DNAzyme technology and cancer therapy: cleave and let die

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Abstract

Novel molecules are constantly being discovered and developed to find better means of managing debilitating and fatal diseases, which include cancer in its multiple forms. Among these molecules, and as a direct consequence of a better understanding of the molecular basis of diseases, are those falling within the class of gene therapeutics. Among these players, deoxyribozymes (DNAzymes) have come a long way from being just another analytic tool available to molecular biologists. Recent studies have shown the potential DNAzymes to serve as drugs both in cell-based assays and preclinical models of cancer. It is anticipated that with the development of smart delivery systems for DNAzymes, better pharmacokinetics and pharmacodynamics will be possible, expediting DNAzyme march toward the clinic. Also, the ability of DNAzymes to yield to such phenomena as light-induced activation may be exploited for targeted therapy. This review documents the rise of DNAzymes in the fight against cancer and serves as a forecast for this promising biotechnology in this context. [Mol Cancer Ther 2008;7(2):243–51]

Introduction

Over the past decade, our appreciation of the pathogenesis of human disease has rapidly evolved particularly due to recent significant advances in genomics, which have shifted the focus of researchers onto the intricate molecular and cellular pathways that constitute a disease. This is in part largely due to the Human Genome Organisation gene sequencing project, and even more so, the almost readily available and applied gene microarray tools available to researchers. These tools have dramatically altered the pace with which tumor samples are analyzed for temporospatial expression of genes on a global scale. The information technology age has also significantly helped in expediting research efforts that were unimaginable even as recently as 5 years ago.

Hand-in-hand with these findings, and probably more importantly, we have acquired the capacity to selectively attenuate the expression of specifically targeted genes both as a means of dissecting molecular function as well as switching genes down or off to modulate disease outcome. As such, strategies to specifically knockdown gene expression have recently received considerable attention, and these include antisense, oligodeoxynucleotide decoys, small interfering RNA, aptamers, and the catalytic ribozymes and DNAzymes. Technologies for gene modulation, once items of sheer curiosity and limited potential, have now given way to an oligonucleotide-based therapeutic. Approved by the Food and Drug Administration in 1998 was Isis Pharmaceuticals’ Vitravene (fomivirsen), a treatment for cytomegalovirus retinitis in immunocompromised patients, such as those with AIDS (1).

A second possible candidate, Genasense (Oblimersen), manufactured by Genta, Inc., was initially intended for use in treating relapsed or refractory chronic lymphocytic leukemia in combination with fludarabine and cyclophosphamide. Genasense is directed against the bcl-2 oncogene. The molecule has also recently acquired orphan drug status for treating melanoma in Australia in combination with decarbazine (2).

However, it is nearly a decade since the approval of the initial antisense drug, and despite the best efforts of large biotechnology corporations to shepherd lead candidates through the development pipeline, there has been a relative paucity of such drugs emerging. However, the ability to specifically target gene expression in such a novel way means that if a potent molecule is found, then it may well be useful against various pathologies as will be discussed below.

Catalytic Nucleic Acids—Brief Overview

Through Watson-Crick–based interactions with complementary sequences mediated by such attributes as flexible binding and discrimination of nucleic acid substrates,
ribozymes perform catalytic reactions with great precision. Because the initial designing of RNA-cleaving catalytic RNA (3), this capability led by ribozymes has rapidly progressed to a limited number of clinical trials (4). As these ribozymes can be encoded and transcribed from DNA, much of this development has been advanced by enhanced vector designs used in other forms of gene therapy that rely on gene overexpression. On the other hand, substantial progress has also been made in the synthesis and delivery of ribozymes (i.e., not within expressible gene vectors). It was a decade later that DNAzymes entered the scene of nucleic acid–mediated catalysis.

These DNAzyme molecules, discovered in 1994 by Ronald Breaker and Gerald Joyce (5), and exemplified by the 10-23 deoxyribozyme (6), essentially combine the benefits of highly sequence-specific RNase-independent RNA destruction, with the relatively stable chemistries used in oligodeoxynucleotide-based antisense reagents. Perhaps a major obstacle in the further development of these technologies is a phenomenon that requires substantial development efforts invested in drugs of various classes, the uphill battle to effect cellular delivery in a targeted manner. This challenge is being met with a multidisciplinary approach with the hope that a greater understanding of each step of this process will enhance DNAzyme pharmacodynamics. Such diverse fields as gene vector design, biomolecular and chemical engineering, drug delivery, pharmaceutics, membrane biophysics, anatomy, and physiology, are coming together to present tangible solutions to enhance DNAzyme efficacy.

