

## Designs and antiviral activity of gene-based drugs

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Nucleic acid-based drugs are a promising class of therapeutic agents that have important clinical applications for the prevention and treatment of viral diseases. This review highlights applications of four main classes of this technology that function primarily by gene suppression. Antisense oligonucleotides have a high level of specificity and affinity for target genes, and have demonstrated anti-gene activity *in vivo*. DNAzymes and Ribozymes are nucleic acid-based catalysts, which can cleave RNA transcripts in a highly sequence-specific manner. Finally, small interfering RNA, with its ability to harness the cells own gene-silencing apparatus, has demonstrated unprecedented anti-gene activity. This review will focus on the design, mode of action and efficacy of these technologies as antiviral agents from *in vitro* activity, to animal protection studies, and beyond to the clinic.

**Key words** antiviral therapeutic; gene; antisense; DNAzyme; ribozyme; siRNA

### 1. Introduction

In the past, the discovery of antiviral chemotherapeutics has been either a serendipitous affair, or the result of an arduous process whereby chemical libraries of hundreds of thousands of compounds are screened for target affinity and efficacy. Indeed, this is a slow and expensive process, and even until recently has produced relatively few therapeutic antiviral drugs. However, the advent of biotechnology and molecular genomics has seen the dawn of an era in which the nucleic acid sequence of small viral genomes can be exploited to target viruses in a highly specific manner. In this review, we outline several antiviral approaches that utilise nucleic acid-based therapeutic agents to block gene expression at the RNA level. Nucleic acids including antisense oligodeoxynucleotides (ODNs), ribonucleic enzymes (ribozymes), DNA enzymes (DNAzymes) and small interfering RNA (siRNA) have been designed and evaluated for their antiviral activities in tissue culture systems and in animal protection studies. These examples provide evidence that nucleic acid-based drugs have the potential to be used as an economical, expedient and powerful approach to antiviral therapy.

### 2. Antisense oligodeoxynucleotides (ODNs)

#### 2.1 Background

In 1978, Zamecnik and Stephenson used a 13-mer antisense ODN complementary to the Rous Sarcoma Virus (RSV) terminal sequences to inhibit RNA translation, virus replication and cell transformation *in vitro* [1, 2]. This provided the first evidence that antisense ODNs could act as antiviral agents. Now, almost 30 years later, and antisense ODNs are at the forefront of a new era in therapeutic innovation in the treatment of a variety of diseases, including those caused by viral infections.

Antisense ODNs are single-stranded, synthetic DNA oligonucleotides often 15-20 nucleotides in length, which hybridise specifically to complementary nucleic acid sequences via Watson-Crick base

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pairing. As such, they are designed in antisense conformation to a desired gene, so that the antisense ODN interacts with the mRNA of the target gene to inhibit its expression. Once bound, the antisense ODN can potentially function in a number of modalities, including passive inhibition of translation, reverse transcription, splicing, polyadenylation, 5'-capping and even nuclear export. More significantly however, the generation of a DNA/RNA heteroduplex renders the target RNA susceptible to endonucleolytic cleavage by RNase H.

## 2.2 Antisense ODNs as Antiviral Agents

Antisense ODNs are designed for antiviral activity by targeting hybridisation to an essential sequence such as the AUG start codon, or by screening a range of antisense sequences in a 'walk' of the viral genome. This can be achieved either in a comprehensive manner, or aided by using RNA structure algorithms to target regions predicted to lack significant secondary structure. In this manner, typically from 5 to 30 antisense ODNs are thus designed and tested for gene suppression using a range of criterion, including *in vitro* translation inhibition, plaque assays, cell viability or proliferation assays, marker gene activity from cells transformed with viral constructs, or by antiviral activity *in vivo* [3]. Sequence specificity, a hallmark of antisense drug design, is evaluated using a range of control oligonucleotides that may include random, sense, missense or scrambled orientation sequences [4]. Using this approach, a number of genes from viruses including the human immunodeficiency virus (HIV), cytomegalovirus (CMV), hepatitis B (HBV), hepatitis C (HCV) and influenza have been studied as potential targets for antiviral antisense activity (see reviews [3, 5]).

Antisense ODNs are the most comprehensively investigated form of gene-based therapy currently in the clinical setting. However for this development, investigators have had to address the inherent susceptibility of phosphodiester bonds to nuclease activity, as this can severely compromise the half-life of ODNs *in vivo*. This involved the development of a range of nuclease-resistant chemical modifications, typically to the phosphate backbone and the 2'-position on the ribose sugar moiety [6].

With the emergence of phosphorothioate (PS) antisense chemistry coinciding with the clinical awareness of HIV, a number of studies were initiated to explore the potential of this technology to target the *rev* and *tat* genes of the HIV-1 genome [7, 8]. While these treatments saw the sequence-specific inhibition of viral replication *in vitro*, a number of other non-sequence related effects were also observed, including the inhibition of virion binding to the surface of host cells [9]. Such findings saw the emergence of Hybridon's anti-HIV-1 drug, GEM91, which entered clinical trials in the early 1990's [10]. This 25-mer antisense PS ODN was designed to inhibit viral replication by targeting the *gag* gene of HIV-1, but despite promising results from Phase I clinical trials, GEM91 was discontinued due to dose-limiting toxicities and inconsistent responses apparent in Phase II of the trials [11]. From this, Hybridon developed GEM92, a "second-generation" mixed backbone (MBO) antisense ODN, which has now also been discontinued.

Further support for the use of antisense ODNs as therapeutics came when Vitravene (ISIS 2922), the first antisense drug to be approved by the United States FDA, was designed to treat AIDS-associated CMV-induced retinitis [12]. Vitravene is a 21-mer PS ODN complementary to the DNA polymerase gene, located in the major immediate-early region of the CMV genome [13]. However, the mechanism of Vitravene's action appears to be complex, including both sense and antisense effects, as well as inhibition of CMV adsorption to the host cells [14]. Pharmacokinetic studies showed detection of Vitravene in the vitreous humour and the retina after intravitreal injection in rabbits and monkeys. The accumulation of the ODN in these tissues was dose-dependent, and the parent drug was detectable for up to 7, but not 14 days after administration [15].

Vitravene is currently being used as a second-line treatment for CMV in cases where the patients are intolerant or unresponsive to previous treatments of first-line drugs such as Foscarnet, Ganciclovir or Cidofovir [16, 17]. However, it should be noted that Vitravene is not capable of resolving CMV-induced retinitis, but only preventing the progression of the ailment by suppressing viral replication [18]. Indeed, Vitravene represents a significant milestone in the development of antisense ODNs as an antiviral agent,

and monitoring its progress in a clinical environment will be important for the consideration of other antisense ODNs as potential antiviral drugs.

