DNAzyme mediated post-transcriptional gene silencing: A novel therapeutic approach

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Abstract

The increasing understanding of the regulatory mechanisms involved in the progression of diseases is opening up opportunities for new therapeutic intervention. DNAzymes, derived by in vitro selection processes, is one such discovery that has potential for selective gene silencing. The first report on DNAzymes came nearly eighteen years ago and since then hundreds of DNA sequences have been isolated in many research laboratories around the world to facilitate many chemical transformations of biological importance. These catalytic molecules have been successfully used against several targets including viruses, cardiovascular diseases, cancers and brain disorders with commendable results. The recent years have witnessed the use of these catalytic molecules for various innovation driven applications ranging from biosensing to gene regulation. This review will discuss the discovery, synthesis and applications of DNAzymes as potential therapeutic molecules.

Introduction

The current advances in molecular genetics have revealed new tools for selective gene silencing. Novel molecules are constantly being discovered and developed to find better means of managing debilitating and fatal diseases, which include cancer and several infectious diseases. The development of catalytic nucleic acids exhibiting selective and specific binding properties towards molecular targets has added important tools for effective down-regulation strategies in chemical biology and medicine (Ellington and Szostak, 1990). Traditionally, DNA molecules were thought to have the sole function of carrying and passing genetic information from one generation to another, but about 30 years ago, however, DNA molecules began to find a new role in the field of materials science (Seeman, 2003). DNAzymes, also known as deoxyribozymes or DNA enzymes, refer to the single-stranded DNA molecules with catalytic capabilities. These nucleic acid molecules are generated de novo by in vitro selection which is a powerful technique that has been routinely used to isolate extremely rare DNA or RNA sequences with a function of interest (e.g. ligand-binding or catalysis) from a large population of single-stranded DNA or RNA molecules. The DNA based drugs are of significant advantages over currently available low molecular weight pharmaceuticals because of their selective recognition of molecular targets and pathways, which imparts tremendous specificity of action. Of all the DNAzymes catalyzing various functions like RNA ligation (Silverman, 2009), carbon–carbon bond formation (Chandra and Silverman, 2008) and the hydrolytic cleavage of DNA, the RNA-cleaving DNAzymes remain the best characterized and likely the most significant class of catalytic DNA molecules currently available. Naturally occurring DNAzymes have not been reported till date and the obvious reason is that, except for few viral genomes and replication intermediates, DNA molecules are predominantly double-stranded.

The recent years have witnessed the successful use of DNAzymes in medicine and therapeutics like in cardiovascular diseases, cancer and brain disorders. These molecules have also shown excellent results in inhibiting the replication of many RNA viruses such as the hepatitis (Oketani et al., 1999), HIV (Singh et al., 2012) and influenza (Kumar et al., 2012).

Post-transcriptional gene silencing (PTGS)

Post-transcriptional gene silencing (PTGS) applies to RNA interference (RNAi) in animals, and to some types of virally- and transgene-induced silencing in plants. The transcription of the gene is unaffected; however, gene expression is lost because mRNA molecules become unstable. A rapid evolution in the field of gene silencing strategies has taken place in the past decade following the enhanced understanding of gene functions in the pathogenesis of a disease. The development of catalytic nucleic acids exhibiting selective and specific binding properties towards molecular targets has added important tools for effective down-regulation strategies in chemical biology and medicine (Ellington and Szostak, 1990).

Among the several range of agents that encompasses gene silencing therapeutics, the DNAzymes (Dz), ribozymes (Rz) and antisense molecules (Illustration 1) have shown proven results in down-regulating the replication of many pathogens in mammalian hosts (Akkina et al., 2003; Santoro and Joyce, 1997). Traditionally, DNA molecules were thought to have the
sole function of carrying and passing genetic information from one generation to another, but about 30 years ago, however, DNA molecules began to find a new role in the field of materials science (Seeman, 2003).

