in Results.

### Cloning and *in vitro* transcription of picornaviral RNA

The 5′ non-translated region of HRV14 was derived from the plasmid pWR3 encoding a full length HRV14 cDNA clone as described.[13] cDNAs corresponding to the 5′ non-translated region of CAV-21 and CBV-3 were obtained by reverse transcription and amplification of viral RNA using the PCR primers TTA AAA CAG CTC TGG GGT TG (bases 1–20) and CCA TTT GCA CTG ACT ATT GTG (bases 714–696) in the case of CAV-21 and TTA AAA CAG CCT GTG GGT TG (bases 1–20) and CCA TTT TGC TGT ATT CAA CTT A (bases 742–724) for CBV-3. Subsequently, the cDNAs were cloned into pcDNA3.1/CT-GFP-TOPO vectors (Invitrogen, Karlsruhe, Germany). The plasmids were linerarized using BamH1 (HRV) and EcoRV (CAV-21, CBV-3) (NEB, Frankfurt/M, Germany), respectively, to allow run-off *in vitro* transcriptions. The RiboMAX large scale production system from Promega (Madison, WI, USA) was employed for the transcription according to the manufacturer's instructions. The resulting transcripts contained 876 (HRV14), 797 (CAV-21) and 825 (CBV-3) bases, respectively.

The approximately 2600 base long VR1 mRNA was obtained as described.[7]

### RNase H assay

100 nM mRNA was incubated with 500 nM oligodeoxynucleotide and 0.4 unit RNase H (Promega) in a final volume of 10 μl of the manufacturer's buffer. To inhibit unspecific degradation of the target molecule, 1 unit RNasin (Promega)/μl was added to the samples. All reactions were quenched after 7.5 minutes by adding EDTA to a final concentration of 83 mM. Full-length mRNA and degradation products were separated on a 1.5% (w/v) agarose-gel stained with ethidium bromide. Band intensities were quantified using Quantity One software (BioRad, München, Germany). To determine RNase H induction by the less well binding inactive DNAzyme, the amount of RNase H was increased to 4 units/reaction, and the reaction was allowed to go on for 30 minutes before quenching.

### Determination of $k_{cat}$ and $K_M$

The 19mer target sequence was radioactively labeled by incubating 1 pmol of RNA with five units of T4 nucleotide kinase (Promega) and 10 μCi of [γ-$^{32}$P]ATP for 90 minutes at 37 °C. Purification of the labeled probe was achieved by electrophoresis on a 12%(w/v) denaturing polyacrylamide gel and elution of the band with 0.3 M sodium acetate(pH 5.5) for 45 minutes at 80 °C. Subsequently, an ethanol precipitation was performed overnight at −20 °C.

DNAzyme (0.1 nM final concentration) and six

different solutions of target RNA to give final concentrations of 1 nM to 250 nM were incubated separately at 85 °C for two minutes and put to 37 °C immediately afterwards. Reactions were conducted under standard conditions for kinetic measurements on DNAzymes (50 mM Tris–HCl(pH 7.0), 10 mM $MgCl_2$). Target RNA solutions contained 500 cpm/μl end-labeled RNA. Cleavage reactions were started by combining DNAzyme and target. For every target concentration, at least four 10 μl aliquots were drawn at different time points during the first 10% of the cleavage reaction. Ongoing cleavage was stopped by addition of 4 μl of 90% (v/v) formamide and 20 nM EDTA. Full-length RNA and degradation products were separated on a 20% polyacrylamide gel. The amount of cleavage was quantified by autoradiography using a Storm 840 Phosphoimager by Molecular Dynamics. Initial velocities were computed for the reaction at each substrate concentration. $k_{cat}$ and $K_M$ values were obtained from a plot of the initial velocities against substrate concentration by fitting hyperbolically using the Microcal Origin Program (Microcal, CA, USA). Values given are means ± standard deviation of three independent experiments.

#### Single turnover kinetics with the long target RNA

DNAzymes were allowed to act on their specific long target mRNAs in an enzyme excess set-up. Measurements were performed in 50 mM Tris–HCl, 10 mM $MgCl_2$; 1 unit/μl RNasin was added to prevent unspecific RNA degradation. After two minutes of denaturation at 65 °C, the respective DNAzyme was cooled down to 37 °C in the heating block. Addition of enzyme to the target solution started the reaction. The final concentrations of the DNAzyme and target RNA were 1 μM and 100 nM, respectively, resulting in a DNAzyme/RNA ratio of 10 : 1. After defined intervals, aliquots were taken and quenched by adding 83 mM EDTA and cooling on ice. The amount of cleavage was analyzed by agarose gel electrophoresis and ethidium bromide staining. Band intensities were quantified with the Quantity One software, followed by fitting with a single exponential decay function to obtain the observed cleavage rate $k_{obs}$ and the amount of RNA predicted to remain uncleaved ($y_0$) using Microcal Origin. Values given are means ± standard deviation of at least three independent experiments.

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