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RESEARCH ARTICLE

An efficient RNA-cleaving DNA enzyme can specifically target the 5′-untranslated region of severe acute respiratory syndrome associated coronavirus (SARS-CoV)

Shuwen Wu[1]
Junqiang Xu[1]
Jiangxia Liu[1]
Xuan Yan[1]
Xiaodong Zhu[2]
Gengfu Xiao[1]
Lunquan Sun[3]**
Po Tien[1,2]*

[1]*Modern Virology Research Center, State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan 430072, China*

[2]*Center for Molecular Virology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China*

[3]*Faculty of Medicine, University of New South Wales, Sydney, 2052, Australia*

*Correspondence to: Po Tien, Modern Virology Research Center, State Key Laboratory of Virology, College of Life Sciences, Wuhan University; Wuhan 430072, China.
E-mail: tienpo@sun.im.ac.cn

**Correspondence to: Lunquan Sun, Faculty of Medicine, University of New South Wales, Sydney, 2052, Australia.
E-mail: lunqsun@optusnet.com.au

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Abstract

Background The worldwide epidemic of severe acute respiratory syndrome (SARS) in 2003 was caused by a novel coronavirus called SARS-CoV. We report the use of DNAzyme (catalytic DNA) to target the 5′-untranslated region (5′UTR) of a highly conserved fragment in the SARS genome as an approach to suppression of SARS-CoV replication. A mono-DNA enzyme (Dz-104) possessing the 10–23 catalytic motif was synthesized and tested both *in vitro* and in cell culture.

Materials and methods SARS-CoV total RNA was isolated, extracted from the SARS-CoV-WHU strain and converted into cDNA. We designed a RNA-cleaving 10–23 DNAzyme targeting at the loop region of the 5′UTR of SARS-CoV. The designed DNAzyme, Dz-104, and its mutant version, Dz-104 (mut), as a control consist of $9 + 9$ arm sequences with a 10–23 catalytic core. *In vitro* cleavage was performed using an *in vitro* transcribed 5′UTR RNA substrate. A vector containing a fused 5′UTR and enhanced green fluorescent protein (eGFP) was co-transfected with the DNAzyme into E6 cells and the cells expressing eGFP were visualized with fluorescence microscopy and analyzed by fluorescence-activated cell sorting (FACS).

Results and conclusions Our results demonstrated that this DNAzyme could efficiently cleave the SARS-CoV RNA substrate *in vitro* and inhibit the expression of the SARS-CoV 5′UTR-eGFP fusion RNA in mammalian cells. This work presents a model system to rapidly screen effective DNAzymes targeting SARS and provides a basis for potential therapeutic use of DNA enzymes to combat the SARS infection. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords DNAzyme; inhibition; SARS-CoV; 5′UTR

Introduction

Severe acute respiratory syndrome (SARS) is a life-threatening form of pneumonia. In the course of a few months, a total of 8422 probable cases of this highly infectious disease had been reported to the WHO by August 2003 [1] including 916 deaths. The disease has been etiologically linked to a novel coronavirus named the SARS-associated coronavirus (SARS-CoV) [2]. Genome sequences of SARS-CoV isolates obtained from a number of index patients have been published and have provided important information on

the phylogeny and variability [3,4]. The viral genome has the characteristics of a linear and positive single-stranded RNA virus about 29.7 kb in length, containing 14 identifiable open reading frames (ORFs) and short untranslated regions at both termini [5].

There are currently no approved antiviral drugs that are highly effective against coronaviruses. Strategies exemplified by the use of antisense RNA or deoxyoligonucleotides [6] were reported to inhibit gene expression by interfering with RNA transcription or translation. Unfortunately, the administration of large amounts of antisense phosphorothioate oligonucleotides has led to a toxicity problem [7].