**DNAzymes**

DNAzymes, also known as deoxyribozymes or DNA enzymes, refer to the synthetic catalytic single-stranded deoxyribonucleic acid molecules that demonstrate precise substrate recognition and are capable of sequence-specific cleavage of mRNA molecules with greater biological stability (Akhtar et al., 2000). The credit goes to Ronald R. Breaker and Gerald F. Joyce who first discovered a Pb2+ dependent RNA cleaving DNAzyme in 1994 (Breaker and Joyce, 1994). The discovery of DNA as an enzyme was unexpected as compared to that of RNA as an enzyme because of the presence of 2'-hydroxyl group in the latter, which was thought to play a central role in ribozyme function. DNAzymes have tremendous potential as gene suppression agents for a variety of therapeutic applications and has high RNA cleavage specificity and activity as compared to the ribozymes, while maintaining the robust chemistry of an oligodeoxynucleotides (Cairns et al., 2002). The central catalytic motif is flanked by two arms (arm I and II) of complementary sequence that bind to the target RNA molecules on a Watson-Crick basis. The catalytic efficiency is dependent on metal ions and breaks the phosphodiester bond between purine and pyrimidine nucleotides (Santoro and Joyce, 1998). Despite its small size, the DNA enzyme has a catalytic efficiency (k cat/K m) of ≈10^8 M^-1 min^-1 under multiple turnover conditions, exceeding that of any other known nucleic acid enzyme (Santoro and Joyce, 1997). Unlike in RNAs, the absence of a 2'-hydroxyl group having the potential to act as a nucleophile makes DNA ≈100,000 fold more stable and ideal for storing genetic information but precluding it from adopting complex secondary and tertiary structure (Breaker, 2000). The DNA motifs confer improved biological stability to DNAzymes as compared to the most active ribozymes, a yet another powerful tool for gene silencing. These DNA based drugs are of significant advantages over currently available low molecular weight pharmaceuticals because of their selective recognition of molecular targets and pathways, which imparts tremendous specificity of action. Of all the DNAzymes catalyzing various functions like RNA ligation, (Silverman, 2009) carbon–carbon bond formation, (Chandra and Silverman, 2008) and the hydrolytic cleavage of DNA, the RNA-cleaving DNAzymes remain the best characterized and likely the most significant class of catalytic DNA molecules currently available. Naturally occurring DNAzymes have not been reported till date and the obvious reason is that, except for few viral genomes and replication intermediates, DNA molecules are predominantly double-stranded.

**Synthesis of DNAzymes**

DNAzymes do not occur naturally but were instead generated de novo by an in vitro selection process and the credit goes to Ronald R. Breaker and Gerald F. Joyce who first discovered a Pb2+ dependent RNA cleaving DNAzyme in 1994 (Breaker and Joyce, 1994). A library of chimeric molecules consisting of a single ribonucleotide embedded with fixed primer domains, a random sequence of 50 oligonucleotides and a 5' biotin moiety were generated. The sample population from these combinatorial libraries was immobilized on a solid support containing streptavidin via a biotin tag. Following streptavidin binding; alkali denaturation removed the nonbiotinylated strand. RNA phosphoester bond were cleaved in the presence of divergent metal cation cofactor which yielded a catalytically active population in the streptavidin column eluant, which was further amplified with PCR and added back to the next round of selection. This approach, on repetition, eventually yielded enough quantities of selected DNA fractions that were then examined for cleavage by electrophoresis, before cloning and finally sequencing (Breaker and Joyce, 1994; Sun et al., 2000). Santoro and Joyce identified two catalytic DNAzyme motifs, 8-17 and 10-23 that have been known to be widely used for gene suppression. Each enzyme motif recognises nucleotide residues within the target RNA resulting in phosphodiester bond cleavage and the generation of two RNA fragments. The sequence of the substrate can be changed without loss of catalytic activity for both the 8-17 and 10-23 DNAzymes, as long as the substrate-binding arms of the enzyme are changed in a complementary manner. Initially divergent cations were used for cleaving the target RNA (Breaker and Joyce, 1994), however, after further developments of this protocol, highly efficient magnesium-dependent DNAzymes capable of cleaving all RNA substrates in biologically relevant environments were identified (Santoro and Joyce, 1997). From this selection experiment, two prototypes were characterized and denoted as the “10-23” and the “8-17” RNA-cleaving DNA enzymes.

**The 10-23 DNAzymes**

The same in vitro selection procedure was used by
Stephen W. Santoro and Gerald F. Joyce in 1997 who discovered a DNAzyme that can cleave RNA phosphodiester bond in RNA substrate and is considered as the general purpose RNA-cleaving DNA enzyme (Santoro and Joyce, 1997). The 10-23 DNAzymes are the most widely used as they have the ability to cleave almost any RNA sequence with high specificity and kinetic efficiency, with rates approaching and even exceeding those of other nucleic acid and protein endoribonucleases. The 10-23 DNAzymes obtained its name from its origin as the 23rd clone from the 10th cycle of in vitro selection. The structure is made up of a catalytically active core of 15 nucleotides, flanked by substrate binding arm. It will cleave any RNA substrate at a phospho-diester bond located between an unpaired purine (A, G) and a paired pyrimidine (U, C) in the presence of Mg2+ producing two cleaved products one with 2'-3' cyclic phosphate and another with 5'-OH at the end (Illustration 2).

