Nucleic Acids-Based Therapeutics in the Battle Against Pathogenic Viruses

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Contents

Abstract For almost three decades, researchers have studied the possibility to use nucleic acids as antiviral therapeutics. In theory, compounds such as antisense oligonucleotides, ribozymes, DNAzymes, and aptamers can be designed to trigger the sequence-specific inhibition of particular mRNA transcripts, including viral genomes. However, difficulties with their efficiency, off-target effects, toxicity, delivery, and stability halted the development of nucleic acid-based therapeutics that can be used in the clinic. So far, only a single antisense drug, Vitravene for the treatment of CMV-induced retinitis in AIDS patients, has made it to the clinic. Since the discovery of RNA interference (RNAi), there is a renewed interest in the development of nucleic acid-based therapeutics. Antiviral RNAi approaches are highly effective in vitro and in animal models and are currently being tested in clinical trials. Here we give an overview of antiviral nucleic acid-based therapeutics. We focus on antisense and RNAi-based compounds that have been shown to be effective in animal model systems.

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Abbreviations

AIDS	Acquired immune deficiency syndrome
AAV	Adeno-associated virus
CMV	Cytomegalovirus
HIV-1	Human immunodeficiency virus type 1
HBV	Hepatitis B virus
HCV	Hepatitis C virus
RSV	Respiratory syncytial virus
SARS	Severe acute respiratory syndrome
RNAi	RNA interference
siRNA	Small interfering RNA
shRNA	Short hairpin RNA
RISC	RNA-induced silencing complex
PS	Phosphorothioate
OMe	2′-*O*-Methyl-RNA
MOE	2′-*O*-Methoxy-ethyl-RNA
PNA	Peptide nucleic acid
LNA	Locked nucleic acid
MF	Morpholino phosphoroamidates

1 Introduction

The most efficacious method to avoid infection with pathogenic viruses is immunization of individuals with prophylactic vaccines. However, for most viruses such vaccines are not available. Antiviral drugs that block replicating virus in infected individuals are therefore of great importance in the battle against virus infections. Ideally, these drugs should induce strong and specific inhibition of virus replication without affecting cellular processes. This type of drugs remained fiction until Zamecnik and Stephenson reported the sequence-specific inhibition of Rous sarcoma virus replication by antisense DNA oligonucleotides in 1978 (Zamecnik and Stephenson 1978). This paper and subsequent reports on the sequence-specific knock-down of genes by antisense technology made a big impression on the scientific community that is comparable to the current RNAi boom. However, the development of antisense-based therapeutics stuttered and almost came to a stop because of problems with toxicity of the oligonucleotides, their instability in serum, and the problem of delivery to the right target cells. A new generation of nucleic acid-based antiviral compounds were subsequently developed, such as ribozymes, DNAzymes, and decoy RNAs, but they faced similar problems. Some of the problems could be solved using chemically modified nucleic acids instead of normal DNA/RNA oligonucleotides. For example, modifications such as phosphorothioate (PS) DNA or 2′-*O*-methyl-RNA (OMe) increased the affinity of the oligonucleotides for their target sequence and increased serum stability (see Fig. 1).
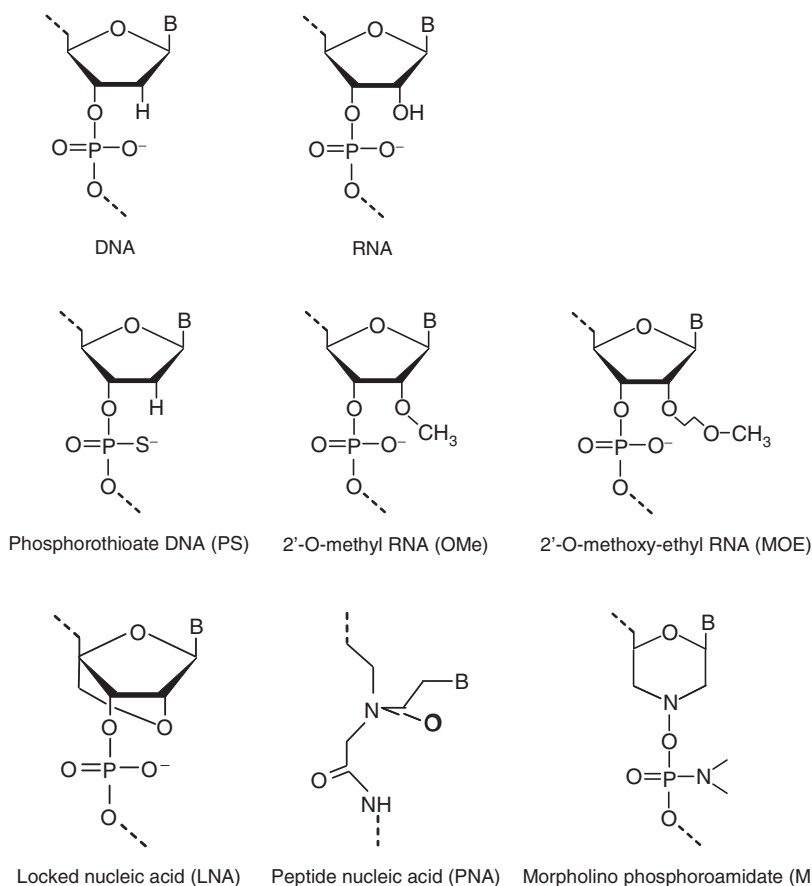
Fig. 1 Chemical structures of backbone modifications used in therapeutic nucleic acid analogs. Shown are the unmodified DNA/RNA chemical structures in addition to a selection of first (PS), second (OMe, MOE), and third generation (PNA, LNA, MF) nucleic acid modifications

