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Review

Brothers in Arms

DNA Enzymes, Short Interfering RNA, and the Emerging Wave of Small-Molecule Nucleic Acid-Based Gene-Silencing Strategies

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The past decade has seen the rapid evolution of small-molecule gene-silencing strategies, driven largely by enhanced understanding of gene function in the pathogenesis of disease. Over this time, many genes have been targeted by specifically engineered agents from different classes of nucleic acid-based drugs in experimental models of disease to probe, dissect, and characterize further the complex processes that underpin molecular signaling. Arising from this, a number of molecules have been examined in the setting of clinical trials, and several have recently made the successful transition from the bench to the clinic, heralding an exciting era of gene-specific treatments. This is particularly important because clear inadequacies in present therapies account for significant morbidity, mortality, and cost. The broad umbrella of gene-silencing therapeutics encompasses a range of agents that include DNA enzymes, short interfering RNA, antisense oligonucleotides, decoys, ribozymes, and aptamers. This review tracks current movements in these technologies, focusing mainly on DNA enzymes and short interfering RNA, because these are poised to play an integral role in antigene therapies in the future. ***(Am J Pathol 2007, 171:1079–1088; DOI: 10.2353/ajpath.2007.070120)***

Over the past century, our appreciation of the pathogenesis of human disease has continued to evolve with corresponding therapeutic developments. In particular, more recent significant advances in genomics have led to a substantial shift away from conventional perceptions and dogma to focus on intricate molecular and cellular pathways regulated by an array of key genes. It is at this interface that nucleic acid molecules are emerging as a potent force in further characterizing important molecular pathways and in defining themselves as a sustainable therapeutic class of agent. The ability to selectively attenuate the expression of specifically targeted genes represents an appealing method of therapy and a means of dissecting molecular function. As such, strategies to specifically knockdown gene expression have received considerable attention.

Paterson et al[1] demonstrated the utility of nucleic acids in modulating gene expression approximately 30 years ago. Zamecnik and Stephenson[2] soon after showed the capacity of antisense molecules to inhibit viral replication. The field of nucleic acid therapeutics has since evolved considerably with numerous gene targets and methods comprising both naturally occurring and synthetic molecules that have been applied *in vitro* and *in vivo* in a variety of contexts with varying degrees of success. Although target selection is clearly vital, the method used in achieving this is of equal importance. Strategies have included DNA enzymes (DNAzymes), siRNA, antisense oligonucleotides, decoys, ribozymes, and aptamers, all of which attenuate gene expression by interfering with cytosolic mRNA or translated protein. Currently, a num-

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ber of these approaches are being evaluated in human and higher animal trials and are poised to offer considerable inroads and additions to our current therapeutic armamentarium where an unmet clinical need exists. Issues that underpin the clinical feasibility of "antigene" nucleic acid strategies are many and include i) that the agent is gene specific and functionally active with temporal relevance to the particular disease process; ii) that the target gene should play a key role in the disease process and that its role should not readily be compensatable by other genes; iii) that target gene inhibition should not adversely influence normal physiological processes; iv) local versus systemic delivery routes; v) the choice of endogenous (eg, gene/viral transcription) or exogenous (eg, synthetic nucleic acid) nucleic acid delivery; vi) the choice of delivery agent (eg, naked DNA, polymer, cationic lipid, PEGylated liposome, protein/nucleic acid chimera, or complex); and vii) that nucleic acid modification/stabilization made to avoid degradation of the agent may contribute to nonspecific (off-target) effects. This review will focus mainly on DNAzyme and siRNA strategies and briefly cover recent developments in antisense oligonucleotides, decoys, ribozymes, and aptamers.

DNAzymes

DNA enzymes (DNAzymes or deoxyribozymes), like ribozymes, may be perceived as gene-specific molecular scissors. Catalytic DNA has not been observed in nature, and all existing molecules have been derived by *in vitro* selection processes similar to those used to identify aptamers (see below). The most well-characterized DNAzyme is the "10-23" subtype comprising a cation-dependent catalytic core of 15 deoxyribonucleotides[3] that binds to and cleaves its target RNA (Figure 1) between an unpaired purine and paired pyrimidine through a de-esterification reaction, producing a 2′,3′-cyclic phosphate terminus and a 5′-hydroxyl terminus. Sequence conservation in the border regions of the catalytic core is important for the maintenance of catalytic activity.[4] This core is flanked by complementary binding arms of 6 to 12 nucleotides in length that confer target mRNA specificity.

