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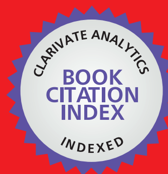

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Chapter 8

Small-molecule Nucleic-acid-based Gene-silencing Strategies

Zhijie Xu and Lifang Yang

Additional information is available at the end of the chapter

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Abstract

Gene-targeting strategies based on nucleic acid have opened a new era with the development of potent and effective gene intervention strategies, such as DNAzymes, ribozymes, small interfering RNAs (siRNAs), antisense oligonucleotides (ASOs), aptamers, decoys, etc. These technologies have been examined in the setting of clinical trials, and several have recently made the successful transition from basic research to clinical trials. This chapter discusses progress made in these technologies, mainly focusing on Dzs and siRNAs, because these are poised to play an integral role in antigene therapies in the future.

Keywords: Gene-targeting strategies, DNAzymes, siRNAs, basic research, clinical trials

1. Introduction

Over the past decade, it is known that the advent of oligonucleotide-based gene inactivation agents have provided potential for these to serve as analytical tools and potential treatments in a range of diseases, including cancer, infections, inflammation, etc. During this time, many genes have been targeted by specifically engineered agents from different classes of small-molecule nucleic-acid-based drugs in experimental models of disease to probe, dissect, and characterize further the complex processes that underpin molecular signaling. Subsequently, 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 deoxyribozymes (DNAzymes, Dzs), ribozymes, siRNAs, ASOs, aptamers, and decoys. This chapter tracks

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current movements in these technologies, focusing mainly on Dzs and siRNAs, because these are poised to play an integral role in antigene therapies in the future.

2. DNAzymes

Among the gene-silencing technologies, Breaker and Joyce, in 1994, used an *in vitro* selection method to identify a special Dz from a random pool of single-stranded DNA to catalyze Pb^{2+}-dependent cleavage of an RNA phosphodiester linkage [1]. Afterward, a number of Dzs were created with the capacity to catalyze many reactions, including the cleavage of DNA or RNA, the modification and ligation of DNA, and the metalation of porphyrin rings. However, because of the low efficiency of RNA cleavage, they are not widely used for biological applications except for 10-23 Dz [2]. The inherent catalytic RNA-cleaving property of Dzs has been used with different mRNA targets as *in vitro* diagnostic and analytical tools, as well as *in vivo* therapeutic agents.

2.1. The possible mechanisms and characteristics of DNAzymes

Dzs of the 10-23 subtype are single-stranded DNA catalysts that comprise a central cation-dependent catalytic core of around 15 deoxyribonucleotides [ggctagctacaacga], and two complementary binding arms of 6–12 nucleotides that are specific for each site along the target RNA transcript [3]. As diagrammed in Figure 1, the enzyme binds the substrate through Watson-Crick base pairing and cleaves a particular phosphodiester linkage located between an unpaired purine and paired pyrimidine in the RNA. This results in the formation of 5′ and 3′ products, which contain a 2′, 3′-cyclic phosphate and 5′-hydroxyl terminus, respectively. Even though the 10-23 Dz can cleave any RY junction, the reactivity of each substrate dinucleotide compared in the same background sequence with the appropriately matched DNAzyme is found to follow the scheme AU = GU > GC >> AC. Murray *et al*. found that when the target site core is an RC dinucleotide, the relatively poor activity could be enhanced up to 200-fold by substituting deoxyguanine with deoxyinosine, which could effectively reduce the strength of Watson-Crick pairing between bases flanking the cleavage site [4].

Due to the simple cleavage-site requirement, Dzs are capable of cleaving any particular mRNAs for multiple turnover by appropriately designing the sequence in the binding arms. Several features make Dzs attractive from a drug developmental viewpoint. For example, these are inexpensive to synthesize, and their small size allows specificity. Moreover, DNAzymes can be rendered more stable by structural modifications, such as phosphorothioate (PS) linkages, locked nucleic acids (LNAs), and 3′-3′ inverted nucleotide end of the DNAzyme [5]. Enhanced biostability, low toxicity, affinity, and versatility suggest great promise for diagnostic and therapeutic applications [6]. Limitations thus far in the development of DNAzymes as novel therapeutics have been delivery and biodistribution, which revolve around poor cellular uptake and stability. Delivery systems depend on the route of administration and the target site. Moreover, an ideal delivery system would facilitate rapid and efficient distribution to the site of action, stability, low toxicity, and efficacy.

