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Identification and characterization of DNAzymes targeting DNA methyltransferase I for suppressing bladder cancer proliferation

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ABSTRACT

Epigenetic inactivation of genes plays a critical role in many important human diseases, especially in cancer. A core mechanism for epigenetic inactivation of the genes is methylation of CpG islands in genome DNA, which is catalyzed by DNA methyltransferases (DNMTs). The inhibition of DNMTs may lead to demethylation and expression of the silenced tumor suppressor genes. Although DNMT inhibitors are currently being developed as potential anticancer agents, only limited success is achieved due to substantial toxicity. Here, we utilized a multiplex selection system to generate efficient RNA-cleaving DNAzymes targeting DNMT1. The lead molecule from the selection was shown to possess efficient kinetic profiles and high efficiency in inhibiting the enzyme activity. Transfection of the DNAzyme caused significant down-regulation of DNMT1 expression and reactivation of p16 gene, resulting in reduced cell proliferation of bladder cancers. This study provides an alternative for targeting DNMTs for potential cancer therapy.

1. Introduction

DNA methylation is a major epigenetic modification that regulates gene expression in the genome of higher eukaryotes by DNA methyltransferases (DNMTs), which gained interest as the most important epigenetic factor [1]. DNMTs promote the covalent addition of a methyl group from S-adenosyl-L-methionine to cytosine, mostly within CpG dinucleotides [2]. In mammals, four members of the DNMT family have been identified: DNMT1, DNMT3A, DNMT3B and DNMT3L, among which DNMT1 is the most abundant and is essential for mammalian development and cancer cell growth [3]. Reactivation of tumor suppressor genes that have been silenced by an epigenetic mechanism is an attractive strategy for cancer therapy. There are several demethylating agents currently being evaluated in preclinical and clinical studies [4]. Despite the efficacy of azacitidine and decitabine, the relatively low specificity of these two drugs along with the substantial toxicity, poor bioavailability and instability in physiological media prompts the need to identify novel and more-specific DNMT inhibitors that do not function via incorporation into DNA [5].

DNAzyme (DNA enzyme or deoxyribozyme) molecules, generated in an in vitro selection system, are DNA sequences with inherit stability and catalytic activity, inducing the target RNA destruction in a highly sequence-specific way [6]. The “10-23” subtype of the DNAzymes contains 15 nucleotides catalytic core, flanked by complementary binding arms of 6–12 nucleotides in length to specifically target mRNA [7]. Contrast to the instability of ribozymes, the relatively stable chemistries of DNAzyme, especially those with structural modification of incorporation of a 3′–3′ inverted nucleotide at the 3′-end and phosphorothioate linkages in the binding arms, make DNAzymes as more promising oligonucleotide agents for drug exploitation. Compared with other oligonucleotide agents, DNAzyme exhibits more advantages as a nucleic acid-based therapeutic agent. For example, DNAzymes, being composed of DNA, are not only easier and less expensive to synthesize, but also much more resistant to degradation than RNA molecules *in vivo*. Accumulating evidence indicates the utility, efficacy, and potency of DNAzymes in a variety of animal models of disease and human cancers, allowing characterization of key molecular pathways underlying pathogenesis and use as a therapeutic agent [8–12]. Recently, well-designed clinical trials demonstrated that a c-jun and EBV-LMP1 targeted DNAzymes were efficacious and safe in patients with melanoma and nasopharyngeal carcinoma [13,14], which resurrects the clinical utility of DNAzymes.

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In the present study, we identified and characterized the DNMT1-targeted DNAzymes using bioinformatics and multiplex in vitro selection platform. These DNAzymes were validated in bladder cancer cells for their target-specific effect on DNA methylation and cell proliferation. The data provide experimental basis for the further preclinical study of the candidate DNAzymes.

2. Materials and methods

2.1. Cells and DNAzyme oligonucleotides

Human bladder cancer cells T24 (ATCC HTB-4) were cultured and maintained according to vendors' recommendations. All the oligonucleotides were made by Oligos Etc Inc (Oregon, USA) and purified by gel electrophoresis for in vitro studies and by HPLC for cell-based assays.

