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Preclinical Anticancer Activity of DNA-based Cleavage Molecules

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ABSTRACT Deoxyribozymes (DNAzymes) are DNA residue-based molecules capable of specific cleavage of complementary mRNA. As such, they are more stable counterparts for the earlier discovered ribozymes. A handful of studies have shown the potential of DNAzymes against cancer both in cell culture and importantly in vivo models. This relatively new molecular entity may progress to clinical trials provided that more extensive testing is carried out at the preclinical stage. While a significant amount of work has gone into chemically stabilizing the molecule, delivery is one area that needs particular attention.

KEYWORDS DNAzyme, Oligonucleotide, Gene therapy, Anticancer, Cancer

INTRODUCTION

Encoded within the nucleotide sequences of genomic DNA are the instructions for protein synthesis. These sequences are transferred to messenger RNAs that are subsequently "read" by ribosomal and transfer RNAs and converted to proteins. Thus, in essence, the natural flow of information stored in DNA (deoxyribonucleic acid) or RNA (ribonucleic acid) can be halted by designing short complementary DNA or RNA oligonucleotides that bind to the nucleic acids. For example, antisense oligonucleotides (whose sequences are complementary to their target genes) have been developed to selectively inhibit a variety of genes with one such molecule (Vitravene) being approved by the FDA as an antiviral agent (Reese & Yan, 2002; Holmlund, 2003). According to Breaker (2004), molecular engineers have only begun to harness the power of nucleic acids that form more complex three-dimensional structures, and apply them as tools for exploring biological systems and as therapeutics. One such nucleic-acid-based molecule is the deoxyribozyme (DNAzyme), a DNA-backbone-based RNA-cleaving entity, which recently has shown various forms of anticancer activity in cell culture, especially in vivo, and is being developed for efficacy against cardiovascular disease indications (reviewed in Dass, 2004a). In light of recent findings in the areas of tumor angiogenesis and enhanced drug

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delivery (reviewed in Dass, 2004b), a combination of redesigning old delivery vehicles and development of novel ones is urgently required to advance this class of drug candidates along the preclinical pipeline into phase I clinical studies.

ACTIVITY OF DNAZYMES ASSESSED IN CULTURED CELLS

In 1997, Santoro & Joyce published results of an in vitro selection procedure to develop a DNA-based enzyme (DNAzyme) that could potentially cleave almost any targeted RNA substrate in a test tube. The enzyme was comprised of a catalytic domain of 15 deoxynucleotides, flanked by two substrate-recognition domains of seven to eight deoxynucleotides each. The RNA substrate was bound through Watson-Crick base pairing and was cleaved at a particular phosphodiester located between an unpaired purine and a paired pyrimidine residue. DNAzyme activity was found to be dependent on the presence of Mg^{2+} ion. By changing the sequence of the substrate-recognition domains, the DNAzyme was demonstrated to cleave synthetic RNAs corresponding to the start codon region of HIV-1 gag/pol, env, vpr, tat, and nef mRNAs.

A multiplex cleavage assay for screening the entire length of a target RNA molecule for deoxyribozyme cleavage sites that are efficient, both in terms of kinetics and accessibility, was subsequently developed (Cairns et al., 1999). The RNA cleaving activity of 80 deoxyribozymes for a model target gene (HPV16 E6, implicated in human cervical cancer), and an additional 60 deoxyribozymes against the rat c-*myc* oncogene target (upregulated in a variety of human cancers such as those of the breast and prostate) were compared. The human papilloma virus (HPV) target was used to characterize the multiplex system and prove its validity. The c-*myc* target, coupled with a smooth muscle cell (SMC) growth assay, allowed an association between in vitro cleavage efficiency and c-*myc* gene suppression in cell culture to be made.

Sun et al., (1999) demonstrated that a DNAzyme targeting the c-*myc* RNA was a potent inhibitor of SMC proliferation, and efficiently cleaved its full-length substrate in vitro and down-regulated c-*myc* gene expression in SMCs. The serum nuclease stability of this molecule was enhanced without much loss of kinetic efficiency by inclusion of a 3'-3'-internucleotide inversion at the 3'-terminal. The extent of SMC suppression was found to be influenced by the length of the substrate binding arms. This correlated to catalytic activity in both the short substrate under multiple turnover conditions and the full-length substrate under single turnover conditions, with the 9+9 base arm molecule producing the greatest activity.

Wu et al., (1999) designed three DNAzymes that specifically cleaved the two variants of the p210 bcr-abl gene (splice 1, b3a2; splice 2, b2a2) and the p190 variant (ela2). Bcr-Abl is implicated in chronic myeloid leukemia (Singhal et al., 2004). Mutated DNAzymes, in which only one critical base had been altered, did not cleave their targets. The cleaving DNAzymes specifically inhibited p210bcr-abl protein expression by K562 cells (which express p210bcr-abl) by about 40%, and inhibited cell growth by more than 50% in a 6-day liquid culture assay. In an assay using freshly isolated CD34+ bone marrow cells from patients with chronic myelogenous leukemia (CML), the DNAzymes specifically inhibited the growth of bcr-abl-positive CFU-Mix colonies by up to 80%.