New hope for nucleic acid-based therapeutics came in 2001 when Elbashir and coworkers showed that synthetic small interfering RNAs (siRNAs) induced sequence-specific knock-down of genes in mammalian cells without triggering the interferon response (Elbashir et al. 2001). RNAi was immediately recognized as a potentially powerful new tool to combat a variety of viruses. Indeed, profound inhibition has been reported for many important human pathogenic viruses, and several potential antiviral siRNA compounds are currently being tested in clinical trials (Haasnoot et al. 2007). Despite these promising data, it is becoming increasingly clear that some of the problems that were earlier encountered by antisense drugs also apply to RNAi-based therapeutics. Although RNAi-mediated silencing is highly sequence-specific, therapeutic siRNAs can cause unwanted silencing of cellular mRNAs (Birmingham et al. 2006; Fedorov et al. 2006; Jackson et al. 2003;

Kleinman et al. 2008). In addition, synthetic siRNAs are also subject to degradation in vivo by nuclease activity. Besides side effects and instability, the efficient and specific delivery of the RNAi inducers to the target cell still requires optimization. Here we summarize the current status of nucleic acid-based antiviral therapeutics. The focus will be on antiviral strategies using antisense and RNAi technology. Additionally, antiviral ribozymes and aptamers will be discussed briefly, with a focus on recent studies. Gene therapy approaches and delivery systems are the subject of Chapter 11 of this book.

2 Antisense-Based Antiviral Therapeutics

Antisense technology is based on the sequence-specific binding of antisense DNA oligonucleotides to complementary sequences in the target mRNA (Van Aerschot 2006). After binding to the target RNA, the oligonucleotide can block translation via steric hindrance of the elongating ribosome or induce RNA cleavage by activation of RNase H, the enzyme that specifically recognizes DNA/RNA duplexes. Although antisense technology can induce profound sequence-specific inhibition of gene expression in some settings, major problems include instability, delivery, and unwanted side effects of the oligonucleotides. Chemical modification of the oligonucleotides was found to improve the efficacy (Fig. 1).

The first generation of chemical modification was designed to enhance nuclease resistance of the oligonucleotide in serum (Stein et al. 1997). The PS modification, in which the oxygen atom in the DNA backbone is replaced by a sulfur atom, has been widely used. The advantage of the PS-modified oligonucleotides is that it allows RNase H cleavage of the target sequence. A disadvantage is that the PS modification introduces a negative charge that causes nonspecific interactions with other molecules, for example, cellular proteins. As a result, PS-modified oligonucleotides are notorious for inducing severe side effects that are toxic for cells. A well-known example of an antiviral PS-modified oligonucleotide is Vitravene for treatment of cytomegalovirus (CMV)-induced retinitis in AIDS patients. This is a 21-nucleotide that inhibits the expression of viral proteins from the major immediate-early transcriptional unit (Vitravene study group, 2002). Until now, Vitravene is the only antisense-based drug that has received FDA approval and is used in the clinic. Although Vitravene provides a marked reduction of disease severity, the compound is currently not widely used mainly because AIDS patients on antiretroviral therapy rarely develop CMV-induced retinitis.

Second generation oligonucleotides are based on modifications of the sugar ring. For example, OMe and $2'$-O-methoxy-ethyl (MOE) modifications (Fig. 1) increase the stability of the oligonucleotide and the affinity for the target mRNA when compared to PS-modified oligonucleotides. In contrast to the PS modification, the OMe and MOE-modified oligonucleotides do not allow cleavage by RNase H, but they rather block translation via steric hindrance of the ribosome during translation. For optimal activity, the oligonucleotide has to be targeted to sequences that are at or

near the translation initiation site. The OMe modification has also been used in combination with PS modifications in the so-called gap-mers, in which the central nucleotides carry the PS modification and the flanking nucleotides the OMe modification (Monia et al. 1993). This approach combines the benefits of both the first and second generation modifications. An example of a second generation-modified antiviral oligonucleotide is GEM-92, which was developed by Hybridon (currently discontinued). GEM-92 is a gap-mer, with OMe modified flanks and PS modified central sequences (Turner et al. 2006).

