Journal of Cellular Biochemistry 98:14–35 (2006)

Antisense Applications for Biological Control

Wei-Hua Pan[1,2] and Gary A. Clawson[1,2]*

[1]Gittlen Cancer Research Foundation, Hershey Medical Center, Departments of Pathology, Biochemistry & Molecular Biology, College of Medicine, Pennsylvania State University, Hershey, Pennsylvania 17033
[2]College of Life Sciences, Nankai University, Tianjin 300071, People's Republic of China

Abstract Although Nature's antisense approaches are clearly impressive, this Perspectives article focuses on the experimental uses of antisense reagents (ASRs) for control of biological processes. ASRs comprise antisense oligonucleotides (ASOs), and their catalytically active counterparts ribozymes and DNAzymes, as well as small interfering RNAs (siRNAs). ASOs and ribozymes/DNAzymes target RNA molecules on the basis of Watson-Crick base pairing in sequence-specific manner. ASOs generally result in destruction of the target RNA by RNase-H mediated mechanisms, although they may also sterically block translation, also resulting in loss of protein production. Ribozymes and DNAzymes cleave target RNAs after base pairing via their antisense flanking arms. siRNAs, which contain both sense and antisense regions from a target RNA, can mediate target RNA destruction via RNAi and the RISC, although they can also function at the transcriptional level. A considerable number of ASRs (mostly ASOs) have progressed into clinical trials, although most have relatively long histories in Phase I/II settings. Clinical trial results are surprisingly difficult to find, although few ASRs appear to have yet established efficacy in Phase III levels. Evolution of ASRs has included: (a) Modifications to ASOs to render them nuclease resistant, with analogous modifications to siRNAs being developed; and (b) Development of strategies to select optimal sites for targeting. Perhaps the biggest barrier to effective therapies with ASRs is the ''Delivery Problem.'' Various liposomal vehicles have been used for systemic delivery with some success, and recent modifications appear to enhance systemic delivery, at least to liver. Various nanoparticle formulations are now being developed which may also enhance delivery. Going forward, topical applications of ASRs would seem to have the best chances for success. In summary, modifications to ASRs to enhance stability, improve targeting, and incremental improvements in delivery vehicles continue to make ASRs attractive as molecular therapeutics, but their advance toward the bedside has been agonizingly slow. J. Cell. Biochem. 98: 14–35, 2006. © 2006 Wiley-Liss, Inc.

Key words: antisense reagents; ribozymes; DNAzymes; SiRNAs; accessible site; biological control

NATURE'S ANTISENSE APPROACH

The importance of "small RNA guides"-21–30 nt RNAs, including microRNAs (miRNAs) and small interfering RNAs (siRNAs)—in regulation of an amazing variety of biological pathways is just now becoming apparent. Production of the various types of small RNAs utilize common pathways, including dsRNA-specific endonucleases (e.g., Dicer), dsRNA binding proteins, and small RNA binding proteins referred to as Argonautes [Zamore and Haley, 2005]. It is now clear that miRNAs represent a large class of riboregulators, which regulate expression of at least one-third to one-half of all human genes [Lewis et al., 2005]. Likewise, the extent to which antisense transcripts are involved in regulation of cellular metabolism is also becoming clearer. Data from the Riken Genome Exploration Research Group, GenomeScience Group, and Fantom Consortium [Katayama et al., 2005] have now shown that a large proportion of the genome (at least 50%) produces transcripts in both sense (S) and antisense (AS) orientations, and that AS transcripts often tie neighboring genes into linked transcriptional units. Expression profiling has generally revealed concordant regulation of A/AS pairs, and further analyses have shown in

*Correspondence to: Gary A. Clawson, PhD, MD, Director, Gittlen Cancer Research Foundation, H059, Hershey Medical Center, Pennsylvania State University, 500 University Drive, Hershey, PA 17033. E-mail: gac4@psu.edu

Received 17 November 2005; Accepted 18 November 2005
DOI 10.1002/jcb.20790

© 2006 Wiley-Liss, Inc.

representative cases that perturbation of the AS RNA transcript alters expression of the S RNA transcript. While A/AS hybrids could have direct RNA interference (RNAi) effects, the concordant regulation observed for most S/AS transcript pairs [Katayama et al., 2005] would seem to argue against this pathway, so that the effects of AS transcription may not even involve dsRNA formation. For example, this appears to be the case of IGF2R [Sleutels et al., 2003] as well as IGF2 [Vu et al., 2003]. siRNA effects encompass direct effects on transcription in the nucleus [Kawasaki and Taira, 2004; Morris et al., 2004; Matzke and Birchler, 2005], as well as heterochromatin formation [Wassenegger, 2005]. In this perspective, we will focus on antisense reagents employed experimentally.

Antisense oligonucleotides (ASO) were first used experimentally to inhibit viral replication in cell culture [Stephenson and Zamecnik, 1978; Zamecnik and Stephenson, 1978]. Since that time, alternative antisense approaches have been developed, including ribozymes (Rz), DNAzymes (Dz), and small interfering RNAs (siRNAs), which employ S/AS RNA regions. This family of antisense reagents (ASR) has become a powerful tool for target validation and therapeutic purposes. ASRs act at the RNA level: ASOs, generally 13–25 nt for experimental purposes, specifically hybridize with their complementary targeted RNAs by Watson-Crick base-pairing. They then induce target RNA degradation by RNase-H, and/or block protein translation. Rzs and Dzs are catalytically active oligonucleotide enzymes that not only bind to, but also cleave, their target RNAs enzymatically. siRNAs contain ~20 nt S/AS RNA regions; they trigger degradation of targeted RNAs via the RNAi-induced silencing complex (RISC; see [Filipowicz, 2005]).

Following the seminal studies of Zamecnik and Stephenson, progress was modest until DNA oligonucleotides became readily available. Once ASOs became widely available, many studies in the 1980's documented their effects on cells in culture, and in the early 1990's ASOs were increasingly tested in in vivo models. Modifications were introduced to modify the phosphodiester links between DNA bases, in efforts to protect against breakdown by nucleases in cells and blood, and various other substitutions were introduced. A phosphorothioated antisense agent, Vitravene^{TM}, developed by Isis Pharmaceuticals, represented the first FDA-approved antisense therapy in 1998. Methods for identifying/optimizing accessible sites within targeted RNAs were being developed, and work was also aimed at delivering the ASOs to certain locations. Sequencing of the human genome has disclosed a plethora of potential targets. We now have literally thousands of targets, with hundreds of preclinical animal studies, and some 20 clinical trials ongoing. Recognition of miRNA regulation of many biological processes, including development, proliferation, apoptosis, carcinogenesis, and the stress response [Croce and Calin, 2005; Eis et al., 2005; Gregory and Shiekhattar, 2005; Hatfield et al., 2005; He et al., 2005; Lu et al., 2005; O'Donnell et al., 2005], is likely to further expand ASR development.

The appeal of antisense approaches is that they potentially provide highly specific, nontoxic reagents for safe and effective therapeutics of a wide variety of diseases, including for example AIDS, Crohn's disease, viral diseases, psoriasis, asthma, cardiovascular disease, and cancers.

ASRS AND THEIR APPLICATIONS

Antisense Oligonucleotides (ASO)

ASOs are in theory designed to specifically modulate the transfer of the genetic information to protein, but the mechanisms by which an ASO can induce a biological effect are subtle and complex. Although some of these mechanisms of inhibition have been characterized, rigorous proof for others is still frequently lacking. Several mechanisms that explain how ASOs inhibit translation have been proposed. The most widely accepted involves the formation of an RNA-ASO duplex through complementary Watson-Crick base-pairing, leading to RNase H-mediated cleavage of the target mRNA [Crooke, 1998; Wu et al., 2004; Galarneau et al., 2005]. Other proposed mechanisms include prevention of mRNA transport, modulation or inhibition of splicing, translational arrest, and formation of a triple helix through ASO binding to duplex genomic DNA, resulting in inhibition of transcription.

RNase H is a ubiquitous enzyme that hydrolyzes the RNA strand of an RNA/DNA duplex. Oligonucleotide-assisted RNase H-dependent reduction of targeted RNA expression can be quite efficient, reaching 80–95% downregulation of protein and mRNA expression. Furthermore,

in contrast to ASOs, which sterically block ribosomal translation, RNase H-dependent ASOs can inhibit protein expression when targeted to many (accessible) regions of the mRNA. Thus, whereas most steric-blocker ASOs are efficient only when targeted to the 5′- or AUG initiation codon region, other ASOs can inhibit protein expression when targeted to widely separated areas in the target RNA [Larrouy et al., 1992; Dean et al., 1994].

The importance of RNase H-induced cleavage of mRNA has been demonstrated in at least four systems, including wheat germ extract [Cazenave et al., 1993], rabbit reticulocyte lysate [Minshull and Hunt, 1986], *Xenopus* oocytes [Shuttleworth and Colman, 1988], and human leukemia cells [Giles et al., 1995]. RNase H-compatible ASO backbones (i.e., those which facilitate degradation of the bound target RNAs by RNase H) include phosphodiesters, phosphorothioates, and 2′-fluoro(2′F)-oligodeoxynucleotides [Wilds and Damha, 2000; Damha et al., 2001]. Other modifications, including methylphosphonates, 2′-methoxy (2′MeO)-oligoribonucleotides, peptide nucleic acids (PNAs), and morpholino oligonucleotides, are not RNase H compatible.

The precise mechanism by which RNase H recognizes duplexes is not well understood. Using chimeric oligonucleotides in which 2′MeO-oligoribonucleotide phosphorothioates were placed at the 3′ and 5′ termini of the oligonucleotide, while the central region remained phosphorothioate oligodeoxyribonucleotide, Monia et al. [1993] demonstrated that a 5-bp region of homology is sufficient to induce RNase H activity. It is unclear if such a remarkable lack of "stringency" also occurs within cells. Despite this caveat, it has been shown that Isis 3521, a 20-mer phosphorothioate ASO targeted to protein kinase C (PKC)-α mRNA, can also downregulate PKC-ζ, with which it shares 11 bases of contiguous homology [Benimetskaya et al., 1998]. This phenomenon of cleavage of non-targeted mRNAs because of partial hybridization may be a major concern when ASOs are used to validate gene function. Furthermore, although the use of chimeric oligonucleotides can suppress this problem, it does not appear to eliminate it altogether [Monia et al., 1993; Giles et al., 1995].

Other oligonucleotide modifications (2′-*O*-alkyl, PNA, and morpholinos) may use different mechanisms to inhibit protein expression, for example, they can inhibit intron excision, a key step in the processing of mRNA. Splicing occurs during the maturation step and can be inhibited by the hybridization of an oligonucleotide to the 5′ and 3′ regions involved in this process [Kole and Sazani, 2001]. Such inhibition can lead to the lack of expression of a mature protein [Giles et al., 1999; Hudziak et al., 2000] or, as numerous reports have shown, to the correction of aberrant splicing and the restoration of a functional protein [Sierakowska et al., 1996; van Deutekom et al., 2001]. As would be expected, most of the ASOs capable of inhibiting splicing are not dependent upon RNase H activity [Giles et al., 1999; Karras et al., 2001; Mann et al., 2001].

Numerous reports in the literature also demonstrate that modified ASOs, which lose the ability to activate RNase H activity, can still efficiently inhibit mRNA translation. This inhibition may be attributable to the disruption of ribosomes or to physically blocking the initiation [Baker et al., 1997] or elongation steps of protein translation. Steric blockade of translation can be demonstrated by the arrest of polypeptide chain elongation. In an in vitro system assay, [Dias et al., 1999] identified a truncated protein after incubating a PNA oligonucleotide with H-*ras* mRNA. This truncated product had the same size as a truncated protein produced by the RNase H-mediated cleavage obtained when using a phosphodiester ASO targeted to the same site.

ASO Therapeutics

Antisense therapeutics has seen its ups and downs since the first antisense trial was conducted for treatment of leukemia in 1992 [Reynolds, 1992], followed by the excitement over FDA approval of the first antisense drug, Vitravene (Fomivirsen), for the treatment of CMV retinitis in 1998 [Roehr, 1998]. Currently, there are about 30 clinical trials of various Phases underway. Cancer is the major focus of these ongoing trials using antisense therapies, although a number of other diseases are also involved (Table I). The targets of ASOs for cancer treatment include genes involved in cell growth, apoptosis, angiogenesis, and metastasis. Unfortunately, a likely limitation for ASO therapeutics for cancer may be the single target approach. Even if the target is successfully inhibited by ASOs, other pathways may be activated and compensate for the

TABLE I. ASOs in Clinical Trials

Product	Company	Target	Disease	Phase
Genasense	Genta	Bcl-2	Cancer	III*
ISIS 2302	ISIS	ICAM-1	Ulcerative colitis	II
ISIS 113715	ISIS	PTP-1B	Diabetes	II
ISIS 301012	ISIS	ApoB-100	High cholesterol	II
ATL-1102	ATL/ISIS	VLA-4	Multiple sclerosis	II
ATL-1101	ATL/ISIS	IGF-1R	Psonasis	I
OXG-011	OncoGeneX/ISIS	Clusterin	Prostate cancer	II
LY2181308	Lilly/ISIS	Survivin	Solid tumors	I
EPI-2010	EpiGenesis	Adenosine A1-R	Asthma	II
GTI 2040	Lorus	RN-Reductase-2	Cancer	II
GTI 2051	Lorus	RN-Reductase-1	Cancer	II
Resten-NG	AVI	NYC	Restenosis	II
Resten-MP	AVI	MYC	Restenosis	II
AVI-4126	AVI	MYC	Cancer, PKD	II
AVI-4020	AVI	WNV	WNV infection	II
AVI-4065	AVI	HCV	Hepatitis C	I/II
CancerVaccine	NovaRx	TGF-β2	NSCLC	II
Gem231	Hybridon	PKA RI	Solid tumors	I/II
Gem92	Hybridon	HIV gag	AIDS	I
LR/INX-3001	Gewirtz et al.	MYB	Chronic myelogenous leuk.	II
HGTV-43	Enzo Biochem	HIV-1	HIV infection	I
VRX496	VIRxSYS	HIV env	HIV infection	II
AEG35156	Aegera	XIAP	Solid tumors	I
AS-EGFR	SOG/NCI	EGFR	Cancer	I

*In multiple trials.