Recently, several RNA-cleaving DNAzymes have been shown to be active in cleaving their substrate RNA under simulated physiological conditions. Two representative catalytic motifs, 10–23 and 8–17 (Figure 1), were reported by Santoro and Joyce through *in vitro* selection from a combinatorial library of DNA sequences [8]. The most efficient DNAzyme, termed '10–23', consists of a conserved catalytic core of 15 nucleotides, flanked by two variable hybridizing arms that can theoretically be designed to be complementary to any target RNA. It can cleave between any unpaired purine and a paired pyrimidine residue, which endow them with tremendous potential for applications in down-regulation of targeted genes. To date, a number of groups have explored the potential of the 10–23 DNAzyme in biological systems. Banerjea and co-workers have attempted to inhibit HIV-1 gene expression by DNAzymes targeted at the HIV-1 *tar, gag, tat/rev* RNA, and coreceptor CCR5 [9–12]. Sun and co-workers targeted the viral sequences from the HPV16 E6 and E7 genes [13] and the *c-myc* gene in smooth muscle cells [14] by DNAzymes. The biological activity of DNAzymes that cleave RNA derived from the *bcr-abl, Egr-1, huntingtin*, the *env* gene of HIV and PB2 mRNA of influenza virus A have also been examined [16–20]. All the reported work demonstrated that DNAzymes can be used as a potential tool to specifically inhibit the disease-related genes.

In this study, we explored the potential use of DNAzymes targeting the SARS 5′UTR fused with the green fluorescent protein (GFP) gene. The DNAzyme designed for this purpose was found to be effective both *in vitro* and *in vivo*, suggesting that the DNAzymes could be as a new class of anti-SARS agent.

Materials and methods

Cells and virus

The African green monkey kidney (Vero E6) cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco Invitrogen Corporation) at 37 °C. The SARS-CoV-WHU strain (Accession No. AY293 850) was maintained and propagated in Vero E6 cells as described [21].

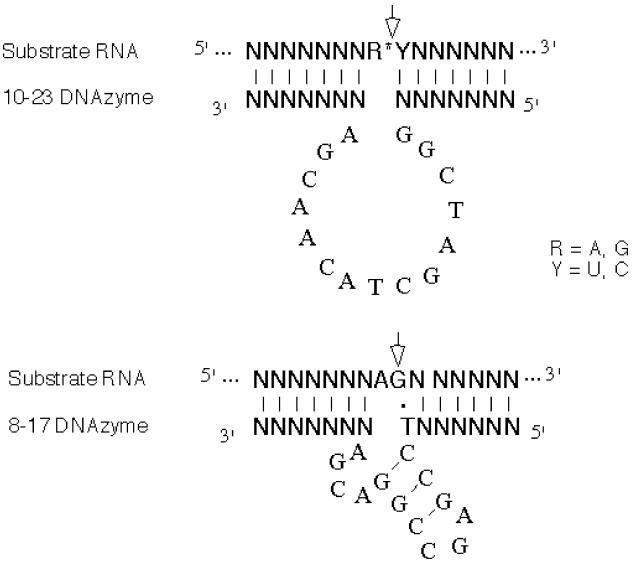

Figure 1. Composition of the 8–17 and 10–23 catalytic motifs. The DNAzyme (lower strand) binds the RNA substrate (top strand) through Watson–Crick pairing. Cleavage occurs at the position indicated by the arrow. R = A or G; Y = U or C

Construction of a SARS 5′UTR-eGFP fusion vector

SARS-CoV total RNA was isolated and extracted from the SARS-CoV-WHU strain. The RNA was converted into cDNA by reverse transcription. The following primers were used to amplify a 264 bp fragment of the 5′UTR of the SARS genome by polymerase chain reaction (PCR): pUTR1, 5′AGAATTCTAGGTTTTTACCTACCCAGG-3′ (EcoRI digestion site underlined); pUTR2, 5′AGGATCC CTTACCTTTCGGTCACACC-3′ (BamHI digestion site underlined).

The conditions for PCR were 94 °C, 2 min; (94 °C, 40 s; 50 °C, 40 s; 72 °C, 2 min) for 30 cycles; followed by 72 °C, 10 min. The amplified product was analyzed by electrophoresis, cloned into pMD18-T PCR cloning vector, then subcloned into the multiple cloning site (MCS) between EcoR1 and BamH1 in peGFP-N1 vector (BD Biosciences Clonetech), and then transformed into *Escherichia coli* strain DH5a. The sequence integrity of the construct was verified by restriction mapping and DNA sequencing.