It was 30 years ago when it was first reported that nucleic acids could be used to down-regulate gene expression via a direct and highly specific interaction with the transcript (7). Thereafter, Stephenson and Zamecnik (8) exhibited the capacity of antisense molecules to inhibit viral replication. Since then, the field of nucleic acid–based therapies has rapidly blossomed with numerous gene targets and methods comprising of both naturally occurring and synthetic molecules that have been tested both in vitro and in vivo against different pathologies with reasonable to dramatic success. Currently, a number of these approaches are being evaluated in human and larger animal trials and are poised to offer better therapies in the future.

Essential criteria dictating the usefulness of a DNAzyme are that the DNAzyme should specifically cleave its target mRNA, the target gene should play a key (noncompensatable) role in the disease process, target gene inhibition should not adversely influence normal physiologic processes, the DNAzyme should be capable of being carried and delivered by a variety of vehicles (if free delivery is suboptimal), the DNAzyme is amenable to chemical modification to avoid degradation, and the DNAzyme should not cause nonspecific (off target) effects. This review will focus exclusively on DNAzyme strategies, and the reader is referred to excellent reviews by others for updates on antisense (9), small interfering RNA (10), and ribozyme (4) technologies and the utility of these molecules against cancer.

Background to DNAzymes
Unlike ribozymes, which have been widely hypothesized to have carried genetic information and performed catalysis during the primordial “RNA World” (11), catalytic DNA has not been observed in nature and all existing molecules have been derived by in vitro selection processes. The most well-characterized DNAzyme is the 10-23 sub-type consisting of a cation-dependent catalytic core of 15 deoxyribonucleotides (6) that binds to and cleaves its target RNA between an unpaired purine and paired pyrimidine through a de-esterification reaction (Fig. 1). Sequence conservation in the border regions of the catalytic core is important for the maintenance of catalytic activity (12). This core is flanked by complementary binding arms of 6 to 12 nucleotides in length, which, via Watson-Crick hybridization, confer specificity for the targeted mRNA. Once the target is bound, catalysis proceeds and the mRNA strand is nicked into two. DNAzymes are purported to be just as efficient as protein enzymes at cleaving RNA (13).

In terms of biological specificity, DNAzymes with nonsense or mismatch sequences in the binding arms, or with point mutations in the catalytic core rendering the DNAzyme enzymatically inactive, can serve as important controls when assessing specificity (Fig. 2). We commonly use the entity of the “scrambled DNAzyme,” in which the nucleotides in the binding arms of the DNAzyme are jumbled. This produces a molecule of identical size and same net charge with a binding sequence that is not matched to the target gene.

The 10-23 DNAzyme, named because of its selection process in vitro, catalyses sequence-specific RNA cleavage in a manner akin to the hammerhead ribozyme and hence has substantial utility as a gene silencing agent. In vitro cleavage experiments have shown that the 10-23 DNAzyme is highly specific and in fact quite sensitive to small changes in target sequence (14, 15). Imperatively, DNAzyme activity is dependent on the prevailing secondary structure of long target RNA at the cleavage site (15). For this reason, it is important to test a range of synthesized molecules to identify those that display a high level of activity against biologically relevant target molecules. The journey from in vitro cleavage kinetics to cell culture assessment (down-regulation of the target gene, plus phenotypic changes) to evaluation in vivo (in clinically relevant models demonstrating efficacy and minimal toxicity) can be arduous and is usually accompanied by a large attrition rate of candidate molecules.

Stability of DNAzyme Molecules
A number of structural modifications have been used to enhance stability as well as improve potency of DNAzymes. An important, commonly used modification is the incorporation of a 3’-5’ inverted nucleotide at the 3’ terminus of the DNAzyme to prevent exonuclease degradation. This can dramatically increase stability of the molecule extending the half-life from minutes to >21 h in human serum as we have shown (16). Additionally, DNAzymes
with this modification can remain functionally intact for at least 24 to 48 h after exposure to serum compared with its unmodified counterpart (17) with little change in the cleavage kinetics (18). Phosphorothioate linkages enhance stability by rendering the oligonucleotide more resistant to endogenous nucleases, and have been used with DNAzymes (19), although such alterations in antisense oligodeoxynucleotides have been associated with toxicity (20), immunologic responsiveness (21), and increased affinity for cellular proteins resulting in sequence-independent effects (22, 23). Thus, phosphorothioate modifications are seldom used for DNAzyme work and have been used with decreasing frequency over the past 5 years.