ASO-mediated inhibition. Another potential problem is that in spite of the fact that major decreases in target RNAs may be achieved, complete inhibition is probably not realistic. For example, one of the most advanced (Phase III) antisense Clinical Trials recently reported disappointing results for Affinitak (an ASO inhibitor of PKC-α) for the treatment of non-small cell lung cancer. In contrast, relatively recent reports have shown promising preliminary results for use of respiratory ASOs (RASONs) for treatment of Asthma [Ball et al., 2003]. It is noteworthy that after extensive efforts at endogenous expression of antisense RNA by plasmids and viral vectors in a variety of disease models, viral delivery of antisense constructs has also advanced to initial testing in patients. VRX496 (a lentivirus vector encoding an ASO targeted to HIV-1 env RNA) started its phase II trial in 2005. Cancer vaccine, a cell therapy using non-small cell lung cancer cell lines genetically engineered to express an ASO targeted to transforming growth factor-β (TGF-β), has also been tested in patients with lung cancer. With the emergence of new generations of modified oligonucleotides, and with development of technologies for delivery and identification of optimal accessible sites, ASO therapeutics are moving closer to fulfilling their potential in the clinic for diseases other than cancer, such as cardiovascular disease, asthma, psoriasis and Crohn's disease.

There are a couple of recent developments in the antisense field. First, Isis Pharmaceuticals and Pfizer are collaborating to develop ASRs using Isis' RNAi technology for treatment of eye diseases. Second, Hybridon is collaborating with Novartis for drug discovery efforts for asthma and allergy targeting the Toll-like receptor 9, wherein Hybridon will contribute TLR9 agonists developed using its ASO technology.

Ribozymes (Rz) and DNAzymes (Dz)

In the early 1980s, Cech et al. [1981] discovered catalytic RNA–RNA enzymes that have been termed ribozymes (Rz; [Kruger et al., 1982; Guerrier-Takada et al., 1983; Khan and Lal, 2003; Puerta-Fernandez et al., 2003]). Although there are different types of Rzs in experimental usage, the most therapeutically relevant class is the hammerhead Rz (hRz), which was identified by comparison of naturally occurring Rz [Symons, 1992]. The modified hRz is fewer than 40 nucleotides long and consists of two substrate-binding antisense arms (which hybridize to the target RNA based on Watson-Crick base pairing), flanking a central catalytic domain. The catalytic activity of hRz cleaves the substrate RNA at the sequence NUH (N, any nucleotide; H, not guanosine). Obstacles to the successful therapeutic application of Rzs include target site identification, nuclease-mediated degradation of the hRz, and limitations in delivery to the target cell. Rzs are

typically expressed in situ from a plasmid vector transfected into the target cell, but they can be produced in vitro or synthesized as oligonucleotides and administered exogenously. Synthetic Rzs, similar to ASOs, can incorporate modified sugars and bases to improve stability by increasing resistance to nucleases. However, this is more problematic with Rzs than with ASOs, because modifications that improve stability dramatically interfere with the catalytic activity. In one common modification of a synthetic hRz, the 3′ end is protected with an inverted thymidine, the fourth nucleotide is a 2′-C-allyl uridine surrounded by five unmodified ribonucleotides, and the remainder of the nucleotides has a 2′MeO-ribose structure. These modifications increase the half-life of the ribozyme in serum from 1 min to 10 days [Grunweller et al., 2003; Kurreck, 2003]. However, we have found that while 3′-inverted Ts dramatically increase stability in serum, they do not increase stability of ASOs within cells (unpublished).

A number of Rzs have reportedly been in early stage (Phase I and II) clinical trials, although little or no follow-up is readily available. This is clearly not a sign of success, and one would guess that delivery issues may be at the heart of the problems. The first clinical trial using a hRz targeted HIV [Rowe, 1996; Brower et al., 1998], and another antiviral Rz was Heptazyme (LY466700) that targeted the HCV genome. ANGIOZYME, an hRz that targets vascular endothelial growth factor, was examined in a phase II trial for treatment of metastatic colorectal cancer, where it was tested in combination with several chemotherapy agents. Another Rz which started in cancer clinical trials, HERZYME, was of a class of modified Rzs (so-called Zinzymes) that has high catalytic activity under physiological Mg^{2+} conditions and cleaves at the triplet YGH (Y = C or U; H, not guanosine), with a half-life in human serum of >100 h. HERZYME had 2′-OMe modifications at all bases except for two unmodified guanosines and two 2′-amino nucleotides. The substrate-binding arms had phosphorothioate bonds and an inverted 3′–3′ deoxyabasic sugar in one substrate recognition arm. HERZYME targets human epidermal growth factor-2, and was tested in a Phase I trial to determine toxicity and efficacy in breast and ovarian cancer patients [Probst, 2000; Kurreck, 2003]. There are now only two Rz clinical trials listed in the Clinical Trials database. A very small (five patient) study, listed as "completed," examined a retroviral construct encoding L-TR/Tat-neo (vs. irrelevant) ribozymes targeting HIV in peripheral blood stem cells which were given to HIV patients with non-Hodgkin's lymphoma (again, no follow-up is readily available). The only current study is evaluating the safety and efficacy of use of "Autologous CD34+ Hematopoietic Progenitor Cells Transduced with Placebo or an Anti-HIV-1 Ribozyme (OZ1) in Patients with HIV-1 Infection." Both of these studies circumvent delivery problems by treating target cells ex vivo.

Another category of site-specific cleaving nucleic agents that has received attention is that of catalytic DNAs. Small DNAs capable of site-specific cleavage of RNA targets were developed via in vitro evolution; DNA enzymes (Dz) apparently do not exist naturally [Santoro and Joyce, 1997, 1998]. Two different catalytic motifs, with different cleavage site specificities, were found via this process. The basic format of Dzs is analogous to that of hRzs, with short antisense regions flanking a central catalytic domain. The most commonly used 10–23 Dzs bind to their RNA substrates via Watson-Crick base pairing with the antisense arms, inducing site-specific cleavage of the bound target RNAs, resulting in 2′, 3′-cyclic phosphate and 5′-OH termini (analogously to hRzs). Cleavage of the target mRNAs results in their rapid destruction, and the Dzs (and Rzs) can recycle and cleave multiple substrates. Dzs are relatively inexpensive to synthesize and have good catalytic properties [Cairns et al., 2002; Emilsson and Breaker, 2002; Khachigian, 2002], making them useful alternatives for either ASOs or Rzs. Several applications of Dzs in cell culture have been published including for example the inhibition of *veg F* mRNA and consequent prevention of angiogenesis [Zhang et al., 2002], and inhibition of expression of the *bcr*/*abl* fusion transcript characteristic of chronic myelogenous leukemia [Wu et al., 1999]. A drawback of Dzs compared to Rzs is that they can only be delivered exogenously, but they can be backbone-modified, perhaps allowing them to be delivered systemically in the absence of a carrier.

Theoretically, Rzs/Dzs would be expected to have a clear advantage over ASOs, since each Rz/Dz-molecule's enzymatic activity could result in cleavage of multiple copies of the target

mRNA, whereas ASOs should be expected to interact with only one target mRNA molecule. A difficulty (in addition to effects on activity), however, is that those modifications that improve the stability of Rzs/Dzs also generally increase the affinity for substrate mRNA (i.e., hybridization strength) and thus drastically reduce the catalytic activity, since release of Rz/Dz after cleavage is generally the rate-limiting step in the catalytic cycle. Another potentially important factor is that Rzs/Dzs require special cleavage sites to trigger the chemical step of the kinetic activity, not only the accessible sites which are free for a simple Watson-Crick base pairing. These considerations are likely to contribute to the fact that few Rzs/Dzs have progressed to clinical trials [Opalinska and Gewirtz, 2002]. Although future modifications or alternative RNA/DNA-based enzymes yet to be described might yield reagents more effective than ASOs, ASOs at present seem to have the upper hand compared to state-of-the-art Rzs/Dzs.

Small Interfering RNAs (siRNA)

RNA interference (RNAi) is increasingly being employed as a strategy to provide stable inhibition of gene expression. RNAi is a gene silencing mechanism that was discovered in *Caenorhabditis elegans*, when injection of double-stranded (ds) RNA was found to cause a specific and potent interference (i.e., down-regulation) of the target gene product [Fire et al., 1998]. This endogenous gene silencing mechanism is a physiological process used by eukaryotes (including mammals) to regulate gene expression by reducing protein production. RNAi can also be exploited as a reverse genetic tool to study the function of genes associated with human disease, and as a therapeutic to treat disease. The recent interest in RNAi has prompted publication of several reviews of the molecular mechanisms responsible for this phenomenon [McManus and Sharp, 2002; Shuey et al., 2002; Caplen, 2003; Cerruti et al., 2003; Dykxhoorn et al., 2003; Sandy et al., 2005; Zamore and Haley, 2005].

Briefly, RNAi is initiated in *C. elegans* (and many other eukaryotic organisms, including *Drosophila*, *Aradopsis*, and mammals) when dsRNA is cleaved by an RNase III-like enzyme (DICER) into 21–23 base-pair (bp) dsRNA products with two nucleotide 3′ overhangs, producing what is known as small interfering RNAs (siRNAs; see McManus and Sharp [2002]; Wall and Shi [2003]). These siRNAs are then unwound and associate with the complementary RNA, through the action of a multiprotein complex known as the RISC, and the target mRNA is cleaved within the region of complementarity to the siRNA. In plants, fungi and *C. elegans*, the process is amplified by an RNA-dependent RNA polymerase and results in continual degradation of the target mRNA. In mammalian cells, however, both dsRNA and siRNA activate slightly different molecular pathways. Long dsRNAs activate endogenous dsRNA-dependent protein kinase (PKR), which leads to phosphorylation of the translation initiation factor EIF2α. The result is a general cellular suppression of protein synthesis that may lead to apoptosis [McManus and Sharp, 2002]. The presence of long dsRNA in mammalian cells also stimulates interferon α and β production (which have pro-apoptotic actions), and activates RNase L, leading to widespread degradation of cellular RNA. Although delivery of siRNA into mammalian cells results in knockdown of target gene expression, the effect is transient because mammalian cells lack the RNA-dependent RNA polymerase that is required to amplify the process [Schwarz et al., 2002; Cerruti et al., 2003; Stein et al., 2003]. For sustained gene knockdown by RNAi in mammalian cells, siRNA can be produced by plasmid vectors stably transfected into cells. The requirement for stable transfection for effective RNAi is likely to be a crucial limitation for clinical utility [Chiu and Rana, 2002; Ramaswamy and Slack, 2002; Scherr et al., 2003; Shi, 2003].

There have been a few attempts to compare the relative efficacies of RNAi and ASOs, although such comparisons are complicated by a number of factors. Although the siRNA duplexes are more stable in cells than a single-stranded antisense DNA molecule [Bertrand et al., 2002], the various chemical modifications to ASOs (discussed below) greatly enhance their intracellular stability. Some groups have reported that siRNA can be used at lower concentrations than ASOs to achieve comparable reductions in gene expression [Bertrand et al., 2002; Grunweller et al., 2003; Miyagishi et al., 2003], although others have found that ASOs and siRNAs have similar potency [Vickers et al., 2003]. There are some problems with various comparisons, however. One such

problem may be that the most efficient target site for an ASO may not function efficiently for an siRNA, and another is that modifications introduced for stability are likely to differ. Further studies are necessary to determine which technology is more efficient, when both the ASO and siRNA are designed with optimized modifications and target site, and to reveal the physiological basis of apparent differences in RNA sequences optimally targeted by ASO and siRNA (see below). In an interesting twist, a combination of ASO and siRNA was recently used to downregulate the expression of P2X3 (a receptor mediating pain signaling). Enhanced reduction in P2X3 mRNA and protein levels was observed when the ASO and siRNA were used in combination [Hemmings-Mieszczak et al., 2003], and this type of mixed treatment may be useful to achieve optimal efficacy while reducing untoward side effects.

The relative therapeutic merits of the two technologies have recently been reviewed [Thompson, 2002; Grunweller et al., 2003; Zender and Kubicka, 2004; Tong et al., 2005]. It appears that ASOs afford a number of advantages over siRNA reagents; these include greater flexibilities in chemistry, lower synthesis costs, and relative ease of delivery to the target cell. Furthermore, present state-of-the-art ASOs are much more resistant to nucleases than current siRNAs, although chemical modification of siRNAs is a burgeoning (perhaps bludgeoning) field. Finally, there exists an extensive literature describing the pharmacokinetics and toxicology of ASOs in animal models, and relevant experience has been gained from a considerable number of clinical trials using ASO reagents. In contrast, preclinical pharmacokinetic and toxicological studies of RNAi therapeutics are only just beginning to appear in the literature [Kurreck, 2003; Gleave and Monia, 2005], and problems with non-specific effects of siRNAs have been noted. For example, siRNA administration has been shown to trigger widespread interferon-mediated [Sledz et al., 2003], and other effects [Frantz, 2003]. Off-target effects have also been clearly documented by gene array expression analysis [Jackson et al., 2003].

ACCESSIBLE SITES FOR ASR TARGETING

Although the AUG start code and 5′-end untranslated region are often arbitrarily used for antisense targeting, in practice, only a few complementary ASOs can successfully hybridize to a targeted mRNA [Tu et al., 1998]. It is assumed that this is largely because of problems of target accessibility, which in turn may be because of the secondary or tertiary mRNA structure and/or to the proteins bound to the RNA.

Computational Prediction

To define the best mRNA hybridization sites, several predictive methods have been developed. Ding and Lawrence [2001] proposed a method based on the determination of the RNA structures using algorithms and thermodynamic and structural properties of the RNA. Zuker (http://www.bioinfo.rpi.edu/applications/mfold/) used a similar approach to select effective ASO sites. A number of groups have used a systematic alignment of computer-predicted secondary structures of local regions of the targeted RNA to identify favorable local target sequences, and then designed more effective ASOs. Using this approach, 17 of the 34 ASOs tested showed significant inhibition (>50%) of ICAM-1 expression in mammalian cells [Patzel et al., 1999; Scherr et al., 2000; Sczakiel, 2000]. Other selection methods have been based on the determination of melting temperatures [Chiang et al., 1991] or the free energies of formation of the ASO/RNA duplexes [Stull et al., 1992; Ding and Lawrence, 1999]. Unfortunately, it is frequently observed that only a small fraction of ASRs engineered using such predictions give rise to significant reductions in target RNA levels within cells.

