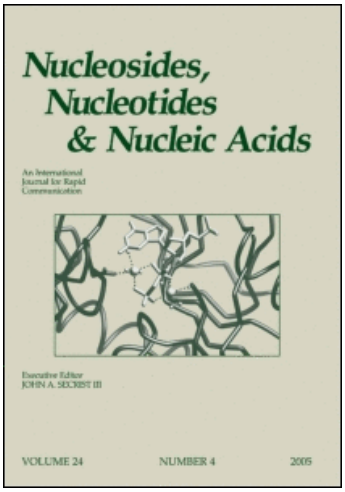

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BRIEF COMMUNICATION: STABILITY AND CATALYTIC ACTIVITY OF NOVEL CIRCULAR DNAZYMES

Georg Seifert, Tillmann Taube, Kriztina Paal, Hagen Graf von Einsiedel, Sven Wellmann, Günter Henze, and Karl Seeger □ *Charité Universitätsmedizin Berlin, Otto-Heubner Center for Pediatric and Adolescent Medicine (OHC), Department of Pediatric Oncology/Hematology, Berlin, Germany*

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□ *DNAzymes represent a new generation of catalytic nucleic acids for specific RNA targeting in order to inhibit protein translation from the specifically cleaved mRNA. The 10–23 DNAzyme was found to hydrolyze RNA in a sequence-specific manner both* in vitro *and* in vivo. *Although single-stranded DNAzymes may represent the most effective nucleic acid drug to date, they are nevertheless sensitive to nuclease degradation and require modifications for* in vivo *application. However, previously used stabilization of DNAzymes by site-specific phosphorothioate (PT) modifications reduces the catalytic activity, and the PTO displays toxic side effects when applied* in vivo. *Thus, improving the stability of DNAzymes without reducing their catalytic activity is essential if the potential of these compounds should be realized* in vivo. *Results: The Circozyme was tested targeting the mRNA of the most common genetic rearrangement in pediatric acute lymphoblastic leukemia* TEL/AML1 *(ETV6/RUNX1). The Circozyme exhibits a stability comparable to PTO-modified DNAzymes without reduction of catalytic activity and specificity and may represent a promising tool for DNAzyme* in vivo *applications. Conclusion: The inclusion of the catalytic site and the specific mRNA binding sequence of the DNAzyme into a circular loop-stem-loop structure (Circozyme) of approximately 70 bases presented here represents a new effective possibility of DNAzyme stabilization.*

Keywords DNAzyme; Stability; Activity; Phosphorothioate; TEL-AML1

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INTRODUCTION

Recently, a new generation of catalytic nucleic acids, termed DNAzymes or deoxyribozymes, was discovered.[1,2] DNAzymes display many advantages compared to ribozymes, which have been extensively studied as a tool for specific inhibition of protein translation in cancer and infectious or genetic diseases.[3,4] DNAzymes are promising tools in order to inhibit protein translation from the specifically cleaved mRNA. They may represent an option for therapeutic application *in vivo*.[5–11] Compared to ribozymes, DNAzymes are inexpensive, easy and fast to synthesize, and, furthermore, the single-stranded DNA molecules exhibit higher stability than single-stranded RNA. Therefore, DNAzymes may represent the most convenient nucleic acid drug to date.[12,13] Nevertheless, DNAzymes are single-stranded DNA and are sensitive to nuclease degradation when applied *in vivo*. Previously, protection of single-stranded DNA, e.g., antisense oligonucleotides, from degradation by serum exo- and endonucleases was achieved by site-specific phosphorothioate (PTO) modifications or structural changes.[14,15] But PTO modifications of ODN are burdened by several disadvantages. In DNAzymes, PTO modifications may reduce the catalytic activity.[8] Furthermore, it has been shown that PTO-modified DNA molecules can react as topoisomerase inhibitors in dividing cells, which may induce malignancies.[16,17] The dosage of PTO-modified DNA is limited by nonspecific toxic side effects.[18–21]

Here, we present a new circular design of a DNAzyme that substantialy increases stability without PTO modifications. This stabilization was achieved by including the 10–23 DNAzyme sequence in a circular closed loop–stem loop structure (Circozyme). To investigate the activity of Circozymes, the DNAzymes were targeted to the *TEL-AML1* fusion mRNA that results from the the most common recurring chromosomal translocation t(12;21)(pl3;q22) in childhood acute lymphoblastic leukemia (ALL).[22–24]

