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Oligonucleotide-Based Antiviral Strategies

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Abstract In the age of extensive global traffic systems, the close neighborhood of man and livestock in some regions of the world, as well as inadequate prevention measures and medical care in poorer countries, greatly facilitates the emergence and dissemination of new virus strains. The appearance of avian influenza viruses that can infect humans, the spread of the severe acute respiratory syndrome (SARS) virus, and the unprecedented raging of human immunodeficiency virus (HIV) illustrate the threat of a global virus pandemic. In addition, viruses like hepatitis B and C claim more than one million lives every year for want of efficient therapy. Thus, new approaches to prevent virus propagation are urgently needed. Antisense strategies are considered a very attractive means of inhibiting viral replication, as oligonucleotides can be designed to interact with any viral RNA, provided its sequence is known. The ensuing targeted destruction of viral RNA should interfere with viral replication

without entailing negative effects on ongoing cellular processes. In this review, we will give some examples of the employment of antisense oligonucleotides, ribozymes, and RNA interference strategies for antiviral purposes. Currently, in spite of encouraging results in preclinical studies, only a few antisense oligonucleotides and ribozymes have turned out to be efficient antiviral compounds in clinical trials. The advent of RNA interference now seems to be refueling hopes for decisive progress in the field of therapeutic employment of antisense strategies.

Keywords Antisense oligonucleotides · Antiviral agents · Ribozymes · RNA interference · RNAi

1 Introduction

In recent years, the prevalence of chronic infections with viruses such as human immunodeficiency virus (HIV) and the hepatitis B and C viruses (HBV and HCV, respectively) has been steadily increasing and new viruses like the severe acute respiratory syndrome (SARS) coronavirus have emerged. Thus, the demand for efficient antiviral treatments is obvious. Currently, approximately 40 small molecular compounds have been approved to treat viral infections, at least half of which are intended for patients with HIV infections (De Clercq 2004). The most prominent class of drugs used to inhibit viral propagation is the group of inhibitors of DNA or RNA synthesis, many of which are nucleoside analogs. These substances, however, are not fully specific for viral polymerases and cause severe side effects upon long-term treatment. For numerous viral infections, effective therapies are lacking altogether.

A strategy for the fast development of specific antiviral agents is therefore desirable. Antisense (AS) strategies employ oligonucleotides (ONs) complementary to a given target RNA. They offer the opportunity to fulfill demand for the development of an antiviral compound as soon as the sequence of a virus is known. In fact, the first AS study, published in 1978, describes the use of an AS ON to inhibit replication of Rous-Sarcoma virus (Zamecnik and Stephenson 1978). The following sections will deal with the use of AS ONs, ribozymes, and small interfering (si)RNAs as antiviral agents. Aptamers will not be addressed here, although there is no doubt about their usefulness to diagnose and treat viral infections (e.g., Darfeuille et al. 2004; De Beuckelaer et al. 1999; overview by McKnight and Heinz 2003). This type of ON is dealt with in the chapters by H.U. Göringer et al., M. Menger et al., M. Sprinzl et al., H. Ulrich, and A.K. Deisingh of the present volume.

2 Antisense Oligonucleotides

AS ONs are typically 15–20 nucleotides in length and bind to their cognate RNA via Watson-Crick base pairing. Since a DNA sequence of this length will statisti-