Locked nucleic acids have been attractive monomers for modifying DNAzymes (24–26) in efforts to increase binding affinity. Locked nucleic acid bases comprise a 2'-O-4-C methylene bridge that locks in a C3'-endo conformation (27), effectively increasing affinity for complementary sequences (28, 29). The advantages of locked nucleic acid include increased thermal stability of duplexes toward complementary DNA or RNA, stability toward 3'-exonucleolytic degradation, and greater solubility due to structural similarities to nucleic acids (30, 31). Locked nucleic acid incorporation into DNAzymes, however, has been found to influence both catalytic activity under single turnover conditions (24, 26) and biological potency (26, 32). Because DNAzymes with an inverted nucleotide at the 3' end are catalytically more efficient compared with their locked nucleic acid–modified counterparts due to a faster product release rate (25, 33), they are still highly recommended for usage.

**Activity of DNAzymes against Cancer Cells In vitro**

Demonstrating cleavage kinetics with DNAzymes in a cell-free system is a valuable way to narrow the choice of molecules one needs to test in in vitro assays, mainly for successful down-regulation of the specific mRNA target. Having successfully done this, one needs to screen the DNAzymes through selective cell-based assays that help identify the effects of such down-regulation on cell physiology. A good starting point assay for selection of anticancerous DNAzymes is that of proliferation, which may indicate that an apoptotic assay needs to be done if positive (reduced cell proliferation) is noted. For more sophisticated assays, such as those for colony formation or invasion through a biological matrix (34), results are more helpful, and may even help to explain findings in subsequent in vivo studies.

The first usage of a DNAzyme against a target in cancer cells was by Wu and colleagues (35) who used three DNAzymes against 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 >95% of chronic myeloid leukemia patients and 20% to 30% of adults with acute lymphoblastic leukemia. 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 chronic myeloid leukemia K562 cells by ~40%, and inhibited cell growth by >50% over a 6-day assay period. Cells were transfected

![Figure 1. DNAzyme mechanistics.](image-url)
with the GS2888 cationic liposomal reagent. In an assay using freshly isolated CD34+ bone marrow cells from patients with chronic myeloid leukemia, the DNAzymes specifically inhibited the growth of bcr-abl-positive colonies by up to 80%.

We examined the interaction between DNAzymes and transfection reagents using a panel of human cancer cell lines (16). Altering DNAzyme size (17-33mer) and chemistry (phosphodiester, 4+4 arms phosphorothioate arms, or 3'-3'–inverted terminus) did not significantly affect uptake into cultured prostate, breast, and lung cancer cells. DNAzyme was distributed primarily in punctate structures surrounding the nucleus and substantial delivery to the nucleus was not observed up to 24 h after initiation of transfection using two cationic reagents, N-[1-(2,3-dioleoylloxyl)propyl]-N,N,N-trimethylammoniummethyl sulfate and Superfect. Incubation in human serum showed that the 3'-inversion greatly increased DNAzyme stability (t1/2 in the order of minutes) compared with the unmodified form (t1/2 in the order of hours). These results confirmed our earlier findings (17). The 3'-inversion–modified DNAzymes remained stable during cellular uptake, and functional oligonucleotide could be extracted from the cells 24 h posttransfection. The modified DNAzyme targeting the c-myc gene showed a more potent antiproliferation effect than did its unmodified sequence.

Cieslak and coworkers (36) designed DNAzymes to β1 and β3 integrin mRNAs. β1 and β3 integrins are important in cell-cell and cell-matrix interactions in the pathogenesis of various tumors and other diseases. In the presence of Mg2+, both DNAzymes cleaved their substrates, synthetic β1 and β3 mRNA fragments, specifically. Although DNAzymes partially modified with phosphorothioate and with 2'-O-methyl groups at both the 5' and 3' ends indicated similar kinetics, they were significantly more potent than the unmodified DNAzymes because of their greater stability. The DNAzymes inhibited microvascular endothelial cell capillary tube formation in fibrin and Matrigel, signaling their usefulness against tumor-induced angiogenesis.