In Vitro Library Selection

Various methods have used combinatorial ASOs to identify hybridization sites directly within the RNA. These sites are revealed by RNase H cleavage [Lloyd et al., 2001; Sohail et al., 2001], microarrays [Mir and Southern, 1999], or MALDI-TOF mass spectrometry [Altman et al., 1999]. Despite the fact these methodologies can be somewhat cumbersome, they may indeed ultimately identify excellent target sites.

Selection of accessible sites from random libraries of oligonucleotides that contained 10^7 different sequences, which cover all possibilities in the sequence length of 10 nucleotides [Birikh et al., 1997; Lima et al., 1997] has been reported. For example, Birikh et al. [1997] used a

completely randomized oligonucleotide library (dN10) in conjunction with RNase H to map sites that are accessible for oligonucleotide binding in an RNA transcript. A number of other studies [Lima et al., 1997; Mir et al., 2001] have also employed RNase H-based strategies. Although the experimental approaches for identification of accessible binding sites offer clear advantages for targeting ASOs, they do not necessarily optimize for Rz cleavage activity (chemical step, k_{cat}, [Campbell et al., 1997; Clouet-d'Orval and Uhlenbeck, 1997]).

Lieber and Strauss [1995] and Lieber and Kay [1996] constructed a hRz library that was targeted to a preselected triplet (GUC) and which contained randomized sequences in the annealing arms, allowing the screening of suitable sites in the target RNA molecule. This procedure identified both the accessible sites and the precise position of cleavable triplets, but reverse transcription, tailing, and polymerase chain reaction (PCR) were necessary to amplify the cleavage products. A number of other reports utilizing random libraries of various types of Rzs have subsequently appeared [Jarvis et al., 1996; Pierce and Ruffner, 1998; Yu et al., 1998; zu Putlitz et al., 1999; Bramlage et al., 2000; Mir et al., 2001], including hRz [Jarvis et al., 1996; Pierce and Ruffner, 1998; Mir et al., 2001]. In general, while these experimental approaches offer potential advantages, they often identify very limited numbers of sites and are generally quite difficult to perform, and various manipulations (i.e., primer extension, etc.) might again bias the results.

We described a SELEX (systematic evolution of ligands by exponential enrichment) method to locate accessible sites within any targeted RNA by systematically isolating guide RNAs from a large pool of random RNA sequences [Pan et al., 2001]. Fifty percent of hRz designed to the identified accessible sites were "highly active" in cleaving their long, structured targets, with k_{cat}/K_m values of around 10^6/M·min. In comparison, none of the hRz directed to target sites predicted by mFold program showed high activity. While effective, this SELEX procedure was quite labor intensive, requiring subsequent PCR and extensive cloning/sequencing.

Subsequently, we constructed an hRz library with randomized annealing arms and fixed 5′/3′-end flanking sequences (which allow recovery of bound species) and utilized it in an iterative fashion. After two rounds of binding under inactive (magnesium-free) annealing conditions, the selected active Rz library was incubated with target, and the sites of cleavage were directly identified on sequencing gels. These selected hRzs generally showed even higher catalytic activity than those targeted based on the SELEX procedure [Pan et al., 2003; Pan and Clawson, 2004].

Using our CLIP [Benedict et al., 1998; Crone et al., 1999; Ren et al., 1999; Norris et al., 2000], SNIP and $SNIP_{AA}$ Rz expression cassettes [Zhang et al., 2002; Pan et al., 2003, 2004], hRz targeted to library selected sites were shown to effectively block hepatitis B virus (HBV) replication within cultured HepG2 cells [Pan et al., 2001], with major decreases in HBSAg secretion, viral mRNAs, and viral DNA. We also documented efficient downregulation of human papillomavirus (HPV) type 16 and 11 E6/E7 transcripts within cultured cells [Pan et al., 2003, 2004]. More importantly, the anti-HBV hRz was further tested in vivo, using an hRz expression construct and a liposomal delivery vehicle specifically targeted to hepatocytes. After injection of $SNIP_{AA}$-Rzs twice a week for 2 weeks, an >80% reduction in liver viral DNA was observed in a transgenic mouse model, with no evidence of toxicity. Immunohistochemical staining for HBV-core antigen showed a similar decrease in the number of hepatocytes staining positively, compounded by a concomitant loss of residual staining intensity [Pan et al., 2004]. This was significant because there are so few examples of successful in vivo applications of Rzs against bona fide naturally occurring, disease-causing organisms.

Somewhat surprisingly, only a small proportion of Dzs targeted to sites identified by the hRz library selection have been found to show "high activity." For example, only one out of eight was highly active against $HPV16_{E6/E7}$ mRNA. Given this difficulty, a Dz library was designed to select optimal accessible sites for Dz cleavage. In comparison to Dz targeted to sites identified using hRz-library selection protocols, the Dzs targeted to Dz-library selected sites generally showed 1–2 orders of magnitude higher activity in vitro, with the best achieving k_{cat}/K_m values on the order of 10^8/M·min. In spite of the perceived inherent advantages of Dzs over ASOs, however, we have generally not observed any increased efficacy with Dzs in cell culture

experiments. The single exception (of perhaps 20) was the most active Dz we have constructed, named Dz_{1628}, which was based on Dz-library selection of woodchuck hepatitis virus (WHV) pre-mRNA. This Dz cleaved essentially 100% of target both in standard in vitro assays and in cell culture, where it clearly showed increased efficacy over the corresponding ASO. However, in collaborative studies under the auspices of an NIH antiviral testing contract (with Dr. Tenant at Cornell), Dz1628 delivered to liver using hepatocyte-targeted liposomes was without appreciable effect on WHV infections (not shown).

Kretschmer-Kazemi Far and Sczakiel [2003] investigated quantitatively the relationship between local target accessibility and the extent of inhibition of the target gene by siRNA. Two sites of ICAM-1 mRNA predicted to serve as accessible motifs, and one site predicted to adopt an inaccessible structure, were chosen to test siRNA constructs for suppression of ICAM-1 gene expression in ECV304 cells. The concentration dependency of siRNA-mediated suppression indicated a >1,000-fold difference between active siRNAs (IC50, 0.2–0.5 nM) versus an inactive siRNA (IC50, 1 μM). This is certainly consistent with the activity pattern of ASOs when relating target suppression to predicted local target accessibility.

Recently, design of a genome-wide siRNA library for targeting siRNAs was facilitated by an artificial neural network approach [Huesken et al., 2005]. An algorithm, trained on a complementary 21 nt guide sequence set, was used to design a genome-wide siRNA collection with 2 "good-activity" siRNAs per gene target. The collection of 50,000 siRNAs was validated by identification of genes involved in the cellular response to hypoxia (see Miyagishi and Taira [2005] for a succinct description). Development of such learning approaches are likely to facilitate future identification of optimized sites for targeting of siRNAs, although one wonders whether linear sequence information will be sufficient.

Library Selection in Cells

Although the in vitro selection methods along with other advantages are likely to produce more efficacious ASRs, the selected accessible sites still leave a gap for cell and animal studies. Specially for siRNA design, even though genome-wide siRNA libraries have been constructed [Berns et al., 2004; Kittler et al., 2004; Kronke et al., 2004; Paddison et al., 2004], it has become clear that shifting of siRNAs by only one or a few nucleotides can significantly affect its silencing function, and multiple factors for siRNA design have not achieved statistical significance [Holen et al., 2002; Xu et al., 2003; Kronke et al., 2004; Saetrom and Snove, 2004; Overhoff et al., 2005]. In addition, the numerous "off-target" effects produced by siRNAs are of concern. In an attempt to circumvent these difficulties, we constructed a double-stranded DNA library which was used to generate an RNA library with multiple copies of approximately 4.4×10^{12} different sequences. The library-RNAs, with a central 21-nt region flanked by defined 5′/3′-ends, were annealed to a target RNA and then separated on native gels. The selected library-RNAs that covered the full-length of any given targeted RNA (by one nucleotide shifting) were reverse-transcribed to cDNAs. The cDNAs were amplified by PCR for siRNAs and by "fold-back"-PCR to construct their complimentary sequences for short airpin RNAs (shRNAs), then inserted to our OPIII-cassette that contains opposing H1/U6 promoters (manuscript in preparation). Using full-length transcripts of human papillomavirus HPV16 E6/E7 mRNA, we found that the selected library-RNAs clearly decreased the fluorescent intensity of an eGFP/HPV16-E6/E7 fusion construct in 293 cells. We are now in the process, using the Flp-In™ system, of selecting optimally active si/shRNAs with a minimum of "off-target" effects.

ASR CHEMISTRIES

ASO Chemistry

ASOs (used experimentally) are unmodified or chemically modified single-stranded DNA molecules that generally are relatively short (13–25 nucleotides) and hybridize (at least in theory) to a unique sequence in the total pool of target RNAs present in cells. Although it is not a complicated matter to synthesize phosphodiester oligonucleotides, their use is limited as they can rapidly be degraded by the intracellular endonucleases and exonucleases, usually via $3' \rightarrow 5'$ activity [Wickstrom, 1986; Akhtar et al., 1991; Eder et al., 1991]. In addition, the degradation products of phosphodiester oligonucleotides, dNMP mononucleotides, may be cytotoxic and also exert antiproliferative effects

[Vaerman et al., 1997; Koziolkiewicz et al., 2001] have demonstrated that the toxic effects of dNMPs can be correlated with mononucleotide dephosphorylation by the cell-surface enzyme ecto-5′-nucleotidase. In human endothelial cells, and in HeLa cells, this enzyme dephosphorylates dNMP to the corresponding nucleoside, which then inhibits the function of other critical proteins, such as thymidine kinase [Kara and Duschinsky, 1969], an event that can result in inhibition of cell growth. For these reasons, DNA phosphodiester ASOs are generally not used in antisense experiments.

Many chemical modifications have been developed in attempts to circumvent these problems (Fig. 1 shows the modified analogs of the phosphodiester bond and ribose moieties, and Fig. 2 shows the backbone modifications).

Phosphodiester bond and ribose modifications. Methylphosphonate oligonucleotides (MePO) were the initial chemically synthesized modified ASOs; they represent an uncharged oligomer, in which a nonbridging oxygen is replaced by a methyl group at each phosphorus in the oligonucleotide chain. Although these MePO-ASOs have excellent stability in biological systems [Miller et al., 1979], the absence of charge reduces their solubility and their cellular uptake [Miller et al., 1981; Blake et al., 1985], which appears to occur predominately via adsorptive endocytosis [Tonkinson and Stein, 1994] not by diffusion through membranes [Shoji et al., 1991]. Whereas the absence of charge eliminates charge–charge repulsions that would ordinarily occur during the formation of an RNA–DNA duplex, MePO linkages are also inherently helix-destabilizing and, most importantly, cannot activate RNase H activity. These features severely restrict their utility as ASO reagents.

Phosphorothioate oligonucleotides (PS) are the most widely studied ASOs, because of their nuclease stability and relative ease of synthesis, in which one of the nonbridging oxygen is replaced by sulfur in the oligonucleotide chain. PS-modified ASOs are highly water-soluble and exhibit increased protein-binding capacity [Levin, 1999], have improved nuclease resistance and cellular uptake [Zamaratski et al., 2001; Kurreck, 2003], and can efficiently recruit RNase H to cleave the target RNA [Zamaratski et al., 2001]. During the last 2 decades, many reports have been published using this backbone to generate antisense effects both in tissue culture and in vivo. These data have led to the introduction of PS-modified ASOs into clinical therapeutic trials. At the present time, the most promising of these seems to be G3139 (aka Genasense, Oblimersen, or Oblimerson), an 18-mer targeted to the initiation codons of *bcl*-2

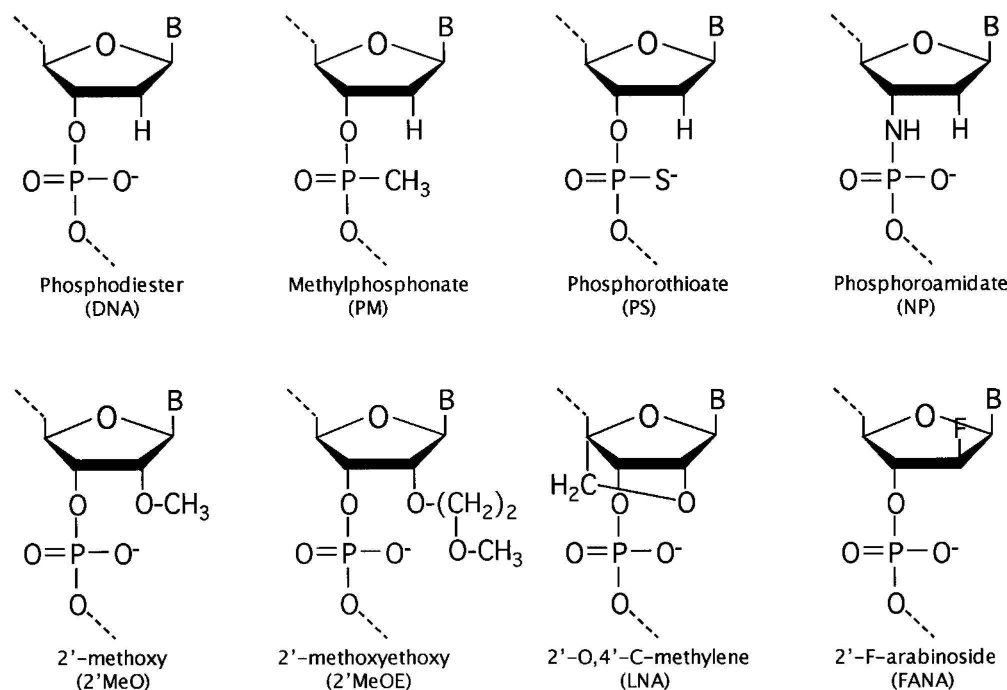

Fig. 1. The modified analogues of the phosphodiester bond and ribose moieties.