2.2. DNAzyme transfection of T24 cells

Approximately 6×10^5 T24 cells were seeded in each 60 mm dish 1 day before transfection. Cells reached approximately 80–90% confluency after being incubated at 37 °C overnight. Lipofectamine was used to transfect DNAzymes into the cells. Following 6 h post transfection, cells were allowed to recover in complete medium overnight and continued to be in culture for up to 48 h before subjected to further analyses.

2.3. In vitro multiplex selection of DNMT1 DNAzymes

A DNMT1 construct was constructed based on pcDNA3 expression vector containing the 4.8 kb coding region of the human DNMT1 (Genebank Accession # NP_001370). This construct was used to generate a 4.8 kb transcript. The transcript was made at 42 °C in high concentrations of NTPs (7.5 mM) using the Ampliscribe T7 Transcription Kit (Epicentre Technologies).

DNAzymes were designed based on "10-23" type (Fig. 1A) and fairly evenly covered the whole coding sequence of DNMT1. The DNMT1 DNAzymes were organized in groups corresponding to approximately 300 bases along the DNMT1 RNA. Fifteen Primer extension primers were designed to extend along the RNA corresponding to each of these groups. The template for each of the primer extension reactions was the DNMT transcript, which was cleaved by each group of DNAzymes. For each cleavage reaction, the transcript and DNAzymes were pre-equilibrated at 37 °C and then added together to give final concentrations of DNAzymes at 0, 5, 50 and 500 nM. The final concentration of transcript in each case was 30 nM. Primer extension was carried out on the cleavage transcripts using the ^{32}P end-labeled primers and Superscript. The resultant fragments were resolved on a 6% PAGE.

2.4. Kinetics analyses

2.4.1. Single-turnover kinetics

Each synthetic substrate was end-labeled with ^{32}P–γ–ATP. Labeled substrate and DNAzyme were pre-equilibrated in reaction buffer at 37 °C for 10 min before being combined at t = 0 to a final concentration of 40 nM and 320 nM respectively. The reaction was stopped at t = 0, 5, 10, 20, 30 and 60 min by mixing 2 μl aliquots of the reaction with an equal volume of stop buffer (90% formamide, 20 nM EDTA and loading dye). The cleaved and uncleaved products were separated on a 16% PAGE and the resultant bands quantified by ImageQuant. The percentage of band intensity in the cleavage products then analyzed graphically in a plot against time. A curve was generated for the data (least-squares) using the equation $\%P = \%P_\infty - C \cdot \exp[-kt]$, where %P is the percentage product, $\%P_\infty$ is the percentage product at t = ∞, C is the difference in %P between t = ∞ and t = 0, and k is the first order rate constant [15]. The first order rate constant (k_{obs}) was used to determine the cleavage efficiency.

2.4.2. Multiple-turnover kinetics

Each DNAzyme and labeled synthetic RNA substrate were pre-equilibrated in reaction buffer at 37 °C for 10 min before being