DNA enzyme design

Based on Santoro and Joyce's report [8], we designed a RNA-cleaving DNAzyme possessing the 10–23 motif. The target site was selected in the loop region of the predicted secondary structure of the 5′UTR. The designed DNAzyme, Dz-104, consists of 9 + 9 arm sequences with a 10–23 catalytic core, and was synthesized using standard chemistry (Sangon, Shanghai, China). The details of the target sequences, the DNAzyme (Dz-104) and the mutant control (Dz-104 (mt)) are shown in Figure 2. As indicated, the A nucleotide of the target gene was left unpaired and

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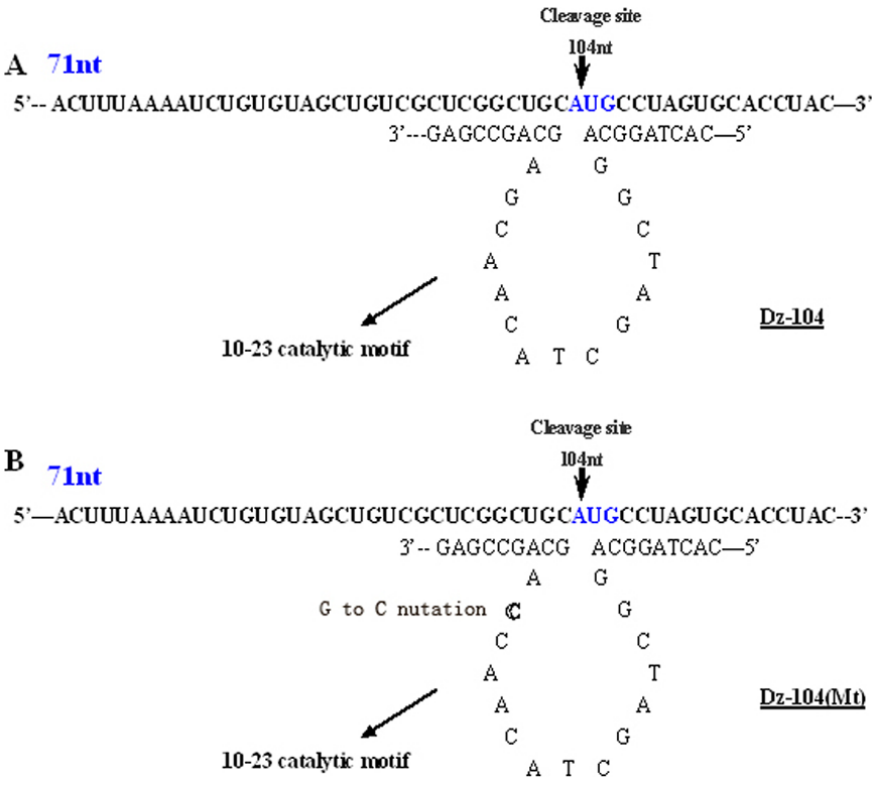

Figure 2. DNA enzyme target sites and two designed DNA enzymes. The target sequence is shown together with (A) active DNAzyme (Dz-104) and (B) the mutated control (Dz-104 (mut))

cleavage is expected to take place after the A. The mutant DNA enzyme (Dz-104 (mt)) possessed a single mutation in the catalytic motif (G to C).

In vitro synthesis of substrate RNA

To generate the substrate RNA for testing DNAzyme cleavage activity *in vitro*, the SARS-CoV 5′UTR was produced by PCR from the recombinant plasmid p5′UTR-eGFP with a forward primer containing a T7 promoter sequence (5′CTGTAATACGACTCACTATAGGGCCAAGTACGCCCCCTATTGA and a reverse primer (5′GTAGTTGCCGTCGTCCTTGA). The amplified fragment is 1000 bp in length and the predicted cleavage site is in the 5′UTR of SARS at the midpoint of the fragment.

The conditions for PCR were 94 °C, 2 min; (94 °C, 40 s; 50 °C, 40 s; 72 °C, 5 min) for 30 cycles; followed by 72 °C, 15 min. The amplified product was analyzed by electrophoresis and then recovered from the agarose gel. Then 1 μg purified DNA template was used for *in vitro* transcription with a T7 transcription kit (JINGMEI BIOTECH, Beijing, China) in a volume of 20 μl as recommended by the manufacturer and incubated for 2 h at 37 °C. Upon completion of the transcription, the DNA template was removed by digestion with DNase at 1 U DNase/1 μg template DNA (TaKaRa Biotechnology, Dalian, China) for 15 min at 37 °C. The transcript RNA was further purified with TE-saturated phenol/chloroform and chloroform/isoamyl alcohol (24 : 1).

