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DNAzyme-based therapeutics for cancer treatment

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Gene-silencing strategies based on catalytic nucleic acids have been rapidly developed in the past decades. Ribozymes, antisense oligonucleotides and RNA interference have been actively pursued for years due to their potential application in gene inactivation. Pioneered by Joyce *et al.*, a new class of catalytic nucleic acid composed of deoxyribonucleotides has emerged via an *in vitro* selection system. The therapeutic potential of these RNA-cleaving DNAzymes have been shown both *in vitro* and *in vivo*. Although they rival the activity and stability of synthetic ribozymes, they are limited by inefficient delivery to the intracellular targets. Recent successes in clinical testing of the DNAzymes in cancer patients have revitalized the potential clinical utility of DNAzymes.

Due to the simple four-nucleotide chemistry and standard Watson–Crick pairing, nucleic acids are appealing targets for exogenous regulation of gene expression. Utilizing hybridization to achieve artificial gene suppression is mostly related to the involvement of single-stranded oligodeoxynucleotides and double-stranded siRNA based approaches, both of which have been developed in laboratory and clinical applications. Other nucleic acids-based technologies may also find applications in this field. Gene suppression mediated by RNA-cleaving catalytic DNA has been one of the promising strategies for such applications. These molecules recognize their targets by hybridization and then cleave the target without assistance of any host-encoded proteins. The nuclease-independent catalytic DNA or DNAzymes open a new field that is beyond the scope of RNAi-dependent siRNA and ribonuclease H-dependent oligodeoxynucleotides.

In 1994, Breaker and Joyce proposed that DNA could have catalytic activity as for ribozymes. However, no naturally occurring DNAzyme was found because DNA always presents as a complete duplex. Thus, they came up with an *in vitro* selection system to obtain DNAzymes that cleaved RNA. The system was based on hydrolytic cleavage of a phosphodiester and nested PCR [1–3]. First, they established a pool of 1014 ssDNA molecules. Each one contained a 5′ biotin moiety, followed by a 50 random deoxyribonucleotides domain which was flanked by fixed sequence. Then these molecules were combined to a streptavidin affinity matrix and washed with buffer to remove the unbound. Next, the same buffer containing certain cation passed through the matrix to cause cation-dependent cleavage of phosphoester and release the catalytic DNAs. These DNAs were collected and amplified by nested PCR, reintroducing the 5′ biotin and target phosphoester. After serveral rounds of selection, DNAzymes were obtained.

Further biochemical characterization revealed that the DNAzymes varied in their sequences while all of them had two basic domains, catalytic domain and substrate-binding domain. Based on the structures, two types of DNAzymes selected via *in vitro* system, the '8–17' DNAzymes and '10–23' DNAzymes were proposed (Figure 1).

The '8–17' DNAzyme was derived from the 17th clone obtained after eight rounds of selective amplification and '10–23 DNAzyme was similarly obtained from the 23rd clone

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Key terms

DNAzyme: A DNA molecule that has the ability to perform catalytic action.

LMP1: A critical oncogenic protein encoded by EBV.

c-jun: A transcription factor that is upregulated in many types of cancers.

after ten-round amplification. A structure of '8–17' DNAzymes have a 13-nt-long catalytic core, which consists of a short internal stem-loop followed by an unpaired region of 4 nt. The stem contained 3 bp and at least two of them were G-C while the loop had a fixed sequence of 5′-AGC-3′. The unpaired region had a sequence of 5′-WCGR-3′ or 5′-WCGAA-3′ (W = A/T, R = A/G). These DNAzymes preferred an rG-dT wobble pair located immediately downstream from the cleavage site. However, altering the length of the stem or the sequence of the loop could lead to noncatalytic activity.

The catalytic core of '10–23' DNAzymes were composed of 15 nt [4–6], the eighth of which was usually T, C or A while a T often provided the highest activity. The substrate-binding domain bound RNA substrates through standard Watson–Crick pairing and these DNAzymes cleaved at unpaired purine and paired pyrimidine.

DNAzymes are more stable in physiological conditions than ribozymes, even extending the half-life from minutes to over 21 h in serum after modification [7]. Also their well-catalytic characteristic make them promising in targeted gene therapy. With known target, one could design corresponding DNAzymes to downregulate the target disease genes. To date, DNAzymes, mainly '10–23' type, have been extensively explored for their ability to validate target gene and therapeutic potentials.