More recently, investigators have also been adopting a MBO approach, helping to reduce the inherent toxicity of the PS backbone without affecting antiviral activity, while also having the potential to enhance binding affinity to the target mRNA, as well as the pharmacokinetic and pharmacological profiles of the agent [19, 20]. One such modification includes the incorporation of 2'-*O*-methyl RNA bases into the antisense ODN. While this provides increased nuclease resistance and mRNA binding affinity, it is important to note that the centre-most 6-8 bases need to lack ribose modification in order to efficiently activate RNase H, and that the stability of the antisense ODN decreases as the number of modified bases increases [21]. Indeed, there are numerous publications in which 2'-*O*-methyl-modified antisense ODNs have been used to successfully inhibit the *in vitro* replication of viruses such as HIV-1 and Hepatitis C Virus [22]. While other MBO ODN modifications are prominent (see reviews [22, 5]), one modification that is potentially advantageous in the clinical setting is 2'-methoxy ethyl modification, which increases the affinity of the ODN for the target RNA, as well as increasing its capacity for serum albumin binding, subsequently increasing its *in vivo* distribution and pharmacokinetic properties [23].

Interestingly, ISIS 14803 (HepaSense), an 18-mer PS ODN targeted to the AUG start codon of HCV, was modified to contain five 5-methylcytidine nucleotides. This modification has been shown to enhance RNA binding affinity, and decrease the immunostimulatory effects of the CpG motifs in the sequence. However, despite initial reports of a reduction in patient's HCV titres [24], results from the Phase I clinical trial of ISIS 14803 were uninspiring, with a four-week treatment of the drug only effecting a reduction in plasma HCV RNA levels in three out of 28 patients [25]. As such, ISIS 14803 has been discontinued [26].

Another more recent MBO ODN becoming popular in antisense design incorporates Locked Nucleic Acids (LNAs). This modification consists of a ribose sugar moiety with a bridging methylene carbon between the 2' and 4' positions of the ribose ring. This conformationally-restricted oligonucleotide possesses enhanced hybridisation kinetics with the capacity to overcome RNA secondary structures, thereby enabling the antisense ODN to function more effectively at a greater number of potential target sites within the viral and human genomes. Indeed, LNAs have been shown to be one of the most potent and effective antisense inhibitors to date [27]. However, despite the improved sequence specificity, nuclease resistance and reduced toxicity, these chimeric DNA/LNA antisense ODNs must retain DNA in order to support RNase H activity. For example, a chimeric species complementary to the conserved dimerisation initiation site (DIS) of the HIV-1 genome has been shown to inhibit viral replication *in vitro* [27].

### 3. Catalytic DNA

#### 3.1 Background

As described above, the most common mechanism for ODN-based suppression is the induction of RNase H, which digests the RNA component of the heteroduplex, thereby leaving the DNA to bind to other target molecules. In an alternative strategy, catalytic DNA independent of RNase H activity has been utilised. Despite the lack of catalytic DNA in nature, an artificial evolution system known as *in vitro* selection has been utilised to produce DNA enzymes (DNAzymes) in the laboratory [28-31]. In an *in vitro* selection system, single stranded DNA free of its complementary strand is able to explore alternative structural forms, some of which are capable of catalytic activity, including site-specific RNA cleavage and ligation [32, 33].

The initial development of this technique saw the selection of DNAzymes capable of cleaving target RNA in the presence of various divalent cations [28]. After further development of this *in vitro* selection protocol, Santoro and Joyce evolved highly efficient magnesium-dependent DNAzymes capable of cleaving all RNA substrates in biologically relevant environments [30]. From this selection protocol, two prototypes were characterised and denoted the "8-17" and the "10-23" RNA-cleaving DNAzymes, both

containing a conserved catalytic domain flanked by two variable binding-domains that provide target specificity through Watson-Crick base pairing. Of significance is the flexibility of these molecules, in particular the 10-23 DNAzyme, which was found to have a minimum sequence requirement of a purine-pyrimidine pair at the site of cleavage, so long as flanking base-pairing was maintained [30].

The 10-23 DNAzyme obtains its name from its origin as the 23rd clone from the 10th cycle of *in vitro* selection [30]. This enzyme has a number of features which endow it with tremendous potential for applications both *in vitro* and *in vivo*. These include its ability to cleave almost any RNA sequence with high specificity and kinetic efficiency, with rates approaching and even exceeding those of other nucleic acid and protein endoribonucleases [30]. This activity is all the more remarkable when considering that it is achievable at concentrations of magnesium in the physiological range. Indeed, the catalytic motif of the 10-23 DNAzyme is highly evolved with very little sequence redundancy, as demonstrated by subsequent attempts to refine its catalytic activity.

In order to optimise the kinetic efficiency of 10-23 DNAzyme catalytic activity, the inverse relationship between the  $K_M$  (the concentration of substrate required for enzyme-mediated cleavage to proceed at half-maximal velocity) and the enzyme-substrate heteroduplex stability must be considered. From this, it can be reasoned that by increasing the length of the substrate-binding domain of the DNAzyme, the hybridisation free energy of the reaction can decrease to optimal levels, such that the increased heteroduplex stability causes a reduction in the  $K_M$  of the reaction. However, it is important to note that the benefit to the overall kinetic efficiency obtained by increasing the binding-domain length becomes limited at the point where increased enzyme-product stability affects catalytic turnover by reducing the rate at which the RNA product is released from the enzyme. Thus, the length of the flanking binding-domains needs to be optimised to a level that produces a favourable  $K_M$  without compromising the catalytic turnover (indicated by the  $K_{cat}$ ). This was demonstrated by an investigation in which DNAzyme substrate-binding domain lengths ranged between 4/4 (base length/arm) and 13/13, where the maximum overall efficiency ( $K_{cat}/K_M$ ) under physiological reaction conditions was observed with an arm length of between 8-9 bp [31]. In some instances, the rate of DNAzyme-catalysed cleavage can be enhanced by asymmetric arm length truncation [34], while additional factors such as the general GC content and specific pyrimidine content of the DNA should also be considered [35-37].

### 3.2 DNAzymes as Antiviral Agents

After being developed in 1997, the demonstration of *in vivo* DNAzyme function created excitement at the potential for these RNA-cleaving enzymes to be used as therapeutic agents [38]. However, the initial optimism surrounding DNAzymes started to dissipate as numerous investigators discovered that a large proportion of DNAzymes failed to cleave their full-length RNA targets efficiently *in vitro* [39]. By taking note of the lessons learned over the previous two decades in regard to antisense ODNs, researchers are now beginning to make significant advancements in the design of DNAzymes, and the prospect of DNAzymes as therapeutic agents is becoming more and more realistic. Indeed, many research groups are currently focusing on the prospect of DNAzymes as therapeutic agents in cancer and cardiovascular disease, and with numerous studies demonstrating antiviral activity against viruses such as HIV-1, HBV, HCV, and influenza A virus, the potential for these agents as antiviral therapeutics is starting to emerge (see reviews [40, 5]).