The 10-23 DNAzyme, named by virtue of its selection process *in vitro*, catalyzes 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 sensitive to small changes in target sequence.[5,6] DNAzyme activity is dependent on the prevailing secondary structure of long-target RNA at the cleavage site.[6] For this reason, it is important to test a range of molecules to identify those that display a high level of activity against biologically relevant target molecules. In terms of biological specificity, an important control in the assessment of DNAzyme antigene efficacy and specificity is the "scrambled DNAzyme," wherein the sequence of nucleotides in the binding arms of the DNAzyme is jumbled while the catalytic core is preserved. This produces a molecule of

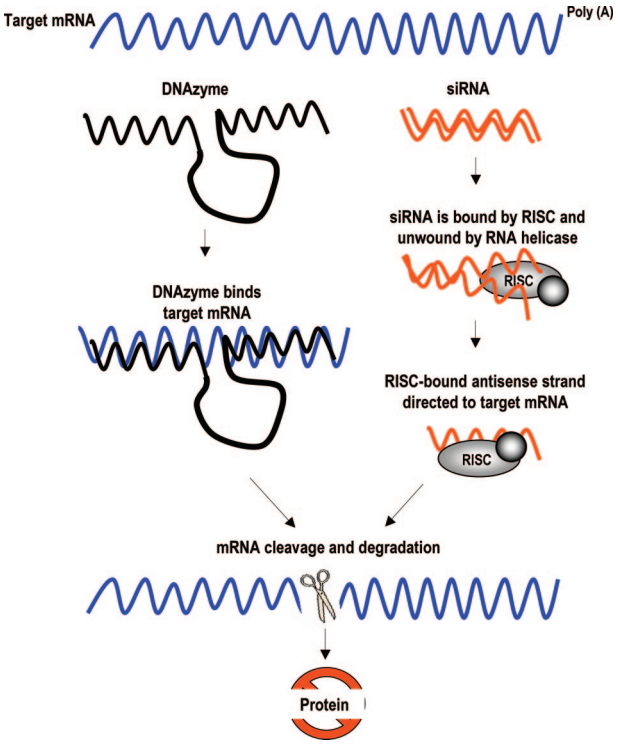

Figure 1. Schematic representation of DNAzyme- and siRNA-mediated degradation of target mRNA. Left: DNAzymes recognize the complementary mRNA sequence of its hybridizing arms via Watson-Crick base pairing and catalyze degradation of the target mRNA, producing two products, one containing a 2′,3′-cyclic phosphate terminus and the other a 5′-hydroxyl terminus. Right: siRNA is recognized by RNA-induced silencing complexes (RISC). RNA helicases unwind the double-stranded siRNA, and the antisense strand guides RISC to the complementary mRNA. Targeted mRNA is cleaved by RISC and rapidly degraded.

identical size, the same percentage composition of nucleic acids, and the same net charge with a binding sequence that is not matched to the target gene. DNAzymes with nonsense or mismatch sequences in the binding arms or with point mutations in the catalytic core that render the DNAzyme enzymatically inactive can serve as additional controls. *In vitro* gene and cell inhibitory applications of the 10-23 DNAzyme are summarized in Table 1.

A number of structural modifications have been used to enhance the stability and to improve the potency of DNAzymes. An important, commonly used modification is the incorporation of a 3′-3′ inverted nucleotide at the 3′ end of the DNAzyme to prevent exonuclease degradation. This can dramatically increase stability of the molecule, extending the half-life from ~70 minutes to >21 hours in human serum.[28] In addition, DNAzymes with this modification can remain functionally intact for at least 24 to 48 hours after exposure to serum compared with its unmodified counterpart[23,28] with little change in the kinetics.[7] Phosphorothioate (PS) linkages, which enhance stability by rendering the oligonucleotide more resistant to endogenous nucleases, have been used with DNAzymes.[29] The introduction of PS modifications may affect cleavage efficiency[28,30] and has been associated with toxicity,[31] immunological responsiveness,[32] and increased affinity for cellular proteins, resulting in sequence-independent effects.[33,34]