Thus the AUG (start codon) of any gene could be an efficient target for this nucleic acid enzyme. The catalytic efficiency (k cat/K m) of this enzyme is found to be ~10^8M⁻¹min⁻¹. It was observed amongst the variable substrate binding domain length, which ranged between 4/4 and 13/13, the maximum overall efficiency (k cat/K m) under physiological reaction conditions was found with an arm length of between 8 and 9 bp (Santoro and Joyce, 1998). The rate of deoxyribozyme-catalyzed cleavage, in some instances, has also been shown to be enhanced by asymmetric arm length truncation in the binding domains. An accessible cleavage site within the secondary structure of the mRNA target is necessary for optimal DNAzyme activity as the stability of the DNA–RNA heteroduplex is necessary for DNAzyme catalytic efficiency (Khachigian, 2000). The kinetic efficiency of any 10-23 deoxyribozyme increases to a maximum with a slight increase of the heteroduplex stability above the minimum threshold level and this efficiency may fall drastically if this stability falls below the threshold by introduction of any mismatch or truncation in the binding domain. The general structure of the 10–23 DNAzyme is shown in Illustration 3.

The 10-23 DNAzymes are more widely used since their catalytic motif is highly generalizable with respect to substrate sequence as compared to the 8-17 motifs. In a study by Volker A Erdmann’s group, each and every residue, one at a time, within the 15 nucleotides catalytic core sequence was deleted to observe the catalytic ability of remaining sequences and it was observed that the Thymine nucleotide at position 8 in the 15 nucleotides sequence was least important followed by cytosine at position 7 (Zaborowska et al., 2005).

The 8-17 DNAzymes

The 8-17 DNAzymes can cleave an RNA sequence at a phospho-diester bond located between an A and G residue in the presence of a divalent cation, with activity following the order Pb2+ >> Zn2+ >> Mg2+. The catalytic domain consists of 13 nucleotides with a four nucleotide loop adjacent to the cleavage site and a stem loop region that resembles the "stem-loop II" region of the hammerhead ribozyme. This stem loop region is essential for the catalysis (Mazumdar et al., 2009). The 8-17 DNAzymes have a special requirement of the nonstandard G–T (substrate–DNAzyme) base pair at the cleavage site, an internal 3 bp stem of which at least two were G–C base pairs, an invariant AGC triloop, and a short unpaired region of sequence WGR (W=A or T; R=A or G or Substitution of a Watson–Crick pair at rG-dT “wobble” pair located immediately downstream from the cleavage site eliminates catalytic activity, thereby making it less popular as compared to the 10-23 DNAzymes (Santoro and Joyce, 1997). A survey of several different combinations of RNA substrate and corresponding DNA-enzyme revealed that the 10-23 motifs was highly generalizable with respect to substrate sequence as compared to the 8-17 motif which further adds to the popularity of 10-23 DNAzymes.

Stability of DNAzymes

One of the major difficulties in DNAzyme mediated gene silencing is the low efficiency of cellular uptake of DNAzyme when supplied externally. 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. Previously, site-specific phosphorothioate (PTO) modifications or structural changes have been employed to provide protection to single-stranded DNA, e.g., antisense oligonucleotides, from degradation by serum exo- and endonucleases (Galderisi et al., 1999), but PTO modifications of oligodeoxynucleotides are often burdened by several disadvantages. In DNAzymes, PTO modifications may reduce the catalytic activity (Wu et al., 1999). Furthermore, it has been shown that PTO-modified DNA molecules can react as topoisomerase inhibitors in dividing cells, which may induce malignancies (Burgin, 2001). The dosage of PTO modified DNA is also limited by nonspecific toxic side effects (Levin, 1999). It has been shown that the DNAzymes can be made...
circular by cloning it in a suitable vector (Illustration 4) so that the DNAzymes would be more stable against nuclease activities (Chen et al., 2004) or by ligating it to a oligonucleotide sequence to make a circozyme (Seifert et al., 2006). These circozymes have shown effective stability for more than two days post transfection as compared to linear DNAzymes which are generally stable for around 24-30 hrs. The DNAzyme stability may also be improved by the addition of a 3–3′ inverted nucleotide at the 3’ end of the DNAzyme. This modification can increase the stability of the molecule and has been shown to extend DNAzyme half life from 70 min to 21 h in human serum (Dass et al., 2002). A development in DNAzyme design is that of the locked nucleic acid (LNA). LNA-modified DNAZymes have higher catalytic rates and sensitivity to target than that of unmodified DNAZymes (Fahmy and Khachigian, 2004). The introduction of hairpin DNAzymes, where stem-loop hairpins are added to the end of the substrate-binding arms, display resistance to nucleolytic degradation for up to 3 days after transfection and produce better gene knockdown than non-hairpin DNAzymes with the same catalytic domain (Abdelgany et al., 2007).