Third generation oligonucleotides are DNA and RNA analogues with extensive modifications of the phosphate backbone and the sugar ring. Examples are peptide nucleic acids (PNA), in which the backbone has been replaced by a peptide linker, morpholino phosphoroamidates (MF), and locked nucleic acids (LNA) (Fig. 1) (Iversen 2001; Larsen et al. 1999; Petersen and Wengel 2003). These modifications provide further improved nuclease resistance and target affinity. In addition, the PNA and MF modifications allow enhanced cellular uptake. To combine all benefits, third generation modifications can be used in combination with unmodified residues in chimaeric gap-mers. LNA modifications are widely used in various applications. It introduces a $2'$-O, $4'$C-methylene bridge that locks the ribose in a C$3'$-endo conformation. This "locked" conformation results in a strong increase in the affinity for the target mRNA (Petersen and Wengel 2003). LNA modifications were reported to cause less toxic side effects when compared to the first and second generation modifications (Wahlestedt et al. 2000). However, LNA-modified oligonucleotides were recently shown to induce profound toxicity in hepatocytes in mice (Swayze et al. 2007).

Despite these intense efforts to test different chemical modifications, there is so far little success in developing potent and safe antivirals. For hepatitis C virus (HCV), McHutchison et al. reported in vivo side effects of a 20-nucleotide PS-modified oligonucleotide (ISIS-14803) (McHutchison et al. 2006). In a test group of 28 patients, only 3 patients responded to the treatment by a reduction in the HCV viral load. The researchers concluded that further studies are needed to evaluate this novel agent and its side effects. Previously, ISIS Pharmaceuticals reported a 3.8 log reduction in plasma virus in patients with chronic HCV infection, using ISIS-14803 (www.isispharm.com).

Warfield et al. reported profound inhibition of Ebola virus in mice and rhesus macaques using antisense MF oligonucleotides (Warfield et al. 2006). Combination of several oligonucleotides targeting mRNAs for VP24, VP35, and the RNA polymerase protected the animals, both therapeutically and prophylactically. The modified oligonucleotides protected 75% of the macaques from lethal Ebola virus infection. Similarly, MF oligonucleotides were recently found to profoundly inhibit the murine hepatitis coronavirus. Treatment protected mice against a normally lethal viral challenge, but there were some indications of toxic effects (Burrer et al. 2007).

A different antiviral approach uses intracellular expression of unmodified antisense molecules corresponding to viral sequences. Such an intracellular immunization approach may require a gene therapy protocol to deliver the antiviral transgene. In a phase I clinical trial, Levine et al. used intracellularly expressed

antisense transcripts to inhibit HIV-1 replication. The advantage of using extended antisense sequences is that multiple viral sites are targeted simultaneously (Levine et al. 2006). A conditionally replicating lentiviral vector was used to transduce CD4 T cells ex vivo for intracellular expression of antisense sequences against the HIV-1 envelope gene. The modified CD4 T cells were infused back into the same patient and were apparently well tolerated in all patients. One of the five patients showed a sustained decrease in viral load (Levine et al. 2006). Alternatively, short antisense molecules against sequences in the HIV-1 gag gene were shown to inhibit virus replication in transduced CEM cells (Gu et al. 2006).

3 Ribozymes as Antiviral Therapeutics

Ribozymes represent a class of natural RNAs that catalyse RNA cleavage and ligation reactions as well as peptide bond formation. In most cases, these reactions are self-processing events that take place in a sequence-specific manner. It was found, however, that ribozymes can be designed to specifically cleave other RNA molecules in *trans* by modification of the substrate recognition domains. Because of their sequence specificity and multiple turnover or catalytic properties, such ribozymes have been extensively studied as potential therapeutics (Peracchi 2004). Of the nine classes of ribozymes, the hammerhead and hairpin ribozymes have received a great deal of attention. These ribozymes are relatively small (50–100 nucleotides) and can direct cleavage of single-stranded target RNA. Many ribozymes that are used in therapeutic applications have been improved by in vitro selection procedures, in which the most active molecules were selected from partially randomized sequences. In vitro selection has also generated DNAzymes that structurally and functionally resemble ribozymes, but that have a DNA instead of RNA backbone (Santoro and Joyce 1997). These DNAzymes can have a catalytic activity similar to that of ribozymes, but they are more stable in vivo.