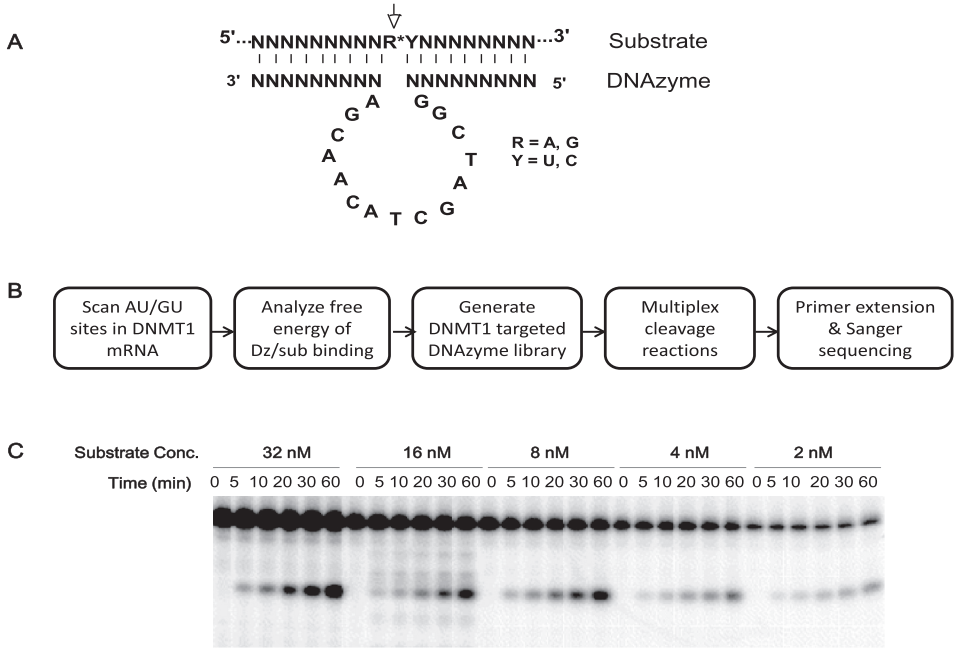

Fig. 1. In vitro selection scheme for DNMT targeted DNAzymes and kinetic analyses. A, schematic diagram of "10-23" DNAzyme. B, multiplex selection of DNAzymes. C, Representative gel of multiple turnover kinetic analysis of DT433.

combined at t = 0. The DNAzyme was at a final concentration of 0.2 nM, and the final concentration of substrate was 2, 4, 8, 16 and 32 nM. The reaction was stopped at t = 0, 5, 10, 20, 30 and 60 min by mixing 2 μl aliquots of the reaction with an equal volume of stop buffer (90% formamide, 20 nM EDTA and loading dye). The cleaved and uncleaved products are separated on a 16% PAGE and the resultant bands quantified by ImageQuant.

The values for k_{obs} (derived from the slope of these time course experiments at each substrate concentration) were used to generate a line of best fit in a modified Eadie-Hofstee plot (k_{obs} versus $k_{obs}/[S]$). In this expression the values for K_M and kcat were given by negative slope of the regression line and the y intercept, respectively.

2.5. DNMT1 enzyme activity assay

DNMT activity was measured using a DNA Methyltransferase Activity/Inhibition Assay Kit (Epigenetek, NY). Briefly, T24 cells were transfected with DNAzymes (1 μM) using Lipofectamine for 48 h. The unclear extracts were prepared for assaying methylation activity of DNMT from the cells with or without DNAzyme treatment. The unique cytosine-rich DNA substrate is stably coated on the strip wells. DNMT enzymes transfer a methyl group to cytosine from S-(5′-Adenosyl)-L-methionine p-toluenesulfonate (Adomet) to methylate DNA substrate. The methylated DNA was recognized with an anti-5-methylcytosine antibody. The ratio or amount of methylated DNA, which was proportional to enzyme activity, was colorimetrically quantified through an ELISA-like reaction. The sequence of DT433 is 5′-GGTTGGTGA GGCTAGCTACAACGA GGTTGTGCT-3′ (catalytic core is underlined). The control DNAzyme was a modified version of DT433 (same arm sequences with an inverted catalytic core as underlined: 5′-GGTTGGTGA AGCAAC ATCGATCGG GGTTGTGCT-3′).