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5'-NNNNNNNNNR*YNNNNNNNNN-3'      Substrate RNA
   |||||||||  ||||||||||
3'-NNNNNNNNN  NNNNNNNNNN-5'      10-23 DNAzyme
Binding arm I G  A  G  G  Binding arm II
             C          C
            A            T
             A          A
              C        G
               A  T  C        R=A, G
                              Y=U, C
```

Figure 1. Secondary structure of the 10-23 DNAzyme–substrate complexes. The 10-23 DNAzyme consists of two variable binding arms, designated arm I and arm II, which flank a conserved 15 base unpaired motif that forms the catalytic core. The only requirement of the RNA substrate is for a core sequence containing an RY junction.

2.2. DNAzymes delivery systems – Past to present

As in all nucleic-acid-based reagents, efficient drug delivery systems (DDSs) to deliver the Dzs to targeting site are highly needed. Furthermore, by adopting DDSs, it could be helpful to solve the obstacles about DNAzymes' stability, biological effects, and toxicity. Several seminal studies have demonstrated that certain DNAzyme delivery systems can efficiently encapsulate DNAzymes and transfect them into cells without clear toxicity. The attempt first involved the microspheres of co-polymers poly (lactic acid) and poly (glycolic acid) (PLGA), which encapsulated the Dzs. PLGA microspheres are able to achieve biphasic release and sustained accumulation of the Dzs [7]. In a second delivery system, a chimeric aptamer–DNAzyme conjugate was generated for the first time using a nucleolin aptamer (NCL-APT) and survivin Dz (Sur_Dz). This conjugate could be used as a specific gene-targeting therapy to kill the targeted cancer cells [8]. A third delivery system is developed and studied based on the cationic liposomal formulation technology. Li *et al.* reported the effect of a c-Jun targeted DNAzyme (Dz13) in a rabbit model of vein graft stenosis after autologous transplantation in a cationic liposomal formulation containing 1,2-dioleoyl-3-trimethylammonium propane (DOTAP)/1,2-dioleoyl- snglycero-3-phosphoethanolamine (DOPE). Dz13/DOTAP/DOPE allows sufficient uptake by the veins and reduces SMC (smooth muscle cell) proliferation and c-Jun protein expression *in vitro*. Meanwhile, a Phase I clinical trial has indicated that it is safe and well tolerated after local administration in skin cancer patients [9]. Finally, due to their low toxicity and no side effects, nanoparticulate systems have spread rapidly and could significantly enhance the efficacy of tumoricidal Dzs. Marquardt *et al.* found that c-Jun targeted Dz13 delivered in this manner is capable of enhanced skin penetration efficiency and cellular uptake with a high reduced degradation of Dz13 in *vitro* [10]. These results indicate that, with more suitable delivery approaches, the biological effects of Dzs would be further increased, and the Dzs could be applied to new subject areas.

2.3. Application of DNAzymes *in vivo* and *in vitro*

Increasing evidence indicates the efficacy and potency of DNAzymes *in vivo* and *in vitro* in a range of disease settings, allowing characterization of key pathogenic pathways and their

potential use as therapeutic agents (Table 1). DNAzymes have been widely applied as a new interference strategy in the treatment of many conditions, including cancer, viral diseases, and vein graft stenosis. For instance, Dz13 targeting the transcription factor c-Jun has shown promise in experimental models of mice infected with H5N1 virus via reducing H5N1 influenza virus replication and decreasing expression of pro-inflammatory cytokines [11]. Furthermore, Dz13/DOTAP/DOPE reduces SMC proliferation and c-Jun protein expression *in vitro*, and inhibits neintima formation after end-to-side transplantation, which may potentially be useful to reduce graft failure [9]. Likewise, Cai *et al.* demonstrated that safe and well-tolerated Dz13 could inhibit tumor growth and reduce lung nodule formation in a model of metastasis [12].

Target	Summary Description on Biological Effects (In Vitro and In Vivo)	Refs.
LMP1	·Inhibiting proliferation and metastasis ·Promoting apoptosis ·Enhancing radiosensitivity	[13-15]
Egr-1	·Inhibiting proliferation and metastasis ·Suppressing tumor growth	[16]
MMP-9	·Inhibiting invasion and metastasis ·Suppressing tumor growth	[17, 18]
IGF-II	·Inhibiting proliferation ·inducing caspase-dependent apoptosis	[19]