In vitro cleavage reaction and kinetic analysis

In cleavage reactions, the purified substrate RNA (100 nM) and the DNAzyme (various concentrations) were mixed in 10 μl of 50 mM Tris-HCl, pH 7.5, in the presence of 10 mM $MgCl_2$ and 1 U RNasin to prevent non-specific RNA degradation. Prior to mixing enzyme and target RNA, both solutions were denatured separately for 2 min at 85 °C. The cleavage reaction was incubated at 37 °C. For a single turnover kinetic experiment, 10-fold excess of DNAzyme was used in the cleavage reaction (1000 nM). In a multiple kinetic reaction, 1/10 concentration of DNAzyme (10 nM) was employed and aliquots were taken after defined intervals during the first 10% of the reaction. The reaction was stopped by adding 83 mM EDTA and cooled on ice. 10× sample buffer (37% formaldehyde and 7% 5×MOPS buffer in formamide) was added to the cleavage reactions prior to being loaded onto an agarose gel. The gel was stained with ethidium bromide and the intensities were quantified with the Quantity One software (Gene Snap). Data were further analyzed by fitting either linearly to obtain the initial velocity v_{init} for substrate excess experiments or with a single exponential decay function to obtain the observed cleavage rate for enzyme excess experiments using Origin (Microcal Software, Northampton, MA, USA). Values given are mean ± standard deviation (SD) of at least three independent experiments.

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Transfection of Vero E6 cells with p5′UTR-eGFP and DNA enzymes

Vero E6 cells (2×10^5) were grown to 60% confluence on a 24-well plate and were then co-transfected with a fixed amount of p5′UTR-eGFP (1 μg) along with varying amounts of DNA enzymes (0.7, 3.5, 7 μg, respectively) for 12 h using Lipofectamine Plus (Invitrogen, USA) in a final volume of 500 μl. After 4 h of incubation in the presence of Lipofectamine Plus, the cells were washed with DMEM without serum and incubated for a further 12 h in the same medium with 10% FBS. Then, the cells expressing eGFP were visualized with fluorescence microscopy (Nikon, Tokyo, Japan).

Fluorescence-activated cell sorting (FACS) analysis of the transfected Vero cells

The transfected cells were trypsinized and collected by centrifugation. The cells were further rinsed with phosphate-buffered saline (PBS), re-suspended in 100 μl 2% polyformaldehyde and kept at 4 °C; then 900 μl PBS were added to each sample. The cells were then analyzed on Beckman Coulter counter for the percentage of the cells expressing GFP.

Real-time PCR assay for quantification of the GFP mRNA expression

At 24 h post-transfection, the cells were collected for total RNA extraction using Trizol according to the manufacturer's instruction. Real-time PCR reactions were carried out in ICycler iQ (BioRad). The primers used in the PCR were: GFP1, 5′CAAGCTGACC CTGAAGTTCA 3′ and GFP2, 5′ATGCGGTTCACCAGGGTGT3′, which gave rise to a 250 bp product of the GFP gene. The standard curve was generated using a serial of 10-fold dilution of pEGFP-N1 or p5′UTR-EGFP (10^{10}, 10^9, 10^8, 10^7, 10^6, 10^5copies). The amplification condition was: room temperature (RT), 70 °C for 5 min; 1×94 °C for 2 min; 45×94 °C for 45 s, 60 °C for 45 s, and 72 °C for 30 s. The mean Ct values were collected and used for analysis.

Results and discussion

Target site selection and DNAzyme design

SARS-CoV belongs to the coronavirus family with a structure similar to the other coronaviruses, which contain S, M, E and N gene products. The virus features a discontinuous transcription of subgenomic mRNAs, regulated by the pre-transcribed leader sequence and the specific TRSs in the 5′UTR of the genome. The 5′UTR sequence contributes to the regulation of the virus gene expression. Most strikingly, there is a high degree of sequence conservation in the 5′UTR region among all the SARS-CoV strains identified thus far, which suggests the functional importance of the region. Targeting the 5′UTR would therefore not only present a feasible strategy, but also potentially avoid the possible emergency of escape mutant viruses.

A 10–23 DNAzyme was designed (Dz-104) with a cleavage site at nucleotide 104. The arm length was chosen as a $9 + 9$ format as previously reported, which gave rise to a much more efficient catalytic activity based on a balance of the hybridization strength ($-\Delta G$) and kinetic turnover [14]. The cleavage site was selected at an AU dinucleotide since a hierarchy of RY reactivity (where R is a purine and Y is a pyrimidine) to the 10–23 DNAzyme follows the general scheme AU = GU ≥ GC ≫ AC [15].