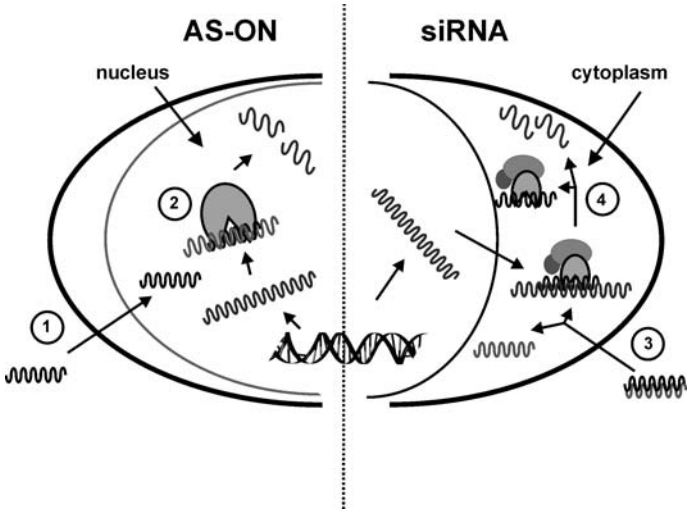

Fig. 1 Comparison of mechanisms of gene silencing by AS ONs and siRNAs. AS ONs (*left*) exert their effect predominantly in the nucleus, whereas siRNAs work mainly in the cytoplasm (*right*). *Left*: An AS ON is transported into the nucleus (*1*) and base-pairs with the complementary sequence of an mRNA. RNase H is recruited to the hybrid helix (*2*) and cleaves the RNA moiety. *Right*: An siRNA double helix reaches the cytoplasm (*3*). One of the strands is incorporated into a protein complex called RISC, while the other strand is discarded. The siRNA guides RISC to a complementary sequence on the target RNA. Upon binding, cleavage of the target molecule is induced (*4*). RISC can go on through multiple rounds of cleavage

cally occur only once in the human genome, the targeted RNA can be considered to be a highly specific receptor for the AS agent. AS ONs are known to act by two distinct mechanisms. (1) In the cell nucleus, the heteroduplex of a DNA ON bound to an RNA is recognized by RNase H, which cleaves the RNA moiety of the hybrid (Fig. 1). The RNA fragments are further degraded by exonucleases, whereas the ON is set free and can bind to new RNA molecules in a multiple turnover manner. (2) In the cytoplasm, AS ONs can disable messenger (m)RNAs and prevent protein synthesis by a steric blockade of the ribosome.

2.1 Development of Efficient Antisense Oligonucleotides

Theoretically, AS ONs can be directed against any region of the targeted RNA. In practice, however, long RNA molecules are known to form complex secondary and tertiary structures. Furthermore, various proteins bind to RNA molecules, precluding ONs from hybridizing. It is therefore necessary to select regions of the targeted RNA that are accessible to the AS ON. Various methods have been developed for this purpose and are summarized in the review by Sohail and Southern (2000).

Another major decision in the development of an AS ON is the choice of a suitable chemistry, i.e., nucleotide modification. Unprotected DNA ONs are degraded in blood serum within a few hours. Therefore, chemically modified building blocks are often used for AS ONs. The most widely employed DNA analogs are phosphorothioates in which one of the nonbridging oxygen atoms of the phosphodiester bond is replaced by a sulfur atom (Fig. 2). ONs consisting of phosphorothioates combine several advantages, including enhanced nuclease resistance and activation of RNase H cleavage (Eckstein 2000). Major disadvantages, however, are their decreased binding affinity to a complementary sequence as compared to an isosequential DNA molecule and their unintended propensity to interact with various proteins. Although binding to plasma proteins improves the pharmacokinetic profile by increasing serum half-life, interactions with other proteins may be disadvantageous and result in toxic side effects that have been observed when higher doses of phosphorothioates were applied (Levin 1999).

Due to the problems associated with phosphorothioates, other types of modifications have been developed. Nucleotides of the second generation carry a methyl or methoxy-ethyl group at the 2′ oxygen of the ribose (Fig. 2). In recent years, numerous nucleic acid analogs have been developed for applications in AS technology (Kurreck 2003). The types of alterations range from substitutions of functional groups of the ribose by fluoro- or amino-groups over bi- or tricycle nucleotides, to a complete replacement of the ribose-phosphate backbone by peptide bonds. Most of these nucleotides are less toxic and have a higher target affinity than phosphorothioates, but they lack the ability to induce RNase H cleavage of the complementary mRNA. Therefore, so-called gapmers have been developed, which consist of modified nucleotides at both ends to protect the ON from exonucleases and a central stretch of DNA or phosphorothioate monomers that is sufficient to activate RNase H.