Table 1. *In Vitro* Applications of 10-23 DNAzymes

Gene	Cell type	Assay conditions	Proliferation assay	Cell death assay	References
c-*myc*	Rat aortic SMCs	SS	Yes	No	7
Transforming growth factor-β1	Rat mesangial cells	SS	No	No	8
Protein kinase C-ϵ	Human pulmonary artery SMCs	SS	No	No	9
bcr-abl	K562 cells	SFC	Yes	Yes	10
survivin	PANC-1 cells	SCC	Yes	Yes	11
ftsZ	DH5alphapro cells	SFC	Yes	No	12
PML/RARa	NB4 cells	SS	Yes	Yes	13
	K562 cells	SS	Yes	Yes	13
PB2	MDBK cells	SD	Yes	No	14
β1 and β3 integrin subunits	EA.hy 926	SCC	No	Yes	15
	K562	SCC	No	Yes	16
HIV-1 TATRev	HeLa	SS	No	Yes	17
	Cos-1	SS	No	Yes	17
	THP-1	SS	No	Yes	17
VEGF receptor-2	BAEC	SCC	Yes	Yes	18
	HUVEC	SCC	Yes	Yes	18
	MDA-MB-435	SCC	Yes	Yes	18
Urokinase-type plasminogen activator receptor	Saos-2	SS	No	Yes	19
Egr-1	MCF-7 cells	SS	Yes	No	20,21
	HMEC-1	SS	Yes	No	20
	Human vascular SMCs	SS	Yes	No	22
	Porcine vascular SMCs	SS	Yes	No	23
	Rat aortic SMCs	SS	Yes	Yes	23
c-Jun	Human vascular SMCs	SS	Yes	No	24
	Porcine vascular SMCs	SS	Yes	No	24
	HMEC-1				
	T79-Squamous CC	SS	Yes	Yes	25
	LK2-Squamous CC	SS	Yes	No	26
		SS	Yes	No	26
VDUP1	H9C2	SCC	No	Yes	27

SS, serum stimulation; SD, serum deprivation; SFC, serum-free conditions; SCC, serum-constant conditions.

Locked nucleic acids (LNAs), more recently, have been attractive monomers for modifying oligonucleotides[35] and DNAzymes,[30,36,37] in an attempt to increase binding affinity. LNA bases comprise a 2′-*O* 4-C methylene bridge that locks in a C3′-endo conformation,[35] which places constraint on the ribose ring, increasing affinity for complementary sequences.[38,39] The advantages of LNAs include increased thermal stability of duplexes toward complementary DNA or RNA, stability toward 3′-exonucleolytic degradation, solubility due to structural similarities to nucleic acids, easy automated synthesis with complete modified LNA or chimeric (LNA/DNA or LNA/RNA) oligonucleotides,[40,41] and straightforward cellular delivery using standard transfection reagents.[31,36,42,43] LNA incorporation into DNAzymes may influence catalytic activity under single-turnover conditions[30,36,37,43] and biological potency.[36,43] DNAzymes with an inverted nucleotide at the 3′ end are catalytically more efficient compared with their LNA-modified counterparts because of a slower product release rate.[30,44]

Accumulating evidence indicates the utility, efficacy, and potency of DNAzymes in a variety of animal models of disease, allowing characterization of key molecular pathways underlying pathogenesis and use as a therapeutic agent. For instance, DNAzymes targeting the "master-regulator" zinc finger transcription factor Egr-1[45,46] have shown promise in experimental models of restenosis via inhibition of smooth muscle cell hyperplasia. We have demonstrated inhibition of neointima formation in the rat carotid artery after both balloon injury (first demonstration of DNAzyme efficacy in an animal model) and carotid artery ligation.[23,47] Furthermore, intracoronary administration of DNAzymes targeting human Egr-1 reduced neointima formation in porcine coronary arteries after stent implantation.[22] Likewise, Egr-1 DNAzymes attenuated neointima formation in human internal mammary arteries *ex vivo*.[48] These and other *in vivo* applications of DNAzymes are summarized in Table 2.

We have also recently evaluated Egr-1 DNAzymes in the setting of myocardial infarction and demonstrated that intramyocardial delivery of Egr-1 DNAzymes in rats undergoing myocardial ischemia-reperfusion resulted in a 50% reduction in infarct size, myocardial neutrophil infiltration, and intercellular cell adhesion molecule-1 (ICAM-1) expression.[51] Four other studies have used DNAzymes to target the myocardium. Itescu and colleagues[55,56] conducted two separate studies in which intramyocardial delivery of DNAzymes targeting PAI-1 in a rodent model of myocardial infarction resulted in a reduction in apoptosis, improved functional recovery, and enhanced myocardial capillary density. The same group also used intramyocardial administration of DNAzymes targeting the vitamin D3 up-regulated protein 1, which promotes cellular oxidative stress, and demonstrated a reduction in cardiomyocyte apoptosis when delivered at the time of myocardial infarction in rats.[27] Iversen et al[57] delivered tumor necrosis factor-α DNAzymes via peritoneal osmotic minipumps in rats with myocardial infarc-