The epidermal growth factor receptor is a receptor tyrosine kinase of the ErbB receptor family that is abnormally activated in many epithelial tumors (37). Beale et al. (38) used DNAzymes that inhibited the growth of epidermal growth factor receptor–overexpressing 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 epidermal growth factor receptor protein expression.

DNAzymes were synthesized to cleave the PML/RARα transcript (39). Of note is that a quadruple transfection protocol was used by this group to attain ~80% down-regulation at the protein level. Two constructs cleaved PML/RARα mRNA efficiently and specifically, and transfected cells exhibited a significant suppression of PML/RARα protein expression. In cell-based assays using acute promyelocytic leukemia cells, the DNAzymes inhibited proliferation and induced apoptosis in these cells.

Latent membrane protein, LMP1, encoded by EBV, is suggested to be one of the major oncogenic factors in EBV-mediated carcinogenesis. DNAzymes targeting LMP1 were transfected (19) into B95-8 cells (established from peripheral blood lymphocytes of a Marmoset monkey and releasing high titers of EBV), which constitutively express LMP1. Tetramethylpyridyl porphyrin was used by the researchers to attain reasonable transfection efficiency. There was significant down-regulation of LMP1 and consequent inhibition of B95-8 cell growth at the G0-G1 checkpoint. There was an interesting concomitant down-regulation of bcl-2 gene expression, suggesting an association between...
LMP1 and apoptosis. As confirmation of apoptosis, the DNAzymes induce the release of cytochrome c from mitochondria.

A DNAzyme targeting human survivin mRNA was evaluated in human pancreatic carcinoma PANC-1 cells (40). The DNAzyme cleaved survivin mRNA efficiently in both a dose- and time-dependent manner. It achieved an ~80% decrease in target protein levels 72 h after a single transfection. It also pushed the cells into apoptosis and inhibited the growth of this cell line.

de Bock and coworkers (41) used three different DNAzymes against urokinase-type plasminogen activator receptor (uPAR) mRNA, and were able to decrease target uPAR transcript in vitro by at least 84%. They also showed an ability to down-regulate uPAR mRNA in the human osteosarcoma cell line SaOS-2 after transfection. One DNAzyme decreased uPAR mRNA within 4 h of transfection and inhibited uPAR protein concentrations by 55% in SaOS-2 cells with a triple transfection protocol. The decrease in uPAR mRNA and protein concentrations reduced SaOS-2 cell invasion in Matrigel. The use of DNAzyme adds a new potential clinical agent for decreasing uPAR mRNA expression and inhibiting cancer invasion and metastasis.

These studies in cell culture show that different cancer gene targets can be successfully down-regulated whether they are endogenous (cell-expressed) or exogenous (virus-expressed) in a variety of cancer cell lines. Some of the effects in fact lead to anticancer activity as judged by phenotypical changes in cells. DNAzymes are amenable to chemical modification, allowing them to be made more resistant against nuclease degradation and can be made to different arm lengths. These findings have helped pave the way for the in vivo evaluation of DNAzymes.

DNAzymes as Antineoplastic Agents In vivo

Although no studies to date have been done in humans, several investigations done in animal disease models have proven the potential applicability of DNAzymes in general and as discussed below, specifically against cancer (summarized in Table 1). Most of the studies have been done to prove whether these catalytic molecules can perform their function in vivo, and thereby extend their usefulness beyond the cell culture stage. Although in vitro assessment of DNAzyme efficiency helps establish gene and sequence specificity, and is an expedited form of screening, important issues such as delivery, pharmacokinetics, metabolism, toxicity, and efficacy can only be examined in vivo. In the past 5 years, considerable progress has been made with these entities.

Cancers have traditionally been treated with agents that act on the neoplastic cells themselves in an attempt to halt proliferation or induce death due to apoptosis or necrosis. The alternative, and more recent approach, is to develop agents that act in an indirect manner against the tumor blood supply, and not the cancers themselves. Cancers are dependent on a blood vasculature for oxygen and nutrients, and for waste removal just like a normal tissue, only more voraciously (42). Thus, various factors mediating angiogenesis induced by tumors have been targeted with DNAzymes to inhibit neoplastic growth.