Due to their favorable properties, locked nucleic acids (LNAs, Fig. 2) have increasingly been used for AS applications in recent years (Jepsen and Wengel 2004; and the chapter by S. Kauppinen et al., this volume). Gapmers consisting of LNA and DNA monomers in the center were found to exhibit desirable properties like improved nuclease stability and enhanced target affinity (Kurreck

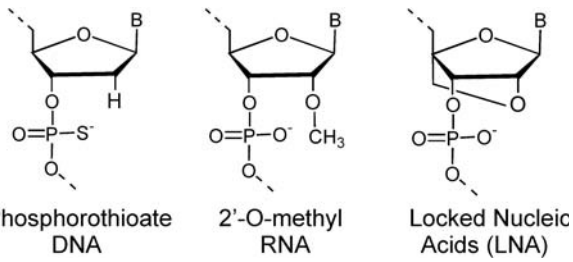

Fig. 2 Modified nucleotides that have been widely used for antisense approaches

et al. 2002) resulting in an almost 200-fold higher potency compared to an isosequential DNA ON (Grunweller et al. 2003).

An important hurdle that has to be overcome for successful AS applications is the cellular uptake of the ONs. DNAs are highly charged molecules that cannot cross hydrophobic membranes efficiently. Therefore, transfection agents are employed to facilitate entry of AS ONs into cells. The most widely used reagents are lipids with positively charged headgroups that neutralize the negative charge of the ONs. The ON–liposome complexes are thought to be taken up by endocytosis. Intracellular release of the ONs can be facilitated by the addition of helper lipids that interfere with the endosomal membrane. Further details about the use of cationic lipids as well as other types of transfection agents are described in a recent overview by Seksek and Bolard (2004).

2.2 Antisense Oligonucleotides as Antiviral Agents

A major advantage of AS ONs is their general applicability, because they can be directed against virtually any RNA of interest. Viruses with RNA genomes are particularly well suited to be targeted by AS ONs, since not only the mRNA but also the genomic RNA can be attacked and, at least theoretically, complete virus clearance can be achieved. In contrast, for DNA viruses or retroviruses with their proviral DNA stably integrated into the host genome, only mRNA (or newly synthesized genomic RNA in the latter case) can be targeted by AS ONs. Thus, only inhibition of virus spreading can be expected, and continuous treatment is required. Nevertheless, AS ONs have successfully been applied to inhibit many viruses of high medical relevance (McKnight and Heinz 2003). Due to space restraints, only a few recent examples can be discussed below to demonstrate the potential of AS ONs in treating virus replication.

Infections with HCV are a major health problem worldwide. Chronic infection with this plus-stranded RNA virus causes liver cirrhosis, liver failure, and hepatocellular carcinoma, often leading to the requirement of liver transplantation. Since current treatment of HCV is unsatisfactory, a need for new, specific anti-HCV drugs stands to reason. AS ONs have therefore widely been used with the intention of inhibiting HCV replication. The 5′-untranslated region (UTR) is one of the most highly conserved regions of the HCV genome and has most frequently been targeted with AS ONs. Since this region is strongly structured, intensive efforts were made to identify accessible target sites. The entire viral cycle of HCV is cytoplasmic and thus AS ONs do not necessarily need to be designed to activate RNase H, which is mainly located in the nucleus. Rather, it has been shown that AS ONs interfering with the assembly of a translation initiation complex on the internal ribosome entry site (IRES) inhibit translation of the viral polyprotein in cell-free translation assays and transfected hepatoma cell lines. Further details of the application of ON-based strategies to inhibit HCV are given in a review by Martinand-Mari et al. (2003).

With more than 40 million infected individuals worldwide, HIV is one of the most severe causes of infectious diseases. Although half of the substances that have been approved for the treatment of viral infections are intended to treat HIV infections, there is still an urgent need for new therapeutic approaches. Current drugs are not only too expensive for patients in poor countries; they also exert severe side effects upon long-term treatment and become ineffective due to the emergence of resistant mutants. Numerous efforts to prevent HIV replication with inhibitory ONs have been published (for a review, see Jing and Xu 2001). Only a few recent examples can be given here. As described for HCV, anti-HIV ONs are not necessarily required to induce virus RNA degradation by RNase H, as they may as well be employed to inhibit essential processes of the viral lifecycle by steric blocking. For example, Arzumanov et al. (2001) developed AS ONs against the HIV-1 *trans*-activating response region (TAR), a 59-residue stem-loop that interacts with the *trans*-activator protein Tat. Steric blockade of this interaction by a chimeric 2′-*O*-methyl RNA/LNA ON prevented full-length HIV transcription. In another study, the HIV-1 dimerization initiation site was chosen to be targeted by AS ONs (Elmén et al. 2004): An LNA/DNA mix-mer directed against this region prevented the dimerization of the genome and inhibited replication of a clinical HIV-1 isolate in a human T cell line.