MATERIALS AND METHODS

In Vitro Synthesis of *TEL-*, *AML1-*, and *TEL-AML1*-mRNA

The target sequences for the DNAzymes were synthesized by *in vitro* transcription of mRNA 600–800 bp in length, coding for *TEL*, *AML1*, and *TEL-AML1*. PCR-amplified sequences of *TEL* (Genbank NM_001987: nucleotide 667–1272), *AML1* (Genbank D43969: nucleotide 174–947) and *TEL-AML1* (Genbank NM_001987: nucleotide 667, Genbank D43969: nucleotide 947) were cloned (pCRII TOPO 4 TA® Cloning; Invitrogen Corp., San Diego, California), isolated (QIAgen Miniprep Kit Qiagen, Hilden, Germany), and sequenced (Big Dye Terminator Cycle Sequencing on ABI

Prism 377 Automated Sequencer, both PE Applied Biosystems, Foster City, California). RNA was synthesized from the plasmid by *in vitro* transcription using RiboMax (Promega, Mannheim, Germany).

In Vitro Cleavage Assay

The *in vitro* cleavage activity of Circozymes and linear DNAzymes was analyzed in a buffer adapted to physiological conditions containing 50 mM Tris-HCL (pH 7.35), 10 mM MgCl, 150 mM NaCl, and 0.01% SDS. RNA (400 nM) and DNAzyme (5 nM) dissolved in buffer were pre-incubated for 10 min at 37°C. The reaction was initiated by mixing the pre-incubated RNA and DNAzyme. The cleavage reaction was performed for 60 min at 37°C and stopped by addition of a formamide buffer. The cleavage products were separated on a 4% polyacrylamide 7 M urea gel, stained with SYBR Gold (MoBiTec, Gottingen, Germany), and visualized on a Fluorimager (Amersham Biosciences, Piscataway, New Jersey). To control for unspecific cleavage activity, the *in vitro*–transcribed mRNA coding for normal *TEL* and *AML1* was incubated with the *TEL-AMLI* specific DNAzyme under the same conditions.

Optimization of the DNAzymes Cleaving Site

Two DNAzymes cleaving the *in vitro*–transcribed *TEL-AMLI* target mRNA at various sites adjacent to the *TEL-AML1* fusion site were investigated (Figure 1). The cleavage activity of the DNAzyme was further optimized by testing different lengths (symmetric and asymmetric) of the

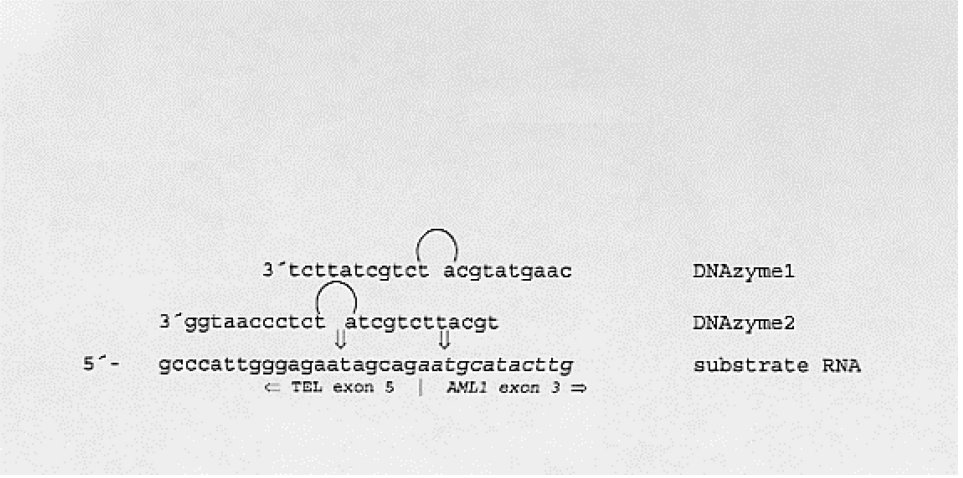

FIGURE 1 DNAzyme cleaving sites adjacent to the TEL-AML1 fusion site with two possible AT-cleaving 10–23 DNAzymes. Different lengths of DNAzyme substrate binding sequences (symmetric and asymmetric) were compared (only one of each is shown).

DNAzyme 3′- and 5′-RNA-binding sequences. Optimization of DNAzyme activity was performed with unstabilized linear DNAzymes (all oligonucleotides: Metabion, Martinsried, Germany).