2.6. Quantitative PCR to measure DNMT1 and p16 expression

Total RNA was extracted from T24 cells with different treatments. RNA samples were reverse transcribed using the SuperScript III First-Strand Synthesis SuperMix for qPCR Kit (Life Technologies) and quantitative real-time PCR (qRT-PCR) analysis was performed using the CFX-96 Real-time PCR System (Biorad). The primer sequences are listed as follows:

DNMT1 F: CCT AGT TCC GTG GCT ACG AGG AGA A
DNMT1 R: TCT CTC TCC TCT GCA GCC GAC TCA
p16 F: TGC AGA TAG ACT AGC CAG GGC
p16 R: CTC GCA GTT CGA ATC TGC AC

2.7. Cell proliferation assay

2×10^5 T24 cells were seeded in 24-well plate for overnight. DNAzyme or control (1 μM) combined with Lipofectamine 2000 transfection reagent and added to proliferating T24 cells for 3 consecutive days. After the final day's incubation the cells were counted and reseeded at equal concentration in a 96 well microtitre plate. After a further incubation for 3 days, the extent of proliferation was measured by MTS assay (Promega).

2.8. Statistical analysis

All of the data were presented as means ± SEM, and a p value less than 0.05 was considered to be statistically significant ($^*p < 0.05$).

3. Results

3.1. DNAzyme target site identification in DNMT1

To target DNAzymes to the DNMT1 mRNA, finding the most accessible sites is not trivial, given the wide range of potential cleavage sites and potential secondary and tertiary structures for this molecule. We previously established a multiplex cleavage assay for screening the entire length of a target RNA molecule for DNAzyme cleavage sites that are accessible [16]. In this study, we utilized the multiplex selection system to identify the most efficient DNMT1-targeted DNAzymes, as outlined in Fig. 1B. To bioinformatically identify all the potential cleavage sites for DNAzymes, all AU and GU sites in the DNMT cDNA were scanned and DNAzymes designed to target all of these sites. DNAzymes predicted to bind the substrate with a free energy (ΔG^0) > −25 kcal/mol were excluded. This left approximately 200 DNAzymes. This number was further reduced by selecting DNAzymes so that they fairly evenly covered the whole coding sequence, and discarded any with potential for G-quartet formation. This resulted in 79 DNAzymes (74 in the coding region + 5 in the UTR). Following cleavage reactions, primer extension was performed to assess both cleavage efficiency and the corresponding sequences of the target sites. Based on the grading according to their overall cleavage ability, 20 DNAzymes across the DNMT1 mRNA were shown in Table 1.

3.2. Biochemical characterization of selected DNAzymes in vitro

From the results from the multiplex selection, five promising DNAzymes (DT370, DT375, DT400, DT410 and DT433) were chosen for single-turnover and multiple-turnover kinetic analyses. In single-turnover kinetics, excessive amount of DNAzymes (320 nM) was incubated with 8-fold less amount of synthetic RNA substrate (40 nM) and the values of k_{obs} for each DNAzyme were obtained (Table 1). Among the DNAzymes tested, DT433 was the most efficient cleaver, with k_{obs} being 0.40. For multiple turnover kinetics, a fixed concentration of DNAzymes (0.2 nM) was incubated with excessive amount of the substrate (2, 4, 8, 16 and 32 nM). An example of a multiple-turnover gel was shown in Fig. 1C. By using a modified Eadie-Hofstee plot, the key kinetic parameters were

Table 1
Cleavage activity of DNMT1 DNAzymes in multiplex selection.

DNAzyme	Position	$-\Delta G^0$ (kcal/mol)	In vitro activity
DT364	238	32.2	−
DT370	**581**	**25.8**	++++
DT374	801	25.6	++
DT375	**839**	**25.8**	++++
DT376	915	24.7	++
DT379	1246	25.1	++
DT380	1302	27	+−
DT400	**2449**	**26**	++++
DT401	2583	32.4	++
DT407	3052	26.1	++−
DT408	3131	30	++
DT410	**3223**	**25.6**	+++
DT411	3385	31.2	++−
DT429	4561	28	+−
DT430	4607	28.3	−
DT431	4672	29.1	+
DT432	4778	28.6	+
DT433	**4816**	**26.3**	+++
DT434	4876	28	+
DT437	5045	27.6	++

−No Cleavage; +−Cleavage at 500 nM only; +Cleavage at 500 nM and faint band at 50 nM; ++Cleavage at 500 nM and 50 nM; +++Cleavage at 500 nM, 50 nM and 5 nM; ++++Strong cleavage at all concentrations.
DNAzymes in bold code were selected for further kinetic analysis.