Sioud & Leirdal (2000) demonstrated enhanced stability of a protein kinase C-α DNAzyme containing phosphorothioate analogues in the antisense arms and in the pyrimidine residues of the catalytic core ($t_{1/2}$ > 90 hours in 50% human serum) and inhibited in vitro cell growth by up to 90% at submicromolar concentrations. Fluorescent versions of the DNAzyme molecules were mainly localized in the nuclei. Most of the DNAzyme-treated cells were killed by apoptosis.

The cellular uptake, intracellular distribution, and stability of 33-mer DNAzymes were closely examined in several cancer (both non-human and human) cell lines by Dass et al. (2002). Altering DNAzyme size (17–33mer) and chemistry (phosphodiester, 4+4 arms phosphorothioate arms, or 3′-3′-inverted terminus) did not significantly affect uptake into cells. DNAzyme was distributed primarily in punctate structures surrounding the nucleus and substantial delivery to the nucleus was not observed up to 24 hours after initiation of transfection using 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and Superfect transfection reagents. Incubation in human serum or plasma demonstra- ted that a 3′-inversion modification greatly increased DNAzyme stability ($t_{1/2}$ approximately 22 hours) in comparison to the unmodified form ($t_{1/2}$ approximately 70 minutes).

The 3′-inversion-modified DNAzymes remained stable during cellular uptake, and catalytically active oligonucleotide could be extracted from the cells 24h post-transfection. In a SMC proliferation assay, the modified DNAzyme targeting the c-*myc* gene showed a more potent anti-proliferation effect than did its unmodified sequence.

Cieslak et al. (2002) designed DNAzymes to β1 and β3 mRNAs containing a 15-deoxynucleotide catalytic domain that was flanked by two substrate recognition segments of 8 and 10 deoxynucleotides for β1 and β3 DNAzymes, respectively. In the presence of Mg^{2+}, both DNAzymes cleaved their substrates, synthetic β1 and β3 mRNA fragments, specifically. Although DNAzymes partially modified with phosphorothioate and 2'-O-methyl groups at both the 5′ and 3′ ends indicated similar kinetic parameters, they were significantly more potent than the unmodified DNAzymes because of their heightened resistance to nuclease degradation. Similar to corresponding antisense oligonucleotides, DNAzymes abolished microvascular endothelial cell capillary tube formation in fibrin and Matrigel. Inhibition of angiogenesis would prove beneficial against a variety of solid and even soft-tissue cancers.

Hjiantoniou et al. (2003) designed a DNAzyme, DZ-TWT, to down-regulate Twist expression. Twist is a basic helix-loop-helix transcription factor that is involved in the regulation of cellular differentiation and apoptosis, and is increasingly being implicated in breast cancer (Yang et al., 2004). The ability of DZ-TWT to cleave mouse Twist mRNA was first shown in a cell-free environment against full-length Twist mRNA. Following transfections of the DZ-TWT in C3H10T1/2 cells, a significant reduction in Twist mRNA levels was observed. This was accompanied by a substantial rise in p21 mRNA levels. Finally, DZ-TWT transfections resulted in an increase of cellular apoptosis, demonstrating the importance of Twist in apoptotic pathways.

Beale et al. (2003) employed scanning arrays to enhance design of DNAzymes targeting the epidermal growth factor receptor (EGFR) mRNA. DNAzymes inhibited the growth of EGFR over-expressing A431 cancer cells in a dose-dependent manner when delivered with cationic lipids. Effects on cell growth were correlated in all cases with concomitant dose-dependent reduction in EGFR protein expression. The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase of the ErbB receptor family that is abnormally activated in many epithelial tumours (Arteaga & Baselga, 2003).

Ackermann et al. (2005) synthesized a DNAzyme against ornithine decarboxylase (ODC), a key enzyme in the biosynthesis of polyamines. It is known that polyamines are necessary components for cell growth, and manipulation of polyamine homeostasis may be an effective strategy for the treatment of a number of disorders, including cancer. DNAzymes able to cleave the target ODC RNA were identified in vitro and further characterized by the effect each had on ODC protein and activity levels using in vitro translated ODC RNA. One of the DNAzymes, DZ IV, which exhibited good activity, was optimized for use in cell culture studies. In HEK 293 cells, DZ IV was able to reduce the amount of translated ODC protein, resulting in approximately 80% reduction in ODC activity, better than ODC antisense. ODC is frequently over-expressed in prostate cancer (Subhi et al., 2004).

The above cell culture studies demonstrate that different cancer gene targets are successful downregulated by DNAzymes whether they were endogenous (cell-expressed) or exogenous (virus-expressed). DNAzymes are amenable to chemical modification, allowing them to be made more resistant against nuclease degradation. They can be made to different lengths with no major fluctuation in efficiency. All these findings have paved the way for in vivo evaluation.