With the enormous capacity to cleave different sequences, the specificity of DNAzymes was one of the first concerns to arise. Due to their impressive catalytic properties, any off-target cleavage could be potentially disastrous. However, the actual substrate specificity of an individual DNAzyme with defined RNA-binding domains appears to be very high, with one investigation showing that any mismatch with the substrate is detrimental to catalytic activity [31]. In our laboratory, we also examined the specificity of 10-23 DNAzymes targeted towards sequences in a relatively conserved segment of the L1 gene from the human papilloma virus (HPV) [41, 42]. In this experiment, we observed the ability of 10-23 DNAzymes to discriminate between sequences that differ by as little as a single nucleotide polymorphism. It should be noted that some mismatches can be tolerated to an extent, particularly those

that produce a “wobble” pair [31, 41]. If however, the difference between the target and non-target substrate lies at the cleavage site, such that the purine-pyrimidine (R-Y) becomes R-R, Y-Y or Y-R, then the DNAzyme would have no activity on the non-target substrate.

As with antisense ODNs, DNAzymes must compete with their targets own prevailing intramolecular base pairing, which can form stable secondary structures. However, an additional requirement for DNAzyme function is the formation of an intermolecular structure that facilitates correct conformation of the DNAzyme’s catalytic core over the purine-pyrimidine cleavage site. So, while most mRNA substrates provide many opportunities for DNAzyme cleavage, finding the sites which are amenable to efficient hybridisation and cleavage is usually a difficult and time consuming task, and involves the empirical testing of many DNAzymes in long folded RNA transcripts *in vitro*. In order to streamline this process, we developed a multiplex approach to target site selection, which allowed the simultaneous analysis of many different DNAzyme cleavage sites in a single reaction [39, 43]. This analysis demonstrated for the first time, that there was a relationship between the *in vitro* cleavage efficiency and the subsequent gene suppression potential of a DNAzyme in cell culture. Indeed, various research groups have since utilised this approach for fast and efficient DNAzyme selection. In order to further increase the flexibility of the 10-23 DNAzyme in constrained target sequences, we also examined nucleotide modifications capable of enhancing the activity of the DNAzyme against various target sites. In this investigation, we demonstrated a hierarchy of natural dinucleotide target reactivity, where  $GU=AU \geq GC \gg AC$ , and utilised nucleotide modification to produce a 200-fold rate enhancement of DNAzyme cleavage of AC target sites under single turnover conditions [44].

However, for any of these approaches to be of value in biological systems, the DNAzyme must avoid nuclease digestion long enough to effect target mRNA cleavage. As a result, as is the case for antisense ODNs, investigators have experimented with modifications to the phosphate backbone, ribose sugar moiety, or to the oligonucleotide structure to endow DNAzymes with properties described previously for antisense ODNs. In one study, modification of the substrate-binding domains with either a 3'-3'-inverted thymidine, PS linkages, 2'-O-methyl RNA, or LNAs, showed both an increase in nuclease resistance and catalytic activity of a DNAzyme complementary to the 5' untranslated region of human rhinovirus 14 [45]. Similarly, we synthesised a DNAzyme to target the HPV 16 E6 gene, and by incorporating a single 3'-terminal nucleotide inversion into the oligonucleotide, the half-life of the DNAzyme was increased from two to 24 hours (unpublished observations). Such a modification has also been shown to increase the efficiency of DNAzyme-mediated gene suppression *in vitro* [46].

The incorporation of LNAs into the substrate-binding arms of DNAzymes is a very exciting prospect [45]. Indeed, this modification expands the potential of DNAzymes to cleave structured RNA targets [47]. This therefore proves beneficial in situations where there is only a small window of opportunity for the possibility of targeting highly conserved segments within and between the RNA genome of multiple pathogenic strains. In such a way, sequences previously refractory to cleavage have been efficiently targeted by DNAzymes [48]. In one study, LNA-modified DNAzymes targeted to the 5' non-coding region (NCR) of HIV-1 were used to block reverse transcription and dimerisation of the HIV-1 RNA template, thereby suppressing HIV-1 replication in cell culture [49]. Such findings are corroborated throughout the literature, and when one considers that LNAs are the most potent and effective antisense inhibitors to date (rivalling that of siRNA silencing), along with their non-toxic profile, there is certainly a strong potential for clinical applications.

## 4. Catalytic RNA

### 4.1 Background

Since the realisation in the early 1980's that RNA molecules possess the potential for catalytic activity, various naturally existing catalytic RNAs have been characterised, including the RNA subunit of RNase P, group I and group II introns, the hepatitis delta virus RNA enzyme (ribozyme), along with the hammerhead and hairpin ribozyme motifs. These catalytic RNA molecules possess, at the very least,

enzymatic cleavage and ligation activities, with the mechanism of catalysis being dependant upon the architecture of the specific ribozyme [50-53]. However, all cleavage reactions follow the same fundamental process – when the ribozyme hybridises to a complementary RNA sequence via Watson-Crick base pairing, site-specific cleavage of the substrate occurs, and the ribozyme dissociates from the RNA cleavage products leaving the catalytic RNA free to bind and cleave other complementary sequences.

Although various ribozymes have been investigated for their therapeutic potential, the hammerhead and hairpin ribozymes, which were initially discovered in plant viroid and virusoids as *cis*-cleaving catalytic motifs, have received much attention due to their inherent simplicity, relatively small size, and the potential to incorporate a variety of flanking motifs without altering their site-specific cleavage capacities. Theoretically, the *trans*-cleaving ribozymes can be designed to cleave any RNA species in a sequence-specific manner, such that the mRNA for any protein associated with a disease can be selectively cleaved. However, it should be noted that while the hammerhead ribozyme cleaves target RNA at a rate 100-fold greater than the ligation reaction, for the hairpin ribozyme, there is much more competition between the cleavage and ligation reactions [54]. As such, being the most extensively characterised ribozyme, the hammerhead ribozyme currently holds much of the hope for the use of ribozyme technology in the inhibition of virus replication, modulation of tumour progression and analysis of cellular gene function.