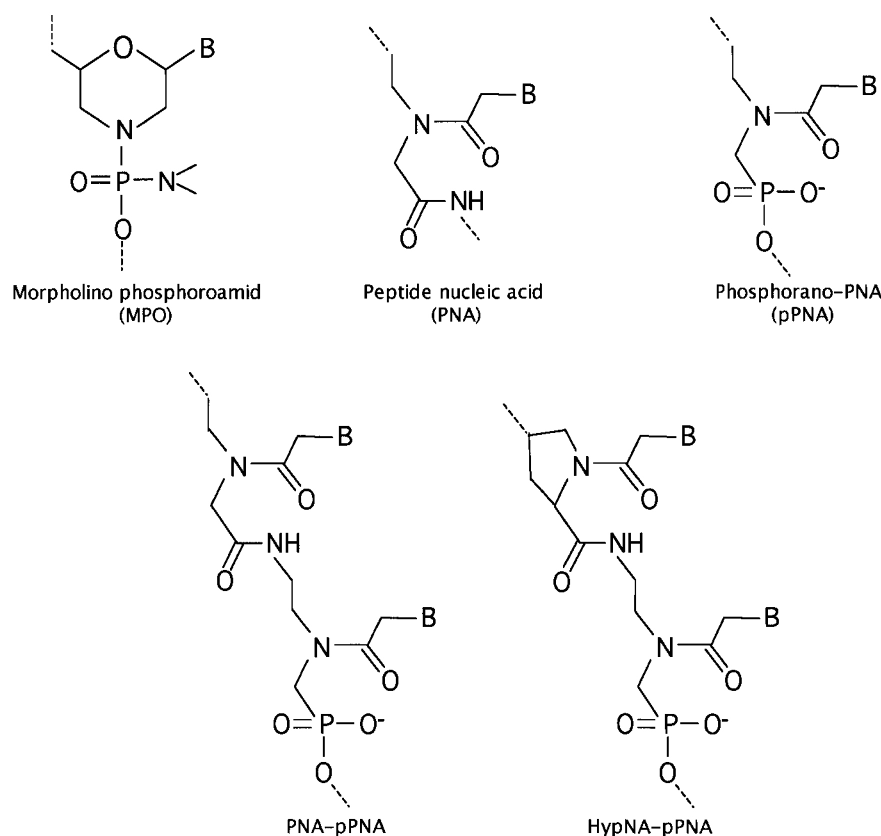

Fig. 2. The analogues of the backbone modifications.

mRNA (now being evaluated clinically for treatment of melanoma, chronic lymphocytic leukemia, and other tumors).

Unfortunately, PS-modified ASOs possess a relatively low binding affinity for target RNA that impacts on their potency in antisense applications. Although the majority of ASOs currently under investigation have PS linkages at all sites, several modifications to the ribose moiety of RNA-based ASOs have also been studied. The two most prevalent are 2′-methoxy (2′MeO)-RNA ASOs and 2′-methoxyethoxy (2′-MeOE) modified ASOs. Others include 2′-*O*, 4′-*C*-methylene bridge nucleic acid (BNA/LNA, [Vester and Wengel, 2004]), 2′-*O*, 4′-*C*-ethylene bridge nucleic acid (ENA, [Takagi et al., 2004; Yagi et al., 2004]), and N3′ → P5′ phosphoroamidate (NP) oligonucleotide which results from the replacement of the oxygen at 3′ position on the ribose moiety by an amine group [Chen et al., 1995; Gryaznov et al., 1995]. These modified oligonucleotides are resistant to degradation by cellular nucleases and hybridize specifically to their target mRNA with higher affinity than the comparable phosphodiester or phosphorothioate ASOs. However, their antisense effects result from RNase H-independent mechanisms. It has been demonstrated that fully modified ASOs (i.e., with 2′ ribose modifications at all nucleotide positions) act predominantly by inhibiting mRNA translation [Crooke, 1999; Fulford et al., 2000]. However, modified ASOs with six to eight DNA bases in the center lacking ribose modification at the 2′ position (known as "GAPmers") efficiently activate RNase H to degrade the target mRNA [Giles and Tidd, 2001; Zamaratski et al., 2001; ten Asbroek et al., 2002]. Like unmodified and fully modified ASOs, GAPmers form stable duplexes with target mRNA, although the stability is decreased with increasing length of the alkyl chain and with an increasing number of modified nucleotides in a sequence. These two chemistries—fully modified RNA oligonucleotides and modified RNA/DNA chimeras (GAPmers)—dominate the antisense drugs currently undergoing clinical trials.

Recently, the stereochemistry at the 2′-position of the sugar of ASOs has been demonstrated to be a key determinant in the target RNA

binding affinity and the activation of RNase H. FANA (2′-deoxy-2′-fluoro-D-arabinonucleic acid) analogs represent the first example of a fully 2′-modified nucleic acid that both has high-affinity RNA binding and that can retain RNase H compatible properties, suggesting that FANA may demonstrate potent intracellular antisense activity [Damha et al., 1998; Noronha et al., 2000; Wilds and Damha, 2000; Trempe et al., 2001]. Comparing the properties of ASOs based on phosphorothioated FANA (PS-FANA) and its GAPmer of the general structure PS-{FANA-DNA-FANA}, the latter was found to have exceptionally potent antisense activity. The antisense activities of the 2′-fluoroarabino GAPmer were sequence-specific and mediated by intracellular RNase H. Importantly, unlike 2′-*O*-methylribose chimeric compounds, the potencies of the 2′-fluoro-arabino chimeras were not limited by the length of the DNA core [Lok et al., 2002].

Backbone modifications. Stable ASOs have also been produced that do not possess the natural phosphate-ribose backbone. PNAs, or peptide nucleic acids [Pooga and Langel, 2001; Braasch and Corey, 2002], are achiral neutral molecules that have a backbone similar to that of poly-amino acids. They have been shown to hybridize with DNA and RNA with greater specificity and tighter binding properties compared to phosphodiester and PS-ASOs, and are resistant to endo- and exonuclease activity as well as protease degradation [Kurreck, 2003]. Because PNAs are not compatible with RNase H mechanisms (or other RNases), the antisense mechanism of PNAs depends on steric blocking ability. PNAs can also bind to DNA and inhibit RNA polymerase initiation and elongation [Hanvey et al., 1992; Boffa et al., 1996; Cutrona et al., 2000], as well as the binding and action of transcription factors, for example nuclear factor B [Vickers et al., 1995]. PNAs can also bind mRNA and inhibit splicing [Karras et al., 2001] or inhibit translation initiation and elongation [Gambacorti-Passerini et al., 1996; Good and Nielsen, 1998; Dias et al., 1999; Mologni et al., 1999, 2001]. Difficulties in use of PNAs in vivo include low solubility, a propensity to aggregate, and reduced cellular uptake due to the neutrality of the PNA molecule, and these drawbacks have limited their clinical development [Kurreck, 2003]. However, gripNA, a modified PNA dimer that is comprised of a backbone of alternating trans-4-hydroxy-L-proline PNA (HypNA) and phosphoro-PNA (pPNA) monomers with the bases attached through methylene carbonyl linkages, has overcome the shortcomings of poor water solubility and the tendency to self-aggregate [van der Laan et al., 1996; Efimov et al., 1998, 1999; Morris et al., 2001].

Another common class of ASOs that is widely used experimentally (and which has representatives in early clinical trials (Phase I/II) is the morpholino phosphorodiamidate oligomers (MPOs). These have a six-membered morpholine ring rather than a ribose, and a nonionic phosphorodiamidate linkage in lieu of phosphodiester bonds. Phosphoramidate-modified ASOs, referred to as morpholinos, have a higher affinity for ssRNA than phosphodiester ASOs [Urban and Noe, 2003]. Morpholinos mediate their antisense effects by blocking protein translation, without recruitment of RNase H [Crooke, 1999; Summerton, 1999]. Nonspecific side effects, including widespread cell death, have been observed when high concentrations of morpholinos (5–8 μM final) were injected into zebrafish embryos ([Heasman, 2002]; and references therein). Nevertheless, morpholinos have displayed limited toxicity in Phase I Clinical Trials [Iversen et al., 2003].

Rz/Dz Chemistry

A first and obvious limit of the hRz is represented by its poor chemical stability. RNA oligonucleotides are hydrolyzed in seconds or minutes in the presence of cellular extracts or blood serum, because of the ubiquitous occurrence of RNAses [Qiu et al., 1998]. However, hRz arms can be protected from degradation by exo- and endonucleases through the introduction of various types of modified nucleotides like ASOs. Generally, when these analogs, such as PS [Shimayama et al., 1993; Heidenreich et al., 1996; Jarvis et al., 1996], 2′MeO [Beigelman et al., 1995; Usman and Blatt, 2000], LNA [Wahlestedt et al., 2000], and MPO modifications [Stein et al., 1997], are introduced solely in the binding arms, effects on catalysis are minimal.

As for protecting the ribozyme core from endonucleases, various studies have shown that RNases attack primarily the pyrimidine nucleotides in the core [Qiu et al., 1998; Heidenreich et al., 1993]. Accordingly, these nucleotides have been substituted with a variety of modified analogues (2′-fluoro, 2′-amino,

2′-MeO, 2′-MeOE etc.) to produce stable hRzs. Contrary to most other modifications in the core, including the 2′-modification of several purine residues [McKay, 1996], 2′-modifications at nucleotides U4 and U7 are apparently compatible with efficient ribozyme function [Pieken et al., 1991]. Mutation of these two residues, in combination with the introduction of other protecting modifications, has led to the development of ribozymes that are both stable and active in vivo, increasing the half-life of the ribozyme from a few minutes to days without serious impairment of catalytic competence [Flory et al., 1996; Parry et al., 1999]. However, when the hRZ was subjected to a systematic 'subtraction mutagenesis' analysis, in which each nucleotide in the ribozyme core was individually replaced with a basic residue, nearly all of these mutations produced huge decreases in catalysis, generally larger than 1,000-fold [Peracchi et al., 1998]. Subsequent work lent further support to the idea of a large-scale pre-catalytic rearrangement [Blount et al., 2002] and showed that the activity of the hRz can be boosted by the introduction of additional stabilizing interactions, as predicted by the 'core folding' model [Khvorova et al., 2003]. The structural instability of the hRz may be an important limit for therapeutic applications, even though it is believed that, within the cell, substrate binding (and release) rather than the cleavage step is rate-limiting for disruption of the target. The high sensitivity of hRz function to structural changes restricts the kind of modifications that can be introduced in the core.

Dzs have offered an answer to some of the limits of conventional ribozymes. First, Dzs, being made of DNA, are easier and less expensive to synthesize, while DNA is much more resistant than RNA to degradation both in vitro and in vivo. In fact, studies have shown that, in contrast to hRzs, very stable Dz constructs can be created via the introduction of a minimal number of modifications, reducing the possibility of nonspecific toxic effects. For example, the 10–23 Dz-motif containing just 30-nt was found to have a half-life of 12–24 h in 100% human serum [Sioud and Leirdal, 2000; Dass et al., 2002]. The use of other stabilizing modifications on the 'arms' has also been explored [Oketani et al., 1999; Warashina et al., 1999; Sioud and Leirdal, 2000; Vester et al., 2002]. In particular, the introduction of LNA [Wahlestedt et al., 2000] seems to increase the Dz efficiency at low concentrations [Vester et al., 2002]. Moreover, mutagenesis studies provide a suggestion as to where stabilizing mutations in the 'core' of Dzs would be best tolerated [Zaborowska et al., 2002].

The superior chemical stability, lower potential toxicity, and improved catalytic efficiency make Dzs substantially more attractive than hRzs as candidates for exogenous applications. A number of Dzs have been used by several groups to inhibit gene expression in cell culture [Sun et al., 1999; Warashina et al., 1999; Toyoda et al., 2000] and in animal models [Santiago et al., 1999; Sorensen et al., 2002; Clawson et al., 2004] by targeting the selective destruction of various mRNAs. Despite these encouraging results, their applications are subject to many of the concerns described above with regard to hRzs. For example, these small nucleic acids do not seem to be structurally robust. One recent study suggested that, under ionic conditions simulating the intracellular milieu, Dzs were only partially folded and showed a largely suboptimal catalytic activity [Cieslak et al., 2003].

siRNA Chemistry

In the past 2–3 years, a number of chemical modifications have been introduced into siRNAs, which preserve their RNAi effects.

For example, activity of siRNAs is preserved with PS linkages at 5′ and 3′ ends, and with 2′-fluoro (2′F) substitutions at pyrimidines [Harborth et al., 2003]. Activity is also maintained with alternating 2′MeO linkages, or with several PS and 2′MeO substitutions at 5′ and 3′ ends [Amarzguioui et al., 2003; Braasch et al., 2003; Hemmings-Mieszczak et al., 2003], although alternating substitutions with PS have been reported to produce cytotoxicity [Harborth et al., 2003]. Activity is also reportedly preserved even when the sense strand is completely modified with PS, 2′MeOE, or 2′F substitutions [Chiu and Rana, 2003]. RNAi activity can even be induced with hybrids comprising sense-DNA/antisense RNA [Hohjoh, 2004], and in fact RNAi activity has actually been reported to be greater and of longer duration using such constructs [Lamberton and Christian, 2003]. The potential drawback to this strategy is that the antisense RNA moiety can be degraded by RNase H [Crooke, 2004], so further modifications (as mentioned above) would seem to be indicated to enhance stability of the antisense RNA.

DELIVERY

In order for an ASR to downregulate gene expression, it must penetrate into the targeted cells. However, the precise mechanisms involved in ASO penetration are not clear. Early work showed that uptake occurs through active transport, which in turn depends on temperature [Loke et al., 1989; Yakubov et al., 1989], the structure and the concentration of the ASO [Vlassov et al., 1994], and the cell line. It is believed that adsorptive endocytosis and fluid phase pinocytosis are the major mechanisms of oligonucleotide internalization, with the relative proportions of internalized material depending on ASO concentration. At relatively low ASOconcentration, it is likely that internalization occurs via interaction with a membrane-bound receptor [Loke et al., 1989; Yakubov et al., 1989], and some of these receptors were purified and/or characterized [de Diesbach et al., 2000; Herz and Strickland, 2001; Emonard et al., 2005]. At relatively high ASO concentration, these receptors are saturated, and the pinocytotic process assumes larger importance. Numerous experiments have demonstrated that the sine qua non of antisense activity appears to be nuclear localization.

