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DNAzyme as a rising gene-silencing agent in theranostic settings

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Decades of biochemical studies have advanced DNA beyond its primary role as genetic blueprint. DNAzymes are single-stranded enzymatic DNA molecules that do not exist in nature. They are ideal candidates for gene silencing owing to their scalability by solid-phase synthesis (without batch variations), reprogrammability by directed evolution and local sequence alterations, compatibility with diverse delivery methods, and capability of achieving high catalytic turnover independent of any auxiliary proteins. With these unique features, various artificially evolved DNAzymes have been employed as theranostic tools in designing biosensors and logic gates, RNA/DNA cleavage and ligation, phosphorylation and dephosphorylation, DNA photorepair, and peptide side-chain modifications, to name but a few (Ponce-Salvatierra et al., 2021). This perspective will focus on the functional aspects and therapeutic potentials of RNA-cleaving DNAzymes.

Modus operandi: The best characterized DNAzymes to date are 8–17 and 10–23; both were discovered and enriched by iterative SELEX cycles from a library of 10^{14} chimeric molecules, each containing a 5′-biotin, 12 target ribonucleotides, and 50 random deoxynucleotides (N_{50}) (Santoro and Joyce, 1997). DNAzymes generally have a catalytic core flanked by two RNA bindings arms for target recognition. Target cleavage is initiated by metal-assisted deprotonation of 2′-hydroxyl in the RNA, generating a 2′,3′-cyclic phosphate upstream and a 5′-hydroxyl downstream, reminiscent of unrepaired "dirty" ends. The 8–17 DNAzyme specifically cleaves at A|G dinucleotide junctions in the presence of Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+} or Pb^{2+}. The compatibility of 8–17 with several biologically relevant cations may benefit its catalytic efficiency at a cellular level. The crystal structure of 8–17 unexpectedly revealed long-range base-pairing events that supported a catalytic pseudoknot. These long-range interactions include a Watson-Crick G6–C12 base pair and a non-canonical Hoogsten-sugar edge A5-G13 base pair (**Figure 1A** and **B**), whose substitutions completely abolish DNAzyme function (Wang et al., 2010). In contrast to 8–17, the 10–23 DNAzyme cuts RNA at any purine-pyrimidine dinucleotide junction (R|Y with R = A/G; Y = U/C), potentially offering a broader range of targetability. The folding of 10–23 preceding catalysis is also driven by increasing ionic concentration but the mechanism remains poorly understood. One structural study suggests that two 10–23 DNAzymes and two RNA targets collectively form a five double-helical bundle, which could yield a catalytically inactive conformation (PDB entry: 1BR3). Despite the apparent topological differences, both 8–17 and 10–23 share highly conserved residues (**Figure 1A** and **C**), and 10–23 might be a structural variant of 8–17 (Wang et al., 2010).

Design considerations: When designing DNAzymes for gene silencing purposes, several factors could impact the experimental outcome. (1) We and others have noticed that the length of RNA binding arms significantly contributes to overall catalytic efficiency (Silverman, 2005; Zhang et al., 2021). Longer arms generally correlate with tighter RNA binding and higher cleavage efficiency, to a point where product release becomes rate-limiting. However, longer binding arms may be more tolerant to mismatches, thus causing off-target cleavage. (2) Chemical modifications may unpredictably affect DNAzyme function. Modification strategies, including backbone phophorothioate linkages, 2′-O-methylribonucleotides, inverted 3′-3′ thymidine nucleotides, peptide nucleic acids (PNAs), or xeno nucleic acid analogues (such as locked nucleic acids [LNAs] and 2′-fluoro-arabinonucleic acids [FANAs]), are often incorporated into DNAzyme to enhance stability and RNA binding affinity and to discourage nuclease degradation. In our experience, a complete substitution of 8–17 binding arms with PNA resulted in poor folding and catalysis, while LNA substitutions at terminal residues retained enzymatic activity (Zhang et al., 2021). In parallel, a recent study shows that a 10–23 hybrid DNA-FANA-TNA (α-L-threofuranosyl nucleic acid) design works most efficiently in multiple turnover kinetics *in vitro* (Wang et al., 2021). (3) Substitutions of non-essential catalytic residues can increase (or decrease) DNAzyme activity and eliminate CpG-motifs that are potentially recognized by Toll-like receptors. (4) Similar to antisense oligonucleotides (ASOs), "naked" DNAzyme should achieve wide distribution in the central nervous system of rodents and non-human primates via intracerebroventricular or intrathecal bolus injections (Jafar-Nejad et al., 2021). Biochemical conjugation of DNAzyme to nanoparticles, DNA origami, gold particles, liposomes, antibodies, sugar moieties (such as GalNAc) or even to itself (divalent DNAzymes) can be readily attainable, further improving its bioavailability and efficacy.