The hammerhead ribozyme consists of three basic components: (1) A highly conserved 22-nucleotide catalytic domain; (2) substrate-binding sequences that flank the susceptible 3',5' phosphodiester bond; and (3) a recognition sequence on the target RNA. The cleavage reaction occurs 3' to this recognition sequence, and requires the presence of divalent metal ions, such as magnesium, which assists RNA folding and participates directly in the cleavage mechanism [55, 56]. This recognition sequence can be represented as a 5'-NHH-3' triplet, where N is any nucleotide, and H can be any nucleotide except G [57]. Comparative studies have also shown the reaction rate ( $K_{cat}$ ) to decrease in the following order: AUC, GUC>GUA, AUA, CUC>AUU, UUC, UUA>GUU, CUA>UUU, CUU [58].

As previously discussed for DNAzymes, the substrate-binding domains of the ribozyme can significantly affect their kinetic properties. However, in comparison to DNAzymes, ribozymes are generally less stable, more susceptible to off-target catalysis, and the overall kinetic efficiency of cleavage is generally slower [59]. Still, the principles of hammerhead ribozyme design reflect those mentioned above for the 10-23 DNAzyme. As such, there must be a compromise with substrate-binding domain length such that catalysis proceeds efficiently without being too detrimental to catalytic turnover [60]. However, due to increased intermolecular stability, an additional consideration for ribozymes is that if the binding-domain length is too long, mismatch base pairing may be tolerated, resulting in off-target effects. In order to combat this, asymmetric arm length truncation has been used, with the 3' arm of the ribozyme being more critical for specificity than that of the 5' arm [61]. Indeed, ribozyme activity is closely related to the arm length in both symmetric and asymmetric models, and this is somewhat dependant on the sequence context [62-65].

#### 4.2 Ribozymes as Antiviral Agents

Since their discovery in the early 1980's, researchers have been investigating the therapeutic potential of ribozymes, with much of the focus surrounding the treatment of cancer, as well as viral infections such as HIV, HBV, HCV, and influenza (see reviews [53, 40]). However, it soon became apparent that the susceptibility of ribozymes to nuclease activity constituted a significant problem, with RNA hydrolysis occurring in minutes or even seconds in the presence of cellular extracts or blood serum [66]. In order to protect the hammerhead ribozyme from nuclease degradation, researchers have adopted the MBO approach that was developed for antisense ODNs. To date, perhaps the most prevalent MBO ribozyme design incorporates a 3'-inverted thymidine, a 2'-C-allyl modification at nucleotide U4, which itself is surrounded by five unmodified ribonucleotides, while the remainder of the nucleotides are 2'-O-methyl-

modified [22]. Such modifications have maintained the catalytic integrity while increasing the *in vivo* half-life of the ribozyme from a few minutes to a few days [67].

Synthetic ribozymes, like DNAzymes and antisense ODNs, have a very low capacity to traverse cellular membranes, and as such their therapeutic application is more reliant on localised delivery to the targeted area [40]. One distinct advantage of an RNA-based agent however, is that they can be stably expressed from a transgene construct. Constitutive or inducible expression, achieved under the transcriptional regulation of a suitable promoter, overrides the issue of nuclease-sensitivity and facilitates the treatment of chronic diseases without the pharmacological drawbacks of continuous oligonucleotide delivery. The expression construct approach therefore significantly increases the therapeutic potential of ribozymes, and has seen the progression of HIV-1-targeting ribozymes to clinical trials. The rationale proposes that if a ribozyme construct can protect CD4<sup>+</sup> T cells from HIV-1 infection and its sequelae in patients, then the decline in the numbers of CD4<sup>+</sup> T cells could be halted or even reversed to some extent, meaning a potential clinical benefit for HIV-infected individuals.

The potential of ribozymes as antiviral therapeutics was given a large boost with promising results arising from two independent Phase I clinical trials. In both of these trials, a retroviral LNL6 vector (a MoMLV-based expression vector) was used to express a ribozyme (Rz2) targeted to the *tat* gene of HIV-1. In the first trial, which focussed on identical twins discordant for HIV infection, healthy CD4<sup>+</sup> lymphocytes from the uninfected twin were transduced with the anti-HIV ribozyme construct, before being expanded *ex vivo* and transfused into the bloodstream of the corresponding HIV-positive twin [68]. In doing so, HIV-1 infection/replication was inhibited, and HIV-1 induced pathogenicity of both T-lymphocyte cell lines and normal peripheral blood T-lymphocytes was reduced [69]. In the second trial, CD34<sup>+</sup> hematopoietic progenitor cells were removed from HIV-positive individuals and transduced with the Rz2/LNL6 construct before being reintroduced back into the individual [70]. Indeed, this resulted in the differentiation of multiple hematopoietic lineages which express the anti-HIV ribozyme. Furthermore, this production of *de novo* anti-HIV T-lymphocytes demonstrated for the first time, the long-term maintenance of a potential therapeutic transgene against HIV.

The incorporation of ribozymes into plasmid constructs also enables the use of tissue specific promoters and subcellular localisation signals that direct ribozyme expression to specific tissues and even specific compartments of the cell [71, 72]. Similarly, “allosteric ribozymes” have been evolved *in vitro* to respond to a second drug, and thus aid in the localisation of ribozyme function to a specific tissue [73, 74].

Like other antisense-based methods of gene suppression, ribozymes must also overcome the secondary and tertiary structures of their target RNA molecules. In fact, some researchers believe that the primary reason for ribozyme failure is due to an inability to overcome the intramolecular stability of their targets and access the RNA cleavage site [5]. In order to identify accessible sites, RNA secondary-structure folding programs have been utilised. However, these predictions are often thwarted in long RNA transcripts. This has seen the development of S1 nuclease and RNase mapping, in which the ability of other nucleases to access target sites is used as a guide to predict hammerhead ribozyme accessibility [75, 76]. The targeting of sites that are generally exposed for potential RNA-RNA or RNA-protein interactions, such as the 5' leader region, *gag* and *tat* genes of the HIV-1 genome, has also aided in the identification of accessible sites [77-81]. In an elegant experiment, the hammerhead domain of a ribozyme was even fused with a helicase-recruiting RNA motif, such that helicase recruitment of the ribozyme to the target site significantly increased target accessibility *in vivo* [82].

While *in vitro* screening provides the simplest and most direct approach for identifying accessible sites, there is not always a correlation between *in vitro* and *in vivo* results. To overcome such problems, various combinatorial approaches have been described. In one experiment, a library of ribozyme genes with random sequences of 13 nucleotides on both sides of the hammerhead were generated. Using RACE technique, cleavage sites were successfully identified, and the ribozyme genes were subsequently re-amplified and cloned [83]. In our laboratory, the activity of a similar random ribozyme library was analysed directly by ligation-mediated PCR [84]. The ribozymes selected in this process were found to have significantly more activity than those selected at random.