In a murine model, a DNAzyme that targets the vascular endothelial growth factor receptor 2 (VEGFR2) mRNA transcript was found to cause an ~75% reduction in tumor size compared with the saline-injected control group (43). Marked cell death in the peripheral regions of the tumor accompanied by a reduction in blood vessel density was noted, being consistent with an antiangiogenic mechanism. Zhang et al. (43) first successfully applied this technology in vivo against tumors with DNAzymes targeting VEGFR-2 attenuating tumor growth in rats. The DNAzyme active against VEGFR-2 was injected intratumorally in a complex formulated from a histidine-lysine polymer.

In our hands, intratumorally administered DNAzymes targeting Egr-1 also showed potent reduction in cancer growth with an associated reduction in tumor angiogenesis (44). A DNAzyme against the zinc finger transcription factor Egr-1 blocked angiogenesis in s.c. Matrigel plugs in mice and in MCF-7 human breast tumors in nude mice. The DNAzymes, complexed with a cationic reagent (Fugene6) did not exhibit any side effects that may occur using antiangiogenic agents, such as emaciation, perturbed wound healing, blood coagulation, or other hematologic variables. Furthermore, the Egr-1 DNAzyme inhibited VEGF-induced neovascularization of the rat cornea. The DNAzyme targeting 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.

DNAzyme activity against cancer is not limited to destruction of the tumor vasculature. We were the first to show the direct anticancer effects due to two different DNAzyme entities. The first report (45) showed that Dz13, targeted against the basic region-leucine zipper protein c-Jun, blocked endothelial cell proliferation, migration, chemoinvasion, and tubule formation. The DNAzymes were complexed with Fugene6 and coadministered with tumor cells. Dz13 inhibited endothelial cell expression and proteolytic activity of matrix metalloproteinase-2, a c-Jun–dependent gene. In vivo, Dz13 inhibited VEGF-induced neovascularization in the rat cornea and B16 melanoma growth in mice. There was a 2.5-fold decrease in tumor volume by Dz13 and a 60% decrease in the number of tumor-associated blood vessels in these mice. The decrease in corneal vascularization due to VEGF stimulus was 75% in the Dz13 group of rats.

A direct antitumor effect was achieved with an Egr-1 DNAzyme complementary to the human Egr-1 mRNA sequence (46). This molecule, DzF, strongly inhibited Egr-1 expression in the MCF7 human breast carcinoma line, which correlated with inhibition of proliferation of these cells when induced by addition of serum back into the growth medium. DzF also inhibited both cellular migration (40%) and chemoinvasion (60%) in these cells. DzF was also capable of significantly reducing s.c. tumor growth when...
injected intratumorally once tumors were palpable at a dose of 10 μg/injection twice a week. At termination, tumors were at least one third the volume of those in the control groups.

We have also more recently shown that the Dz13 DNAzyme is capable of reducing not only solid melanoma growth, but squamous cell carcinoma growth in mice as well (47). Dz13 down-regulated c-Jun expression in T79 cells and also inhibited their growth in a dose-dependent manner. In nude mice, when Dz13 was co-injected with T79 cells s.c. into the midback, tumor volumes in the Dz13-treated group decreased by 80%, and the blood vessel number in examined tumor sections were reduced by 40%. Importantly, the levels of c-Jun were significantly (80%) reduced by Dz13. The Dz13 molecule is a case in point in that once a potent down-regulation agent is found, with its inherent sequence-specific knockdown, then it may be found to possess applicability across various pathologies that are caused by expression or overexpression of the targeted gene. Further evidence of the antiangiogenic properties of Dz13 is shown by its inhibition of retinal neovascularization in mice induced by exposure to hyperoxia-normoxia (44). Dz13 also has the capacity to inhibit restenosis (48) and inflammation (49) in a variety of animal models consistent with c-Jun–targeting activity.

These studies collectively show the potential of DNAzymes as sequence-specific molecular tools. The number of studies using DNAzymes have increased quite substantially in the past 3 years (50, 51) with DNAzyme activity assessed in a variety of clinically relevant models. This will undoubtedly continue in coming years.