survivin	·Inhibiting proliferation ·Promoting apoptosis	[8]
β-integrin	·Inhibiting invasion and metastasis ·Blocking angiogenesis	[20]
VEGFR-1	·Blocking angiogenesis ·Suppressing tumor growth	[21]
DNMT1	·Inhibiting proliferation	[22]
Bcl-XL	·Promoting apoptosis ·Enhancing Taxol chemosensitivity	[23]
c-Jun	·Inhibiting proliferation ·Restraining virus replication and host inflammation ·Suppressing tumor growth	[9, 11, 12]
BCR-ABL T315I	·Overcoming imatinib resistance based on BCR-ABL T315I Mutation	[24]
EGFR T790M	·Overcoming EGFR T790M mutant-based TKI resistance	[25]
TXNIP	·Attenuating oxidative stress, renal fibrosis, and collagen deposition	[26]

Table 1. *In vivo* and *in vitro* applications of 10-23 DNAzymes

As is well known, treatment resistance is one of the leading causes of tumor recurrence. We have recently evaluated Dz1 targeting latent membrane protein 1 (LMP1) in the setting of nasopharyngeal carcinoma model and demonstrated that injected intratumorally DZ1 with fuGENE 6 in nude mice inoculating LMP1-positive cells resulted in a significant inhibition of tumor growth and an enhanced radiosensitivity. Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) showed that DZ1 reduces the angiogenesis and microvascular permeability [13]. Other studies have used DNAzymes to target the other key genes in cancer therapy. DNAzyme targeting the Bcl-XL gene significantly sensitized a panel of cancer cells to apoptosis and further to reverse the chemoresistant phenotype [23]. Due to a secondary mutation at T790M in the epidermal growth factor receptor (EGFR), most of nonsmall-cell lung cancer (NSCLC) patients will eventually develop resistance to tyrosine kinase inhibitors (TKIs) treatment. Allele-specific silencing of EGFR T790M expression and downstream signaling by DNAzyme DzT could suppress the growth of xenograft tumors derived from $H1975^{TM/LR}$ cells, indicating that DzT is capable of overcoming EGFR T790M mutant-based TKI resistance [25]. In a similar way, Kim *et al.* developed the DNAzyme that specifically targets the site of the point mutation (T315I), conferring imatinib resistance in BCR–ABL mRNA. Cleavage of T315I-mutant ABL mRNA by DNAzyme could significantly induce apoptosis and inhibit proliferation in imatinib-resistant BCR-ABL-positive cells [24].

2.4. DNAzymes in clinical trials

The favorable properties of 10-23 Dzs, such as their enhanced biological stability, negligible side effects, and lack of immunogenicity, have paved the way for Dzs to enter clinical trials [17]. Up to now, Dzs to three targets have been undergoing clinical trials and at least one of them has proved its therapeutic efficacy in Phase II trials (Table 2). These results further show the potential of Dzs therapeutic approach for the treatment of diseases and represent a major advance in this field.

As we have found that LMP1-targeted Dz1 could effectively inhibit the growth and enhance the radiosensitivity of NPC cells both *in vivo* and *in vitro*, we investigated the antitumor and radiosensitizing effects of Dz1 in NPC patients for the first time [27]. Being safe and well tolerated, a randomized and double-blind clinical study was conducted in 40 NPC patients, who received Dz1 or saline intratumorally in conjunction with radiation therapy. In a 3-month follow-up, compared with the saline control group, the mean tumor regression and undetectable EBV-DNA copy number in the DZ1 group is significantly higher. Molecular imaging analysis found that Dz1 was tested to accelerate the decline of K^{trans}, generally recognized as a marker of tumor blood flow and permeability [28].

The nuclear transcription factor c-Jun is preferentially expressed in a range of cancers. Dz13 cleaves at the G1311U junction in human c-jun mRNA and exerts its antitumor activity via induction of apoptosis, inhibition of angiogenesis, and the induction of adaptive immunity [11]. A phase I first-in-human trial is conducted to determine the safety and tolerability of Dz13 in nine patients with basal-cell carcinoma (BCC), who received a single intratumoral injected

Target	Disease	Phase	Trial ID	Refs.
LMP1	Nasopharyngeal carcinoma	Phases I/II Completed	NCT01449942	[27, 28]
c-Jun	Nodular basal-cell carcinoma Melanoma with satellite or in-transit metastasis	Phases I Completed Phase I/Ib Ongoing	ACTRN12610000162011 ACTRN12613000302752	[29]
GATA-3	Asthma	Phases I Completed	NCT01470911	[30-33]