Dz-104 cleavage of SARS-CoV 5′UTR RNA

To test the cleavage activity of Dz-104, we conducted *in vitro* cleavage analyses using a 1 kb RNA transcript that was transcribed from a T7-containing primer-based PCR fragment of the 5′UTR. The cleavage point was in the middle of the transcript; thus the two bands of the cleavage product would appear at the same position in a gel. In a single turnover assay where 10-fold access of Dz-104 was used, the substrate RNA was efficiently cleaved with the expected size of the cleavage products (Figure 3A) and the observed rate was 0.064 min^{-1} (Figure 3B). Under a multiple turnover condition, the substrate was cleaved with an initial velocity at 0.4 nM min^{-1} (Figure 3C). When the mutant DNAzyme (Dz-104 (mt)) was subjected to the cleavage assay under the same conditions, it did not show any cleavage (Figure 3A, lower panel). These results demonstrated that the designed DNAzyme was chemically active in cleaving its substrate RNA with a capacity to turnover under the *in vitro* conditions. It also suggested that the local RNA structures where the Dz-104 targeted are accessible under the simulated physiological conditions.

Establishment of a model system for DNAzyme targeting in cells

To examine the efficacy of the designed DNAzymes, it is crucial to have a sensitive and meaningful system to perform the assay. We chose to use a fusion transcript of the 5′UTR and GFP gene as a model system. In this system, the DNAzyme target was linked to the 5′ end of the GFP gene, which provided a versatile assay to monitor DNAzyme activity in cells (Figure 4). When the DNAzyme cleavage occurred, it was anticipated that the truncated fusion RNA would be subjected to the nuclease degradation; therefore, the level of the GFP expression would be reduced, which could be monitored by fluorescence microscopy.

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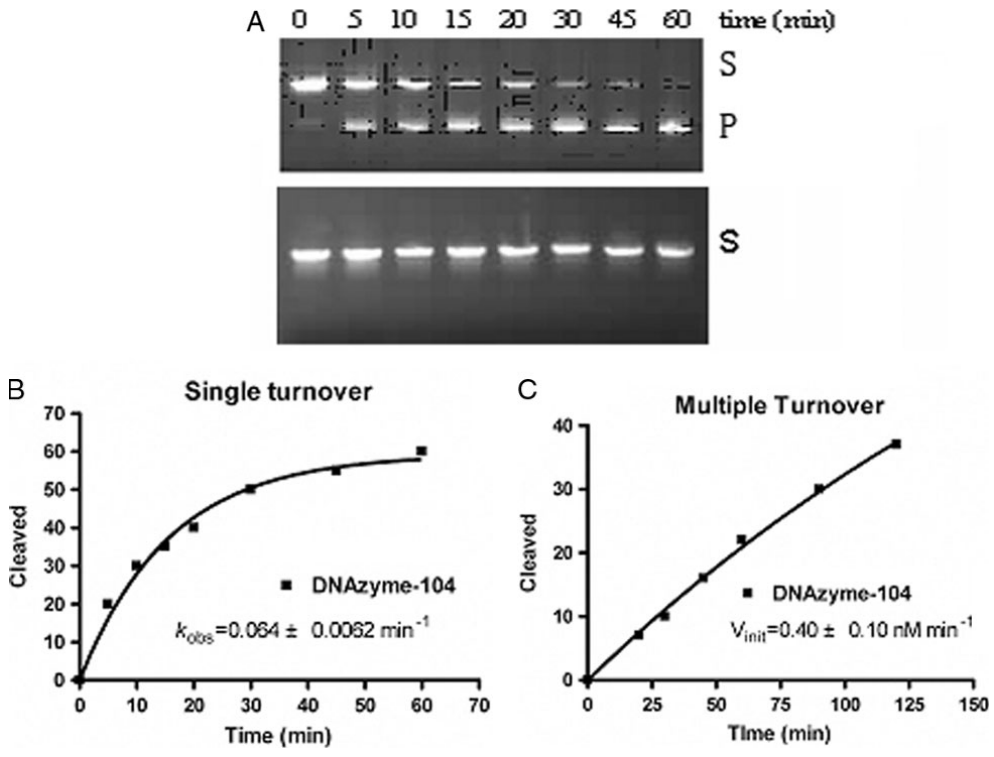