Numerous reports have demonstrated that naked ASOs are internalized poorly by cells whether or not they are negatively charged [Bennett et al., 1992; Stein et al., 1993; Gray et al., 1997]. More specifically, naked oligonucleotides tend to localize in endosomes/lysosomes, where they are unavailable for antisense purposes. Having said that, there is nevertheless a recent report showing efficacious application of short hairpin siRNAs targeting Hepatitis C virus using a simple hydrodynamic transfection of liver cells via tail vein injection of mice [Wang et al., 2005].

To improve cellular uptake and oligonucleotide spatial and temporal activity, a range of techniques and transporters have been developed. Simultaneously, the use of these vehicles increases the stability of oligonucleotides against nuclease digestion and permits the use of lower doses (perhaps 10-fold) of ASOs.

The major obstacle to effective antisense therapies in vivo is clearly effective delivery to target cells. The first generation of delivery vehicles developed were liposomes, which are colloid vesicles generally composed of bilayers of phospholipids admixed with various agents and/or cholesterol. Liposomes can be neutral or cationic, depending on the nature of the phospholipids. The nucleic acid can be easily encapsulated in the liposome interior, which contains an aqueous compartment, or be bound to the liposome surface by electrostatic interactions [Mahato, 2005]. Cationic liposomes, because of their positive charge, have a relatively high affinity for cell membranes, which are negatively charged under physiological conditions. As these vehicles use the endosomal pathway to deliver oligonucleotides into cells, certain "helper" (fusogenic) molecules have been added into the liposomes to allow the oligonucleotides to escape from the endosomes; examples include species such as chloroquine, 1,2-distearoyl-*sn*-glycero-3-phosphocholine, and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine, DLinDMA, etc. These "helper" molecules ultimately induce endosomal membrane destabilization, allowing leakage of the ASO, which then appears to be actively transported in high concentration to the nucleus [Fraley et al., 1981; Felgner et al., 1994; Farhood et al., 1995; Ma and Wei, 1996]. There are also reports that some ASOs (i.e., those containing PS modifications) actually shuttle between nucleus and cytoplasm [Lorenz et al., 2000], and siRNAs can be exported from the nucleus by exportin-5-dependent processes [Yi et al., 2003], so nuclear localization seems to be a dynamic process.

The use of other cationic polymers, including poly-L-lysine [Clarenc et al., 1993; Stewart et al., 1996], PAMAM dendrimers [Bielinska et al., 1996], polyalkylcyanoacrylate nanoparticles [Chavany et al., 1992; Zobel et al., 1997], and polyethyleneimine [Boussif et al., 1995], have been also developed for drug delivery. Nucleic acids interact with these vectors via electrostatic interactions. Activity has been demonstrated in various cell lines [Chavany et al., 1992; Hughes et al., 1996] and in a nude mice model [Schwab et al., 1994], but unfortunately, these polyamines, which appear to cause endosomal rupture via a "molecular sponge" mechanism, tend to be somewhat toxic and are less commonly in use than are the cationic liposomes.

Therapeutically relevant advances have recently been described with siRNA targeting HBV. [Morrissey et al., 2005a,b] employed a chemically-modified siRNA, wherein all 2′OH

residues on the RNA were replaced with 2′F, 2′OMe, or 2′H groups, and 1-3 ribonucleotides were placed on the 5′ end of the antisense strand [Morrissey et al., 2005b]. The siRNAs were then incorporated into stable nucleic acid-lipid particles (SNALPs), which consisted of a lipid bilayer of cationic and fusogenic lipids. The SNALPs were in turn coated with a diffusible polyethylene glycol–lipid conjugate, which provided a neutral hydrophilic exterior and stabilized the formulation, preventing rapid systemic clearance. This formulation was very effective against HBV in an in vivo mouse model, and demonstrated a number of advantages, including an extended duration of RNAi effects in vivo, decreased relative dose, and reduced dosing frequency. Continued future formulation development will clearly be the key to effective clinical applicability of ASR, although targets other than liver may prove much more problematic.

All of these cationic delivery systems internalize ASOs via an endocytotic mechanism. In alternative ways to avoid the resulting compartmentalization problems, consideration has also been given to modulating plasma membrane permeability. By using basic peptides, one can increase ASR passage through the plasma membrane by a receptor- and transporter-independent mechanism. As these peptides have membrane translocation properties, covalent coupling with an ASR can increase the latter's penetration into the cell, delivering them directly into the cytoplasm and ultimately the nucleus. Several of these peptides, such as the *Drosophila melanogaster* homeotic transcription factor, the Antennapedia peptide [Derossi et al., 1998], and the Tat protein of HIV-1 [Vives et al., 1997], have been identified and studied. In another example, using fluoresceinylated ASOs coupled to the E5CA peptide (which corresponds to the NH2-terminal segment of the HA2 subunit of the influenza virus agglutinin protein), [Pichon et al., 1997] demonstrated that oligonucleotides were rapidly taken up by cells and diffused into the nucleus.

PROSPECT

ASRs represent important research tools, and evolutions of their design, including chemical modifications and improved target site selection, have increased their efficacy. However, while toxicity (at least with ASOs) has not been a problem, effective clinical therapeutics have not been developed. This most likely reflects relatively poor systemic delivery (with the possible exception of liver). While continued incremental improvements in liposomal vehicles, as well as development of new nanoparticle delivery platforms [Luo et al., 2004; Kukowska-Latallo et al., 2005], particularly the intriguing nanoparticles based on branched DNA polystyrene (see Chiarello [2005]) may well rectify delivery problems down the road, we suggest that topical applications of ASRs with suitable formulations offers a better chance of therapeutic success for the next few years. Successful development will also likely depend upon the validity of the animal models used.

REFERENCES

Akhtar S, Kole R, Juliano RL. 1991. Stability of antisense DNA oligodeoxynucleotide analogs in cellular extracts and sera. Life Sci 49:1793–1801.

Altman RK, Schwope I, Sarracino DA, Tetzlaff CN, Bleczinski CF, Richert C. 1999. Selection of modified oligonucleotides with increased target affinity via MALDI-monitored nuclease survival assays. J Comb Chem 1:493–508.

Amarzguioui M, Holen T, Babaie E, Prydz H. 2003. Tolerance for mutations and chemical modifications in a siRNA. Nucleic Acids Res 31:589–595.

Baker BF, Lot SS, Condon TP, Cheng-Flournoy S, Lesnik EA, Sasmor HM, Bennett CF. 1997. 2′-*O*-(2-Methoxy)-ethyl-modified anti-intercellular adhesion molecule 1 (ICAM-1) oligonucleotides selectively increase the ICAM-1 mRNA level and inhibit formation of the ICAM-1 translation initiation complex in human umbilical vein endothelial cells. J Biol Chem 272:11994–12000.

Ball HA, Sandrasagra A, Tang L, Van Scott M, Wild J, Nyce JW. 2003. Clinical potential of respirable antisense oligonucleotides (RASONs) in asthma. Am J Pharmacogenomics 3:97–106.

Beigelman L, McSwiggen JA, Draper KG, Gonzalez C, Jensen K, Karpeisky A, Modak A, Matulic-Adamic J, DiRenzo A, Haeberli P, Sweedler D, Tracz D, Grimm S, Wincott F, Thackray V, Usman N. 1995. Chemical modification of hammerhead ribozymes. J Biol Chem 270:25702–25708.

Benedict CM, Pan W, Loy SE, Clawson GA. 1998. Triple ribozyme-mediated down-regulation of the retinoblastoma gene. Carcinogenesis 19:1223–1230.

Benimetskaya L, Takle GB, Vilenchik M, Lebedeva I, Miller P, Stein CA. 1998. Cationic porphyrins: Novel delivery vehicles for antisense oligodeoxynucleotides. Nucleic Acids Res 26:5310–5317.

Bennett CF, Chiang MY, Chan H, Shoemaker JE, Mirabelli CK. 1992. Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. Mol Pharmacol 41:1023–1033.

Berns K, Hijmans EM, Mullenders J, Brummelkamp TR, Velds A, Heimerikx M, Kerkhoven RM, Madiredjo M,

Nijkamp W, Weigelt B, Agami R, Ge W, Cavet G, Linsley PS, Beijersbergen RL, Bernards R. 2004. A large-scale RNAi screen in human cells identifies new components of the p53 pathway. Nature 428:431–437.

Bertrand JR, Pottier M, Vekris A, Opolon P, Maksimenko A, Malvy C. 2002. Comparison of antisense oligonucleotides and siRNAs in cell culture and in vivo. Biochem Biophys Res Commun 296:1000–1004.

Bielinska A, Kukowska-Latallo JF, Johnson J, Tomalia DA, Baker JR, Jr. 1996. Regulation of in vitro gene expression using antisense oligonucleotides or antisense expression plasmids transfected using starburst PAMAM dendrimers. Nucleic Acids Res 24:2176–2182.

Birikh KR, Heaton PA, Eckstein F. 1997. The structure, function and application of the hammerhead ribozyme. Eur J Biochem 245:1–16.

Blake KR, Murakami A, Spitz SA, Glave SA, Reddy MP, Ts'o PO, Miller PS. 1985 . Hybridization arrest of globin synthesis in rabbit reticulocyte lysates and cells by oligodeoxyribonucleoside methylphosphonates. Biochemistry 24:6139–6145.

Blount KF, Grover NL, Mokler V, Beigelman L, Uhlenbeck OC. 2002. Steric interference modification of the hammerhead ribozyme. Chem Biol 9:1009–1016.

Boffa LC, Morris PL, Carpaneto EM, Louissaint M, Allfrey VG. 1996. Invasion of the CAG triplet repeats by a complementary peptide nucleic acid inhibits transcription of the androgen receptor and TATA-binding protein genes and correlates with refolding of an active nucleosome containing a unique AR gene sequence. J Biol Chem 271:13228–13233.

Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. Proc Natl Acad Sci USA 92: 7297–7301.

Braasch DA, Corey DR. 2002. Novel antisense and peptide nucleic acid strategies for controlling gene expression. Biochemistry 41:4503–4510.

Braasch DA, Jensen S, Liu Y, Kaur K, Arar K, White MA, Corey DR. 2003. RNA interference in mammalian cells by chemically-modified RNA. Biochemistry 42:7967–7975.

Bramlage B, Luzi E, Eckstein F. 2000. HIV-1 LTR as a target for synthetic ribozyme-mediated inhibition of gene expression: Site selection and inhibition in cell culture. Nucleic Acids Res 28:4059–4067.

Brower V, Chahine K, Dorey E, Dove A, Francisco M, Hodgson J, Michael A, Marshall A. 1998. All clear for HIV-targeting ribozyme in phase II. Nat Biotechnol 16:123.

Cairns MJ, Saravolac EG, Sun LQ. 2002. Catalytic DNA: A novel tool for gene suppression. Curr Drug Targets 3:269–279.

Campbell TB, McDonald CK, Hagen M. 1997. The effect of structure in a long target RNA on ribozyme cleavage efficiency. Nucleic Acids Res 25:4985–4993.

Caplen NJ. 2003. RNAi as a gene therapy approach. Expert Opin Biol Ther 3:575–586.

Cazenave C, Frank P, Busen W. 1993. Characterization of ribonuclease H activities present in two cell-free protein synthesizing systems, the wheat germ extract and the rabbit reticulocyte lysate. Biochimie 75:113–122.

Cech TR, Zaug AJ, Grabowski PJ. 1981. In vitro splicing of the ribosomal RNA precursor of Tetrahymena: Involvement of a guanosine nucleotide in the excision of the intervening sequence. Cell 27:487–496.

Cerruti NR, Keshavamurthy S, Tomsovic S. 2003. Exploring classical phase space structures of nearly integrable and mixed quantum systems via parametric variation. Phys Rev E Stat Nonlin Soft Matter Phys 68:056205.

Chavany C, Le Doan T, Couvreur P, Puisieux F, Helene C. 1992. Polyalkylcyanoacrylate nanoparticles as polymeric carriers for antisense oligonucleotides. Pharm Res 9: 441–449.

Chen JK, Schultz RG, Lloyd DH, Gryaznov SM. 1995. Synthesis of oligodeoxyribonucleotide N3′–>P5′ phosphoramidates. Nucleic Acids Res 23:2661–2668.

Chiang MY, Chan H, Zounes MA, Freier SM, Lima WF, Bennett CF. 1991. Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two distinct mechanisms. J Biol Chem 266:18162–18171.

Chiarello K. 2005. Size matters: New DNA buckball building blocks could help delivery drugs. Pharm Technol 29:32.

Chiu YL, Rana TM. 2002. RNAi in human cells: Basic structural and functional features of small interfering RNA. Mol Cell 10:549–561.

Chiu YL, Rana TM. 2003. siRNA function in RNAi: A chemical modification analysis. Rna 9:1034–1048.

Cieslak M, Szymanski J, Adamiak RW, Cierniewski CS. 2003. Structural rearrangements of the 10–23 DNAzyme to beta 3 integrin subunit mRNA induced by cations and their relations to the catalytic activity. J Biol Chem 278: 47987–47996.

Clarenc JP, Degols G, Leonetti JP, Milhaud P, Lebleu B. 1993. Delivery of antisense oligonucleotides by poly (L-lysine) conjugation and liposome encapsulation. Anticancer Drug Des 8:81–94.

Clawson GA, Miranda GQ, Sivarajah A, Xin P, Pan W, Thiboutot D, Christensen ND. 2004. Inhibition of papilloma progression by antisense oligonucleotides targeted to HPV11 E6/E7 RNA. Gene Ther 11:1331–1341.

Clouet-d'Orval B, Uhlenbeck OC. 1997. Hammerhead ribozymes with a faster cleavage rate. Biochemistry 36:9087–9092.

Croce CM, Calin GA. 2005. miRNAs, cancer, and stem cell division. Cell 122:6–7.