	Atopic dermatitis	Phases I Completed Phases I Completed Phases II Completed Phase IIa Completed Phases I Completed	NCT01554319 NCT01577953 NCT01743768 EUCTR2012-003570-77-DE NCT02079688	
		Phases IIa Ongoing		
	Ulcerative colitis Chronic obstructive pulmonary disease	Phases I/II Ongoing Phase IIa Pending	NCT02129439 DRKS00006087	
	Atopic eczema	Phase IIa Ongoing	EUCTR2013-001091-38-DE	

Table 2. Clinical trials of DNAzymes in anti-diseases therapy

dose of Dz13 (10, 30, or 100 ìg) [29]. Followed-up over four weeks, c-Jun expression is reduced in all nine participants. Meanwhile, Dz13 could significantly promote apoptosis and stimulate inflammatory and adaptive immune responses in the tumors. Among the participants, five patients have a reduction in histological tumor depth. These results indicated that Dz13 possibly could represent a future treatment option for BCC prior to excision by surgery.

The transcription factor GATA-3 plays an important role in the regulation of Th2-mediated immune mechanisms such as in allergic bronchial asthma, and the DNAzyme hgd40 has been shown to specifically and selectively reduce expression of GATA-3 mRNA. Turowska *et al.* found that hgd40 is evenly distributed in inflamed asthmatic mouse lungs within minutes after single dose application, and could slowly eliminate from lung tissue with the goal to minimize accumulation and to ensure continued exposure for efficacy [32]. Safety pharmacology studies showed that with no observable adverse event, hgd40 has a highly favorable toxicity profile when administered by aerosol inhalation at the therapeutic doses [33]. With good safety and tolerability in the phase I program [31], a randomized, double-blind, placebo-controlled, multicenter clinical trial of hgd40 was conducted in patients with allergic asthma, who had biphasic early and late asthmatic responses after laboratory-based allergen provocation [30]. After each study drug administered by inhalation once daily for 28 days, hgd40 significantly attenuates both late and early asthmatic responses and improves lung function. Moreover, the Th2-regulated inflammatory responses are also attenuated.

These studies, taken together, further demonstrate the potential use of DNAzymes as gene-targeting drugs. As Dzs are safe and well tolerated in humans, there is a good chance that we may witness the Dzs reaching the clinic in the near future.

3. Small interfering RNA

Small interfering RNA (siRNA), first discovered in plants and *Caenorhabditis elegans* and later in mammalian cells, is a member of a family of noncoding RNAs (ncRNAs) that affect and regulate gene transcriptional and posttranscriptional silencing [34]. This sequence-specific gene-silencing phenomenon could cause mRNA to be effectively broken down after transcription, resulting in no obvious translation. SiRNA represents an emerging therapeutic approach against diseases for *in vivo* and *in vitro* studies, and along with novel drug delivery techniques, the challenge of siRNA-based therapeutics is only now being optimized. These discoveries led to a surge in interest in harnessing siRNA for biomedical research and drug development.

3.1. The possible mechanisms and challenges of siRNAs

SiRNAs, synthetic mediators of RNA interference (RNAi), are basically dsRNA molecules designed specifically to silence expression of target genes. Cytoplasmic dsRNA molecules are considered unusual and are substrate for endonuclease Dicer, an RNase III family member. Vertebrate-specific TAR (HIV trans-activator RNA) RNA-binding protein (TRBP) and protein kinase R-activating protein (PACT) help Dicer to identify and dice dsRNA into about 21 bp fragments with 2 nucleotides overhangs at each end, generating the siRNA. Then recognized by an important enzyme Argonaute 2 (AGO2), siRNA of 21-23 nucleotides are incorporated into an RNA-induced silencing complex (RISC). RNA helicases unwind the double-stranded siRNA. The sense strand of the double-stranded siRNA is cleaved during the formation of the RISC complex, and the antisense strand guides RISC to the complementary target mRNA, which is rapidly degraded by RISC (Figure 2) [35, 36].