Applications of DNAzymes

10?23 DNAzyme, owing to its RNA phosphodiesterase activity and having ubiquitous cleavable site had been widely used in silencing several genes of therapeutic interest.

Inhibition of Viral replication

The recent years have witnessed the successful use of DNAzymes to inhibit the replication of many RNA viruses such as the hepatitis (Oketani et al., 1999), HIV (Singh et al., 2012) and influenza (Kumar et al., 2012). Several strategies are being explored for developing the antiviral agents against highly pathogenic viruses. Both the sub-genomic as well as full-length replicon systems have been targeted by antiviral candidates (Blight et al., 2002). Although there are several known sites for DNAzyme activity on the influenza virus mRNA, most of them do not work efficiently and cleave poorly or not at all when used against a longer mRNA length, however in a recent study, authors have shown the down-regulation of the short mRNA length of influenza A virus by novel DNAzyme (Kumar et al., 2013). Interference in the initial obligatory step of RNA translation have direct consequences on viral replication. The successful post-transcriptional gene silencing results obtained by several authors have paved new pathway to an improved and effective management of many respiratory infections like influenza and RSV.

Treatment of cardiovascular diseases

Marked improvement in the pharmacotherapies and mechanical treatments has taken place in recent years, yet the cardiovascular disease remains a principal cause of morbidity and mortality worldwide (Mark et al., 2007). Early growth response (Egr-1) is a transcription factor which is required for SMC (smooth muscle cell) and endothelial cell recovery from mechanical injury in vitro. SMC proliferation is a characteristic of neointima formation following angioplasty. ED5, a DNAzyme targeting the A816U junction within the start codon of rat Egr-1 was developed by Santiago et al. (Santiago et al., 1999), which cleaved a short synthetic Egr-1 RNA substrate and in vitro transcribed mRNA of various lengths, in a sequence-specific manner. ED5 inhibited the upregulation of Egr-1 protein and thus inhibited neointima formation.

In another study, the Egr?1 silencing through intracoronary delivery of a targeting DNAzyme at the time of reperfusion following acute myocardial ischemia decreased myocardial inflammation and apoptosis leading to improved cardiac function (Rayner et al., 2013).

PAI-1 (plasminogen activator inhibitor-1) is one of the marker of cardiovascular disease, levels of which are elevated in patients with restenosis, atherosclerosis and acute myocardial Infarction (AMI). DNAzyme against PAI-1 has been shown to inhibit the transforming growth factor β-mediated PAI-1 stimulation in endothelial cells and also decrease the PAI-1 expression (Xiang et al., 2004).

Cancer treatment

The bcr-abl oncogene, also known as the Philadelphia chromosome (Ph), is detected in >95% of patients with chronic myelogenous leukemia (CML) and in 20%–30% of adults with acute lymphoblastic leukemia (ALL). A study demonstrated that the DNAzymes targeting bcr-abl efficiently cleave only their respective bcr-abl substrates, which led to down-regulation of protein expression and significant inhibition of the proliferation of Ph+ malignant cells (Wu et al., 1999).

DNAzymes have tremendously been used in cancer research. The DNAzymes against the Egr-1 has been shown to suppress tumour growth in breast cancer (Mitchell et al., 2004). The Aurora kinase A gene has been successfully targeted by specific DNAzymes to inhibit the proliferation and metastasis, promoting apoptosis and down-regulating telomerase activity in prostate cancer (Qu et al., 2008).

The role of DNAzymes have also been well established in skin cancer wherein the authors have
demonstrated the capacity of a DNAzyme-targeting c-jun mRNA, Dz13, to inhibit growth of two common skin cancer types—basal cell and squamous cell carcinomas—in a therapeutic setting with established tumors (Cai et al., 2012).