Antiproliferative *DNAzymes*

Uncontrolled proliferation is a typical feature of cancer cells. To extend the normal life span and escape from the cell cycle, excessive signals promoting cell survive are always activated. PI3K-AKT signaling is thought to be one of the most important pathways, which makes it an ideal target for DNAzymes. Yang *et al*. designed five DNAzymes, based on the analysis of sequences, thermodynamics and site distribution within the Akt1 gene. One of the DNAzymes, namely Dz2, strongly inhibited Akt1 mRNA and protein expression in the nasopharyngeal carcinoma (NPC) cells CNE1-**LMP1**, and markedly suppressed NPC xenograft growth in nude mice. Mechanistically, Dz2 was shown to affect multiple key tumorigenic processes *in vitro* and *in vivo* by downregulating AKT1 expression [8]. There was also a recent study showing that the AKT1-specific DNAzymes significantly inhibited cell proliferation, induced apoptosis and inhibited invasion in thyroid tumor SW597 cells [9]. These DNAzymes acted via the mechanisms of inhibiting cellular proliferation by direct suppression of AKT1 expression.

The inhibition of DNA methyltransferases (DNMTs) may lead to demethylation and expression of the silenced tumor suppressor genes. Wang *et al*. utilized a multiplex selection system and generated some efficient RNA-cleaving DNAzymes targeting DNMT1. Introduction of the DNAzymes caused significant downregulation of DNMT1 expression and reactivation of p16 gene, resulting in reduced cell proliferation of bladder cancers [10]. This study suggests that DNAzyme targeting of epigenetic modifying enzymes may provide a novel strategy for epigenetic inactivation of the genes that caused the uncontrolled proliferation.

c-jun, a basic leucine-zipper (bZIP) protein and prototypic member of AP-1 transcription factor, was upregulated in a variety of cancers. Previous work demonstrated that Dz13 could well target c-jun and effectively inhibit tumor growth [11]. Further studies examined the dose range, sustained effect after cessation of therapy and biodistribution of Dz13, showing that Dz13 was safe and tolerated in animal models [12].

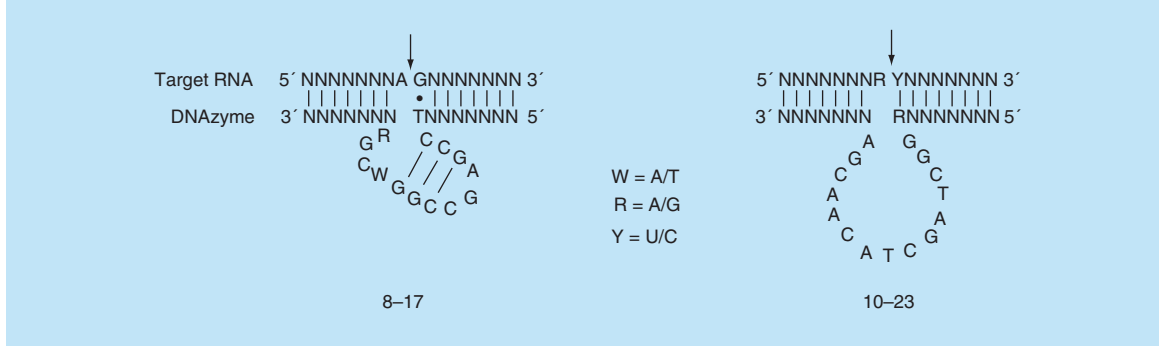

Figure 1. Structures of '8–17' and '10–23' DNAzymes. The substrate binding domain binds RNA through Watson–Crick pairing. The catalytic domain cleaves targets at the site indicated by the arrow.

In this study, the dermal tumor models of squamous cell carcinoma and basal cell carcinoma were developed and Dz13 injected twice a week intratumorally. The effect of Dz13 sustained during the injection in a dose-dependent manner. No tumor growth was observed even 10 days after cessation of therapy when injected with 100 μg, comparing to 10 and 20 μg Dz13-treated mice. They also provided the evidence that the observed effect was dependent on the catalytic domain of the DNAzyme and the adaptive immune system of the host. To evaluate the drug potential of Dz13, biodistribution and toxicology analysis were implemented. Transient liver accumulation of Dz13 was detected after intratumoral administration, while no changes of liver function were observed. Pharmacokinetics of Dz13 via intratumoral, intravenous and intradermal routes revealed a favorable profile for clinical use. GLP-compliant plasma distribution, repeat-dose toxicology and local tolerance studies all indicated Dz13 was well tolerated, with no abnormal clinical signs, necropsy findings or adverse effects [12].