Table 2. *In Vivo* Applications of DNAzymes

Gene	Model	Applications	Reference
c-Jun	Rabbits, rats, mice	Restenosis, neovascularization, inflammation, tumor growth	24–26,49,50
Egr-1	Pigs, rats, mice	Restenosis, tumor growth, neovascularization, ureteral obstruction	20–23,36,47,51,52
Xylosyltransferase-1	Mice	Spinal regeneration	53
Transforming growth factor-β1	Rats	Glomerulonephritis	8
mPer1	Mice	Morphine addiction	54
PAI-1	Rats	Myocardial infarction	55 56
Vitamin D3 up-regulated protein 1	Rats	Myocardial infarction	27
Tumor necrosis factor-α	Rats	Congestive cardiac failure	57
VEGF-2	Mice	Tumor growth	18

tion-induced heart failure and demonstrated improved hemodynamic performance compared with controls.

DNAzymes targeting a second immediate-early gene, the leucine zipper transcription factor c-Jun, also show promise. We have demonstrated that c-Jun DNAzymes (Dz13) play a modulatory role in the inflammatory process by disrupting the expression of key downstream molecules, including the cell adhesion molecules ICAM-1 and VCAM-1, and E-selectin and VE-cadherin.[49] Dz13 rapidly abolished the processes of leukocyte rolling, adhesion, and extravasation in response to interleukin-1β stimulation in a rat microcirculation model. Intranasal administration of Dz13 abolished pulmonary inflammation in a murine lung sepsis model and joint inflammation in a murine arthritis model after intra-articular delivery. This represents a key area for future therapeutic exploitation because inflammation is integral in the pathogenesis of many diseases and current strategies are still far from optimal.

By exploiting the reliance of cancers on angiogenesis for growth, various transcription factors mediating this process have been targeted successfully to inhibit tumor growth. Zhang et al[18] first applied this technology *in vivo* against tumors with DNAzymes targeting vascular endothelial growth factor (VEGF) receptor 2 attenuating tumor growth in rats. Intratumorally administered DNAzymes targeting Egr-1 also demonstrated potent reduction in tumor growth with an associated reduction in tumor angiogenesis.[20] A direct antitumor effect was achieved with a humanized Egr-1 DNAzyme.[21] Furthermore, the Egr-1 DNAzyme inhibited *de novo* VEGF-induced neovascularization of the rat cornea.[25] Dz13, targeting c-Jun, attenuated solid melanoma and squamous cell carcinoma growth in mice in part via its suppression of tumor angiogenesis.[25,26] Further evidence of the antiangiogenic properties of Dz13 is demonstrated by its inhibition of corneal neovascularization in rats[25] and retinal neovascularization in mice induced by exposure to hyperoxia-normoxia.[49] Although *in vitro* assessment of DNAzyme efficacy helps establish gene and sequence specificity and facilitates high-throughput screening, the clinical utility of these agents, like any other potential therapeutic, can only be gauged in animal models, where important issues such as delivery, biodistribution, pharmacokinetics, metabolism, toxicity, and pharmacodynamics can be explored. Dz13, for example, has the capacity to inhibit restenosis,[24] angiogenesis,[25] tumor growth,[25,26] and as already discussed, inflammation[49] in animal models consistent with its activity in a variety of *in vitro* systems.

DNAzymes have been used in a variety of other animal models. For example, DNAzymes targeting transforming growth factor-β1, important in extracellular matrix accumulation, delivered by injection into the renal artery followed by electroporation in a rat anti-Thy-1 model of glomerulonephritis led to reduced extracellular matrix accumulation.[8] DNAzymes have also proved efficacious in the CNS when delivered via the intracerebroventricular route in a study in mice examining the role of the circadian "clock gene" mPer-1 in morphine addiction. Investigators found less morphine dependence in mice whose mPer-1 expression had been attenuated with mPer-1-targeting DNAzymes compared with those that did not.[54] The potential role of DNAzymes in augmenting spinal regeneration was explored in a study in which DNAzymes were designed to disrupt the enzyme xylosyltransferase-1, which is important in glycosylating the protein backbone of proteoglycans. The investigators found enhanced axonal regeneration in newborn rats with DNAzyme treatment and reduced glycosaminoglycan chains on proteoglycans.[53] These studies, taken together, demonstrate the potential of DNAzymes as gene-specific molecular tools. That DNAzymes possess a number of advantages over other gene-silencing techniques, including lower production cost and relative serum stability, makes these attractive therapeutic candidates en route to the clinic.