Working on a different class of viruses, Yuan et al. (2004) have recently described the inhibition of coxsackievirus B3 (CBV-3) replication in cardiomyocytes and in mouse hearts. CBV-3 is a member of the plus-stranded picornavirus family. It can infect multiple organs of humans and is considered to be one of the major causes of viral myocarditis, which may develop into dilated cardiomyopathy and eventually lead to heart failure. No specific antiviral treatments exist for this important pathogen to date. The authors found AS ONs targeting the proximal terminus of the 3′-UTR to effectively inhibit CBV-3 replication in a cardiomyocyte cell line. The antiviral activity of the AS ON was further evaluated in a CBV-3 myocarditis mouse model, and a significant decrease of viral replication and virus titers was observed.

The examples given above as well as numerous further studies demonstrate the potential of AS ONs to act as specific antiviral agents. The AS approach is particularly appealing for diseases for which no satisfactory specific treatment is available. It is therefore not surprising that a rather high percentage of the AS ONs currently being tested in clinical trials are intended to treat patients with viral infections.

2.3 Antisense Oligonucleotides in Clinical Trials

Approximately 20 AS ONs have reached the stage of clinical testing, and one AS drug is currently on the market (a comprehensive overview is given in Crooke 2004). Here, we will focus only on those AS ONs that are directed against viral targets (Table 1): Isis Pharmaceuticals is working on a phosphorothioate AS

Table 1 Antisense oligonucleotides in clinical development to treat viral infections according to Crooke (2004) and companies' Web pages

Drug	Company	Virus	Status
Vitravene	Isis	CMV	Approved
ISIS 14803	Isis	HCV	Phase II
CpG 7909	Coley	HBV	Phase I/II
CpG 10101	Coley	HCV	Phase I/II
GPI-2A	Novopharm	HIV-1	Phase I
GEM92	Hybridon	HIV-1	Phase I
MBI 1121	Hybridon	HPV	Phase I

CMV, human cytomegalovirus; HBV, hepatitis B virus; HCV, hepatitis C virus; HPV, human papillomavirus; HIV, human immunodeficiency virus

ON targeting the IRES of HCV. In a phase II trial, patients with chronic HCV infection were treated with the AS ON, and several individuals experienced significant viral titer reductions. Further AS ONs were investigated with respect to their ability to treat HIV infections: GPI-2A, developed by Novopharm Biotech, exerted strange adverse effects in a phase I trial, most likely due to the cationic liposomal formulation used as delivery system. Hybridon is developing second-generation AS ONs targeting the gag gene of HIV-1 (GEM92) and the mRNA of the E1 protein of human papillomavirus (MBI 1121). Phase I trials with these ONs showed promising safety results and confirmed the possibility of second-generation AS ONs to be delivered orally. Further ONs are being tested in patients with HBV and HCV infections (CpG 7909 and CpG 10101 by Coley Pharmaceutical and 1018-ISS by Dynavax Technologies). The mechanism of action of the latter ONs is thought to be activation of the immune response via Toll-like receptors that recognize CpG motives rather than a classical AS mechanism (Agrawal and Kandimalla 2004).

The first AS drug approved by the U.S. Food and Drug Administration (FDA)—in 1998—is a phosphorothioate ON named Vitravene (Fomivirsen). This antiviral ON targets the RNA of the immediate-early mRNA of the human cytomegalovirus (CMV) mRNA and is intended to treat CMV-induced retinitis in immunodeficient patients with acquired immunodeficiency syndrome (AIDS). A major drawback of this drug is its mode of application, since it must be injected intravitreally. Although Vitravene meets an important need for affected patients, it is only of minor commercial significance. In late 2004 a second ON-base drug was approved by the FDA: Macugen is an aptamer that targets the vascular endothelial growth factor (VEGF) and provides an anti-angiogenic treatment for patients with the wet form of age-related macular degeneration, an eye disease that leads to loss of central vision.