Though siRNAs can efficiently silence target gene expression in a sequence-specific manner, many challenges, including rapid degradation, poor cellular uptake, off-target effects and immune response, need to be addressed in order to carry these molecules into clinical trials [37, 38]. For example, Chung *et al*. illustrated the underappreciated off-target effects of siRNA gene knockdown technology. Hepatitis C Virus (HCV) depends on a core MOBKL1B (Mps one binder kinase activator-like 1B)–NS5A peptide complex to complete its life cycle. However, without the absence of MOBKL1B, siRNA of MOBKL1B still has off-target inhibitory effects on virus replication [39]. Researchers have tried to develop modified method to reduce the disadvantages. By using the default parameters in siDirect 2.0 Web server (http://siDirect2.RNAi.jp/), at least one qualified siRNA for >94% of human mRNA sequences in the RefSeq database can be designed [40]. In addition, chemical modifications have been shown to protect siRNAs from nuclease degradation without interfering with siRNA-silencing efficiency [37]. Thus, improvements in rational design strategies might have the potential to make the siRNAs

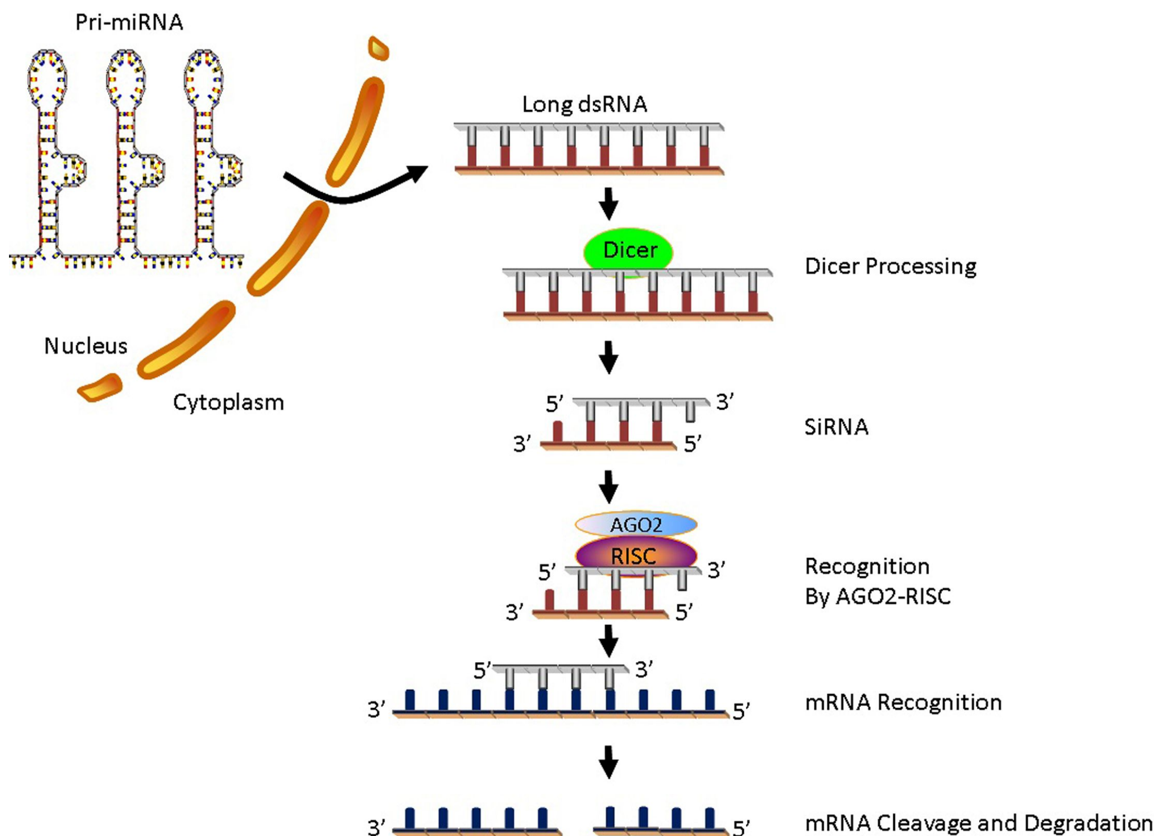

Figure 2. The process of siRNA-mediated degradation of target mRNA in eukaryotic cells. siRNA is recognized by AGO2 and incorporated into the RISC. After that, RNA helicases unwind the double-stranded siRNA, and the anti-sense strand guides RISC to the complementary targeted mRNA, which is cleaved by RISC and rapidly degraded.

more effective in the near future and to open the door to development of highly effective and safe therapeutics for clinical applications.

3.2. SiRNAs delivery systems

Delivery of siRNAs to target tissues is impeded by many barriers at different levels. As possible drugs in the near future, targeted delivery of siRNAs provides remarkable opportunities for accelerating RNAi-based high-performance treatments. The success of siRNAs-based delivery systems may be dependent upon uncovering a delivery route and sophisticated delivery carriers. In this regard, Fujita *et al.* have reported a powerful platform (PnkRNA™ and nkRNA®) to promote naked RNAi approaches through inhalation without delivery vehicles in lung cancer xenograft models. This modified local drug delivery system could offer a promising strategy for enhancing RNAi effects in cancer therapy [41]. In addition, with high binding specificity, nucleic acid aptamer represents a different promising tool for selective delivery siRNAs to cancer cells or tissues, resulting in increasing the therapeutic efficacy as well as reducing toxicity [42]. Likewise, the latest studies in using cell-penetrating peptides (CPPs) combined with molecular cargos, including liposomes, polymers, nanoparticles, and so on, have indicated that for the delivery of siRNAs, the combination strategy can remit the

reduced internalization efficiency caused by neutralization [43]. However, each transfection process needs to be optimized because of cell density, siRNA concentration, transfection reagents, etc.

3.3. Application of siRNAs – From the bench to the clinic

The discovery of RNA interference (RNAi) was approximately 20 years ago, and opened up a new mechanism for gene-silencing therapeutics. Kim *et al.* evaluated the inhibition effect on Notch1 expression by siRNA, and found that Notch1-targeted-siRNA could result in retarded progression of inflammation, bone erosion, and cartilage damage in collagen-induced arthritis (CIA) mice by efficiently inhibiting the expression of Notch1 in mRNA level [44]. Cao *et al.* demonstrated that after silencing the expression of vascular endothelial growth factor (VEGF) by siRNA, the number of living cells on the gel and the mucosa thickness are significantly decreased *in vivo*, which indicated siRNA-targeting VEGF may be useful as a convenient therapeutic option for chronic rhinosinusitis [45]. Similarly, VEGF-siRNA decreases the vessel-forming ability and exhibited no testable cytotoxicity by significantly decreasing the expression of VEGF mRNA and protein [46].

To date, given the progress of basic research, there are examples of clinical trial projects based on RNAi technology against cancer and other diseases. SiRNA therapeutics is now well poised to enter the clinical formulary as a new class of drugs in the near future. In an open-label phase I/IIa study in the first-line setting of fifteen patients with nonoperable locally advanced pancreatic cancer (LAPC), an siRNA drug (G12D) against KRAS, a Kirsten ras oncogene homolog from the mammalian ras gene family, is well tolerated, safe, and demonstrated a potential therapeutic efficacy to the patients enrolled, when combined with chemotherapy. However, five participants