Therapeutic ribozymes have been used both as synthetic RNA that is transfected into cells and as intracellularly expressed RNA. Because unmodified ribozymes are rapidly degraded in serum, synthetic ribozymes require chemical modification to extend the half life and to improve the therapeutic potential of ribozymes (Gonzalez-Carmona et al. 2006; Jakobsen et al. 2007). Antiviral ribozymes have been extensively tested in gene therapy settings (Haasnoot et al. 2007). In these studies, the ribozyme is placed under control of a suitable promoter and inserted in a vector to transduce the target cells. Anti-HIV-1 ribozymes have been studied in several clinical trials. Macpherson et al. have tested an anti-tat ribozyme in a phase I clinical trial (Macpherson et al. 2005). In preclinical studies, this ribozyme was shown to decrease HIV-1 replication and virus-induced pathogenicity in T cell lines and peripheral blood T-lymphocytes. In the clinical trial, CD4$^+$ T-lymphocytes were transduced ex vivo using a retroviral vector for expression of the ribozyme. The transduced cells were given back to the HIV-positive patients. This gene therapy was found to be safe and was shown to inhibit HIV-1 replication. Li and coworkers have

used a ribozyme in combination with an siRNA and a decoy RNA to inhibit HIV-1 replication (Li et al. 2003, 2005b). The ribozyme is targeted towards the mRNA for the cellular CCR5 protein, which functions as essential coreceptor for HIV-1 entry. The decoy is the viral TAR RNA hairpin structure, which is supposed to bind the viral Tat protein and cellular cofactors that are needed for viral gene expression. CD34$^+$ hematopoietic progenitor cells were also transduced with a retroviral vector for the expression of the ribozyme. These cells differentiated normally into mature macrophages and showed significant resistance to viral infection. When the ribozyme was used in combination with the Tat/Rev siRNA and the TAR decoy, the transduced cells showed long-term viral resistance in vitro and the transduced hematopoietic stem cells developed normally into T cells in SCID-hu mice. These cells were able to resist HIV-1 infection ex vivo (Anderson et al. 2007).

4 Aptamers and Decoy RNAs as Antiviral Therapeutics

In contrast to other nucleic acid-based antiviral strategies, decoy RNAs or small RNA/DNA aptamers act via specific binding to a viral target protein rather than to viral RNAs. The interaction of the decoy with the target protein prevents it from being active and hence blocks virus replication (James 2007). The HIV-1 TAR decoy described earlier is a well-known example. Another decoy RNA to inhibit HIV-1 corresponds to the Rev responsive element (RRE). Interaction of the RRE with the viral Rev protein is required for the transport of single and unspliced viral RNAs from the nucleus to the cytoplasm. Overexpression of an RRE decoy sequesters the Rev protein, thereby blocking nuclear export of viral RNAs and thus virus replication (Bahner et al. 1996). This RRE decoy has earlier been tested in a phase I clinical trial in which CD34$^+$ stem cells were transduced ex vivo with a retroviral vector for the expression of the RNA inhibitor (Kohn et al. 1999). There was no evidence that expression of the decoy affected cell function, but there were also no detectable changes in the subject's plasma HIV-1 level.

Besides overexpression of a decoy RNA that resembles a natural viral RNA motif, there is also an interest in the use of artificial small nucleic acid aptamers that have a high affinity for a certain target protein. These aptamers were identified in large randomized pools of sequences by repetitive rounds of selection for binding with the highest affinity to the target protein (James 2007). The early targets for aptamer development were viral polymerases. For example, potent aptamers were selected that inhibit HIV-1 reverse transcriptase (RT) and HCV RNA polymerase (Bellecave et al. 2003; Burke et al. 1996; Tuerk et al. 1992). These aptamers are not similar to any viral sequences, indicating that the high affinity binding aptamers do not necessarily have to be analogues of natural substrates. It has been shown that HIV-1-specific RT aptamers can potently inhibit RT enzymes from multiple HIV-1 subtypes, suggesting that these aptamers can provide broad protection (Held et al. 2007). However, naturally resistant virus strains exist against some aptamers, which means that these antiviral should be used in combination with other HIV-1 inhibitors.