A recent *in vitro* study[58] has related DNAzyme catalysis using a short synthetic substrate with cell death as a measure of biological activity, making comparisons with published data on Dz13 and several other DNAzymes using different cleavage conditions, biological systems, and methodologies. Although oligonucleotides with some motifs, particularly those containing runs of multiple guanines, can induce nonspecific cellular effects[59,60] such as by interacting with particularly abundant cellular proteins, conclusions cannot be drawn in the absence of experiments appropriate for the targeted gene (eg, inducible immediate-early genes versus constitutively expressed genes) and without proper characterization of phenotypic effects (eg, problematic correlation of cell-free cleavage experiments with short substrates and cytotoxicity, synonymity of proliferation with survival and

toxicity, lack of stimulating conditions, or translation *in vivo*) as was the case in that study.[58] Dz13 activity nonetheless was found to be sequence- and dose-dependent, and the oligonucleotide lacked quadruplex structure.[58] Unexpected toxic side effects have not been observed *in vivo*, in the numerous animal models of disease treated with this DNAzyme to date (Table 2).

Although singular 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 tumor stasis, a combinatorial approach may be preferable in efforts to prevent neoplastic cells evolving mechanisms to avoid single agent-based therapy.[61] These strategies might also be used as adjuncts with conventional therapies such as thrombolytic agents (eg, urokinase-type plasminogen activator and warfarin) or antiproliferatives (eg, taxol and rapamycin).

siRNA

The advent of RNA interference (RNAi) as a gene-silencing strategy represents an exciting development in the field of small-molecule nucleic acid-based therapeutics. RNA interference was first described in 1998 by the 2006 Nobel Laureates Andrew Fire and Craig Mello, who demonstrated double-stranded RNA-mediated degradation of target mRNA in *Caenorhabditis elegans* and has subsequently been demonstrated in diverse eukaryotes. Short interfering RNA (siRNA) of 21 to 23 nucleotides processed by the RNase III family member Dicer are incorporated into an RNA-induced silencing complex (RISC). The sense strand of the double-stranded siRNA is cleaved during the formation of the RISC complex.[62] RNA helicases unwind the double-stranded siRNA, and the antisense strand guides RISC to the complementary target mRNA, which is cleaved by RISC (Figure 1).[63–65] siRNA avoids the problem of long double-stranded RNA-mediated activation of the interferon pathway, which can shutdown general protein synthesis and cause nonspecific mRNA degradation in mammalian cells,[66] although siRNAs synthesized from the T7 RNA polymerase system have been found to trigger interferon responses in a variety of cell lines.[67] Short-hairpin RNAs transcribed from RNA polymerase II or III promoters from plasmid- and virus-based vectors provide alternative strategies for RNA-mediated gene silencing.[68] Vector-based short-hairpin RNA are processed by Dicer into siRNA duplexes. These strategies have been recently applied in mammalian cancer genetics[69] and models of neurodegeneration,[70] and vector libraries are now commercially available for high-throughput screens.[71] Mechanisms of RNAi-mediated gene silencing in mammalian systems have been reviewed elsewhere.[71,72]

Some of the major concerns arising with siRNA applications *in vivo*, as with all small-molecule nucleic acid agents, are tissue specificity and the ability to withstand degradation by nucleases. The latter is of particular significance because the molecule has to "survive" within the host if it intends to provide lasting effects in the biological milieu. Tissue-specific delivery continues to present a key challenge for small-molecule nucleic acid therapeutics.[73] Although *in vivo* application of siRNA has attracted attention particularly in cancer therapeutics, systemic delivery would provide more clinical appeal than local intratumoral administration. It is becoming more common to formulate (polyplex or nanoplex) siRNA to incorporate compounds, ligands, or peptides to achieve tissue specificity and nuclease resistance, thus eliminating, as much as possible, non-tissue-specific uptake of siRNA. The targeted tissue would include the tumor itself, inhibiting cell proliferation or the neovasculature, inhibiting angiogenesis, and starving the tumor of a blood supply. Kim et al[74] have nanoplexed an siRNA to the polymer TargeTran, comprising a branched polyethylenimine, polyethylene glycol, and arginine-glycine-aspartate peptide sequence.[75] A nanoimmunodelivery system devised by Pirollo et al[76] demonstrated specific uptake of 6-FAM-labeled nanoplexed siRNA in primary tumors 20 minutes after systemic delivery. Furthermore, the nanoplexed siRNA, composed of an anti-transferrin receptor antibody, specifically penetrated deep into the tumors. Specificity for the tumor tissue was conferred by a lack of fluorescence (6-FAM) by blood vessels surrounding the tumor, suggesting uptake of siRNA by tumor cells and not endothelial cells.