experienced serious adverse events [47]. In addition, a recent systematic analysis of a new RNAi therapeutic agent based on cationic lipoplexes containing chemically stabilized siRNAs, called Atu027, which silences expression of protein kinase N3 in the vascular endothelium in patients with advanced solid tumors. In one case of 24 patients, the study showed that Atu027 is tolerated up to 0.180 mg/kg, and no obvious dose-dependent toxicities are observed [48]. Likewise, the results from another case of 34 patients showed that Atu027 is safe in patients with advanced solid tumors, with 41% of patients having stable disease for at least 8 weeks [49]. Also, because SYL040012 is an siRNA designed to specifically silence β adrenergic receptor 2 (ADRB2) currently under development for glaucoma treatment *in vivo* and *in vitro* [50], a phase I clinical trial of SYL040012 with 30 healthy subjects having intraocular pressure (IOP) below 21 mmHg was conducted [51]. This trial found that administration of SYL040012 over a period of 7 days significantly reduced IOP values regardless of the dose used, was well tolerated locally and had no local or systemic adverse events. Thus, taken together, these clinical studies conducted on siRNAs in the past few years indicate that safe and effective target gene knockdown is achievable. Though targeting any individual gene might lead to unanticipated clinical toxicity that could stop the development of any individual siRNA drug, we anticipate a rapid expansion of clinical trials for multiple clinical indications.

4. Antisense oligonucleotides

Antisense oligonucleotide, first recognized in 1978 by Zamecnik and Stevenson, is a small synthetic piece of DNA (usually 15–18 mer in length) that can bind complementary RNA by Watson-Crick base pairing. ASOs can target most RNA transcripts and have emerged as the ideal therapeutic agents for a broad number of diseases [52, 53]. Upon binding to their target, ASOs can modulate the intermediary metabolism of RNA by the recruitment of endogenous RNase H1 to interfere with RNA function [54]. Human RNase H1 is a ubiquitous enzyme that hydrolyzes the duplex formed between a DNA containing ssASO and target RNA through its N-terminus RNA-binding domain. In order to cleave the RNA in the duplex, the RNase H1 catalytic domain needs at least 5 consecutive DNA/RNA base pairs, and cleavage usually occurs within 7–10 nucleotides from the 5′-end of the RNA. After cleavage, the exposed phosphate on the 5′-end and hydroxyl on the 3′-end are recognized, and the RNA is subsequently degraded by cellular nucleases. At some point after RNase H1 cleaves the RNA, the ssASO is released and is available to reengage another transcript.

Even though much progress has been made in the ASO field so far, there are still many questions that might result in nonspecific effects. One of the principle challenges for success is efficacious delivery to target organs. Because initial ASO molecules are either of low affinity or low membrane permeability, they suffered from poor solubility and rapid degradation by nucleases. In the field, many studies to improve the therapeutic potential of ASOs have focused on chemical modifications to either improve nuclease resistance, such as 2′-O-methoxyethyl (2′-MOE), or to facilitate cellular uptake, like phosphorothioate backbone that improves membrane penetration [55, 56]. Moreover, too many heparin-binding cell surface proteins have been identified to bind the phosphorothioate oligo with nanomolar affinity. The delivery of ASO drug, encapsulating with materials ranging from cationic lipids to dendrimers to alginate/chitosan nanoparticles, has reached new heights of clinical acceptance [52].

Over the past several years, antisense oligonucleotide-based targeted therapy has emerged rapidly. Interest in the field has ramped-up dramatically, as numerous ongoing clinical trials are evaluating the treatment effect on diseases with ASOs. Antisense oligonucleotide sodium LY2181308 (LY2181308), hybridizing to the human survivin mRNA, is well tolerated in patients with acute myeloid leukemia (AML). In combination with chemotherapy, LY2181308 does not cause additional toxicity, though 1/16 patients had incomplete responses, and 4/16 patients had cytoreduction [57]. Thus, future clinical trials are needed to further confirm its clinical benefit. In another open-label, parallel-group study, reducing factor XI levels by a