Preclinical applications in neurodegeneration: There is no effective treatment for neurodegeneration to date. Given the recent clinical setbacks faced by ASOs, DNAzymes may provide an alternative, protein-independent route to gene silencing. Earlier studies have demonstrated the feasibility of reducing toxic RNA species using 10–23 DNAzymes in Huntington's disease and multiple sclerosis. However, these results were largely obtained from biochemical cleavage of purified RNA or cells that were not specific to disease etiology. We recently designed a repeat-based, LNA-modified 8–17 DNAzyme and used it to target expanded mRNA/protein species across several polyglutamine neurodegenerative diseases (Zhang et al., 2021). We discovered that (1) DNAzyme could invade CAG RNA hairpins to initiate cleavage and the catalytic efficiency was ≈ 40% higher with Ca^{2+} than with Mg^{2+}; (2) DNAzyme was highly efficacious in neuronal-like cells and excitatory cortical neurons (90% target clearance); (3) allele-specificity could be achieved in certain patient-derived cells; (4) no significant off-target cleavage was observed on > 60 mRNAs containing short CAG-repeats; (5) DNAzyme was stable in mouse brain for at least 1 month and successfully cleared high molecular weight polyglutamine proteins *in vivo*; (6) DNAzyme treatment rescued cell proliferation defects even beyond the normal cell level, which is in direct contrast to elevated cell death by repeat-based ASO treatment; (7) no toxicity (such as LNA-induced hepatotoxicity), gliosis, or elevated animal death were observed *in vivo*. Further optimization of 8–17 core and binding arms, elevated dosing regimen, and packaging with ionizable lipids to escape endosome may benefit the efficacy of our lead DNAzyme *in vitro* and *in vivo*.

DNAzyme in clinical trials: To date, three variants of 10–23 DNAzyme have been evaluated in clinical trials. (1) The inverted thymidine-modified SB010 (hgd40) DNAzyme was designed to target the master transcription factor GATA-3 in patients who suffer from allergen-induced asthma in Germany. GATA-3 is essential for T-helper 2 cell differentiation and the subsequent cytokine production. In this double-blind, multicenter clinical trial (Krug et al., 2015), treatment of patients (21 drug *vs.* 19 placebo, 10 mg daily inhalation for 28 days) attenuated early and late asthmatic response by 11% (*vs.* 10% increase in placebo) and 34% (*vs.* 1% increase in placebo) respectively. SB010 is currently studied in phase I/II clinical trials for ulcerative colitis and atopic dermatitis, but its indication can potentially extend to cancer therapies, since GATA-3 is mutated in > 10% of breast cancers. (2) The Dz13 DNAzyme was designed to target c-Jun whose function has been implicated in tumorigenesis. In the DISCOVER trial (Cho et al., 2013) – the first reported human trial on DNAzyme – nine patients who suffered from basal-cell carcinoma were injected intratumorally with Dz13 (10, 30 and 100 µg doses, 3 patients per dose). The expression of c-Jun mRNA was reduced in all nine patients, and tumor depth was decrease in 56% of