## 5.Small interfering RNA (siRNA)

### 5.1Background

The gene-based inhibitors discussed thus far all function by counteracting the natural gene expression pathway. However, recent times have seen the emergence of a very powerful and specific gene silencing approach, in which double-stranded RNA (dsRNA) exploits the natural ability of cells to silence gene expression through a mechanism known as RNA interference (RNAi). This highly conserved, but until recently unidentified system for post-transcriptional gene regulation, is thought to be part of an ancient defence mechanism to protect cells against unwanted proliferation of opportunistic nucleic acids that may occur, for example, during transposition or viral infection.

Since the discovery in 1998 that dsRNA is recognised by cells as a signal for potent and specific post-transcriptional gene silencing (PTGS), there has been a flurry of research activity in order to identify and characterise the host factors and biochemical pathways of this mechanism. Indeed, while the complexities of RNAi are still being uncovered today (see reviews [85, 86]), the fundamental junctures are well understood. Within the nucleus, long dsRNA transcripts are cleaved by the RNase III endonuclease Droscha. The shortened RNA duplexes are then actively exported from the nucleus to the cytoplasm, where Dicer, another RNase III endonuclease, processes the dsRNA into small (~21-mer) effector molecules known as small interfering RNA (siRNA). The siRNA molecules are then incorporated into a multiprotein effector complex known as the RNA-induced silencing complex (RISC). This RISC selectively retains one strand of the siRNA (the other is discarded), which acts to guide the RISC to complementary RNA sequences. If the complementarity between the siRNA and the target RNA is perfect, the RISC mediates cleavage of the target RNA. However, the RISC can also be guided to sites of imperfect complementarity, causing a partial suppression of gene expression primarily through the inhibition of translation. This effect is more typical of the gene repression induced by animal microRNAs (see reviews [85, 87]), and is thought to be the main cause of “off-target” activity in the application of siRNAs [88].

Mammalian cells also utilise additional mechanisms to prevent the unwanted proliferation of opportunistic nucleic acids. This protection is provided by a highly developed immune response, in which the presence of dsRNA leads to the induction of apoptosis through a variety of mechanisms. This therefore precludes the application of full-length dsRNA for gene silencing in most mammalian cells. However, dsRNA molecules shorter than 30 base pairs will, in most cases, evade detection by this cellular surveillance network. Importantly, these small inhibitory RNAs were themselves shown to be sufficient to elicit the gene silencing effect [89]. This revelation paved the way for siRNAs to be used not only as an experimental tool, but also as a therapeutic agent.

When designing siRNAs, it is important to consider a few issues. From early experiments with synthetic siRNAs, it has been demonstrated that siRNA function depends upon the presence of 3' dinucleotide overhangs at each end of the duplex [88]. This is a characteristic of RNase III-mediated cleavage, and while the sequence of these overhanging bases does not appear to have any impact on gene silencing, it is interesting to note that siRNAs generated from endoribonuclease III cleavage (esiRNAs) perform better than synthetic siRNAs. Indeed, an siRNA targeted towards the polymerase gene of HBV was shown to reduce HBsAg gene expression by 33%, while its expressed counterpart reduced the genes expression by 90% [90]. Another important design consideration involves the introduction of strand uptake bias to ensure the appropriate RNA strand enters the RISC [91]. Normally, strand asymmetry is attributed to a RISC-associated helicase, which preferentially unwinds the siRNA duplex from the end with least resistance in terms of inter-strand hydrogen bonding [92]. The strand with the lowest relative hybridisation strength at the 5' end is retained by the RISC (guide strand), while the passenger strand is discarded and eliminated. In order to promote the loading bias of the desired guide strand into the RISC, subtle sequence alterations can be introduced to the 3' end of the passenger strand, such that the 5' terminus of the guide strand becomes relatively destabilised, and hence preferentially loaded into the

RISC [93, 94]. These modifications to the siRNA duplex both maximise functionality and minimise off-target effects.

## 5.2 siRNAs as Antiviral Agents

Synthetic siRNA are an attractive nucleic acid-based technology platform for rapid antiviral drug development, and a number of laboratories wasted no time in establishing these credentials with a host of proof-of-concept studies (see reviews [95, 5]). These produced promising results, demonstrating both suppression of viral gene expression and replication *in vitro*. HIV in particular has been a focal point for this development, where synthetic siRNAs targeting the long terminal repeats (LTRs) and accessory protein genes *vif* and *nef* were found to be capable of inhibiting HIV-1 infection with a 30-50 fold reduction in virus production [96]. Similarly, cells transfected with siRNAs specific for the polio virus genome were found to produce very low titres of the virus post infection compared with untreated controls [97]. siRNAs against influenza A virus nuclear protein (NP) and polymerase (PA) RNA sequences were found to suppress mRNA, cRNA and vRNA levels in both cell culture and chicken embryos [98]. These siRNAs were also found to be protective against various strains of the virus, including the highly pathogenic Avian N1 and N7 in mouse challenge assays [99].

However, for the therapeutic potential of siRNAs to be realised, the capacity of these molecules to function as antiviral agents *in vivo* must also be established. This question brings the field back to the issue of susceptibility of RNA to nucleases. Fortunately, some of the lessons learned from antisense ODN, DNAzyme, and in particular ribozyme technology, have expedited the application of stabilised siRNA molecules *in vivo*. A leading example of this technology has been used to fortify siRNA used to target HBV in a mouse model. These siRNA molecules were heavily modified, such that all but three 2'OH groups on the guide strand were replaced by either 2'fluorine, 2'O-methyl or 2'deoxy moieties. Systemic delivery of these molecules by daily intravenous injection was found to suppress HBV replication [100]. However, while daily dosing of these unformulated molecules was required for this effect, a fusogenic liposome or stable nucleic-acid-lipid particle formulation was subsequently found to achieve the same level of potency with a weekly injection regimen [101]. In addition to an enhanced pharmacokinetic profile, the lipid formulation also displayed reduced immunostimulatory activity.

In addition to the direct delivery mode, siRNAs can also be expressed from a transgene. Indeed, expression of small RNA – as with the ribozyme – can now be achieved in a huge range of formats from a variety of vector and promoter systems. In the most established approach, small RNA hairpin-encoding genes are expressed under the control of a pol III promoter such as the H1 or U6 [102, 103]. As with endogenous siRNA production, the hairpin RNA product is then processed by Dicer, which converts it to a functional siRNA. These short hairpins (<30bp) are usually small enough to evade the activation of protein kinase R (PKR) and the interferon pathway. In an alternative approach, siRNA can be expressed from a convergent promoter system consisting of two different opposing pol III promoters, which generate both the antisense and passenger strands of the siRNA from opposite strands of the same short template [104]. Long RNA hairpins encoding multiple siRNAs have also been expressed from a vector, provided that the passenger-strand encoding segment contains mismatches with its corresponding antisense at regular intervals (4-8 bp) [105]. Such an approach has been used to target the *tat*, *rev* and *nef* genes of HIV-1, resulting in the successful inhibition of HIV replication [106].