Recently, Cho and coworkers designed a noncontrolled, nonrandomized, nonblinded, dose-escalating Phase I clinical trial (ACTRN12610000162011) to assess the safety of Dz13 and the tolerance of patients [13]. Nine patients were divided into three groups, injected with 10, 30 and 100 μg Dz13 intratumorally, respectively, and monitored at 24 h, 7 days, 14 days and 28 days post dosing. No severe adverse events or significant systemic exposure were detected. Tumors excised at day 14 had lower c-jun expression comparing to predose biopsy. Five patients even had decreased tumor depth and immune and inflammatory cell populations increased, indicating the local immunity. This study demonstrated the feasibility of clinical use of the c-jun-targeted DNAzyme.

In addition, Dass *et al*. found that downregulating c-jun with Dz13 could inhibit tumor progression in osteosarcoma and liposarcoma models due to increased apoptosis in cancer cells [14,15]. When combined with doxorubicin, Dz13 was shown to be able to chemosensitize osteosarcoma cells to the chemotherapy [16].

DNAzymes targeting apoptosis pathways

It has been suggested that apoptosis is one of the major mechanisms of cell death in response to cancer therapy. The Bcl-2 family of proteins is among the most studied molecules in the apoptotic pathway and elevated in a broad range of human cancers, indicating that these molecules may have a role in raising the apoptotic threshold in a broad spectrum of cancerous disorders. DNAzymes targeting Bcl-xL were shown to effectively downregulate the target gene expression and caused suppression of tumor growth in xenograft mouse models [17]. Importantly, the Bcl-xL-DNAzymes could sensitize the tumor cells to chemotherapy and overcome Taxol resistance [17]. Targeting other genes involved in apoptosis, such as survivin and IGF-II, by DNAzymes was also shown to be a viable approach to cancer growth suppression [18,19].

DNAzymes for antimetastasis

Metastasis is one of the hallmarks for malignant cancers. Destroy of the basement membrane and invasion of cancer cells to extracellular matrix are key steps during the process of tumor metastasis. Matrix metalloproteinase (MMP), especially the MMP-9, is a critical regulator in extracellular matrix degradation, which makes it a potential DNAzyme target in metastasis inhibition. Yang *et al*. demonstrated that a DNAzyme-targeting MMP9 significantly suppressed the invasion and migration of lung cancer cells (A549) [20]. Hallett designed anti-MMP-9 DNAzyme (AM9D) and evaluated its effect in breast cancer cells (MDA-MB-231) and in MMTV-PyMT transgenic breast cancer mouse model, showing its inhibitory effect on tumor growth and metastasis [21]. Further studies of AM9D on the systemic distribution, pharmacokinetics and safety of intravenous administration in the same mouse model demonstrated that AM9D accumulated the most in tumor and then blood and liver. Its initial clearance in tumor was approximately 50% slower than other organs. No significant adverse effects and abnormal organ function were observed [22]. All these data clearly warranted a further clinical evaluation of AM9D.

DNAzymes for antiangiogenesis

Angiogenesis is a complicated process, including vascular endothelial cell migration, proliferation and matrix degradation. It is thought to be a critical event in tumorigenesis since the newly generated blood vessel could supply oxygen and nutrient to the deep inside of solid tumors to help fight against the hostile microenvironment [23,24]. Thus, interfering blood vessel formation could be used to block tumor progression [25].

As the elevated expression of VEGF and its receptors have been closely correlated with tumor vascularity, progression and metastasis, targeting of VEGF/VEGFRs becomes quite a worthwhile strategy for cancer treatment. Zhang *et al*. reported that a VEGFR2-targeted DNAzyme induced apoptosis and markedly inhibits endothelial cell growth compared with controls. After the fourth injection of a tumor model, there was nearly a 75% decrease of tumor size, accompanied by a reduction in blood vessel density [26].

Key terms

VEGFR1: An important signal receptor that is involved in angiogenesis.

Radiosensitizer: An agent that makes tumor cells more sensitive to radiation therapy.

Shen *et al*. obtained an effective anti-VEGFR1 DNAzyme (DT18) via a comprehensive *in vitro* selection of DNAzymes for their activity to cleave the VEGFR-1 mRNA [27]. In a rat corneal vascularization model, DT18 significantly inhibited blood vessel formation. In a mouse melanoma model, DT18 markedly suppressed B16 tumor growth. Further evaluation of DT18 effect on human NPC demonstrated a significant inhibition of tumor growth by downregulation of VEGFR-1 expression in NPC tumor tissues. Molecular imaging analysis using MRI showed that the tumor microvascular permeability was reduced, which was the first *in vivo* evidence to suggest that the anti-VEGFR1 DNAzyme had impacts on tumor vasculature.

In addition, transfection of Dz13 targeting c-jun into microvascular endothelial cells blocked cell proliferation, migration and MMP2 expression were blocked. *In vivo*, Dz13 also inhibited neovascularization in a rat cornea model, which provided the direct evidence linking c-jun and angiogenesis [11].