Table 2
Single-turnover and Multiple-turnover kinetics results.[a]

Dz	Single-turnover kinetics		Multiple-turnover kinetics		
	Cleavage (%)	k_{obs} (/min)	Vmax (pM/min)	K_M (nM)	kcat (/min)
DT370	48.46	0.25	40.00	70.60	0.20
DT375	45.82	0.16	18.20	13.10	0.09
DT400	67.38	0.27	116.20	84.43	0.58
DT410	54.72	0.20	7.60	5.42	0.04
DT433	88.78	0.40	294.00	55.10	1.47

[a] Data were generated from the average of two independent experiments. Dz, DNAzymes.

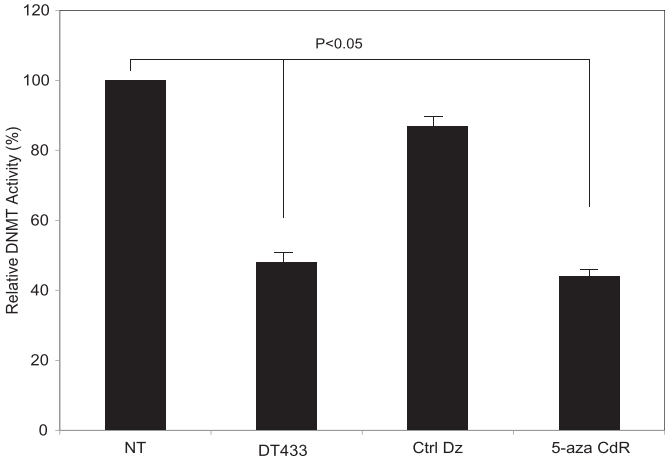

Fig. 2. Effect of DT433 on DNA methyltransferase expression and activity. T24 cells were transfected with the DNAzyme and controls (1 μM) for 48 h. Nuclear proteins were extracted and total DNMT activity was measured using EpiQuik™ DNA Methyltransferase Activity Assay Kit. 5-aza CdR was used as a positive control. Results from three experiments are presented.

generated (Table 2), showing that all the DNAzymes possessed multiple-turnover capacity with DT433 being the most efficient cleaver.

3.3. Effect of DNMT1-targeted DNAzymes on DNMT enzymatic activity

Based on the in vitro cleavage data, we selected DT433 for further analyses for its biochemical activities. We first measured the effect of the DT433 on DNMT activity of methylating cytosine-rich DNA substrate. Since DNMTs predominantly locate in nuclei, the nuclear extracts from DT433-transfected and control cells were used for the enzyme activity assay. As shown in Fig. 2, DT433 caused approximately 50% reduction in methylation activity, which

Table 3
Effect of inhibition of DNMT1 on T24 cell proliferation.

Treatment	[a]Suppression of cell proliferation (%)
DT433	58.5 ± 1.27*
Control DNAzyme	11.4 ± 0.99
5-Aza	43.5 ± 2.01*

*$p < 0.05$, compared with control DNAzyme.
[a] Values were obtained from triplicate wells of each sample from three independent experiments. Suppression of cell proliferation = (untreated − treated)/untreated.

was equivalent to the effect by DNMT1 inhibitor 5′Aza. This effect was not seen in the control DNAzyme-treated cells, suggesting that DT433 can be efficiently transfected into the cells, entered nuclei and cleaved target RNA, leading to decreased DNMT1 enzymatic activity.