second-generation antisense oligonucleotide FXI-ASO (ISIS 416858) is an effective method for prevention of postoperative venous thromboembolism. With respect to the risk of bleeding, FXI-ASO received once daily appeared to be safe [58]. In another phase II trial, compared with those who received placebo, the participants with Crohn's disease who received SMAD7 ASO Mongersen (formerly GED0301) had significantly higher rates of remission and clinical response [59]. Even more important, mipomersen, an antisense agent targeted to apolipoprotein B, has recently received FDA (United States Food and Drug Administration) approval for the treatment of familial hypercholesterolemia (http://www.fda.gov/newsevents/newsroom/

pressannouncements/ucm337195.htm). This compelling therapeutic potential powerfully supports further clinical investigations of ASOs in subjects in the near future.

5. Ribozymes

Ribozymes, also termed catalytic RNA, are highly structured RNA sequences that can be engineered to specifically cleave target RNA molecules, similar to the action of protein enzymes. However, unlike protein ribonucleases, ribozymes cleave only at a specific location, using base-pairing and tertiary interactions to help align the cleavage site within the catalytic core. The general mechanism of ribozymes is as follows: a 2′-oxygen nucleophile attacks the adjacent phosphate in the target RNA backbone, resulting in cleavage products with 2′, 3′-cyclic phosphate and 5′ hydroxyl termini [60].

Since ribozymes were accidentally discovered in 1982, it has been shown that RNA can act in at least two ways in biology: as genetic material and as a biological catalyst. Examples of ribozymes include the hammerhead ribozyme, the Leadzyme, and the hairpin ribozyme. In the last several years, crystal structures of these ribozymes have been determined, providing detailed views of the tertiary folds of these RNAs [60, 61], which would be modulated allosterically to increase specificity of ribozyme action.

Compared to other therapeutical RNAs such as siRNAs, the current therapeutic efficacy of ribozymes remains low due to their limited specificity, and structural instability [62]. And furthermore, the amount of free Mg^{2+} in the intracellular environment plays a critical limitation role for the catalytic activity [63]. To date, gene-therapy-based studies have focused upon developing strategies to stabilize ribozymes and transfect them into live cells. Rouge *et al.* reported the concept of ribozyme-spherical nucleic acid (SNA) conjugates and found that these conjugates could allow high cellular uptake of ribozymes, with favorable catalytic activity and stability [64]. Paudel et al. studied the effect of molecular crowding agents, like polyethylene glycol (PEG), on the folding and catalysis of ribozymes. They demonstrated that PEG favors the formation of the docked structure, which increases ribozymes' activity. In addition, Mg^{2+}-induced folding in the presence of PEG occurs at concentrations ~ 7-fold lower than in the absence of PEG [65].

Up to now, at least two clinical trials have positively showed the safety, feasibility, and long-term stability of using ribozymes targeted to different mRNAs, such as HIV (human immunodeficiency virus) elements [66] and VEGF-1 [67]. However, the transduction efficiency left room for improvement. In a phase II cell-delivered gene transfer clinical trial, 74 HIV-1 infected adults enrolled randomly received a tat/vpr specific ribozyme OZ1 or placebo. This study showed that OZ1-based gene therapy is safe, and has modest efficacy. In the future, modifications would aim to increase the lymphocyte recovery in order to enhance the therapeutic effect [68]. Another phase II trial of RPI.4610, an antiangiogenic ribozyme targeting the VEGFR-1 mRNA, also demonstrated a well-tolerated safety profile but lacked the clinical efficacy, which results in precluding this drug from further development [69]. Thus, insuffi-

cient success suggests that further investigation of allosteric regulation is essential to advance the drug development.

6. Aptamers