DNAzymes targeting oncogenic viruses

It has been reported that nearly 12% of cancers have been causally linked to human oncogenic viruses, such as EBV for lymphomas and NPCs, HBV for liver cancers and HPV for cervical cancers. Some of viral proteins are directly involved in the dysregulation of cellular processes leading to tumor progression. Targeting the oncoproteins presents a great potential for the virus-associated cancers as these proteins do not exist in host cells, thus avoiding nonspecific toxicity. There have been a number of studies using DNAzymes to target the virus oncoproteins [28–31]. Here, we take an example of the EBV-targeted DNAzyme to illustrate the feasibility of the strategy for cancer treatment.

EBV-encoded LMP1 is considered to be a critical oncogenic factor in EBV-NPC [32]. It works as a constitutively activated TNF receptor (TNFR) and activates several intracellular signaling pathways, such as NF-κB, AP-1 and JAK/STAT pathways [33–36]. Lu and Ke designed DNAzymes targeting LMP1 and revealed that these DNAzymes could inhibit LMP1 expression and repress cell proliferation [29,37]. They also showed that downregulation of LMP1 by the DNAzyme (Dz1) led to decrease in Bcl-2 expression and increase in cytochrome c release from mitochondria, which suggested a direct link between LMP1 and cell apoptosis [29,38]. It was also reported that Dz1 could cause cell cycle arrest via affecting DNA damage repair [39]. Further studies established Dz1 as a radiosensitizer as shown both in cells and *in vivo* via interfering the LMP1-activated signaling pathways [38,40,41]. Extensive preclinical studies demonstrated that Dz1 had a favorable PK profile and low toxicity, indicating targeting LMP1 with DNAzymes could be a promising remedy when treating EBV-associated cancers, such as NPC. Based on the above studies, a randomized and double-blind clinical study was conducted, in which 40 NPC patients receiving Dz1 or saline combined with radiation therapy participated. After 3 months follow-up, results revealed that the DZ1 group had a higher tumor regression rate than the control. Clinical readouts indicated that Dz1-treatment caused change of tumor microvascular permeability measured by DCE-MRI and a lower EBV DNA copy in patient serum. All the data indicated Dz1 could be used as a safe and effective adjuvant for NPC radiotherapy [42].

Conclusion & future perspective

Comparing to ribozymes, antisense oligonucleotide and RNA interference, DNAzymes had many advantages. First, it is comprised deoxyribonucleotides, which are more stable and easy to synthesize, and chemical modifications of DNAzymes could further enhance the stability in serum without affecting the catalytic activity [43,44]. Second, DNAzymes exhibit greater substrate flexibility than conventional and hammerhead ribozymes since their substrate binding domain could vary with the target sequence as long as the catalytic core is unchanged [45–48]. All these advantages make DNAzymes promising therapeutic agents for cancer therapy. Recently a similar technology CRISPR that cuts DNA in a manner similar to RNAi is emerging. While it has shown its potential for gene modification, further biological and clinical validations are needed for possible cancer treatment.

Nevertheless, obstacles for DNAzyme therapy still remain as for other nucleic acid-based therapeutics. Key issues are efficient delivery and finding the targets within tumor microenvironment. DNAzymes are required to travel from the administration site to circulation, go through the tumor stroma and specifically recognize the cancer cells. To date, attempts at delivery systems for DNAzymes have been made by conjugation of DNAzymes to polymers or proteins to facilitate the cellular uptake [49]. With further technology development in drug delivery and deepening understanding of tumor biology, DNAzyme therapy for cancers should be realized through a correct choice of the targets and a suitable disease setting in which the DNAzyme can be directly applied.

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Executive summary

- DNAzymes could be chemically modified without affecting their catalytic activity and become more resistance to the nuclease degradation *in vivo*. They cleave the targets at specific site, inhibit their expression effectively and then repress tumor growth.
- Oncogenes often mutate or express at high level in cancer cells and are thought to be a driver for tumorigenesis and cancer development. Activated oncogenes could block programmed apoptosis and promote cell survive through various signaling pathways. DNAzymes targeting these oncogenes could suppress cell proliferation, metastasis, angiogenesis and tumor growth.
- Two recent clinical studies demonstrated that the DNAzymes are safe and effective in patients, revealing their therapeutic potential in clinical settings.
- As for other nucleic acid-based drugs, DNAzyme technology is also facing some challenges, such as efficient delivery and precise targeting. Further work is needed in biologically relevant models to address these issues before the full potential of DNAzymes in cancer therapy is realized.

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