5 Antiviral RNAi-Based Strategies

RNAi has only recently been added to the list of nucleic acid-based therapeutics. In eukaryotic cells, RNAi plays an important role in the regulation of gene expression via microRNAs (miRNA). These are small noncoding RNAs that are expressed as primary miRNAs (pri-miRNAs) and processed by the cellular proteins Drosha and Dicer into mature miRNA of 21–25 nucleotides (Fig. 2). In mammals, miRNAs typically direct translational inhibition of cellular mRNAs bearing complementary sequences in the 3′ UTR (Grimson et al. 2007). RNAi has also been proposed to play a role in the cellular defense against viruses and silencing of transposable elements by generation of virus-specific siRNAs (Bennasser et al. 2005; Soifer et al. 2005; Triboulet et al. 2007; Yang and Kazazian 2006). Recently, it was shown that transposable elements in mouse oocytes are potently silenced by RNAi-related
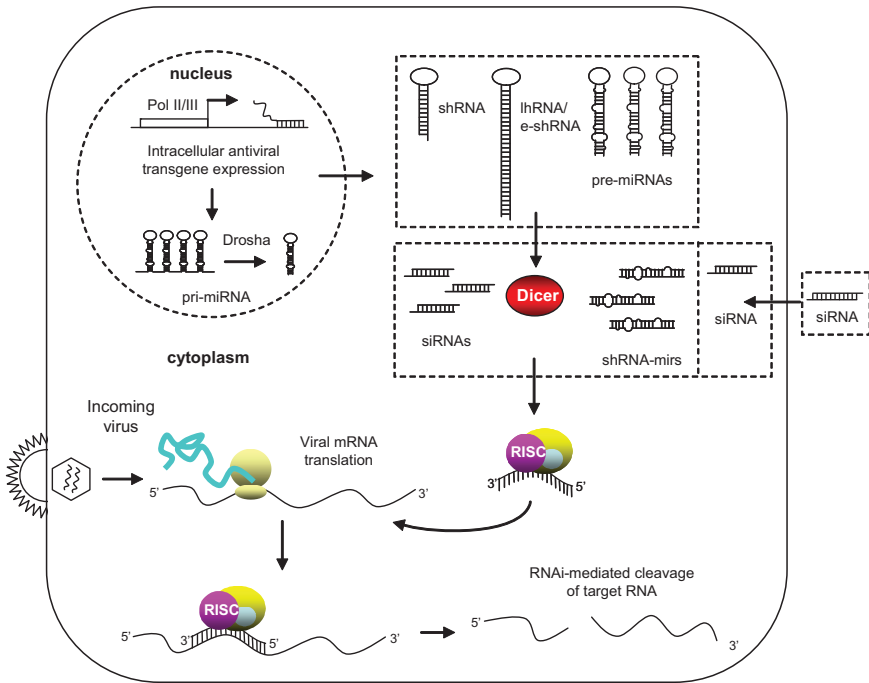

Fig. 2 RNAi inducers used in antiviral strategies. In general, RNAi is induced either by transfection of synthetic siRNAs into cells, or by stable or transient intracellular expression of double-stranded siRNA precursors (shRNA, e-shRNA, lhRNA, or pri-miRNAs). After transcription in the nucleus shRNAs, lhRNAs and e-shRNAs are exported to the cytoplasm and subsequently diced into mature siRNAs. Pri-miRNAs modified to encode antiviral siRNAs first undergo cleavage by Drosha before they are exported to the cytoplasm. Here the antiviral pre-miRNAs (also called shRNA-miRs) are processed by Dicer into the mature miRNAs. After loading of the antisense strand of the siRNAs/miRNAs into RISC, the complex will target and cleave viral transcripts bearing the complementary sequences

mechanisms involving small RNAs (Tam et al. 2008; Watanabe et al. 2008). Both miRNAs and siRNAs are loaded into the RNA-induced silencing complex (RISC) and target RNAs for, respectively, translational repression or destruction (Fig. 2). Although the antiviral function of RNAi is well established in plants, insects, and nematodes, it is still debated whether RNAi has a similar role in mammals, but the circumstantial evidence is growing in support of this idea (Andersson et al. 2005; Bennasser et al. 2005; Cullen 2006; Grimson et al. 2007; Soifer et al. 2005; Tam et al. 2008; Voinnet 2005; Watanabe et al. 2006b, 2008; Yang and Kazazian 2006).

The discovery that exogenously delivered synthetic siRNA and intracellularly expressed short hairpin RNA (shRNA) trigger gene silencing in mammalian cells has made RNAi a powerful technique for generating genetic knock-downs (Brummelkamp et al. 2002; Elbashir et al. 2001). It also allowed the development of RNAi-based therapeutics for prevention of virus transmission or treatment of virus replication. An overview of therapeutic strategies used to induce antiviral RNAi responses is shown in Fig. 2. Synthetic siRNA duplexes used to induce RNAi are generally 21 nucleotides long with 2-nucleotide $3'$ overhangs and are modeled after the natural Dicer cleavage products. Once the antisense strand (guide) of the antiviral siRNA is loaded into RISC, the complex will target the viral RNAs in a sequence-specific manner. Alternatively, transient transfection of plasmids that express antiviral shRNAs is also used to induce RNAi. These shRNAs are typically 19–29 base pairs long, with a small apical loop and a $3'$-terminal UU overhang. The shRNAs are usually expressed in the nucleus under control of a polymerase III promoter (U6 or H1) and are translocated to the cytoplasm by Exportin-5. The shRNAs are processed in the cytoplasm by Dicer into functional siRNAs.

The technology of shRNA-induced RNAi has been significantly improved by using shRNA variants that more closely resemble natural miRNAs. This second generation shRNAs (shRNA-mirs) can be designed using the increased knowledge on the biochemistry of the RNAi mechanism and miRNA biogenesis. These shRNA-mirs are expressed as larger transcripts under control of polymerase II promoters, and contain bulged nucleotides and internal loops as present in pri- and pre-miRNAs (Boden et al. 2004; Du et al. 2006; Liu et al. 2008; Silva et al. 2005). Compared to shRNAs, the shRNA-mirs show significantly increased activity.