**DNAZymes in brain and muscle disorders**

A study on Huntington’s disease has been done using specific DNAzymes for destruction of the mutant huntingtin mRNA using a specific DNAzyme that was able to cleave the mutant huntingtin mRNA in a sequence-specific manner. The mRNA silencing led to significant reduction of mutant huntingtin protein expression in mammalian cells (Yen et al., 1999).

These catalytic nucleic acids have been also used in Glioblastomas that are the most frequent malignant brain tumors, which can progress from lower grade gliomas or arise de novo. The DNAzymes have been exploited to tackle glioblastoma through RNA targeted strategies (Benson et al., 2008).

The muscle acetylcholine receptor (AChR) is expressed at the neuromuscular junction, and plays the critical role in nerve to muscle signal transmission. A number of mutations have been detected in the AChR subunit gene which affect receptor function and give rise to slow channel congenital myasthenic syndrome. AChR have been targeted by specific DNAzymes with successful results (Abdelgany et al., 2005).

**DNAZymes and nanotechnology**

DNAzymes have shown great promise as a general platform for detecting metal ions, as many metal-specific DNAzymes can be obtained using in vitro selection. Recently authors have demonstrated the use of gold nanoparticles (AuNP) with DNAzymes. These DNAzyme-AuNP probe could readily enter cells and served as a metal ion sensor within a cellular environment, making it the first demonstration of DNAzymes as intracellular metal ion sensors (Weichen et al., 2013). This method can also be applied to the detection of other metal ions using other DNAzymes selected through in vitro selection.

**DNAZymes and biosensing applications**

Heavy metal ion contamination can often pose significant hazards to the health of the general public and the environment. Isolated from combinatorial in vitro selection experiments, DNAzymes often possess high metal selectivity, such as the 8-17 and the classic lead-dependent DNAzyme (Breaker and Joyce, 1994). These RNA-cleaving DNAzymes are specific for a wide range of metal ions, have small catalytic domains, and have a fast reaction rate. Combined with the high stability and the low cost of synthesis for DNA, DNAzymes represent good candidates for biosensing applications (Lu, 2002). Similarly, metal ions such as lead or copper can be detected by nucleic acid strands that become catalytic when they bind around metals. Once catalytically active, these metal-dependent DNAzymes cleave a specific part of a DNA sequence, which acts as a fluorescent signal advertising the metal's presence.

**Conclusions**

DNAzymes have been shown to be catalyzing various functions like RNA ligation, carbon–carbon bond formation and the hydrolytic cleavage of DNA. The RNA-cleaving DNAzymes remain the best characterized and likely the most significant class of catalytic DNA molecules currently available. These synthetic catalytic nucleic acids have gained importance in several research areas including cancer (Wu et al., 1999), cardiovascular diseases (Khachigian et al., 2002), allergy and various other disorders (Yen et al., 1999). In case of influenza viruses, the annual formulation of vaccine is necessary to manage the disease burden owing to frequent mutations in the viral genome. The DNAzymes provides promising approach to inhibit the replication of these viruses at post transcriptional level and thereby paving new dimensions in the management of epidemics and occasional pandemics due to influenza A viruses. DNAzymes are, thus, promising tools for mRNA targeting in order to inhibit protein translation in experimental settings and there might be possibilities for therapeutic applications in vivo.

**References**

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Illustrations

Illustration 1

MicroRNA biogenesis and RNA-induced gene silencing. In the cytoplasm, Pre-miRNA is processed by Dicer to the mature micro RNA duplex. The duplex loads onto Argonaut ribonucleases in the RISC complex and separates. One of the mature miRNA strands mediates small interfering RNA silencing by degrading the target mRNA or interfering with translation. The DNAzyme and ribozymes bind to their target in gene specific manner and cleave the target mRNA.
RNA-phosphodiesterase activity of 10-23 DNAzyme in presence of divalent metal ion results into two cleaved products, one with 2′,3′-cyclic phosphate and another with 5′-OH at the end.
Illustration 3

Composition of the 10-23 and 8-17 DNAzyme catalytic motifs. The DNA enzyme (bottom 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.
Structure of the circular DNAzymes and their RNA target. Two circular DNAzymes were designed to cleave the TEM spectrum β-lactamase mRNA at the indicated sites in the initiation and coding regions, respectively. They were constructed through cloning 10-23 DNAzyme into M13mp18 vector. The arrows indicate the cleavage sites of the substrates.