Stabilization of the Linear DNAzymes

Different PTO modifications for stabilization were tested: three PTO at the 5′- and 3′-end of the DNAzyme, complete PTO modification of the RNA binding sequence and PTO modification of the RNA binding sequence together with PTO modification 3′ of every pyrimidin base in the catalytic sequence (Figure 2).

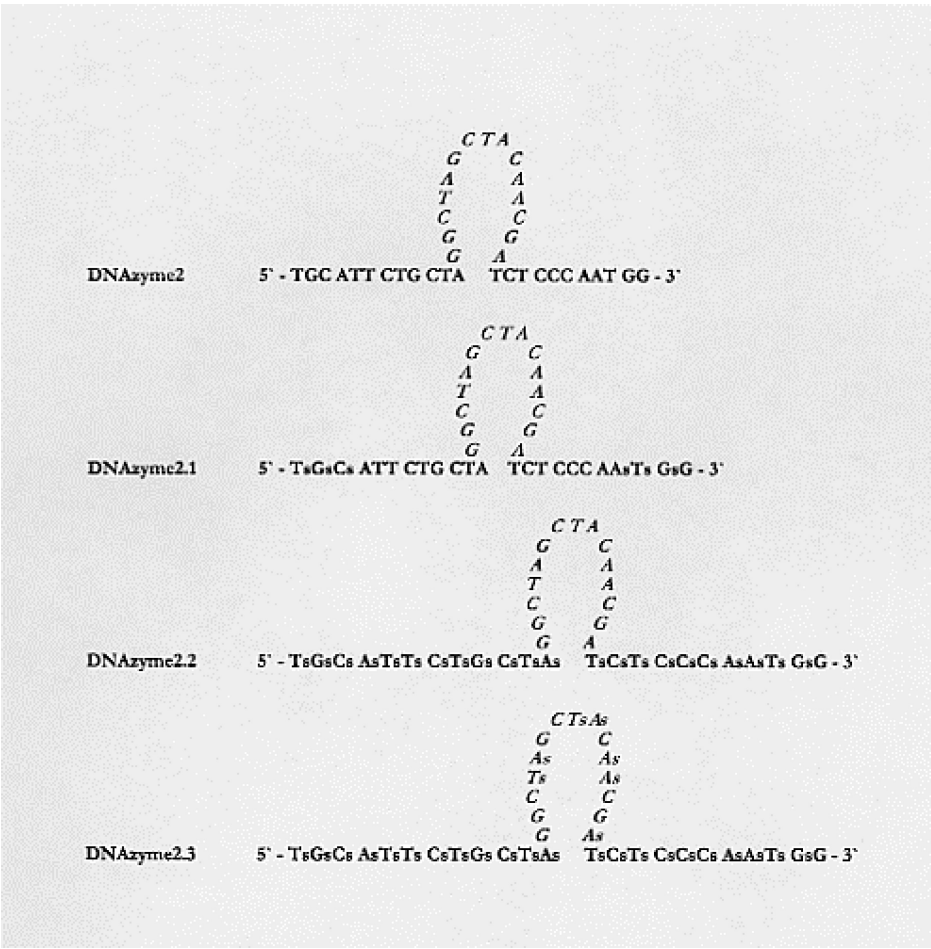

FIGURE 2 DNAzymes with different grades of PTO modification for stabilization. s: Phosphorothioate; *italic:* catalytical sequence.