The issue of whether siRNA diminishes over time because of degradation or dilution due to rapidly dividing cells was addressed by Bartlett and Davis.[77] siRNA polyplexed to transferrin, targeting the luciferase gene, specifically inhibited luciferase activity in nondividing hepatocytes in mice. Inhibition of luciferase activity after a bolus intravenous injection lasted 4 weeks, suggesting that stability of siRNA was not a limiting factor in gene silencing. Schiffelers et al[78] systemically delivered PEGylated siRNA to an arginine-glycine-aspartate peptide ligand specific to the VEGF receptor-2 transcript. They demonstrated a reduction in tumor progression of pre-established tumors xenografted in mice, and tumor regression was paralleled by a reduction in blood vessel formation surrounding the tumor.[78] Atelocollagen, a highly purified type I collagen, complexed with siRNA confers increased resistance to nucleases, efficiency in transducing cells and prolonged gene silencing.[79] Intratumoral injections of siRNA complexed with atelocollagen targeting VEGF inhibited tumor growth and tumor angiogenesis over 40 days after four repeated injections every 10th day.[80] The effectiveness of locally delivered VEGF siRNA in tumors was further complemented by Takeshita et al,[81] who systemically delivered atelocollagen siRNA. They demonstrated that atelocollagen complexed to siRNA improved cellular uptake in tumor tissue sixfold compared with naked siRNA with efficient inhibition of metastatic tumor growth in bone tissue.[81] More importantly, systemic delivery of atelocollagen siRNA failed to elicit an interferon or interleukin-12 response *in vivo*.

Local and systemic delivery of siRNA directed against target genes responsible for the progression of disease has been successful in animal models.[82] Furthermore, siRNA-

mediated specific cleavage of targeted mRNA, *in vitro* and *in vivo*, has also been successfully demonstrated.[83,84] Local delivery of siRNA has been used as a preventative for ocular neovascularization for the treatment of age-related macular degeneration and diabetic retinopathy.[74,85,86] VEGF or its receptors have been prime targets of siRNA for exerting antiangiogenic effects. Local, conjunctival, or periocular administration of siRNA targeting murine VEGF receptor-1 has been compared with systemic delivery.[74,84] A reduction in ocular neovascularization was observed by both groups. Shen et al[84] used a mouse model of laser-induced choroidal neovascularization (CNV), whereas Kim et al[74] used herpes simplex virus DNA containing bioactive CpG motifs to stimulate VEGF and subsequent neovascularization. More potent inhibition of ocular vascularization was evident through local administration rather than the systemic route. Furthermore, Kim et al[74] delivered a cocktail of siRNAs targeting VEGF, VEGF receptor-1, and VEGF receptor-2 at a 1:1:1 ratio and observed synergistic effects. More potent inhibition of ocular neovascularization and corneal VEGF protein and mRNA was achieved with this cocktail compared with siRNA targeting each transcript separately. Subretinal delivery of siRNA targeted to murine VEGF transcript inhibited choroidal neovascularization after laser photocoagulation.[85] Tolentino et al[86] locally delivered siRNA targeting VEGF to inhibit laser-induced choroidal neovascularization in nonhuman primates. Single intravitreal injection of Cand5 (VEGF siRNA) inhibited choroidal neovascularization in a dose-dependent manner and was sustained for 36 days with no evidence of inflammation, cataract formation, retinal detachment, or vitreous hemorrhage. Lipid-based systems may be useful for the systemic intracellular delivery of siRNA. Zimmerman et al,[87] for example, delivered apolipoprotein B siRNA encapsulated in stable nucleic acid lipid particles into nonhuman primates by intravenous injection, reducing levels of apolipoprotein B mRNA and protein, serum cholesterol, and low-density lipoprotein. The clinical interrogation of siRNA has commenced. There are at least two siRNA molecules in clinical trials. For example, Cand5 is in Phase II trials for age-related macular degeneration (Acuity Pharmaceuticals, Philadelphia, PA), and siRNA targeting the VEGF-receptor-1 (SiRNA-027) has successfully completed phase I (Merck & Co., Inc., Whitehouse Station, NJ) for the same condition.[84,86]

Antisense Oligonucleotides, Decoys, Ribozymes, and Aptamers

The wave of small-molecule nucleic acid-based gene-silencing strategies includes a mix of old and new players, such as antisense oligonucleotides, oligonucleotide decoys, ribozymes, and aptamers. These molecules differ in their mechanisms of action, and many are under clinical development for a wide range of disorders.