Aptamers, single-stranded deoxyribonucleic acid or ribonucleic acid oligonucleotides, are generated by an *in vitro* selection process called SELEX (systematic evolution of ligands by exponential enrichment). They can bind their target molecules with high specificity and selectivity, indicating the probable therapeutic and diagnostic applications for diseases like cancer, inflammatory diseases, etc. [70, 71]. Because aptamers contain some advantages over antibodies and other conventional small-molecule therapeutics, such as high specificity, flexible modification, and low adverse effect, they have been shown as a valuable substitute to protein antibodies [72]. Moreover, the strategies developed to chemically modify backbone can further improve affinity and bioavailability of aptamers [73]. Higher affinity and specificity could be simultaneously achieved by the genetic-algorithms-based ISM (in silico maturation) [74].

The properties above have paved the way to further studies on introduction of aptamers to preclinical and clinical applications. Based on previous data showing antitumor activity of AS1411, a first-in-class quadruplex DNA aptamer targeting nucleolus, a phase II trial found that AS1411 appears to have dramatic and durable responses in enrolled patients with metastatic renal cell carcinoma, even though about 34% participants have AS1411-related mild adverse events [75]. Malik *et al.* further discovered that AS1411-linked gold nanospheres (AS1411-GNS) could markedly promote superior cellular uptake by cancer cells and increase antiproliferative/cytotoxic effects, with no signs of toxicity [76]. Likewise, other clinical trials on aptmers targeting FIX (Coagulation Factor IX) [77], vWF (von Willebrand factor) [78], and TFPI (tissue factor pathway inhibitor) [79] respectively, all show that aptamers are well tolerated, safe, and represent a new promising target therapy. However, as for some side effects, further clinical investigations are warranted to better define the clinical indications, safety, efficacy, and optimal dosing strategy.

7. Decoys

Unlike antisense oligonucleotide approaches that target mRNA, decoys are short, double-stranded DNA molecules that compete with specific binding sites of transcription factors to prevent their binding at target promoters, in order to inhibit gene expression at pretranscription level. Since decoys are DNA, they are more stable and easy to handle than RNA-based intervention strategies [80]. Some methods, including the locked nucleic acid (LNA) introduced at the 3′-end [81] and chimeric decoys containing discrete binding sites [82], can increase decoys nuclease resistance and specificity. So far, numerous of studies have indicated that decoys are suited for novel potential therapeutic for combating cancer [80] and infectious

diseases [83]. NOTCH1 decoy, a human IgG Fc consisting Notch1 extracellular domain inhibits tumor angiogenesis and growth by blocking Jagged-dependent activation of Notch signaling. Although well tolerated to mice for three weeks, NOTCH1 decoy treatment causes adverse severe gastrointestinal effects [84]. As above, the STAT3 (signal transducers and activators of transcription 3) decoy oligonucleotide represents another possible single-agent approach to targeting both the tumor and vascular compartments in murine tumor xenografts mediated through the inhibition of both STAT3 and STAT1 [85, 86]. Collectively, these findings point to decoys as highly attractive agents in gene-targeted therapy.

8. Concluding remarks

Gene-targeting strategies based on nucleic acid have opened a new era with the development of potent and effective gene intervention techniques, such as DNAzymes, ribozymes, siRNA, ASOs, aptamers, decoys, etc. It is demonstrated that these technologies have versatility and potency in disrupting pathophysiologically important pathways by silencing the target gene with relative specificity *in vivo* and *in vitro*. Numerous investigative works by several laboratories have been made in these fields. Although some clinical trials have proved the effectiveness of these techniques, only a few antisense drugs have been approved by the FDA for clinical purposes. The main difficulties on the way to develop successful nucleic acid drugs are as follows: how to ensure efficient and controlled delivery, prolonged target-specific action, and no adverse effects. If the challenges outlined above can be overcome, these molecules would prove to be valuable agents for economical and practical new therapies for diseases in the near future.

Author details

Zhijie Xu and Lifang Yang*

*Address all correspondence to: yanglifang99@hotmail.com

Cancer Research Institute, Central South University, Changsha, China

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