One of the advantages of using an expression system is the ability to achieve stable expression of multiple siRNA molecules capable of targeting multiple conserved sites simultaneously. This is particularly important against viral pathogens such as HIV, which are highly proficient at generating escape mutants. The advantage of a combinatorial approach already has some support from a study using a triple combination of a short-hairpin RNA targeting *tat/rev*, a nucleolar localising Tat decoy, and a CCR5 chemokine receptor-targeting ribozyme, which was found to inhibit HIV-1 replication in primary haematopoietic cells [107].

With gene silencing typically observed at concentrations well below those required for antisense reagents, it is not surprising that this novel gene-silencing platform has exploded onto the scene with an

unprecedented passage from preclinical studies to clinical trial. It is likely to only be a matter of time before we see the delivery of anti-HIV siRNAs into lymphocytes using the technique discussed previously for the anti-HIV ribozyme in clinical trials. Alternatively, lymphocytes collected via aphaeresis could be transduced *ex vivo* with the use of a retroviral or lentiviral vector [108]. Sirna Therapeutics, Inc also have an anti-HBV siRNA in preclinical development, as well as an anti-HCV siRNA (siRNA-034) that is currently being manufactured for Phase I clinical trials. Alnylam Pharmaceuticals, Inc have already demonstrated safety in Phase I clinical trials of its intranasally delivered anti-RSV siRNA (ALN-RSV01), and are about to initiate Phase II trials with an inhaled formulation of the compound. However, it is important to consider that if the endogenous RNAi pathway becomes saturated with these therapeutic agents, they may inadvertently be compromising the function of other clients of this pathway, such as the microRNA family.

## 6. Conclusion

Nucleic acid-based therapeutic drugs represent a promising new class of antiviral agents. They offer significant therapeutic advantages over conventional antiviral drugs due to their inherent versatility and specificity. This review has highlighted the key principles behind the utilisation of these nucleic acid-based inhibitors, along with the range of applications for which oligonucleotides are seeking employment in antiviral therapy. Antisense ODNs, DNazymes, ribozymes and siRNA represent four main classes of nucleic acid-based antiviral agents being explored for future clinical development. However, in order for these agents to expand into the clinical setting, a number of key issues need to be addressed. Target-site selection for all of these technologies remains an important consideration, although a number of strategies now exist for finding potent congeners. Biological stability also remains a challenge despite remarkable advances in nucleic acid chemistry, which has led to the development of second and third generation oligonucleotide chemistry that offers both greater stability and fewer side effects. However, without doubt the greatest challenge facing all of these technologies is delivery and transport into intracellular sites of infection. While formulation improvements are making a tremendous impact, such as liposomes that can protect oligonucleotides from nuclease degradation and assist with target localisation, there is still plenty of development needed for these technologies to reach their targets efficiently. With the intense research focusing on these issues, it may only be a matter of time before nucleic acid-based drugs reach their full potential, and become a significant force in the treatment of viral infections.

## References

- [1] P. C. Zamecnik and M. L. Stephenson, Proc Natl Acad Sci U S A **75**, 1 (1978)
- [2] M. L. Stephenson and P. C. Zamecnik, Proc Natl Acad Sci U S A **75**, 1 (1978)
- [3] A. Alama, F. Barbieri, M. Cagnoli and G. Schettini, Pharmacol Res **36**, 3 (1997)
- [4] C. A. Stein, Antisense Nucleic Acid Drug Dev **8**, 2 (1998)
- [5] A. Tafach, T. Bassett, D. Sparanese and C. H. Lee, Curr Med Chem **13**, 8 (2006)
- [6] R. L. Juliano, S. Alahari, H. Yoo, R. Kole and M. Cho, Pharm Res **16**, 4 (1999)
- [7] M. Matsukura, G. Zon, K. Shinozuka, M. Robert-Guroff, T. Shimada, C. A. Stein, H. Mitsuya, F. Wong-Staal, J. S. Cohen and S. Broder, Proc Natl Acad Sci U S A **86**, 11 (1989)
- [8] J. Lisziewicz, D. Sun, V. Metelev, P. Zamecnik, R. C. Gallo and S. Agrawal, Proc Natl Acad Sci U S A **90**, 9 (1993)
- [9] K. Yamaguchi, B. Papp, D. Zhang, A. N. Ali, S. Agrawal and R. A. Byrn, AIDS Res Hum Retroviruses **13**, 7 (1997)
- [10] S. Agrawal, Trends Biotechnol **14**, 10 (1996)
- [11] Hybridon News release, July 25, (1997)
- [12] W. G. Nichols and M. Boeckh, J Clin Virol **16**, 1 (2000)
- [13] R. F. Azad, V. B. Driver, K. Tanaka, R. M. Croke and K. P. Anderson, Antimicrob Agents Chemother **37**, 9 (1993)
- [14] K. P. Anderson, M. C. Fox, V. Brown-Driver, M. J. Martin and R. F. Azad, Antimicrob Agents Chemother **40**, 9 (1996)