3
Ribozymes

In the early 1980s, the research groups of Thomas Cech and Sidney Altman discovered RNA molecules that possess catalytic activity in the absence of any protein moiety. These *ribo*nucleic acids with en*zym*atic properties were named ribozymes. Meanwhile, several classes of ribozymes have been identified that can roughly be divided into large ribozymes consisting of several hundreds to thousands of nucleotides and small ribozymes that range from 30 to about 150 nucleotides in length (Doudna and Cech 2002). In addition to being fascinating objects of basic research, some ribozymes have been employed for medical purposes (for reviews, see Sullenger and Gilboa 2002; Schubert and Kurreck 2004). Here, we will focus on the use of ribozymes as antiviral agents.

Hammerhead and hairpin ribozymes are the most intensively studied and widely used ribozymes to date. Both classes were originally isolated from plant pathogens. Their application as molecular tools became possible only after the development of variants capable of cleaving target RNAs in a multiple turnover manner. Secondary structures of a hammerhead ribozyme targeting the HIV-1 tat gene and a hairpin ribozyme directed against a site in the 5′ long terminal repeat of HIV-1 are depicted in Fig. 3a and b, respectively. As described for AS ONs, ribozymes bind to their specific target RNA by Watson-Crick base pairing. In addition, they possess the capability of cleaving a complementary RNA molecule without the aid of cellular proteins.

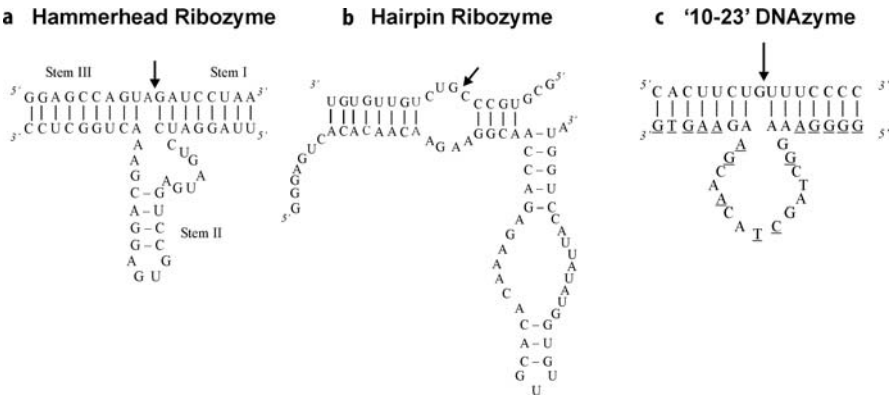

Fig. 3a–c Secondary structure of (**a**) a hammerhead ribozyme targeting HIV-1 *tat* gene (Ngok et al. 2004); (**b**) a hairpin ribozyme engineered to cleave a site in the 5′ long terminal repeat of HIV-1 (Ojwang et al. 1992); and (**c**) a 10–23 DNAzyme against a consensus sequence in the 5′-UTR of picornaviruses (Schubert et al. 2003). The optimized DNAzyme containes 2′-*O*-methyl RNA monomers at the underlined positions. *Arrows* indicate cleavage sites

3.1
Development of Active Ribozymes

The challenges that have to be faced when developing therapeutic ribozymes are similar to those for the optimization of efficient AS ONs, but some additional difficulties have to be met. At first, a suitable target site has to be identified. This region has to be accessible to the ribozyme employed and, in addition, it has to fulfill certain sequence requirements for efficient cleavage by ribozymes. For example, the hammerhead ribozyme cleaves preferably after AUG or GUC triplets, whereas the 10–23 DNAzyme described below processes any junction of a purine and pyrimidine.

The second important step is to stabilize the ribozyme, since RNA molecules are even more susceptible to nucleolytic degradation than DNA ONs. This task is complicated by the fact that the introduction of modified nucleotides very often leads to a severe loss of catalytic activity. Beigelman and colleagues (1995) performed a systematic optimization study for hammerhead ribozymes and developed a design with modified nucleotides in all positions except for five essential ribonucleotides. This molecule retained high catalytic activity and displayed a serum half-life of more than 10 days. It has also been used in clinical trials, as will be described in Sect. 3.3.