ANTICANCER DNAZYME FUNCTION ASSESSED IN VIVO

Several studies performed in animal models of disease have proven the applicability of DNAzymes in general and as discussed below, specifically against cancer. Most of the studies have been performed to prove whether these catalytic molecules can perform their function in vivo, and thereby extend their usefulness beyond the cell culture stage. In the past five years, rapid progress has been made with these entities, allowing some preclinical researchers to believe that a phase I study with a DNAzyme is not far from reality.

In a murine model, a DNAzyme that targets the vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) mRNA transcript was found to cause an approximately 75% reduction of tumor size compared with the saline-injected control group (Zhang et al., 2002). Marked cell death in the peripheral regions of the

tumor accompanied by a reduction in blood vessel density was noted, being consistent with the antiangiogenic mechanism of the DNAzyme.

A catalytically active c-*jun* DNAzyme (Dz13) cleaved c-*jun* mRNA and inhibited inducible c-Jun protein expression in vascular SMCs (Khachigian et al., 2002). Dz13 blocked vascular SMC proliferation with potency exceeding its non-catalytic antisense equivalent. Moreover, Dz13 abrogated SMC repair following scraping injury in vitro and intimal thickening in injured rat carotid arteries in vivo.

Recently, a DNAzyme targeting a specific motif in the 5' untranslated region of the Egr-1 mRNA, inhibited Egr-1 protein expression, microvascular endothelial cell replication and migration, and microtubule network formation on basement membrane matrices (Fahmy et al., 2003). These DNAzymes blocked angiogenesis in subcutaneous Matrigel plugs in mice and inhibited xenografted MCF-7 human breast carcinoma growth in nude mice. Egr-1 DNAzymes suppressed tumor growth without influencing body weight, wound healing, blood coagulation, or other hematological parameters. Such a lack of side-effects points to the potential utility of these catalytic oligonucleotides for in vivo therapeutic applications.

Zhang et al. (2004) demonstrated that Dz13, targeted against the basic region-leucine zipper protein c-Jun (linked to cell proliferation, transformation, and apoptosis), blocked endothelial cell proliferation, migration, chemoinvasion, and tubule formation. Dz13 inhibited endothelial cell expression and proteolytic activity of matrix metalloproteinases (MMP-2), a c-Jun-dependent gene. In vivo, Dz13 inhibited VEGF-induced neovascularization in the rat cornea and solid melanoma growth in mice.

Mitchell et al. (2004) used DNAzymes against the human Egr-1 mRNA, and showed that this factor plays a central role in breast cancer proliferation, migration, chemoinvasion, and xenograft growth in nude mice. DNAzyme inhibition of tumor progression of breast carcinoma cells was sequence-specific and EGR-1 transcription-independent. In cell culture, Egr-1 DNAzymes inhibited breast carcinoma cell migration and chemoinvasion in microchemotaxis chambers. Egr-1 binds to the promoters of many genes whose products influence cell movement and replication in the artery wall. Several earlier studies have shown applicability of DNAzymes against Egr-1 in non-cancer models, mainly cardiovascular disease models (Santiago et al., 1999; Lowe et al., 2001, 2002).

FUTURE PROSPECTS FOR DNAZYMES

DNAzymes targeting specific cancer-related genes can inhibit multiple key tumorigenic processes in vitro and in vivo and may serve as useful anti-cancer agents. What remains to be done is development and testing of enhanced ways to deliver these macromolecules in ways that localize them to the neoplastic site at doses within the therapeutic window (reviewed in Dass, 2004b). Such large carriers as liposomes and microparticles, macromolecular carriers like dendrimers, and small molecular carriers like porphyrins and cyclodextrins are being developed to increase delivery of oligonucleotides to diseased sites in preclinical studies (Dass, 2002). However, these carriers are mostly being tested in cell culture transfection studies. Many of the issues of delivery and toxicity pertinent to DNAzymes are also closely relevant to other forms of nucleic acid-based molecules.

More preclinical studies are now needed to prove whether the DNAzyme molecule can be treated like an anticancer drug in highly clinically-relevant disease models by experienced researchers committed and able to move this new molecule forward into clinical trials. Of value would be a close look at the successes and failures of other oligonucleotide compounds (Arora et al., 2004; O'Neill et al., 2004), the major one being antisense which is now in various stages of clinical testing, with one compound already being approved by the FDA—Vitravene for cytomegalovirus-mediated ocular disease.

SUMMARY

Deoxyribozymes (DNAzymes), DNA-based versions of ribozymes, have progressed from being a novel oligonucleotide entity to a possible drug molecule in a relatively short period of time. Several reports prove the anticancer potential of this class of compounds both against cultured cancer cells and various in vivo models. However, more preclinical studies, with better delivery vehicles, need to be done to test the true capability of this new molecule against an old foe—cancer.

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