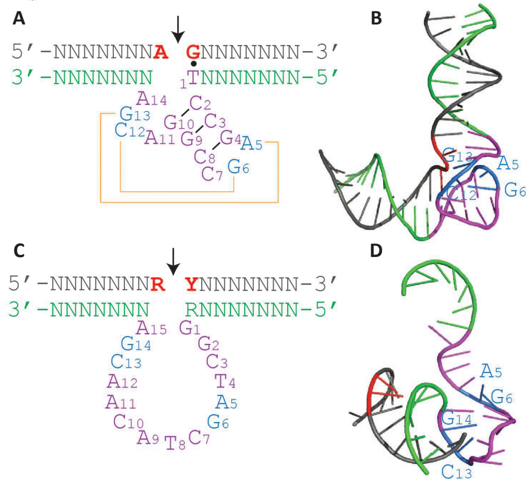

Figure 1 | Sequence and structure of 8–17 (A and B) and 10–23 (C and D) DNAzymes (PDB entries 5XMA and 1BR2).
The RNA binding arms are highlighted in green, catalytic core in purple, highly conserved residues in blue, cleavage sites in red, and RNA targets in grey. R = A/G; Y = U/C. It should be noted that the 10–23 DNAzyme structure is extracted from a 5-double helical bundle which could represent a catalytically inactive state (Nowakowski et al., 1999).

patients. Infiltration of immune cells was also stimulated. (3) The phosphorothioated Dz1 DNAzyme was designed to target Epstein-Barr virus-LMP1 in Chinese patients with nasopharyngeal carcinoma (Cao et al., 2014). Forty LMP1-positive patients received an intratumoral injection of 6 mg Dz1 (n = 20) or saline (n = 20) in conjunction with radiation therapy. The tumor regression rate at week 12 was significantly higher in the Dz1 group vs control, and the level of Epstein-Barr virus DNA copy was significantly lowered by Dz1 treatment. Taken together, DNAzyme shows clinical efficacy without drug-related adverse effects in all three clinical trials. The efficient delivery of nebulized DNAzyme across the nuclease-rich mucosa layer into the lungs is particularly attractive as it offers a direct administration route for pulmonary diseases such as COVID-19. We have developed several 8–17 DNAzymes that target highly conserved RNA regions across 5 viral essential genes; these DNAzymes are insensitive to viral mutations.

In conclusion, DNAzyme is gaining exponential growth as potent biocatalysts alongside proteins and ribozymes. Compared to protein-based gene-silencing agents, DNAzyme may excel at allele-specific cleavage by placing the single nucleotide polymorphism at the recognition site; this could be challenging for ASO or siRNA due to thermodynamic constrains of nucleotide hybridization and mismatch tolerance. One of the major challenges associated with DNAzyme applications is the high divalent cation requirement for maximal activity. For instance, DNAzyme can approach a catalytic efficiency (kcat/km) of 10^9 min^{-1} M^{-1} at 10–50 mM Mg^{2+} concentration, but the total Mg^{2+} concentration in mammalian cells ranges from 17 to 20 mM. In addition, the cytosolic free Mg^{2+} concentration is estimated around 1 mM (Yamaguchi and Ishikawa, 2008). We think that DNAzyme could function as a Mg^{2+} sink and carry out catalysis with physiologically relevant ion influx. This is because Mg^{2+} as a DNA stabilizing counterion can reach 1–2 M concentration between DNA helix axes of 2–4 nm. With improved SELEX methods, new DNAzymes favor low ionic strengths (or monovalent cations such as Na^+ (Torabi et al., 2015)) can be artificially selected. In addition, rule-based algorithms combined with RNA 3D modeling and high throughput screening will streamline DNAzyme rational design and target selection. Another hurdle for DNAzyme therapy is delivery. In light of Moderna and BioNTech mRNA vaccines, DNAzyme (untreated or pre-soaked in cations) can be packaged in liposomes and released from endosome by ionizable lipids. Altering liposome composition or conjugating liposome with ligands will facilitate tissue specificity and the crossing of the blood-brain barrier. Furthermore, the use of moloney mouse leukemia virus reverse transcriptase allows vector-based DNAzyme expression that is compatible with viral delivery (Li et al., 2010). DNAzyme continues to expand its therapeutic frontiers, such as gene editing (e.g., PNA-assisted DNAzymes to generate double-stranded breaks) and epigenetic modification (e.g., m6A *vs* unmodified A at cleavage site) – and serves as a valuable tool in monotherapy or adjuvant therapy to tackle incurable diseases.

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