Crone TM, Schalles SL, Benedict CM, Pan W, Ren L, Loy SE, Isom H, Clawson GA. 1999. Growth inhibition by a triple ribozyme targeted to repetitive B2 transcripts. Hepatology 29:1114–1123.

Crooke ST. 1998. Molecular mechanisms of antisense drugs: RNase H. Antisense Nucleic Acid Drug Dev 8: 133–134.

Crooke ST. 1999. Molecular mechanisms of action of antisense drugs. Biochim Biophys Acta 1489:31–44.

Crooke ST. 2004. Progress in antisense technology. Annu Rev Med 55:61–95.

Cutrona G, Carpaneto EM, Ulivi M, Roncella S, Landt O, Ferrarini M, Boffa LC. 2000. Effects in live cells of a c-myc anti-gene PNA linked to a nuclear localization signal. Nat Biotechnol 18:300–303.

Damha MJ, Wilds CJ, Noronha A, Brukner I, Borkow G, Arion D, Parniak MA. 1998. Hybrids of RNA and arabinonucleic acids (ANA and 2′F-ANA) are substrates of ribonuclease H. J Am Chem Soc 120:12976–12977.

Damha MJ, Noronha AM, Wilds CJ, Trempe JF, Denisov A, Pon RT, Gehring K. 2001. Properties of arabinonucleic acids (ANA & 20′F-ANA): Implications for the design of antisense therapeutics that invoke RNase H cleavage of RNA. Nucleosides Nucleotides Nucleic Acids 20:429–440.

Dass CR, Saravolac EG, Li Y, Sun LQ. 2002. Cellular uptake, distribution, and stability of 10-23 deoxyribozymes. Antisense Nucleic Acid Drug Dev 12:289–299.

de Diesbach P, Berens C, N'Kuli F, Monsigny M, Sonveaux E, Wattiez R, Courtoy PJ. 2000. Identification, purification and partial characterisation of an oligonucleotide receptor in membranes of HepG2 cells. Nucleic Acids Res 28:868–874.

Dean NM, McKay R, Condon TP, Bennett CF. 1994. Inhibition of protein kinase C-alpha expression in human A549 cells by antisense oligonucleotides inhibits induction of intercellular adhesion molecule 1 (ICAM-1) mRNA by phorbol esters. J Biol Chem 269:16416–16424.

Derossi D, Chassaing G, Prochiantz A. 1998. Trojan peptides: The penetratin system for intracellular delivery. Trends Cell Biol 8:84–87.

Dias N, Dheur S, Nielsen PE, Gryaznov S, Van Aerschot A, Herdewijn P, Helene C, Saison-Behmoaras TE. 1999. Antisense PNA tridecamers targeted to the coding region of Ha-ras mRNA arrest polypeptide chain elongation. J Mol Biol 294:403–416.

Ding Y, Lawrence CE. 1999. A bayesian statistical algorithm for RNA secondary structure prediction. Comput Chem 23:387–400.

Ding Y, Lawrence CE. 2001. Statistical prediction of single-stranded regions in RNA secondary structure and application to predicting effective antisense target sites and beyond. Nucleic Acids Res 29:1034–1046.

Dykxhoorn DM, Novina CD, Sharp PA. 2003. Killing the messenger: Short RNAs that silence gene expression. Nat Rev Mol Cell Biol 4:457–467.

Eder PS, DeVine RJ, Dagle JM, Walder JA. 1991. Substrate specificity and kinetics of degradation of antisense oligonucleotides by a 3′ exonuclease in plasma. Antisense Res Dev 1:141–151.

Efimov VA, Choob MV, Buryakova AA, Kalinkina AL, Chakhmakhcheva OG. 1998. Synthesis and evaluation of some properties of chimeric oligomers containing PNA and phosphono-PNA residues. Nucleic Acids Res 26:566–575.

Efimov VA, Buryakova AA, Chakhmakhcheva OG. 1999. Synthesis of polyacrylamides N-substituted with PNA-like oligonucleotide mimics for molecular diagnostic applications. Nucleic Acids Res 27:4416–4426.

Eis PS, Tam W, Sun L, Chadburn A, Li Z, Gomez MF, Lund E, Dahlberg JE. 2005. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. Proc Natl Acad Sci USA 102:3627–3632.

Emilsson GM, Breaker RR. 2002. Deoxyribozymes: New activities and new applications. Cell Mol Life Sci 59:596–607.

Emonard H, Bellon G, de Diesbach P, Mettlen M, Hornebeck W, Courtoy PJ. 2005. Regulation of matrix metalloproteinase (MMP) activity by the low-density lipoprotein receptor-related protein (LRP). A new function for an "old friend." Biochimie 87:369–376.

Farhood H, Serbina N, Huang L. 1995. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. Biochim Biophys Acta 1235:289–295.

Felgner JH, Kumar R, Sridhar CN, Wheeler CJ, Tsai YJ, Border R, Ramsey P, Martin M, Felgner PL. 1994. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. J Biol Chem 269:2550–2561.

Filipowicz W. 2005. RNAi: The nuts and bolts of the RISC machine. Cell 122:17–20.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391:806–811.

Flory CM, Pavco PA, Jarvis TC, Lesch ME, Wincott FE, Beigelman L, Hunt SW, 3rd, Schrier DJ. 1996. Nuclease-resistant ribozymes decrease stromelysin mRNA levels in rabbit synovium following exogenous delivery to the knee joint. Proc Natl Acad Sci USA 93:754–758.

Fraley R, Straubinger RM, Rule G, Springer EL, Papahadjopoulos D. 1981. Liposome-mediated delivery of deoxyribonucleic acid to cells: Enhanced efficiency of delivery related to lipid composition and incubation conditions. Biochemistry 20:6978–6987.

Frantz S. 2003. Studies reveal potential pitfalls of RNAi. Nature Rev Drug Discov 2:763–764.

Fulford AJ, Harbuz MS, Jessop DS. 2000. Antisense inhibition of pro-opiomelanocortin and proenkephalin A messenger RNA translation alters rat immune cell function in vitro. J Neuroimmunol 106:6–13.

Galarneau A, Min KL, Mangos MM, Damha MJ. 2005. Assay for evaluating ribonuclease H-mediated degradation of RNA-antisense oligonucleotide duplexes. Methods Mol Biol 288:65–80.

Gambacorti-Passerini C, Mologni L, Bertazzoli C, le Coutre P, Marchesi E, Grignani F, Nielsen PE. 1996. In vitro transcription and translation inhibition by anti-promyelocytic leukemia (PML)/retinoic acid receptor alpha and anti-PML peptide nucleic acid. Blood 88:1411–1417.

Giles RV, Tidd DM. 2001. The direction of ribonucleases H by antisense oligodeoxynucleotides. Methods Mol Biol 160:157–182.

Giles RV, Spiller DG, Green JA, Clark RE, Tidd DM. 1995. Optimization of antisense oligodeoxynucleotide structure for targeting bcr-abl mRNA. Blood 86:744–754.

Giles RV, Spiller DG, Clark RE, Tidd DM. 1999. Antisense morpholino oligonucleotide analog induces missplicing of C-myc mRNA. Antisense Nucleic Acid Drug Dev 9:213–220.

Gleave ME, Monia BP. 2005. Antisense therapy for cancer. Nat Rev Cancer 5:468–479.

Good L, Nielsen PE. 1998. Antisense inhibition of gene expression in bacteria by PNA targeted to mRNA. Nat Biotechnol 16:355–358.

Gray GD, Basu S, Wickstrom E. 1997. Transformed and immortalized cellular uptake of oligodeoxynucleoside phosphorothioates, 3′-alkylamino oligodeoxynucleotides, 2′-*O*-methyl oligoribonucleotides, oligodeoxynucleoside methylphosphonates, and peptide nucleic acids. Biochem Pharmacol 53:1465–1476.

Gregory RI, Shiekhattar R. 2005. MicroRNA biogenesis and cancer. Cancer Res 65:3509–3512.

Grunweller A, Wyszko E, Bieber B, Jahnel R, Erdmann VA, Kurreck J. 2003. Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2′-*O*-methyl RNA, phosphorothioates and small interfering RNA. Nucleic Acids Res 31:3185–3193.

Gryaznov SM, Lloyd DH, Chen JK, Schultz RG, DeDionisio LA, Ratmeyer L, Wilson WD. 1995. Oligonucleotide N3′−>P5′ phosphoramidates. Proc Natl Acad Sci USA 92:5798–5802.

Guerrier-Takada C, Gardiner K, Marsh T, Pace N, Altman S. 1983. The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. Cell 35:849–857.

Hanvey JC, Peffer NJ, Bisi JE, Thomson SA, Cadilla R, Josey JA, Ricca DJ, Hassman CF, Bonham MA, Au KG, et al. 1992. Antisense and antigene properties of peptide nucleic acids. Science 258:1481–1485.

Harborth J, Elbashir SM, Vandenburgh K, Manninga H, Scaringe SA, Weber K, Tuschl T. 2003. Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. Antisense Nucleic Acid Drug Dev 13:83–105.

Hatfield SD, Shcherbata HR, Fischer KA, Nakahara K, Carthew RW, Ruohola-Baker H. 2005. Stem cell division is regulated by the microRNA pathway. Nature 435:974–978.

He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ, Hammond SM. 2005. A microRNA polycistron as a potential human oncogene. Nature 435:828–833.

Heasman J. 2002. Morpholino oligos: Making sense of antisense? Dev Biol 243:209–214.

Heidenreich O, Pieken W, Eckstein F. 1993. Chemically modified RNA: Approaches and applications. Faseb J 7: 90–96.

Heidenreich O, Xu X, Swiderski P, Rossi JJ, Nerenberg M. 1996. Correlation of activity with stability of chemically modified ribozymes in nuclei suspension. Antisense Nucleic Acid Drug Dev 6:111–118.

Hemmings-Mieszczak M, Dorn G, Natt FJ, Hall J, Wishart WL. 2003. Independent combinatorial effect of antisense oligonucleotides and RNAi-mediated specific inhibition of the recombinant rat P2X3 receptor. Nucleic Acids Res 31:2117–2126.

Herz J, Strickland DK. 2001. LRP: A multifunctional scavenger and signaling receptor. J Clin Invest 108: 779–784.

Hohjoh H. 2004. Enhancement of RNAi activity by improved siRNA duplexes. FEBS Lett 557:193–198.

Holen T, Amarzguioui M, Wiiger MT, Babaie E, Prydz H. 2002. Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. Nucleic Acids Res 30:1757–1766.

Hudziak RM, Summerton J, Weller DD, Iversen PL. 2000. Antiproliferative effects of steric blocking phosphorodiamidate morpholino antisense agents directed against c-myc. Antisense Nucleic Acid Drug Dev 10:163–176.

Huesken D, Lange J, Mickanin C, Weiler J, Asselbergs F, Warner J, Meloon B, Engel S, Rosenberg A, Cohen D, Labow M, Reinhardt M, Natt F, Hall J. 2005. Design of a genome-wide siRNA library using an artificial neural network. Nat Biotechnol 23:995–1001.

Hughes JA, Aronsohn AI, Avrutskaya AV, Juliano RL. 1996. Evaluation of adjuvants that enhance the effectiveness of antisense oligodeoxynucleotides. Pharm Res 13:404–410.

Iversen PL, Arora V, Acker AJ, Mason DH, Devi GR. 2003. Efficacy of antisense morpholino oligomer targeted to c-myc in prostate cancer xenograft murine model and a Phase I safety study in humans. Clin Cancer Res 9:2510–2519.

Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS. 2003. Expression profiling reveals off-target gene regulation by RNAi. Nat Biotechnol 21:635–637.

Jarvis TC, Wincott FE, Alby LJ, McSwiggen JA, Beigelman L, Gustofson J, DiRenzo A, Levy K, Arthur M, Matulic-Adamic J, Karpeisky A, Gonzalez C, Woolf TM, Usman N, Stinchcomb DT. 1996. Optimizing the cell efficacy of synthetic ribozymes. Site selection and chemical modifications of ribozymes targeting the proto-oncogene c-myb. J Biol Chem 271:29107–29112.

Kara J, Duschinsky R. 1969. Inhibition of thymidylate kinase and DNA synthesis in HeLa cells by 5′-deoxythymidine. Biochim Biophys Acta 186:223–225.

Karras JG, Maier MA, Lu T, Watt A, Manoharan M. 2001. Peptide nucleic acids are potent modulators of endogenous pre-mRNA splicing of the murine interleukin-5 receptor-alpha chain. Biochemistry 40:7853–7859.

Katayama S, Tomaru Y, Kasukawa T, Waki K, Nakanishi M, Nakamura M, Nishida H, Yap CC, Suzuki M, Kawai J, Suzuki H, Carninci P, Hayashizaki Y, Wells C, Frith M, Ravasi T, Pang KC, Hallinan J, Mattick J, Hume DA, Lipovich L, Batalov S, Engstrom PG, Mizuno Y, Faghihi MA, Sandelin A, Chalk AM, Mottagui-Tabar S, Liang Z, Lenhard B, Wahlestedt C. 2005. Antisense transcription in the mammalian transcriptome. Science 309:1564–1566.

Kawasaki H, Taira K. 2004. Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. Nature 431:211–217.

Khachigian LM. 2002. DNAzymes: Cutting a path to a new class of therapeutics. Curr Opin Mol Ther 4:119–121.

Khan AU, Lal SK. 2003. Ribozymes: A modern tool in medicine. J Biomed Sci 10:457–467.

Khvorova A, Lescoute A, Westhof E, Jayasena SD. 2003. Sequence elements outside the hammerhead ribozyme catalytic core enable intracellular activity. Nat Struct Biol 10:708–712.

Kittler R, Putz G, Pelletier L, Poser I, Heninger AK, Drechsel D, Fischer S, Konstantinova I, Habermann B, Grabner H, Yaspo ML, Himmelbauer H, Korn B, Neugebauer K, Pisabarro MT, Buchholz F. 2004. An endoribonuclease-prepared siRNA screen in human cells identifies genes essential for cell division. Nature 432:1036–1040.

Kole R, Sazani P. 2001. Antisense effects in the cell nucleus: Modification of splicing. Curr Opin Mol Ther 3:229–234.