Although singular DNAzyme targets have been used in biological systems thus far, it is possible that greater biological efficacy may be achieved using combinations of DNAzymes and/or other small-molecule nucleic acid strategies targeting the same factor or multiple factors. In cancer for instance, there is increasing realization that for effective and lasting tumor regression, resulting in increased patient quality of life, a combinatorial approach may be preferable in efforts to prevent neoplastic cells evolving mechanisms to avoid single agent–based therapy, which in itself may prove quite toxic (58). A tumor, by its very nature, is full of cells that are either quite different, or have the capacity to mutate to become quite different, some of these cells then acquiring the ability to resist chemotherapy. These strategies may include such combinations as DNAzymes against an oncogene together with the use of frontline chemotherapy. Such strategies have been successful in proving the efficacy (59, 60) of the bcl-2 antisense molecule, G3139 (now Genasense).

The fact that DNAzymes possess a number of other advantages over other gene-silencing agents, including lower production cost, ease of chemical modification, and relative serum stability, makes them attractive therapeutic candidates for further development, and clinical evaluation. A key challenge to the use of DNAzymes as anticancer reagents is delivery to the site of disease.

**DNAzyme Delivery—Facing the Beast of a Challenge**

The most important issue facing the therapeutic use of DNAzyme is tissue delivery after systemic administration. The DNAzyme needs to travel from its site of administration (Fig. 3), through the bloodstream, out of the circulation, through the tumor stroma, and acting against the high interstitial pressure common in tumors, directly into the cancer cells. Then, it must find its target mRNA and be able to annul its message. DNA itself can act as a scaffold for the manufacture of some rather smart nanotechnologies, and this technology

### Table 1. In vivo applications of DNAzymes in animal models of cancer

<table>
<thead>
<tr>
<th>mRNA target</th>
<th>Animal</th>
<th>Xenograft</th>
<th>Delivery mode</th>
<th>Dose regimen</th>
<th>Anticancer activity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR-2</td>
<td>Mouse</td>
<td>MDA-MB-435</td>
<td>Intratumoral</td>
<td>Four injections of 30 μL, each of 2.9 μg of DNAzyme and 1.092 nmol his-lys polymer</td>
<td>75% reduction in tumor size, cell death in tumor periphery, reduction in blood vessel density</td>
<td>(44)</td>
</tr>
<tr>
<td>Egr-1</td>
<td>Mouse</td>
<td>MCF-7</td>
<td>Intratumoral</td>
<td>Twice a week, injections of 20 μL, each of 20 μg of DNAzyme and 1 μL Fugene-6 reagent injected s.c.</td>
<td>80% reduction in tumor size, reduction in blood vessel density</td>
<td>(45)</td>
</tr>
<tr>
<td>c-Jun</td>
<td>Mouse</td>
<td>B16</td>
<td>Comixed with cell inoculation</td>
<td>Commencement of experiment, 750 μg of Dz comixed with 2.5 μL Fugene-6 reagent injected s.c.</td>
<td>60% reduction in tumor size, reduction in blood vessel density</td>
<td>(46)</td>
</tr>
<tr>
<td>Egr-1</td>
<td>Mouse</td>
<td>MDA-MB-231</td>
<td>Intratumoral</td>
<td>When tumors were palpable, single injection of 10 μL, of 10 μg of free DNAzyme</td>
<td>67% reduction in tumor size</td>
<td>(47)</td>
</tr>
<tr>
<td>c-Jun</td>
<td>Mouse</td>
<td>T79</td>
<td>Comixed with cell inoculation</td>
<td>Commencement of experiment, 50 μg of Dz comixed with 2.5 μL Fugene-6 reagent injected s.c.</td>
<td>90% reduction in tumor size</td>
<td>(48)</td>
</tr>
</tbody>
</table>
reaches far back as 12 years ago (52, 53). With the aid of the “sticky ends” of DNA, dispersed three- and four-way DNA junctions and other structural blocks can be connected into large repeating structures, on which other entities such as nanoparticles or proteins can be deposited to form quite intricate and conforming patterns (54, 55). DNA has also been conjugated to inorganic nanoparticles to form structures with a limited number of particles or cross-linked aggregates of these particles (56, 57). When the potential of DNA to form such organized microstructures is taken into consideration, then the development of such delivery vehicles for DNAsymes presents an exciting challenge, the enormity notwithstanding.