Antisense Oligonucleotides

Antisense oligonucleotides (ASOs) are single-stranded segments of DNA or RNA generally 15 to 25 bp in length. Although their precise mechanism of action is not fully understood, their function is mediated by interaction with target mRNA via hydrogen bonding, blocking translation into protein by steric hindrance of ribosomal movement along the transcript, or by activation of endogenous RNase H for targeted destruction of the DNA/RNA heteroduplex, resulting in mRNA degradation.[88] Unmodified ASO molecules are prone to degradation, and their negative charge makes cellular membrane penetration inefficient. As such, these molecules have evolved with a variety of modifications that enhance stability and efficacy. These have included the PS backbone modification, which increases oligonucleotide half-life. However, the introduction of PS into the backbone of ASOs increases the propensity of nonspecific interaction with other proteins, resulting in sequence-independent phenotypic effects or cytotoxicity.[33,34] High concentrations of PS ASOs can also bind and inhibit DNA polymerases and RNase H.[89,90] To eliminate off-target effects spurred by the introduction of PS ASOs, other substitutes have since been made. LNAs, described above, have been incorporated into the backbone of ASOs as LNA/DNA gapmers, increasing both target binding affinity and, more importantly, stability.[40,41,91,92] An alkyl modification, such as an 2′-*O*-alkyl modification to the ribose ring (2′-*O*-methyl or 2′-*O*-methoxyethoxy) averts the need for PS modifications, providing stability and efficacy.[93] 3′-3′-inverted T modifications have also remarkably increased oligonucleotide stability.[23,24,94] Takei et al[94] demonstrated greater stability of ASOs with 5′- and 3′-inverted T additions compared with PS-ASOs. In addition, 5′- and 3′-inverted T-modified ASOs inhibited tumor growth more effectively compared with PS ASOs after intratumoral injection. Comparison of siRNA with ASOs, each targeting green fluorescent protein, revealed more efficient inhibition by the siRNA in both cell culture and in mice.[95]

Around 50 clinical studies have used antisense strategies spanning a variety of disease processes, including cancer, cardiovascular disease, inflammation, and infection.[96] Fomivirsen, or Vitravene, which targets the immediate-early RNA encoded by human CMV DNA, has been approved by the United States Food and Drug Administration for use in humans in treatment of CMV retinitis via intravitreal administration.[97] Other antisense approaches that are currently entering Phase III trials include the ICAM-1 antisense Alicaforsen, which has shown promise in the treatment of inflammatory bowel disease when administered as a retention enema.[98,99] Recently, in a Phase III trial, the addition of an antisense oligonucleotide targeting protein kinase C-α, Aprinocarsen, to a standard chemotherapeutic regimen for advanced non-small-cell lung carcinoma failed to improve outcomes.[100] The bcl-2 oligonucleotide oblimersen, or Genasense, is currently in phase II/III for a variety of cancers, including chronic lymphocytic leukemia, acute myelocytic leukemia, melanoma, and multiple myeloma, and has been administered via intravenous and subcutaneous routes.[101]

Decoys

In contrast to antisense approaches that target mRNA, oligonucleotide decoys are short, double-stranded DNA molecules that contain binding elements for a variety of protein targets that competitively inhibit promoter binding and gene expression. Several types of decoys have been developed, including unmodified oligonucleotide duplexes, α-β-anomeric oligonucleotides, duplexes with methylphosphonate- and phosphorothioate-modified bonds, and circular dumbbell double-stranded oligodeoxynucleotides.[102] Morishita et al[103] demonstrated suppression of neointima formation with a decoy oligonucleotide to E2F in a rat model of carotid injury. This was extended further in a rabbit model of vein-conduit arterial bypass grafting in cholesterol-fed rabbits with a reduction in the incidence of neointima formation and atherosclerosis at 6 months in animals treated *ex vivo* with the E2F decoys.[104] Following from the PREVENT trial, which established feasibility and safety of the E2F decoy Edifoligide in infra-inguinal vascular bypass surgery,[105] the recently reported Phase III PREVENT IV study evaluated the efficacy of Edifoligide in preventing vein graft failure in patients undergoing coronary artery bypass grafting. Although safe and well tolerated, no significant improvements in graft failure rate or angiographic appearances of vein grafts at 12 months was achieved.[106] Whether the established effects of Edifoligide on smooth muscle cell proliferation translates into longer-term benefits in this context remains to be seen. Other issues affecting the potential clinical use of molecules include susceptibility to nuclease degradation, propensity to induce a host immunological response, and cell transfection difficulties necessitating higher concentration requirements.