Koziolkiewicz M, Gendaszewska E, Maszewska M, Stein CA, Stec WJ. 2001. The mononucleotide-dependent, nonantisense mechanism of action of phosphodiester and phosphorothioate oligonucleotides depends upon the activity of an ecto-5′-nucleotidase. Blood 98:995–1002.

Kretschmer-Kazemi Far R, Sczakiel G. 2003. The activity of siRNA in mammalian cells is related to structural target accessibility: A comparison with antisense oligonucleotides. Nucleic Acids Res 31:4417–4424.

Kronke J, Kittler R, Buchholz F, Windisch MP, Pietschmann T, Bartenschlager R, Frese M. 2004. Alternative approaches for efficient inhibition of hepatitis C virus RNA replication by small interfering RNAs. J Virol 78: 3436–3446.

Kruger K, Grabowski PJ, Zaug AJ, Sands J, Gottschling DE, Cech TR. 1982. Self-splicing RNA: Autoexcision and autocyclization of the ribosomal RNA intervening sequence of Tetrahymena. Cell 31:147–157.

Kukowska-Latallo JF, Candido KA, Cao Z, Nigavekar SS, Majoros IJ, Thomas TP, Balogh LP, Khan MK, Baker JR, Jr. 2005. Nanoparticle targeting of anticancer drug improves therapeutic response in animal model of human epithelial cancer. Cancer Res 65:5317–5324.

Kurreck J. 2003. Antisense technologies. Improvement through novel chemical modifications. Eur J Biochem 270:1628–1644.

Lamberton JS, Christian AT. 2003. Varying the nucleic acid composition of siRNA molecules dramatically varies the duration and degree of gene silencing. Mol Biotechnol 24:111–120.

Larrouy B, Blonski C, Boiziau C, Stuer M, Moreau S, Shire D, Toulme JJ. 1992. RNase H-mediated inhibition of translation by antisense oligodeoxyribonucleotides: Use of backbone modification to improve specificity. Gene 121:189–194.

Levin AA. 1999. A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. Biochim Biophys Acta 1489:69–84.

Lewis BP, Burge CB, Bartel DP. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120:15–20.

Lieber A, Kay MA. 1996. Adenovirus-mediated expression of ribozymes in mice. J Virol 70:3153–3158.

Lieber A, Strauss M. 1995. Selection of efficient cleavage sites in target RNAs by using a ribozyme expression library. Mol Cell Biol 15:540–551.

Lima WF, Brown-Driver V, Fox M, Hanecak R, Bruice TW. 1997. Combinatorial screening and rational optimization for hybridization to folded hepatitis C virus RNA of oligonucleotides with biological antisense activity. J Biol Chem 272:626–638.

Lloyd BH, Giles RV, Spiller DG, Grzybowski J, Tidd DM, Sibson DR. 2001. Determination of optimal sites of antisense oligonucleotide cleavage within TNFalpha mRNA. Nucleic Acids Res 29:3664–3673.

Lok CN, Viazovkina E, Min KL, Nagy E, Wilds CJ, Damha MJ, Parniak MA. 2002. Potent gene-specific inhibitory properties of mixed-backbone antisense oligonucleotides comprised of 2′-deoxy-2′-fluoro-D-arabinose and 2′-deoxyribose nucleotides. Biochemistry 41:3457–3467.

Loke SL, Stein CA, Zhang XH, Mori K, Nakanishi M, Subasinghe C, Cohen JS, Neckers LM. 1989. Characterization of oligonucleotide transport into living cells. Proc Natl Acad Sci USA 86:3474–3478.

Lorenz P, Misteli T, Baker BF, Bennett CF, Spector DL. 2000. Nucleocytoplasmic shuttling: A novel in vivo property of antisense phosphorothioate oligodeoxynucleotides. Nucleic Acids Res 28:582–592.

Lu C, Tej SS, Luo S, Haudenschild CD, Meyers BC, Green PJ. 2005. Elucidation of the small RNA component of the transcriptome. Science 309:1567–1569.

Luo D, Han E, Belcheva N, Saltzman W. 2004. A self-assembled modular DNA delivery system mediated by silica nanoparticles. J Control Release 95:333–341.

Ma DD, Wei AQ. 1996. Enhanced delivery of synthetic oligonucleotides to human leukaemic cells by liposomes and immunoliposomes. Leuk Res 20:925–930.

Mahato RI. 2005. Water insoluble and soluble lipids for gene delivery. Adv Drug Deliv Rev 57:699–712.

Mann CJ, Honeyman K, Cheng AJ, Ly T, Lloyd F, Fletcher S, Morgan JE, Partridge TA, Wilton SD. 2001. Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. Proc Natl Acad Sci USA 98:42–47.

Matzke MA, Birchler JA. 2005. RNAi-mediated pathways in the nucleus. Nat Rev Genet 6:24–35.

McKay DB. 1996. Structure and function of the hammerhead ribozyme: An unfinished story. Rna 2:395–403.

McManus MT, Sharp PA. 2002. Gene silencing in mammals by small interfering RNAs. Nat Rev Genet 3:737–747.

Miller PS, Yano J, Yano E, Carroll C, Jayaraman K, Ts'o PO. 1979. Nonionic nucleic acid analogues. Synthesis and characterization of dideoxyribonucleoside methylphosphonates. Biochemistry 18:5134–5143.

Miller PS, McParland KB, Jayaraman K, Ts'o PO. 1981. Biochemical and biological effects of nonionic nucleic acid methylphosphonates. Biochemistry 20:1874–1880.

Minshull J, Hunt T. 1986. The use of single-stranded DNA and RNase H to promote quantitative 'hybrid arrest of translation' of mRNA/DNA hybrids in reticulocyte lysate cell-free translations. Nucleic Acids Res 14:6433–6451.

Mir KU, Southern EM. 1999. Determining the influence of structure on hybridization using oligonucleotide arrays. Nat Biotechnol 17:788–792.

Mir AA, Lockett TJ, Hendry P. 2001. Identifying ribozyme-accessible sites using NUH triplet-targeting gapmers. Nucleic Acids Res 29:1906–1914.

Miyagishi M, Taira K. 2005. siRNA becomes smart and intelligent. Nat Biotechnol 23:946–947.

Miyagishi M, Hayashi M, Taira K. 2003. Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. Antisense Nucleic Acid Drug Dev 13:1–7.

Mologni L, Nielsen PE, Gambacorti-Passerini C. 1999. In vitro transcriptional and translational block of the *bcl-2* gene operated by peptide nucleic acid. Biochem Biophys Res Commun 264:537–543.

Mologni L, Marchesi E, Nielsen PE, Gambacorti-Passerini C. 2001. Inhibition of promyelocytic leukemia (PML)/retinoic acid receptor-alpha and PML expression in acute promyelocytic leukemia cells by anti-PML peptide nucleic acid. Cancer Res 61:5468–5473.

Monia BP, Lesnik EA, Gonzalez C, Lima WF, McGee D, Guinosso CJ, Kawasaki AM, Cook PD, Freier SM. 1993. Evaluation of 2′-modified oligonucleotides containing 2′-deoxy gaps as antisense inhibitors of gene expression. J Biol Chem 268:14514–14522.

Morris MC, Depollier J, Mery J, Heitz F, Divita G. 2001. A peptide carrier for the delivery of biologically active proteins into mammalian cells. Nat Biotechnol 19:1173–1176.

Morris KV, Chan SW, Jacobsen SE, Looney DJ. 2004. Small interfering RNA-induced transcriptional gene silencing in human cells. Science 305:1289–1292.

Morrissey DV, Blanchard K, Shaw L, Jensen K, Lockridge JA, Dickinson B, McSwiggen JA, Vargeese C, Bowman K, Shaffer CS, Polisky BA, Zinnen S. 2005a. Activity of stabilized short interfering RNA in a mouse model of hepatitis B virus replication. Hepatology 41:1349–1356.

Morrissey DV, Lockridge JA, Shaw L, Blanchard K, Jensen K, Breen W, Hartsough K, Machemer L, Radka S, Jadhav V, Vaish N, Zinnen S, Vargeese C, Bowman K, Shaffer

CS, Jeffs LB, Judge A, MacLachlan I, Polisky B. 2005b. Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. Nat Biotechnol 23:1002–1007.

Noronha AM, Wilds CJ, Lok CN, Viazovkina K, Arion D, Parniak MA, Damha MJ. 2000. Synthesis and biophysical properties of arabinonucleic acids (ANA): Circular dichroic spectra, melting temperatures, and ribonuclease H susceptibility of ANA.RNA hybrid duplexes. Biochemistry 39:7050–7062.

Norris JS, Hoel B, Voeks D, Maggouta F, Dahm M, Pan W, Clawson G. 2000. Design and testing of ribozymes for cancer gene therapy. Adv Exp Med Biol 465:293–301.

O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. 2005. c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435:839–843.

Oketani M, Asahina Y, Wu CH, Wu GY. 1999. Inhibition of hepatitis C virus-directed gene expression by a DNA ribonuclease. J Hepatol 31:628–634.

Opalinska JB, Gewirtz AM. 2002. Nucleic-acid therapeutics: Basic principles and recent applications. Nat Rev Drug Discov 1:503–514.

Overhoff M, Alken M, Far RK, Lemaitre M, Lebleu B, Sczakiel G, Robbins I. 2005. Local RNA target structure influences siRNA efficacy: A systematic global analysis. J Mol Biol 348:871–881.

Paddison PJ, Silva JM, Conklin DS, Schlabach M, Li M, Aruleba S, Balija V, O'Shaughnessy A, Gnoj L, Scobie K, Chang K, Westbrook T, Cleary M, Sachidanandam R, McCombie WR, Elledge SJ, Hannon GJ. 2004. A resource for large-scale RNA-interference-based screens in mammals. Nature 428:427–431.

Pan WH, Clawson GA. 2004. Identification of efficient cleavage sites in long-target RNAs. Methods Mol Biol 252:125–144.

Pan WH, Devlin HF, Kelley C, Isom HC, Clawson GA. 2001. A selection system for identifying accessible sites in target RNAs. Rna 7:610–621.

Pan WH, Xin P, Bui V, Clawson GA. 2003. Rapid identification of efficient target cleavage sites using a hammerhead ribozyme library in an iterative manner. Mol Ther 7:129–139.

Pan WH, Xin P, Morrey JD, Clawson GA. 2004. A self-processing ribozyme cassette: Utility against human papillomavirus 11 E6/E7 mRNA and hepatitis B virus. Mol Ther 9:596–606.

Parry TJ, Cushman C, Gallegos AM, Agrawal AB, Richardson M, Andrews LE, Maloney L, Mokler VR, Wincott FE, Pavco PA. 1999. Bioactivity of anti-angiogenic ribozymes targeting Flt-1 and KDR mRNA. Nucleic Acids Res 27:2569–2577.

Patzel V, Steidl U, Kronenwett R, Haas R, Sczakiel G. 1999. A theoretical approach to select effective antisense oligodeoxyribonucleotides at high statistical probability. Nucleic Acids Res 27:4328–4334.

Peracchi A, Karpeisky A, Maloney L, Beigelman L, Herschlag D. 1998. A core folding model for catalysis by the hammerhead ribozyme accounts for its extraordinary sensitivity to abasic mutations. Biochemistry 37:14765–14775.

Pichon C, Freulon I, Midoux P, Mayer R, Monsigny M, Roche AC. 1997. Cytosolic and nuclear delivery of oligonucleotides mediated by an amphiphilic anionic peptide. Antisense Nucleic Acid Drug Dev 7:335–343.

Pieken WA, Olsen DB, Benseler F, Aurup H, Eckstein F. 1991. Kinetic characterization of ribonuclease-resistant 2′-modified hammerhead ribozymes. Science 253:314–317.

Pierce ML, Ruffner DE. 1998. Construction of a directed hammerhead ribozyme library: Towards the identification of optimal target sites for antisense-mediated gene inhibition. Nucleic Acids Res 26:5093–5101.

Pooga M, Langel U. 2001. Targeting of cancer-related proteins with PNA oligomers. Curr Cancer Drug Targets 1:231–239.

Probst JC. 2000. Antisense oligodeoxynucleotide and ribozyme design. Methods 22:271–281.

Puerta-Fernandez E, Romero-Lopez C, Barroso-delJesus A, Berzal-Herranz A. 2003. Ribozymes: Recent advances in the development of RNA tools. FEMS Microbiol Rev 27:75–97.

Qiu L, Moreira A, Kaplan G, Levitz R, Wang JY, Xu C, Drlica K. 1998. Degradation of hammerhead ribozymes by human ribonucleases. Mol Gen Genet 258:352–362.

Ramaswamy G, Slack FJ. 2002. siRNA. A guide for RNA silencing. Chem Biol 9:1053–1055.

Ren L, Schalles SL, Pan W, Isom CE, Loy' SE, Lee J, Benedict CM, Pickering MT, Norris JS, Clawson GA. 1999. Construction and deployment of triple ribozymes targeted to multicatalytic proteinase subunits C3 and C9. Gene Ther Mol Biol 3:257–269.

Reynolds T. 1992. First antisense drug trials planned in leukemia. J Natl Cancer Inst 84:288–290.

Roehr B. 1998. Fomivirsen approved for CMV retinitis. J Int Assoc Physicians AIDS Care 4:14–16.

Rowe PM. 1996. Ribozymes enter clinical trials for HIV-1 treatment. Lancet 348:1302.

Saetrom P, Snove O, Jr. 2004. A comparison of siRNA efficacy predictors. Biochem Biophys Res Commun 321:247–253.

Sandy P, Ventura A, Jacks T. 2005. Mammalian RNAi: A practical guide. Biotechniques 39:215–224.

Santiago FS, Lowe HC, Kavurma MM, Chesterman CN, Baker A, Atkins DG, Khachigian LM. 1999. New DNA enzyme targeting Egr-1 mRNA inhibits vascular smooth muscle proliferation and regrowth after injury. Nat Med 5:1264–1269.

Santoro SW, Joyce GF. 1997. A general purpose RNA-cleaving DNA enzyme. Proc Natl Acad Sci USA 94:4262–4266.