Another possibility to induce RNAi is intracellular expression of long hairpin RNAs (lhRNAs) (Akashi et al. 2005; Konstantinova et al. 2006; Liu et al. 2007; Nishitsuji et al. 2006; Watanabe et al. 2006a; Weinberg et al. 2007). In contrast to transfection of dsRNA larger than 30 bp, intracellular expression does not seem to induce the interferon response. Efficient inhibition by lhRNAs has been reported for HIV-1, HCV, and HBV. Besides conventional lhRNAs, extended-shRNAs (e-shRNAs) have also been successfully used to trigger a potent RNAi response. These e-shRNAs encode individually effective siRNAs that are stacked to form a lhRNA structure (Liu et al. 2007). The potential advantage of a lhRNA or e-shRNA is the generation of multiple siRNAs from a single precursor molecule, which may prevent viral escape.

5.1 Chemically Modified siRNAs

Similar to antisense oligonucleotides, synthetic siRNAs are relatively instable in vivo due to degradation by nuclease activity. The siRNA half life must be increased to be effective as antiviral therapeutic. This would allow treatment with infrequent administration of small amounts of siRNAs, which also increases the prophylactic potential of siRNAs. In case of influenza virus, individuals at risk could be treated with such stabilized antiviral siRNAs during the seasonal epidemic and thus avoid infection. Chemical modifications that were earlier used for antisense compounds have been successfully used to increase the half life of siRNAs in vivo while retaining their activity (Morrissey et al. 2005a, b; Rana 2007). For example, introducing a PS-linkage at the $3'$ end renders siRNA resistant to $3'$ exonuclease activity, whereas OMe modification gives resistance to endonucleases. However, some of the modifications that increase the stability have a negative effect on the siRNA activity (Czauderna et al. 2003). Modification of the $5'$ end resulted in loss of siRNA activity, emphasizing its importance. Modifications in the antisense (passenger) strand can stabilize the siRNA, without a negative effect on the activity of the sense (guide) strand. LNA modification dramatically increases the potency of siRNA in vitro and in vivo without toxicity (Elmen et al. 2005).

5.2 RNAi-Mediated Inhibition of HIV-1 and Virus Escape

Numerous research groups have studied RNAi as an alternative method to block HIV-1, using both transient transfection of synthetic siRNAs and stably expressed shRNAs (Arrighi et al. 2004b; Das et al. 2004; Jacque et al. 2002; Lee et al. 2002; Martinez et al. 2002; Novina et al. 2002; Qin et al. 2003; Surabhi and Gaynor 2002; Ter Brake et al. 2006; Westerhout et al. 2006). Long-term virus suppression by an RNAi gene therapy approach is perhaps best suited to target HIV-1 and other viruses that cause a chronic infection. Lenti-, retro-, and adeno-associated virus (AAV) vectors can be used to stably transduce cells with constructs that express an antiviral shRNA, resulting in cellular resistance to the virus. Transduction of CD34$^+$ hematopoietic stem cells with anti-HIV-1 RNAi constructs ex vivo and grafting these cells back into the patient will result in a resistant cell population that might reconstitute the patients' immune system (Berkhout 2005; see also Chapter 11).

Prolonged in vitro culturing of resistant cells with HIV-1 resulted in the emergence of viral escape variants that are no longer targeted by the siRNAs. These RNAi escape variants contain point mutations or deletions within the siRNA target sequences (Das et al. 2004). In addition, an escape virus was described with a mutation outside the target sequence. This mutation triggered escape by inducing a conformational change in the local RNA secondary structure that rendered the target sequence inaccessible for RISC (Westerhout et al. 2005). Escape from RNAi has also been reported for poliovirus, hepatitis A virus, HBV, and HCV (Boden et al. 2003; Das et al. 2004; Gitlin et al. 2005; Kusov et al. 2006; Ter Brake and

Berkhout 2005; von Eije et al. 2008; Westerhout et al. 2005; Wilson and Richardson 2005; Wu et al. 2005b). These combined results show that a single siRNA therapy is not sufficient to obtain long-term inhibition of virus replication. Escape from RNAi is reminiscent of the evolution of drug resistant HIV-1 variants in patients on antiretroviral therapy. Only the combined use of multiple HIV-1 drugs (highly active antiretroviral therapy or HAART) can permanently block virus replication without the emergence of resistant variants. Similarly, the combined expression of multiple siRNAs would be required to persistently block virus replication. This combinatorial approach should ideally target viral sequences that are essential and well conserved among different virus strains. The emergence of HIV-1 escape variants was delayed in cells expressing two siRNAs when compared to cells expressing a single siRNA (Ter Brake et al. 2006). In cells expressing four potent siRNAs, no viral escape was observed (Ter Brake et al. 2008).

lhRNAs have also been used to induce an anti-HIV-1 RNAi response (Barichievy et al. 2007; Konstantinova et al. 2006, 2007; Liu et al. 2007). These lhRNAs can be processed into multiple effective siRNAs, thus preventing the chance of viral escape. Although lhRNA have been shown to effectively inhibit HIV-1 replication, there is currently no data on their ability to prevent viral escape. The use of multiple siRNAs or lhRNAs should take into account the increased danger of side effects due to interference with cellular miRNA processing and function.