Figure 3. ***In vitro*** **cleavage of SARS-CoV 5′UTR by DNAzyme-104. (A) Gel analysis of the cleaved product with Dz-104 (upper panel) and Dz-104 (mut) (lower panel). S, substate RNA; P, cleavage products. (B) Kinetic profile of DNAzyme-104 under single turnover conditions with a 10-fold enzyme excess. (C) Kinetic profile of DNAzyme-104 under multiple turnover conditions with a 10-fold substrate excess. Y axis represents the % of cleaved product**

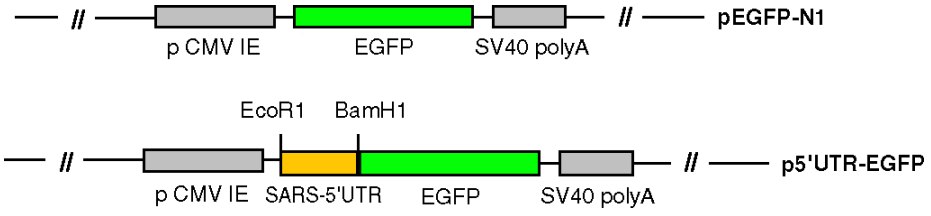

Figure 4. Fusion construct of SARS-CoV 5′UTR and eGFP. The construct is based on the peGFP-N1 vector with insertion of the 5′UTR of SARS at EcoR1 and BamH1 sites at the 5′ end of the eGFP gene. The resultant construct is designated as p5′UTR-eGFP

When the p5′UTR-eGFP construct was transfected into Vero E6 cells, the expression of the eGFP gene could be easily observed, but appeared to be a slightly weaker than the parental vector peGFP-N1 (Figure 5). This minor change could be due to the effect of the extra 5′UTR sequences on the promoter activity or transcription efficiency of the GFP gene. However, the system should be sensitive enough to be used for assays of the DNAzyme activity.

Effect of Dz-104-mediated cleavage of 5′UTR-eGFP fusion RNA on eGFP expression in Vero E6 cells

The advantage of using GFP is that it facilities the direct observation of the target gene expression by fluorescence microscopy. To demonstrate that the designed DNAzyme could target the mRNA with the SARS 5′UTR and suppress the eGFP expression, we co-transfected Vero E6 cells with p5′UTR-eGFP and Dz-104 at different ratios. The data showed that co-transfection of p5′UTR-EGFP/Dz-104, compared to the Dz-104 (mt) control, significantly decreased the fluorescent level, while it had no effect on the GFP in the cells co-transfected with pEGFP-N1 that contained no 5′UTR sequence (Figure 5). The effect of Dz-104 on eGFP expression was shown to be dose-dependent and the transfection procedure had no effect on cell growth (data not shown).

To confirm the DNAzyme effect on GFP expression, the percentage of the GFP-expressing cells was measured using flow cytometry. The results showed that there was a dose-dependent reduction in the number of GFP+ cells that were transfected with the p5′UTR-eGFP and Dz-104 (Table 1). This reduction was not seen in the cells transfected with either the parental vector pEGFP-N1 or mutant DNAzyme Dz-104 (mut).

To determine if the effect of Dz-104 on the GFP expression was due to a cleavage of the 5′UTR-GFP