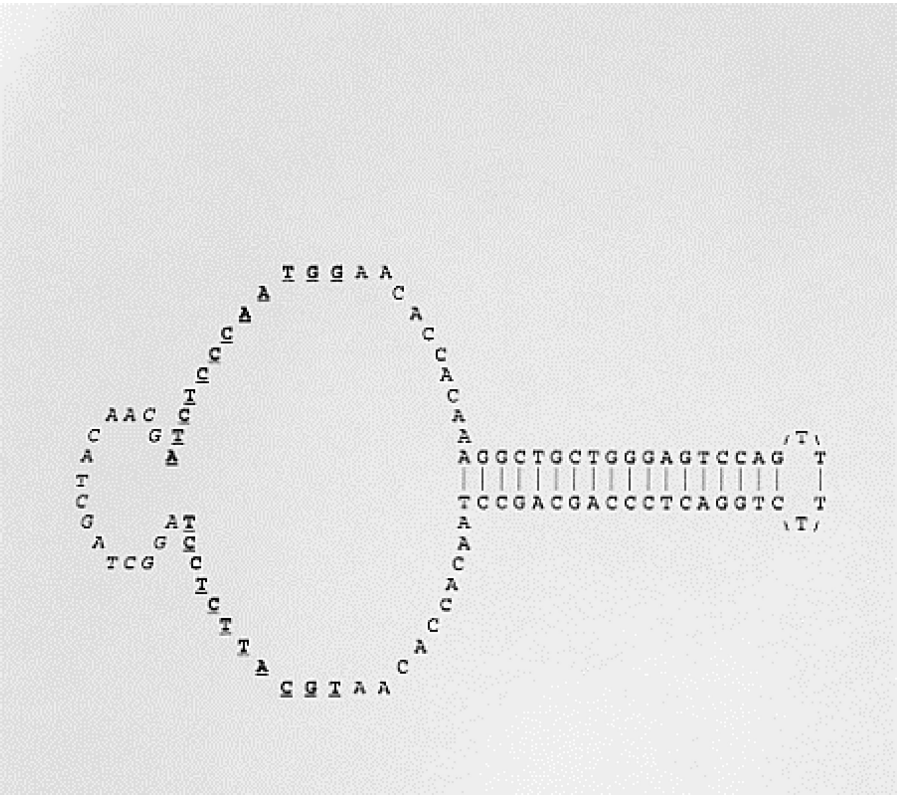

FIGURE 3 Structure of the Circozyme. *italic:* Catalytical sequence; **bold**/underlined: RNA substrate binding sequence; normal: stabilizing sequence of the Circozyme with stem-loop structure.

Synthesis of the Circozymes

Circular DNAzymes, Circozymes (Figure 3), were generated by ligating two chemically synthesized oligonucleotides. One oligonucleotide contained the respective DNAzyme. Both oligonucleotides were 5′ phosphorylated. Equimolar amounts of both oligonucleotides were ligated at a DNA concentration of 0.55 mg/ml using 10 Weiss units of T4 DNA ligase (Fermentas AB, Vilnius, Lithuania) per milligram of 5 DNA. Unligated DNA was digested using 25 U/mg DNA T7 DNA Polymerase (Fermentas AB). The products were purified by ion-exchange chromatography on DMAE and precipitated with ethanol.

Stability Test

The different PT-stabilized DNAzymes and the Circozyme were incubated at 37°C for 0, 3, 6, 9, 11, 24, 33, and 53 h in 1640 RPMI (Biochrome, Berlin, Germany) containing 10% FCS (Biochrome). The concentration of the DNAzymes and the Circozymes was 200 nM. DNAzymes and Circozyme

were separated on 15% polyacrylamide 7 M urea gels stained with SYBR Gold and visualized on the Fluorimager.

RESULTS

Stability of the Circozyme and the Linear PT-Modified DNAzymes

Incubation of DNAzymes in serum revealed marked differences regarding the stability of the non-modified DNAzyme, the various PTO-modified DNAzymes and the Circozyme (Figure 4). Non-modified linear DNAzymes are degraded rapidly and no full-length DNAzyme was detectable after 24 h. PTO modifications increase the stability of DNAymes. Interestingly, the Circozyme exhibits a comparable stability. Full-length Circozymes and PTO-modified DNAzymes were detectable after more than two days of incubation (Figure 4).

Cleavage Activity of the Circozyme

For further investigation the DNAyzme 2 was chosen because it exhibited higher *TEL-AML1* cleavage activity than DNAzyme 1. When different