To prevent viral escape from RNAi, an alternative strategy is to target host factors that are essential for viral replication. However, there are few host factors that can be targeted without affecting host cell viability (Arrighi et al. 2004a; Liu et al. 2004; Ping et al. 2004; Qin et al. 2003; Zhou et al. 2004). For HIV-1, a well known example is the CCR5 coreceptor for HIV-1. This coreceptor is important for virus entry, yet mutation of the CCR5 gene does not affect the fitness of the host (Liu et al. 1996; Samson et al. 1996). Recently, Brass et al. (2008) published a large-scale siRNA screen to identify host factors required for HIV-1 replication (Brass et al. 2008). More than 250 HIV-dependency factors were identified, some of which may represent potential targets for therapy.

5.3 RNAi-Based Strategies Against HBV

Despite the availability of an effective HBV vaccine, the virus is still a major health problem with approximately 350 million persons infected worldwide. Hepatitis an infection of the liver that is caused by a variety of RNA viruses (hepatitis A virus, hepatitis B virus, hepatitis C virus). RNAi has been used to inhibit HBV replication both in vitro and in vivo (Carmona et al. 2006; Ely et al. 2008; Hamasaki et al. 2003; Klein et al. 2003; Konishi et al. 2003; Weinberg et al. 2007; Ying et al. 2003). HBV is a member of the Hepadnaviridae and its genome is a 3.2-kb double-stranded circular DNA. Synthetic siRNAs and shRNA expression constructs showed potent inhibition of HBV replication in mice (Chen et al. 2005, 2007; Giladi et al. 2003; McCaffrey et al. 2003; Morrissey et al. 2005b; Shin et al. 2006; Wu et al. 2005b;

Xuan et al. 2006; Ying et al. 2007). The RNAi-based therapy needs to be provided for a long period because HBV causes a chronic infection of the liver. Transduction of the hepatocytes in vivo with a vector expressing anti-HBV shRNAs could provide such long-term protection. Chen et al. have indeed demonstrated long-term inhibition of HBV in mice transduced with an AAV vector to deliver the shRNA inhibitor. HBV DNA, mRNA, and protein levels were reduced in the liver of the transgenic mice, whereas the amount of circulating virus dropped by 2–3 logs. Virus inhibition was still apparent 120 days after vector administration (Chen et al. 2007). In addition, lhRNAs and shRNA-mirs could efficiently inhibit HBV replication in vitro and in mice (Ely et al. 2008; Weinberg et al. 2007).

5.4 RNAi-Based Strategies Against Respiratory Viruses

Respiratory viruses are perhaps best suited to target with RNAi-based therapeutics, because the upper airways and lungs are relatively easy to reach as target tissues. Already several studies reported therapeutic effects in animal models and in clinical trials. For example, siRNAs against influenza virus were found to reduce virus titers in the lungs of infected mice when siRNAs were administered through hydrodynamic intravenous injection (Ge et al. 2004). Similarly, virus titers were reduced when mice were given DNA vectors, either intravenously or intranasally, that express antiviral shRNAs (Ge et al. 2004). Moreover, reduced virus-induced mortality was scored in treated mice, even when the siRNA was delivered after virus infection, suggesting that siRNAs can act both in a prophylactic manner and in the treatment of established infections. In another study, synthetic siRNAs also protected mice against a lethal influenza virus challenge (Tompkins et al. 2004). This siRNA treatment induced a broad protection against the pathogenic avian influenza A viruses of serotypes H5 and H7.

Another respiratory virus for which RNAi therapeutics are being developed is respiratory syncytial virus (RSV). In fact, RSV was the first virus for which RNAi-mediated inhibition was demonstrated (Bitko and Barik 2001) and synthetic siRNAs are currently entering phase II clinical trials. RSV belongs to the paramyxoviridae and is an enveloped, nonsegmented, negative-strand RNA virus. RSV is a major cause of respiratory illness in both the upper and lower respiratory tract, typically leading to common cold-like symptoms that in some cases – especially in young children – lead to more serious illness such as bronchiolitis and pneumonia. In mice, replication of RSV and also human parainfluenza virus could be blocked by synthetic siRNAs (Bitko et al. 2005). Synthetic siRNAs and intranasal administration of plasmids expressing shRNAs resulted in a significant decrease of the RSV load (Zhang et al. 2005). Similar to siRNA-induced inhibition of influenza virus, the siRNAs against RSV were effective both before and after infection with RSV. Alnylam has presented the results of a phase I clinical trial with its leading siRNA drug candidate ALN-RSV01 (http://www.alnylam.com/). The drug was found to be safe and well tolerated when administered intranasally in two phase I clinical studies

(DeVincenzo et al. 2008). Phase II clinical trials in naturally infected RSV patients were initiated in April 2008. ALN-RSV01 is expected to advance into the pediatric patient population by the second half of 2008.