Santoro SW, Joyce GF. 1998. Mechanism and utility of an RNA-cleaving DNA enzyme. Biochemistry 37:13330–13342.

Scherr M, Rossi JJ, Sczakiel G, Patzel V. 2000. RNA accessibility prediction: A theoretical approach is consistent with experimental studies in cell extracts. Nucleic Acids Res 28:2455–2461.

Scherr M, Morgan MA, Eder M. 2003. Gene silencing mediated by small interfering RNAs in mammalian cells. Curr Med Chem 10:245–256.

Schwab G, Chavany C, Duroux I, Goubin G, Lebeau J, Helene C, Saison-Behmoaras T. 1994. Antisense oligonucleotides adsorbed to polyalkylcyanoacrylate nanoparticles specifically inhibit mutated Ha-ras-mediated cell proliferation and tumorigenicity in nude mice. Proc Natl Acad Sci USA 91:10460–10464.

Schwarz DS, Hutvagner G, Haley B, Zamore PD. 2002. Evidence that siRNAs function as guides, not primers, in

the *Drosophila* and human RNAi pathways. Mol Cell 10:537–548.
Sczakiel G. 2000. Theoretical and experimental approaches to design effective antisense oligonucleotides. Front Biosci 5:D194–D201.
Shi Y. 2003. Mammalian RNAi for the masses. Trends Genet 19:9–12.
Shimayama T, Nishikawa F, Nishikawa S, Taira K. 1993. Nuclease-resistant chimeric ribozymes containing deoxyribonucleotides and phosphorothioate linkages. Nucleic Acids Res 21:2605–2611.
Shoji Y, Akhtar S, Periasamy A, Herman B, Juliano RL. 1991. Mechanism of cellular uptake of modified oligodeoxynucleotides containing methylphosphonate linkages. Nucleic Acids Res 19:5543–5550.
Shuey DJ, McCallus DE, Giordano T. 2002. RNAi: Gene-silencing in therapeutic intervention. Drug Discov Today 7:1040–1046.
Shuttleworth J, Colman A. 1988. Antisense oligonucleotide-directed cleavage of mRNA in *Xenopus* oocytes and eggs. Embo J 7:427–434.
Sierakowska H, Sambade MJ, Agrawal S, Kole R. 1996. Repair of thalassemic human beta-globin mRNA in mammalian cells by antisense oligonucleotides. Proc Natl Acad Sci USA 93:12840–12844.
Sioud M, Leirdal M. 2000. Design of nuclease resistant protein kinase calpha DNA enzymes with potential therapeutic application. J Mol Biol 296:937–947.
Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR. 2003. Activation of the interferon system by short-interfering RNAs. Nat Cell Biol 5:834–839.
Sleutels F, Tjon G, Ludwig T, Barlow DP. 2003. Imprinted silencing of Slc22a2 and Slc22a3 does not need transcriptional overlap between Igf2r and Air. Embo J 22:3696–3704.
Sohail M, Hochegger H, Klotzbucher A, Guellec RL, Hunt T, Southern EM. 2001. Antisense oligonucleotides selected by hybridisation to scanning arrays are effective reagents in vivo. Nucleic Acids Res 29:2041–2051.
Sorensen DR, Leirdal M, Iversen PO, Sioud M. 2002. Combination of endostatin and a protein kinase Calpha DNA enzyme improves the survival of rats with malignant glioma. Neoplasia 4:474–479.
Stein CA, Tonkinson JL, Zhang LM, Yakubov L, Gervasoni J, Taub R, Rotenberg SA. 1993. Dynamics of the internalization of phosphodiester oligodeoxynucleotides in HL60 cells. Biochemistry 32:4855–4861.
Stein D, Foster E, Huang SB, Weller D, Summerton J. 1997. A specificity comparison of four antisense types: Morpholino, 2′-*O*-methyl RNA, DNA, and phosphorothioate DNA. Antisense Nucleic Acid Drug Dev 7:151–157.
Stein P, Svoboda P, Anger M, Schultz RM. 2003. RNAi: Mammalian oocytes do it without RNA-dependent RNA polymerase. Rna 9:187–192.
Stephenson ML, Zamecnik PC. 1978. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. Proc Natl Acad Sci USA 75:285–288.
Stewart AJ, Pichon C, Meunier L, Midoux P, Monsigny M, Roche AC. 1996. Enhanced biological activity of antisense oligonucleotides complexed with glycosylated poly-L-lysine. Mol Pharmacol 50:1487–1494.
Stull RA, Taylor LA, Szoka FC, Jr. 1992. Predicting antisense oligonucleotide inhibitory efficacy: A computational approach using histograms and thermodynamic indices. Nucleic Acids Res 20:3501–3508.
Summerton J. 1999. Morpholino antisense oligomers: The case for an RNase H-independent structural type. Biochim Biophys Acta 1489:141–158.
Sun LQ, Cairns MJ, Gerlach WL, Witherington C, Wang L, King A. 1999. Suppression of smooth muscle cell proliferation by a c-myc RNA-cleaving deoxyribozyme. J Biol Chem 274:17236–17241.
Symons RH. 1992. Small catalytic RNAs. Annu Rev Biochem 61:641–671.
Takagi M, Morita K, Nakai D, Nakagomi R, Tokui T, Koizumi M. 2004. Enhancement of the inhibitory activity of oatp antisense oligonucleotides by incorporation of 2′-*O*,4′-C-ethylene-bridged nucleic acids (ENA) without a loss of subtype selectivity. Biochemistry 43:4501–4510.
ten Asbroek AL, van Groenigen M, Nooij M, Baas F. 2002. The involvement of human ribonucleases H1 and H2 in the variation of response of cells to antisense phosphorothioate oligonucleotides. Eur J Biochem 269:583–592.
Thompson JD. 2002. Applications of antisense and siRNAs during preclinical drug development. Drug Discov Today 7:912–917.
Tong AW, Zhang YA, Nemunaitis J. 2005. Small interfering RNA for experimental cancer therapy. Curr Opin Mol Ther 7:114–124.
Tonkinson JL, Stein CA. 1994. Patterns of intracellular compartmentalization, trafficking and acidification of 5′-fluorescein labeled phosphodiester and phosphorothioate oligodeoxynucleotides in HL60 cells. Nucleic Acids Res 22:4268–4275.
Toyoda T, Imamura Y, Takaku H, Kashiwagi T, Hara K, Iwahashi J, Ohtsu Y, Tsumura N, Kato H, Hamada N. 2000. Inhibition of influenza virus replication in cultured cells by RNA-cleaving DNA enzyme. FEBS Lett 481:113–116.
Trempe JF, Wilds CJ, Denisov AY, Pon RT, Damha MJ, Gehring K. 2001. NMR solution structure of an oligonucleotide hairpin with a 2′F-ANA/RNA stem: Implications for RNase H specificity toward DNA/RNA hybrid duplexes. J Am Chem Soc 123:4896–4903.
Tu GC, Cao QN, Zhou F, Israel Y. 1998. Tetranucleotide GGGA motif in primary RNA transcripts. Novel target site for antisense design. J Biol Chem 273:25125–25131.
Urban E, Noe CR. 2003. Structural modifications of antisense oligonucleotides. Farmaco 58:243–258.
Usman N, Blatt LM. 2000. Nuclease-resistant synthetic ribozymes: Developing a new class of therapeutics. J Clin Invest 106:1197–1202.
Vaerman JL, Moureau P, Deldime F, Lewalle P, Lammineur C, Morschhauser F, Martiat P. 1997. Antisense oligodeoxyribonucleotides suppress hematologic cell growth through stepwise release of deoxyribonucleotides. Blood 90:331–339.
van der Laan A, Strömberg R, van Boom J, Kuyl-Yeheskiely E, Efimov V, Chakhmakhcheva O. 1996. An approach towards the synthesis of oligomers containing a *N*-2-hydroxyethyl-aminomethylphosphonate backbone: A novel PNA analogue. Tetrahedron Letters 37:7857–7860.
van Deutekom JC, Bremmer-Bout M, Janson AA, Ginjaar IB, Baas F, den Dunnen JT, van Ommen GJ. 2001. Antisense-induced exon skipping restores dystrophin

expression in DMD patient derived muscle cells. Hum Mol Genet 10:1547–1554.

Vester B, Wengel J. 2004. LNA (locked nucleic acid): High-affinity targeting of complementary RNA and DNA. Biochemistry 43:13233–13241.

Vester B, Lundberg LB, Sorensen MD, Babu BR, Douthwaite S, Wengel J. 2002. LNAzymes: Incorporation of LNA-type monomers into DNAzymes markedly increases RNA cleavage. J Am Chem Soc 124:13682–13683.

Vickers TA, Griffith MC, Ramasamy K, Risen LM, Freier SM. 1995. Inhibition of NF-kappa B specific transcriptional activation by PNA strand invasion. Nucleic Acids Res 23:3003–3008.

Vickers TA, Koo S, Bennett CF, Crooke ST, Dean NM, Baker BF. 2003. Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. A comparative analysis. J Biol Chem 278:7108–7118.

Vives E, Brodin P, Lebleu B. 1997. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem 272:16010–16017.

Vlassov VV, Balakireva LA, Yakubov LA. 1994. Transport of oligonucleotides across natural and model membranes. Biochim Biophys Acta 1197:95–108.

Vu TH, Chuyen NV, Li T, Hoffman AR. 2003. Loss of imprinting of IGF2 sense and antisense transcripts in Wilms' tumor. Cancer Res 63:1900–1905.

Wahlestedt C, Salmi P, Good L, Kela J, Johnsson T, Hokfelt T, Broberger C, Porreca F, Lai J, Ren K, Ossipov M, Koshkin A, Jakobsen N, Skouv J, Oerum H, Jacobsen MH, Wengel J. 2000. Potent and nontoxic antisense oligonucleotides containing locked nucleic acids. Proc Natl Acad Sci USA 97:5633–5638.

Wall NR, Shi Y. 2003. Small RNA: Can RNA interference be exploited for therapy? Lancet 362:1401–1403.

Wang Q, Contag CH, Ilves H, Johnston BH, Kaspar RL. 2005. Small hairpin RNAs efficiently inhibit hepatitis C IRES-mediated gene expression in human tissue culture cells and a mouse model. Mol Ther 12:562–568.

Warashina M, Kuwabara T, Nakamatsu Y, Taira K. 1999. Extremely high and specific activity of DNA enzymes in cells with a Philadelphia chromosome. Chem Biol 6:237–250.

Wassenegger M. 2005. The role of the RNAi machinery in heterochromatin formation. Cell 122:13–16.

Wickstrom E. 1986. Oligodeoxynucleotide stability in subcellular extracts and culture media. J Biochem Biophys Methods 13:97–102.

Wilds CJ, Damha MJ. 2000. 2′-Deoxy-2′-fluoro-beta-D-arabinonucleosides and oligonucleotides (2′F-ANA): Synthesis and physicochemical studies. Nucleic Acids Res 28:3625–3635.

Wu Y, Yu L, McMahon R, Rossi JJ, Forman SJ, Snyder DS. 1999. Inhibition of bcr-abl oncogene expression by novel deoxyribozymes (DNAzymes). Hum Gene Ther 10:2847–2857.

Wu H, Lima WF, Zhang H, Fan A, Sun H, Crooke ST. 2004. Determination of the role of the human RNase H1 in the pharmacology of DNA-like antisense drugs. J Biol Chem 279:17181–17189.

Xu Y, Zhang HY, Thormeyer D, Larsson O, Du Q, Elmen J, Wahlestedt C, Liang Z. 2003. Effective small interfering RNAs and phosphorothioate antisense DNAs have different preferences for target sites in the luciferase mRNAs. Biochem Biophys Res Commun 306:712–717.

Yagi M, Takeshima Y, Surono A, Takagi M, Koizumi M, Matsuo M. 2004. Chimeric RNA and 2′-*O*, 4′-C-ethylene-bridged nucleic acids have stronger activity than phosphorothioate oligodeoxynucleotides in induction of exon 19 skipping in dystrophin mRNA. Oligonucleotides 14:33–40.

Yakubov LA, Deeva EA, Zarytova VF, Ivanova EM, Ryte AS, Yurchenko LV, Vlassov VV. 1989. Mechanism of oligonucleotide uptake by cells: Involvement of specific receptors? Proc Natl Acad Sci USA 86:6454–6458.

Yi R, Qin Y, Macara IG, Cullen BR. 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 17:3011–3016.

Yu Q, Pecchia DB, Kingsley SL, Heckman JE, Burke JM. 1998. Cleavage of highly structured viral RNA molecules by combinatorial libraries of hairpin ribozymes. The most effective ribozymes are not predicted by substrate selection rules. J Biol Chem 273:23524–23533.

Zaborowska Z, Furste JP, Erdmann VA, Kurreck J. 2002. Sequence requirements in the catalytic core of the "10–23" DNA enzyme. J Biol Chem 277:40617–40622.

Zamaratski E, Pradeepkumar PI, Chattopadhyaya J. 2001. A critical survey of the structure-function of the antisense oligo/RNA heteroduplex as substrate for RNase H. J Biochem Biophys Methods 48:189–208.

Zamecnik PC, Stephenson ML. 1978. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. Proc Natl Acad Sci USA 75:280–284.

Zamore PD, Haley B. 2005. Ribo-gnome: The big world of small RNAs. Science 309:1519–1524.

Zender L, Kubicka S. 2004. SiRNA based strategies for inhibition of apoptotic pathways in vivo—Analytical and therapeutic implications. Apoptosis 9:51–54.

Zhang L, Gasper WJ, Stass SA, Ioffe OB, Davis MA, Mixson AJ. 2002. Angiogenic inhibition mediated by a DNAzyme that targets vascular endothelial growth factor receptor 2. Cancer Res 62:5463–5469.

Zobel HP, Kreuter J, Werner D, Noe CR, Kumel G, Zimmer A. 1997. Cationic polyhexylcyanoacrylate nanoparticles as carriers for antisense oligonucleotides. Antisense Nucleic Acid Drug Dev 7:483–493.

zu Putlitz J, Yu Q, Burke JM, Wands JR. 1999. Combinatorial screening and intracellular antiviral activity of hairpin ribozymes directed against hepatitis B virus. J Virol 73:5381–5387.