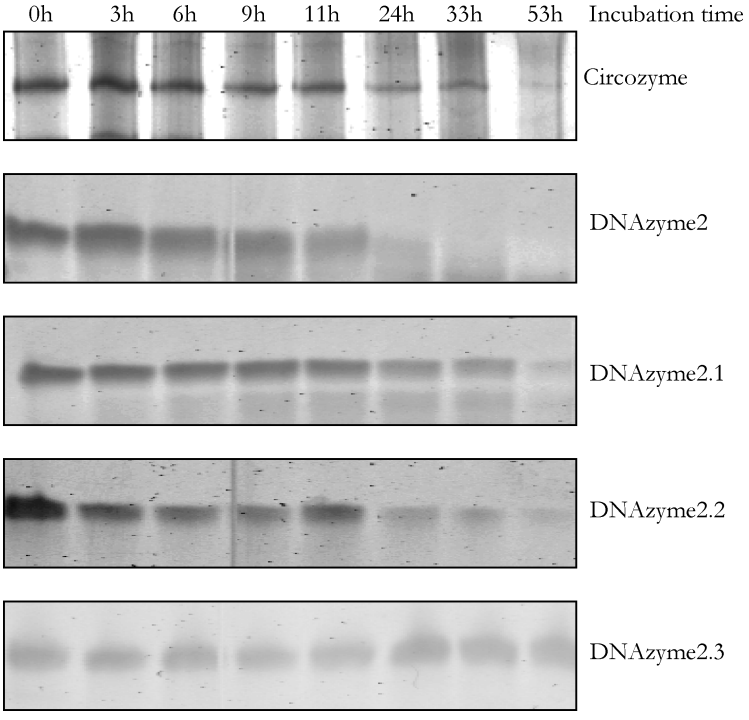

FIGURE 4 Stability of DNAzyme and Circozyme. Linear DNAzymes with and without different phosphorothioate modifications and circular DNAzymes were incubated for 0–53 h at 37°C in medium containing 10% FCS. Separation in PAGE shows reduction of intact DNAzyme with increased incubation time. Unmodified DNAzymes are only stable for a few hours and fragmented after 24 h. The Circozyme shows stability comparable to the PTO-modified DNAzymes.

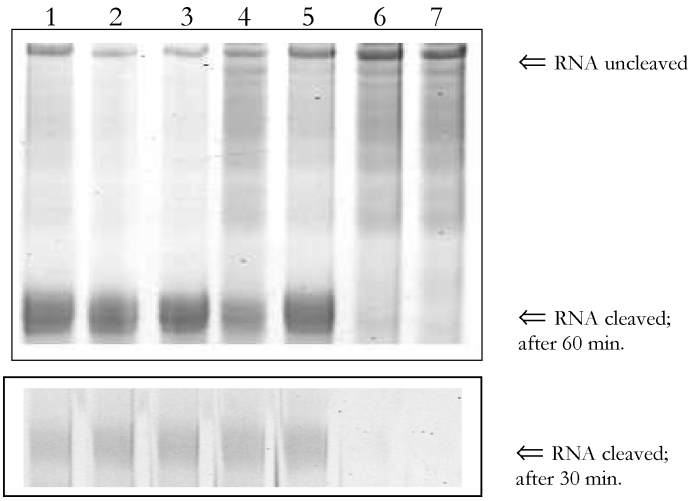

FIGURE 5 Investigation of DNAzyme activity. Lane 1: DNAzyme 2; lane 2: DNAzyme 2.1; lane 3; DNAzyme 2.2; lane 4: DNAzyme 2.3; lane 5: Circozyme; lane 6: inactive Circozyme; lane 7: target RNA. PTO modified DNAzymes lose activity with the increasing numbers of PTO groups. The cleavage activity of the Circozyme is comparable to the activity of conventional stabilized DNAzymes.

lengths of substrate binding sequences were tested an asymmetric design with 9 bases at the 5′-end and 12 bases at the 3′-end showed the highest cleavage activity (data not shown). A comparable catalytic activity of the Circozyme and DNAzymes 2, 2.1, and 2.2 could be shown (Figure 5). PTO modifications in the catalytic sequence (DNAzyme 2.3) significantly reduced the DNAzyme activity (Figure 5). Cleavage specificity was tested by incubation of mRNA coding for *TEL* and *AML1* with the optimized DNAzymes, no cleavage of these control RNAs could be detected (data not shown).

DISCUSSION

DNAzymes are promising tools for mRNA targeting in order to inhibit protein translation in experimental settings and there might be possibilities for therapeutic applications *in vivo*.[5–11] Yet the use of DNAzymes *in vivo* is limited by their stability under physiological conditions. Commonly used PTO modifications to stabilize single-stranded DNA *in vivo* are known for dosage-dependent nonspecific toxicities and may react as topoisomerase inhibitors.[16–21] Furthermore, PT modifications of DNAzymes can reduce the catalytic activity of the compounds.

Therefore, novel alternatives to stabilize the DNAzymes *in vivo* are needed. The presented inclusion of the optimized catalytic DNAzyme sequence into the circular loop–stem loop structure of the Circozyme combines several advantages. The stability of DNAzymes is comparable to PTO-modified single-stranded DNA molecules. The cleavage activity of Circozymes is unaffected compared to the unmodified linear DNAzyme,

while PTO modifications might reduce the DNAzyme acitivity. And, aiming at an *in vivo* use of DNAzymes, the most important advantage of the circular structure might be that toxic side effects due to PTO modifications are avoided.

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