RNAi against the severe acute respiratory syndrome coronavirus (SARS-coronavirus) and other coronaviruses has also been studied extensively. Both siRNAs and plasmid-derived shRNAs were effective in blocking virus replication in cell culture infections (He et al. 2003; Lu et al. 2004; Ni et al. 2005; Pyrc et al. 2006; Wang et al. 2004; Wu et al. 2005a; Zhang et al. 2004). Potent siRNA inhibitors against the SARS-coronavirus spike and pol mRNAs were also tested for efficacy and safety in a rhesus macaque SARS model. The siRNAs were administered intranasally as prophylactic, concurrent with viral challenge, or early post-exposure (Li et al. 2005a). Similar to the mice studies with anti-influenza siRNAs and anti-RSV siRNAs, both prophylactic and therapeutic effects were observed. siRNA treatment caused a significant reduction in viral RNA levels, SARS-like symptoms, and lung histopathology. These combined findings suggest that low dosages of inhaled or intravenously administered siRNAs or shRNA plasmids may provide an easy and efficient basis for prophylaxis against respiratory viruses in certain situations – for example, a pandemic influenza threat – or antiviral therapy of already infected individuals.

5.5 RNAi Side Effects and Off-Target Effects

RNAi is a highly specific tool for inducing gene knock-down. However, studies show that high expression of siRNA/shRNAs can induce interferon responses and other unwanted side effects (Bridge et al. 2003; Jackson and Linsley 2004). Cellular transcripts containing partial complementarity with an siRNA can also be targeted for RNAi-mediated knock-down (Birmingham et al. 2006; Fedorov et al. 2006; Jackson et al. 2003). These off-target effects are of great concern in the development of safe RNAi-based antivirals, because they will have an impact on cell viability (Fedorov et al. 2006; Jackson and Linsley 2004). High concentrations of siRNA/shRNAs may also interfere with cellular miRNA processing and function. This problem is caused by the fact that shRNA processing uses the cellular pathway for miRNA processing. In particular, Exportin-5, which is required for nuclear export and stability of miRNAs, was found to be a limiting factor in the RNAi pathway that could be saturated by exogenous shRNAs. In extreme cases, such side effects can have severe consequences, as illustrated by the mice that died after treatment with an AAV vector for overexpression of shRNAs in the liver (Grimm et al. 2006). This study also showed that there is an shRNA-threshold for toxicity. Because of our limited knowledge on cellular miRNA function, we should not underestimate the impact of shRNAs on these processes. Moderate expression of highly active shRNAs is essential for the development of safe RNAi therapeutics, and candidate shRNA/siRNA inhibitors should be screened for off-target effects. Inducible promoters are useful to regulate shRNA expression to avoid off-target effects. To avoid

toxic off-target effects of antiviral RNAi therapeutics, viruses should ideally be targeted transiently, locally, and with a low dosage. This could be realized for acute respiratory viral infections, but treatment of chronic viral infections may require a durable gene therapy approach. An elegant strategy to reduce potential off-target effects of the siRNA passenger strand is the use of so-called small internally segmented interfering RNAs (sisiRNA) (Bramsen et al. 2007). These sisiRNAs are composed of an intact antisense strand complemented with two shorter 10–12 nt sense strands. Because of its segmented nature the passenger strand is inactive, whereas the antisense strand can still efficiently induce a potent RNAi response.

Delivery of the siRNAs to the right target cells will also limit the impact of potential off-target effects. Progress has been made by using nanoparticles loaded with siRNAs. These particles, ranging between 25–40 nm in diameter, can be used to load high concentrations of siRNAs into a protected environment. The nanoparticles can be targeted to specific cells or tissues by decorating their surface with ligands that target specific cell surface receptors. Ligands such as transferrin, Her2 antibodies, folate, and integrin-targeting peptide have already been successfully used to target siRNA particles to cells bearing the corresponding receptors (Guo et al. 2006; Hu-Lieskovan et al. 2005; Schiffelers et al. 2004; Tan et al. 2007). These results are promising and will contribute to the further development of safe and efficient siRNA delivery systems.

6 Conclusions

The development of nucleic acid-based therapeutics is not as straightforward as researchers had initially anticipated. Stability, toxicity, specificity, and delivery of the compounds continue to be challenging issues that need further optimization. In recent years, researchers have come up with intricate solutions that have greatly improved the efficacy of potential antisense, ribozyme, as well as RNAi-based therapeutics. Clinical trials for all these types of nucleic acid-based therapeutics are underway. So far, data from several trials and studies in animal models look promising, in particular, the therapies that trigger the RNAi pathway. However, history has shown that compounds that do well in phase I or phase II clinical trials may still fail in phase III. A striking example is the nonspecific suppression of angiogenesis by siRNA via toll-like receptor 3 (Kleinman et al. 2008). It will become clear in the near future which compounds will make it as a new class of antiviral therapeutics.

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