Ribozymes

Ribozymes are catalytically active RNA molecules capable of site-specific cleavage of target mRNA and, unlike DNAzymes, can occur naturally. Like DNAzymes and ASOs, ribozymes need access to their binding sites in the target RNA. Several subtypes have been described; those most commonly studied are hammerhead and hairpin ribozymes,[44] which differ in their catalytic response to changes in solvent pH rather than their capacity to bind and ligate cleavage products or reliance on metal ions.[107] Ribozyme catalytic activity and stability can be improved by substituting deoxyribonucleotides for ribonucleotides at noncatalytic bases.[108] Chimeric DNA-RNA hammerhead ribozymes targeting platelet-derived growth factor A-chain mRNA have been shown to inhibit intimal thickening in balloon-injured rat carotid arteries after local delivery,[109] whereas those targeting transforming growth factor-β protect against renal injury in hypertensive rats after systemic (intraperitoneal) delivery.[110] Clinically, ribozymes have been explored therapeutically in several small trials. Hammerhead anti-HIV ribozymes have been used in T-lymphocyte expansion strategies *ex vivo* followed by infusion into patients.[111–114] Hammerhead ribozymes targeting a highly conserved portion of 5′-untranslated region of hepatitis C virus HEPTAZYME[115] showed promise in phase I and II trials. However, because of toxicological concerns, the study was suspended.[116] Ribozymes have also been evaluated as potential adjuncts in cancer therapy. These include the synthetic antiangiogenic ANGIOZYME, which targets the VEGF receptor VEGF R1 (Flt-1) in a variety of solid tumors,[117] and HERzyme, which targets human epidermal growth factor-2 overexpressed in breast and ovarian cell carcinoma.[118]

Aptamers

Finally, aptamers (from the Latin *aptus*, "to fit") are synthetic oligonucleotide ligands that have been derived by *in vitro* selection from a combinatorial library of nucleic acid sequences that like decoys (but unlike antisense approaches) bind their target protein with high affinity and specificity, inhibiting function. The clinical appeal of aptamers has been enhanced by the introduction of chemical modifications, such as substitutions of the 2′-OH groups of the ribose backbone to provide resistance against enzymatic degradation.[119] Pegaptanib, an RNA aptamer targeting $VEGF_{165}$, has been evaluated in patients with neovascular age-related macular degeneration. Intravitreal delivery of this agent results in less visual loss and other clinically relevant improvements as early as 6 weeks, and this agent has been approved by the United States Food and Drug Administration for use against age-related macular degeneration.[120] RNA and DNA aptamers have also been developed that inhibit HIV-1 function by directly interfering with key proteins at critical stages in the viral replication cycle.[121] Other antiviral aptamers under development include those targeting hepatitis C virus and influenza virus.[122] More recently, DNA or RNA molecules have been selected based on their capacity to bind targets with high affinity and specificity using the systematic evolution of ligands by exponential enrichment combinatorial oligonucleotide library-based *in vitro* selection approach.[123]

Parting Remarks

Gene targeting using nucleic acid strategies has now entered a new era with the evolution of stable, potent, and effective molecules. In particular, DNAzymes, siRNA, and antisense oligonucleotides by virtue of their relative specificity and stability have enabled precise targeting of genes regulating pivotal processes in the pathogenesis of disease, providing an exciting class of potential therapeutic tool and a means of understanding complex transcriptional and molecular pathways. Current studies have demonstrated their versatility and potency in disrupting pathophysiologically important pathways, via a variety of different delivery routes with relative specificity of action and *in vivo* stability.

With the ongoing identification of new genes and an appreciation of their regulatory pathways and pathological roles, small-molecule antigene strategies have not only emerged as an important molecular approach to delineate the functions of these genes but also are now a clinical reality inching closer to mainstream therapeutics. Progress over the next few years will determine the feasibility of

small-molecule nucleic acids to silence disease-causing genes in man specifically and with minimal toxicity.

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