3.4. DT433 inhibition of DMNT1 expression and re-activation of p16

To examine if DT433 could specifically inhibit the target gene expression, quantitative PCR was performed to the cells transfected with DT433 and control. Results showed that administration of DT433 into T24 cells significantly reduced the level of the DNMT1 mRNA compared with the control DNAzyme (Fig. 3A).

p16 has been known as a tumor suppressor gene and hypermethylation of the p16 promoter was observed in various tumors including bladder cancer [17]. We next investigated if down-regulation of DNMT1 could impact on the p16 expression by demethylation of the promoter. It was found that DT433 treatment of T24 cells led to an increase in p16 mRNA level, which was comparable to the effect of 5-Aza (Fig. 3B). Thus, DNAzyme mediated suppression of DNMT-1 caused reactivation of p16. Since p16 plays an important role in cell cycle control, we then further examined the effect of reactivation of p16 on cell proliferation by MTS assay. As shown in Table 3, treatment of T24 cells with DT433 or 5-Aza significantly inhibited cell proliferation, in comparison with either untreated or control DNAzyme treated cells. Together, targeting DNMT1 with DNAzyme could reactivate p16 expression, which subsequently resulted in suppression of cell proliferation.

4. Discussion

The past decades have seen the rapid evolution of gene-silencing strategies based on nucleic acids, among which DNAzymes with extraordinary RNA cleavage activity have already demonstrated their capacity for gene suppression both in vitro and *in vivo*, as well as in clinical settings. In the present study, we demonstrate another example of the DNAzyme potential for cancer therapy through careful choices of a therapeutic target within the mRNA that encodes DNMT1.

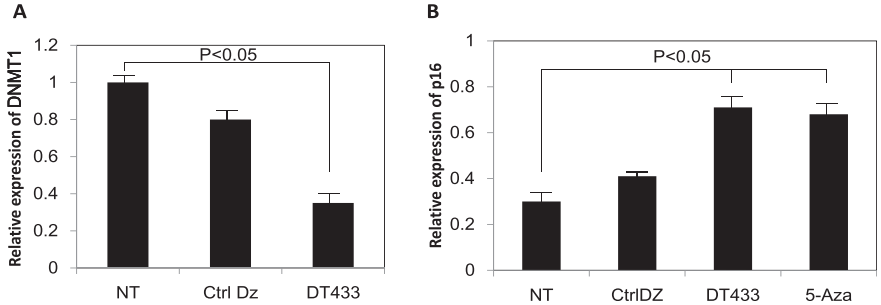

Fig. 3. Effect of DT433 on expression of DNMT1 and p16. T24 cells were transfected with the DNAzyme and controls (1 μM) for 48 h. Total RNA was extracted for qPCR analysis of DNMT1 expression (A) and p16 expression (B). Data were from three independent experiments.

Tremendous efforts have made to develop drugs that can be used to modify epigenetics via changing DNA methylation. One example is the known methylating drug 5-aza-2′-deoxycytidine, which has been around for many years, but had limited utility as it was associated with serious complications [18]. Although it is once more being used in the treatment of lung cancer in its ability to inhibit DNA methylation, clear clinical benefits are still awaited [19]. Thus, while widely recognized that DNMT1 is a drugable target, finding a suitable drug candidate that is both efficacious and less toxic is still challenging. Here, we performed a comprehensive selection of the potential DNAzymes against DNMT1 mRNA and obtained a lead molecule DT433. This molecule efficiently inhibited the DNMT1 expression and enzyme activity, and reactivation of p16 leading to suppression of T24 cell proliferation. This study provides an alternative for demethylation approach to cancer treatment by using a sequence-specific DNAzyme targeting DNMT1.

Taken together, evolving DNAzyme technologies present exciting opportunities not only for use in the validation of target gene function in research, but also for use in appropriate clinical settings. However, like other nucleic acid-based drugs, the most significant consideration for the potential therapeutic use of DNAzymes is the delivery of these molecules to the site of action in the target cell. Considering the high specificity and kinetic efficiency of the anti-DNMT1 DNAzymes, further preclinical validation of the lead molecule DT433, in conjunction with optimization of a targeted delivery system such as nanoparticles [20], is warranted.

Conflict interest

The authors declare no conflict of interest.

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Transparency document

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