- [15] J. M. Leeds, S. P. Henry, S. Bistner, S. Scherrill, K. Williams and A. A. Levin, *Drug Metab Dispos* **26**, 7 (1998)
- [16] S. T. Croke, *Antisense Nucleic Acid Drug Dev* **8**, 2 (1998)
- [17] C. M. Perry and J. A. Balfour, *Drugs* **57**, 3 (1999)
- [18] E. De Clercq, *J Clin Virol* **30**, 2 (2004)
- [19] A. A. Levin, *Biochim Biophys Acta* **1489**, 1 (1999)
- [20] S. Agrawal, Z. Jiang, Q. Zhao, D. Shaw, Q. Cai, A. Roskey, L. Channavajjala, C. Saxinger, R. Zhang, L. S. Channavajjala, A. Eidsath and W. C. Saxinger, *Proc Natl Acad Sci U S A* **94**, 6 (1997)
- [21] E. Zamaratski, P. I. Pradeepkumar and J. Chattopadhyaya, *J Biochem Biophys Methods* **48**, 3 (2001)
- [22] T. L. Jason, J. Koropatnick and R. W. Berg, *Toxicol Appl Pharmacol* **201**, 1 (2004)
- [23] T. P. Prakash, M. Manoharan, A. M. Kawasaki, A. S. Fraser, E. A. Lesnik, N. Sioufi, J. M. Leeds, M. Teplova and M. Egli, *Biochemistry* **41**, 39 (2002)
- [24] ISIS News release Jun 18, (2001)
- [25] J. G. McHutchison, K. Patel, P. Pockros, L. Nyberg, S. Pianko, R. Z. Yu, F. A. Dorr and T. J. Kwok, *J Hepatol* **44**, 1 (2006)
- [26] N. Georgopadakou, *Expert Opin Investig Drugs* **16**, 1 (2007)
- [27] J. Elmen, H. Y. Zhang, B. Zuber, K. Ljungberg, B. Wahren, C. Wahlestedt and Z. Liang, *FEBS Lett* **578**, 3 (2004)
- [28] R. R. Breaker and G. F. Joyce, *Chem Biol* **1**, 4 (1994)
- [29] R. R. Breaker and G. F. Joyce, *Chem Biol* **2**, 10 (1995)
- [30] S. W. Santoro and G. F. Joyce, *Proc Natl Acad Sci U S A* **94**, 9 (1997)
- [31] S. W. Santoro and G. F. Joyce, *Biochemistry* **37**, 38 (1998)
- [32] R. R. Breaker, *Nat Biotechnol* **15**, 5 (1997)
- [33] Y. Li and R. R. Breaker, *Curr Opin Struct Biol* **9**, 3 (1999)
- [34] M. J. Cairns, T. M. Hopkins, C. Witherington and L. Q. Sun, *Antisense Nucleic Acid Drug Dev* **10**, 5 (2000)
- [35] N. Sugimoto, S. Nakano, M. Katoh, A. Matsumura, H. Nakamuta, T. Ohmichi, M. Yoneyama and M. Sasaki, *Biochemistry* **34**, 35 (1995)
- [36] L. Ratmeyer, R. Vinayak, Y. Y. Zhong, G. Zon and W. D. Wilson, *Biochemistry* **33**, 17 (1994)
- [37] J. I. Gyi, A. N. Lane, G. L. Conn and T. Brown, *Biochemistry* **37**, 1 (1998)
- [38] F. S. Santiago, H. C. Lowe, M. M. Kavurma, C. N. Chesterman, A. Baker, D. G. Atkins and L. M. Khachigian, *Nat Med* **5**, 11 (1999)
- [39] M. J. Cairns, T. M. Hopkins, C. Witherington, L. Wang and L. Q. Sun, *Nat Biotechnol* **17**, 5 (1999)
- [40] A. Peracchi, *Rev Med Virol* **14**, 1 (2004)
- [41] M. J. Cairns, A. King and L. Q. Sun, *Nucleic Acids Res* **28**, 3 (2000)
- [42] M. J. Cairns and L. Q. Sun, *Methods Mol Biol* **252**, (2004)
- [43] M. J. Cairns and L. Q. Sun, *Methods Mol Biol* **252**, (2004)
- [44] M. J. Cairns, A. King and L. Q. Sun, *Nucleic Acids Res* **31**, 11 (2003)
- [45] S. Schubert, D. C. Gul, H. P. Grunert, H. Zeichhardt, V. A. Erdmann and J. Kurreck, *Nucleic Acids Res* **31**, 20 (2003)
- [46] C. R. Dass, E. G. Saravolac, Y. Li and L. Q. Sun, *Antisense Nucleic Acid Drug Dev* **12**, 5 (2002)
- [47] B. Vester, L. H. Hansen, L. B. Lundberg, B. R. Babu, M. D. Sorensen, J. Wengel and S. Douthwaite, *BMC Mol Biol* **7**, (2006)
- [48] S. Schubert, J. P. Furste, D. Werk, H. P. Grunert, H. Zeichhardt, V. A. Erdmann and J. Kurreck, *J Mol Biol* **339**, 2 (2004)
- [49] M. R. Jakobsen, J. Haasnoot, J. Wengel, B. Berkhout and J. Kjemis, *Retrovirology* **4**, (2007)
- [50] J. Haseloff and W. L. Gerlach, *Nature* **334**, 6183 (1988)
- [51] A. Hampel and R. Tritz, *Biochemistry* **28**, 12 (1989)
- [52] J. J. Rossi, *Curr Opin Biotechnol* **3**, 1 (1992)
- [53] A. U. Khan and S. K. Lal, *J Biomed Sci* **10**, 5 (2003)
- [54] L. A. Hegg and M. J. Fedor, *Biochemistry* **34**, 48 (1995)
- [55] K. F. Blount and O. C. Uhlenbeck, *Annu Rev Biophys Biomol Struct* **34**, (2005)
- [56] S. C. Dahm and O. C. Uhlenbeck, *Biochemistry* **30**, 39 (1991)
- [57] A. R. Kore, N. K. Vaish, U. Kutzke and F. Eckstein, *Nucleic Acids Res* **26**, 18 (1998)
- [58] T. Shimayama, S. Nishikawa and K. Taira, *Biochemistry* **34**, 11 (1995)
- [59] T. K. Stage-Zimmermann and O. C. Uhlenbeck, *Rna* **4**, 8 (1998)
- [60] K. J. Hertel, D. Herschlag and O. C. Uhlenbeck, *Biochemistry* **33**, 11 (1994)
- [61] K. J. Hertel, D. Herschlag and O. C. Uhlenbeck, *EMBO J* **15**, 14 (1996)