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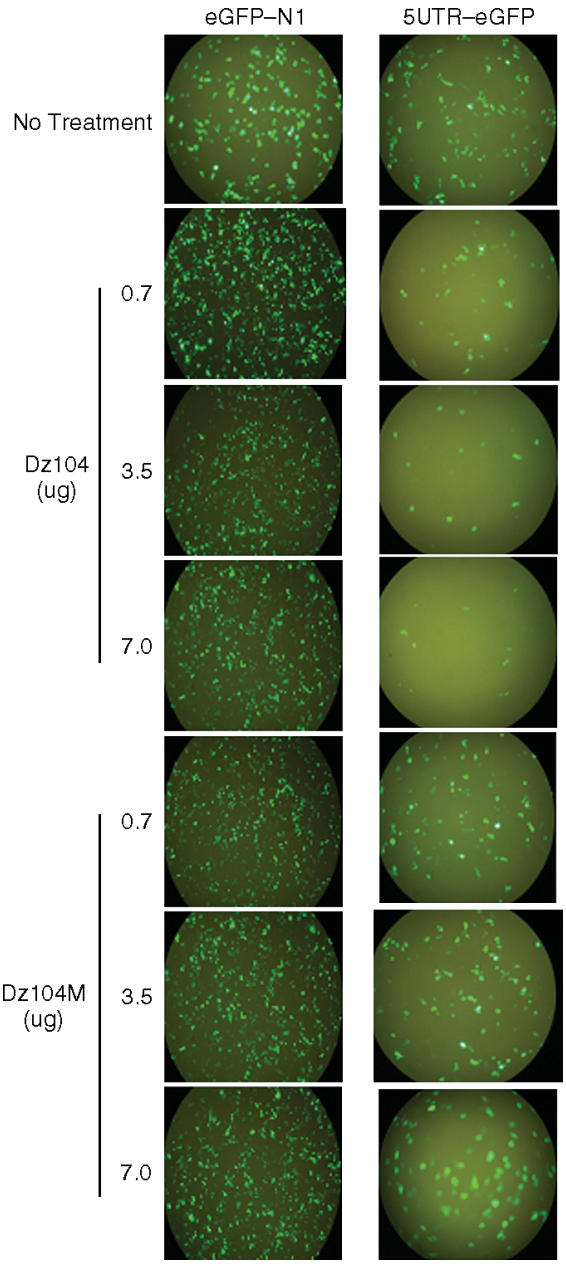

Figure 5. Dz-104-mediated reduction of GFP expression. Dz-104 or Dz-104 (mut) was co-transfected with 1 μg of peGFP-N1 (left panel) or p5′UTR-eGFP (right panel) into Vero cells. Various concentrations of the DNAzyme or its control are shown as 0.7, 3.5 and 7 μg. The experiments were performed twice and one set of representative data are presented here (100×)

mRNA, real-time PCR was performed to measure the level of the target RNA. It was shown that Dz-104 treatment of the p5′UTR-eGFP-transfected cells led to a marked reduction in the target mRNA level, as evidenced in Table 2. This demonstrates that DNAzyme elicits the degradation of the 5′UTR-EGFP mRNA sequence in a sequence-specific manner, resulting in suppression of the eGFP gene expression.

In the present study, the DNAzyme was co-transfected with the target vector into Vero cells. Although this co-transfection did not fully mimic the cells infected with the virus, it did show that the DNAzyme could enter the cells

Table 1. FACS analysis of the transfected Vero cells

		Percentage of fluorescent cells (%)*	
Vector	Dosage (μg)	Dz-104	Dz-104 (mt)
eGFP-N1	0.0	69.5	69.5
	0.7	68.6	71.6
	3.5	66.8	68.5
	7.0	69.3	67.0
5′UTR-eGFP	0.0	57.6	57.6
	0.7	25.8	55.6
	3.5	17.0	56.8
	7.0	9.1	58.2

*The data were the mean of two independent experiments.

Table 2. GFP mRNA expression determined by real-time PCR

		Ct value	
Vector	Dosage (μg)	Dz-104	Dz-104 (mt)
eGFP-N1	0.0	11.64	11.64
	0.7	13.03	11.53
	3.5	11.94	9.59
	7.0	11.60	11.77
5′UTR-GFP	0.0	16.66	16.66
	0.7	19.64	13.84
	3.5	22.67	16.01
	7.0	23.80	16.64

Data were an average of two independent experiments.

and find the target RNA. A previous study showed that the DNAzyme transfected into mammalian cells remained stable and biologically active up to 24 h [22], which supports our results, that sufficient DNAzymes had been transfected into the cells and functioned as a target-specific gene suppressor.

Taken together, our study shows that the DNAzyme can efficiently cleave the 5′UTR of SARS-CoV both *in vitro* and in cells, implying that the 5′UTR may well be a good target for molecular targeting. The study also presents a convenient system for screening other nucleic-acid-based agents for inhibition of SARS replication. Since DNAzymes have inherent physicochemical advantages over other nucleic-acid-based agents, such as higher target flexibility, minimal chemical modification, relative resistance to nuclease degradation and low cost of synthesis, they may be a clinically appealing new class of anti-SARS agent.

Acknowledgements

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