The use of nanobiotechnology has blossomed into a large field, and some of this technology has trickled into the field of gene therapy as well. Solid nanoparticles formulated from such materials as atelocollagen (61), chitosan nanoparticles (62), and polypropylene imine (63) have serve as alternative approaches to liposomes (64). It may well be that biophysical devices, such as those used for delivery of peptides (65), could aid DNAzyme delivery to the diseased site and minimize normal tissue exposure.

Thus far, only atelocollagen has been injected systemically and shown to deliver an small interfering RNA sequence efficiently to growing tumors in mice (61). This study used a metastatic prostate cancer model where tumors deposit in the bone of mice. A series of three injections were made, each with 50 μg of small interfering RNA against the reporter gene luciferase. Also, this group showed the ability of this technology to effect efficacy by using two targets that showed promise in in vitro assays. However, this technology is still in its early days, and more rigorous testing of its applicability to DNAsymes is surely warranted.

Small interfering RNA–encapsulating chitosan nanoparticles developed by Howard and coworkers (62) were capable of down-regulating a target gene in the bronchiole epithelial cells of mice after intranasal administration. Such an approach would be highly valuable for lung cancers or those that metastasize to the lungs such as osteosarcoma (66). To directly address the latter, two orthotopic spontaneously metastasizing models for osteosarcoma have recently been developed to evaluate the potential of novel therapeutic agents and delivery vehicles to target secondary pulmonary tumors (34, 67). Chitosan is an abundant material that is catching the eye of gene therapists (68) and has been shown to efficiently deliver plasmids via the oral route (69).

Figure 3. Route taken by free DNAzyme to down-regulate target gene in targeted cancer cells. 1, DNAzyme is administered via the blood supply. 2, DNAzyme is carried by blood circulation to the site of the lesion and other tissues. 3, DNAzyme extravasates through the capillaries and moves toward the cancer cells. 4, DNAzyme neutralizes its targeted mRNA in the nucleus or in the cytoplasm.
Recent developments suggest that light might be useful as an activation device for DNAzymes and that perhaps some degree of targeting could arise by harnessing this phenomenon. This technology, however, has not progressed beyond the in silico stage, where light is directed to DNAzymes in a cell-free system to test whether better activity can be attained. Liu and Sen (70) elegantly showed that depending on the strategic positioning (whether in the substrate binding arms or within the catalytic core) of azobenzene residues within the DNAzyme, the DNAzyme could either be induced to cleave or repressed when visible or UV light was used. Azobenzene can convert between its cis and trans forms as light is altered from UV to the visible range of the electromagnetic spectrum. The 5- to 6-fold higher rates of cleavage inducible by light is an astonishing feat, and there are plans to emulate this within cells. Ting and colleagues (71) used a photoactivatable adenosine analogue to show that when stimulated by UV light, cleavage activity was enhanced at least 5-fold, especially at 60 min. However, both the above two chemical modifications result in loss in enzymatic activity when compared with the nonmodified (parent) DNAzyme.

To address this, Asanuma and coworkers (72) reported the enhancement of the inherent cleavage activity of active DNAzymes by the covalent attachment of an intercalator such as azobenzene via an amide bond to D-threoninol, which inserts into the DNA backbone using typical phosphoramidite chemistry. The azobenzene chemistry gave a 3-fold increase in DNAzyme activity in a cell-free system over the parent molecule; moreover, its activity was repressible when exposed to UV light. A more recent approach, using nitropiperonyloxymethyl chemistry, which is altered (deprotected) by UV light, was used to synthesize DNAzymes that could potentially be activated in specific areas in cells or in the body (73).

Albeit DNAzyme technology is in its relative infancy, progress thus far suggests the emergence of an exciting era of gene modulation, one that may be able to dramatically change bioactivity in vivo of gene modulation, one that may be able to dramatically change bioactivity in vivo of gene modulation, one that may be able to dramatically change bioactivity in vivo of gene modulation, one that may be able to dramatically change bioactivity in vivo of gene modulation, one that may be able to dramatically change bioactivity in vivo of gene modulation, one that may be able to dramatically change bioactivity in vivo of gene modulation, one that may be able to dramatically change bioactivity in vivo of gene modulation, one that may be able to dramatically change bioactivity in vivo of gene modulation, one that may be able to dramatically change bioactivity in vivo of gene modulation, one that may be able to dramatically change bioactivity in vivo of gene modulation, one that may be able to dramatically change bioactivity in vivo.
Molecular Cancer Therapeutics

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