- [62] O. Heidenreich, F. Benseler, A. Fahrenholz and F. Eckstein, *J Biol Chem* **269**, 3 (1994)
- [63] M. Scherr, M. Grez, A. Ganser and J. W. Engels, *J Biol Chem* **272**, 22 (1997)
- [64] M. Sioud, *Nucleic Acids Res* **25**, 2 (1997)
- [65] P. Crisell, S. Thompson and W. James, *Nucleic Acids Res* **21**, 22 (1993)
- [66] L. Qiu, A. Moreira, G. Kaplan, R. Levitz, J. Y. Wang, C. Xu and K. Drlica, *Mol Gen Genet* **258**, 4 (1998)
- [67] C. M. Flory, P. A. Pavco, T. C. Jarvis, M. E. Lesch, F. E. Wincott, L. Beigelman, S. W. Hunt, 3rd and D. J. Schrier, *Proc Natl Acad Sci U S A* **93**, 2 (1996)
- [68] D. Cooper, R. Penny, G. Symonds, A. Carr, W. Gerlach, L. Q. Sun and J. Ely, *Hum Gene Ther* **10**, 8 (1999)
- [69] J. L. Macpherson, M. P. Boyd, A. J. Arndt, A. V. Todd, G. C. Fanning, J. A. Ely, F. Elliott, A. Knop, M. Raponi, J. Murray, W. Gerlach, L. Q. Sun, R. Penny, G. P. Symonds, A. Carr and D. A. Cooper, *J Gene Med* **7**, 5 (2005)
- [70] R. G. Amado, R. T. Mitsuyasu, G. Symonds, J. D. Rosenblatt, J. Zack, L. Q. Sun, M. Miller, J. Ely and W. Gerlach, *Hum Gene Ther* **10**, 13 (1999)
- [71] E. Bertrand, D. Castanotto, C. Zhou, C. Carbonnelle, N. S. Lee, P. Good, S. Chatterjee, T. Grange, R. Pictet, D. Kohn, D. Engelke and J. J. Rossi, *Rna* **3**, 1 (1997)
- [72] N. S. Lee, E. Bertrand and J. Rossi, *Rna* **5**, 9 (1999)
- [73] N. Piganeau, A. Jenne, V. Thuillier and M. Famulok, *Angew Chem Int Ed Engl* **40**, 19 (2001)
- [74] H. Porta and P. M. Lizardi, *Biotechnology (N Y)* **13**, 2 (1995)
- [75] G. Knapp, *Methods Enzymol* **180**, (1989)
- [76] G. N. Pavlakis, R. E. Lockard, N. Vamvakopoulos, L. Rieser, U. L. RajBhandary and J. N. Vournakis, *Cell* **19**, 1 (1980)
- [77] M. Weerasinghe, S. E. Liem, S. Asad, S. E. Read and S. Joshi, *J Virol* **65**, 10 (1991)
- [78] J. O. Ojwang, A. Hampel, D. J. Looney, F. Wong-Staal and J. Rappaport, *Proc Natl Acad Sci U S A* **89**, 22 (1992)
- [79] K. M. Lo, M. A. Biasolo, G. Dehni, G. Palu and W. A. Haseltine, *Virology* **190**, 1 (1992)
- [80] L. Q. Sun, J. Pyati, J. Smythe, L. Wang, J. Macpherson, W. Gerlach and G. Symonds, *Proc Natl Acad Sci U S A* **92**, 16 (1995)
- [81] N. Sarver, E. M. Cantin, P. S. Chang, J. A. Zaia, P. A. Ladne, D. A. Stephens and J. J. Rossi, *Science* **247**, 4947 (1990)
- [82] M. Warashina, T. Kuwabara, Y. Kato, M. Sano and K. Taira, *Proc Natl Acad Sci U S A* **98**, 10 (2001)
- [83] A. Lieber and M. Strauss, *Mol Cell Biol* **15**, 1 (1995)
- [84] Y. Kim, M. J. Cairns, R. Marouga and L. Q. Sun, *Cancer Gene Ther* **10**, 9 (2003)
- [85] D. P. Bartel, *Cell* **116**, 2 (2004)
- [86] V. N. Kim, *Mol Cells* **19**, 1 (2005)
- [87] V. N. Kim, *Nat Rev Mol Cell Biol* **6**, 5 (2005)
- [88] S. M. Elbashir, W. Lendeckel and T. Tuschl, *Genes Dev* **15**, 2 (2001)
- [89] S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl, *Nature* **411**, 6836 (2001)
- [90] B. Xuan, Z. Qian, J. Hong and W. Huang, *Virus Res* **118**, 1-2 (2006)
- [91] A. Khvorova, A. Reynolds and S. D. Jayasena, *Cell* **115**, 2 (2003)
- [92] D. S. Schwarz, G. Hutvagner, T. Du, Z. Xu, N. Aronin and P. D. Zamore, *Cell* **115**, 2 (2003)
- [93] G. Hutvagner, *FEBS Lett* **579**, 26 (2005)
- [94] A. Birmingham, E. M. Anderson, A. Reynolds, D. Ilesley-Tyree, D. Leake, Y. Fedorov, S. Baskerville, E. Maksimova, K. Robinson, J. Karpilow, W. S. Marshall and A. Khvorova, *Nat Methods* **3**, 3 (2006)
- [95] C. Chakraborty, *Curr Drug Targets* **8**, 3 (2007)
- [96] J. M. Jacque, K. Triques and M. Stevenson, *Nature* **418**, 6896 (2002)
- [97] L. Gitlin, S. Karelsky and R. Andino, *Nature* **418**, 6896 (2002)
- [98] Q. Ge, M. T. McManus, T. Nguyen, C. H. Shen, P. A. Sharp, H. N. Eisen and J. Chen, *Proc Natl Acad Sci U S A* **100**, 5 (2003)
- [99] S. M. Tompkins, C. Y. Lo, T. M. Tumpey and S. L. Epstein, *Proc Natl Acad Sci U S A* **101**, 23 (2004)
- [100] D. V. Morrissey, K. Blanchard, L. Shaw, K. Jensen, J. A. Lockridge, B. Dickinson, J. A. McSwiggen, C. Vargeese, K. Bowman, C. S. Shaffer, B. A. Polisky and S. Zinnen, *Hepatology* **41**, 6 (2005)
- [101] D. V. Morrissey, J. A. Lockridge, L. Shaw, K. Blanchard, K. Jensen, W. Breen, K. Hartsough, L. Machemer, S. Radka, V. Jadhav, N. Vaish, S. Zinnen, C. Vargeese, K. Bowman, C. S. Shaffer, L. B. Jeffs, A. Judge, I. MacLachlan and B. Polisky, *Nat Biotechnol* **23**, 8 (2005)
- [102] T. R. Brummelkamp, R. Bernards and R. Agami, *Science* **296**, 5567 (2002)
- [103] C. P. Paul, P. D. Good, I. Winer and D. R. Engelke, *Nat Biotechnol* **20**, 5 (2002)
- [104] N. Tran, M. J. Cairns, I. W. Dawes and G. M. Arndt, *BMC Biotechnol* **3**, (2003)

- [105] H. Akashi, M. Miyagishi, T. Yokota, T. Watanabe, T. Hino, K. Nishina, M. Kohara and K. Taira, *Mol Biosyst* **1**, 5-6 (2005)
- [106] P. Konstantinova, W. de Vries, J. Haasnoot, O. ter Brake, P. de Haan and B. Berkhout, *Gene Ther* **13**, 19 (2006)
- [107] M. Li, H. Li and J. J. Rossi, *Ann N Y Acad Sci* **1082**, (2006)
- [108] A. Banerjea, M. J. Li, G. Bauer, L. Remling, N. S. Lee, J. Rossi and R. Akkina, *Mol Ther* **8**, 1 (2003)