To date, only catalytically active ONs composed of RNA have been discovered in nature. Enzymatic DNA ONs, referred to as DNAzymes, DNA enzymes, or deoxyribozymes, however, have been obtained by in vitro selection, since they can be expected to be more stable against nucleases. The most prominent representative of this class of molecules is the so-called '10–23' DNAzyme (Santoro and Joyce 1997), the secondary structure of which is shown in Fig. 3c.

Although catalytically active ONs consisting of DNA have a higher intrinsic resistance against nucleolytic degradation, they still need further protection for applications in biological systems. We have systematically improved the properties of a 10–23 DNAzyme targeting the 5′-UTR of the human rhinovirus (Schubert et al. 2003). The resulting DNAzyme, containing modified nucleotides (underlined) at both termini and in the catalytic core, is depicted in Fig. 3c. It displays a tenfold increased catalytic activity under multiple turnover conditions and a substantially improved resistance against endo- and exonucleases.

The DNAzyme was directed against a consensus sequence of picornaviruses, which occurs not only in the rhinovirus but also in various polio-, echo-, entero-, and coxsackieviruses. Surprisingly, the unmodified DNAzyme that cleaves the rhinovirus RNA with reasonable rates was incapable of cleaving coxsackievirus A21 RNA, despite full sequence homology and a similar overall structure. After the introduction of nucleotides with high target affinity (2′-*O*-methyl RNA and LNA), however, the DNAzyme regained the capability of cleaving the coxsackievirus RNA with high catalytic turnover rates by successfully competing with local structures of the target RNA (Schubert et al.

2004). The modified DNAzyme can now be used to degrade a broad range of picornavirus RNAs. Furthermore, we demonstrated that the strategy of introducing nucleotides with high target affinity may generally be applicable to cleave seemingly unsuitable target sites.

As described for AS ONs, cellular uptake of ribozymes and deoxyribozymes has to be enhanced by the use of transfection reagents. Ribozymes, however, cannot only be exogenously applied as chemically presynthesized molecules, but they can also be encoded on plasmids and expressed endogenously. This opens the road to the use of viral vectors that have been employed for gene therapeutic purposes, e.g., retroviruses, adenoviruses, and adeno-associated viruses.

3.2
Cleavage of Viral RNA by Ribozymes

Ribozymes and deoxyribozymes have widely been used to inhibit virus replication in cell culture and in vivo. Here, only a few examples will be discussed; a comprehensive overview has recently been given by Peracchi (2004). Early on, various regions of HIV-1 have been targeted by hammerhead and hairpin ribozymes (e.g., Zhou et al. 1994; Ojwang et al. 1992). Meanwhile, the protective effect of ribozymes against HIV-1 infection has also been demonstrated in vivo: In a SCID-hu mouse model, $CD34^+$ hematopoietic progenitor cells transduced with anti-HIV-1 *tat-rev* or *env* hammerhead ribozymes as well as Rev aptamers were significantly protected against HIV-1 infection upon challenge (Bai et al. 2002). The 10–23 DNAzyme has also been successfully employed to inhibit HIV-1 by either directly targeting the viral RNA (Zhang et al. 1999) or by preventing virus entry by downregulation of the CCR5 coreceptor (Goila and Banerjea 1998).

In addition to HIV-1, hepatitis B and C viruses have been major targets of antiviral ribozymes. For example, a chemically stabilized hammerhead ribozyme has been designed that targets the highly conserved 5′-UTR of HCV. In cell culture, it inhibited replication of an HCV/poliovirus chimera by up to 90% (Macejak et al. 2000). This ribozyme, dubbed Heptazyme, has subsequently been employed in clinical trials as will be outlined below. A different approach has been developed to generate a ribozyme targeting HBV by expression of a triple-ribozyme cassette that undergoes intracellular self-processing (Pan et al. 2004). This sophisticated construct was suitable to reduce viral DNA in the liver of transgenic mice by greater than 80%, as measured 2 weeks after infection. Furthermore, a DNAzyme containing phosphoroamidate nucleotides was capable of inhibiting the replication of influenza A viruses by more than 99% (Takahashi et al. 2004).

3.3 Ribozymes in Clinical Trials

Several ribozymes have already been tested in clinical trials (summarized in Sullenger and Gilboa 2002; Schubert and Kurreck 2004). Most of these studies were intended to treat viral infections, while some ribozymes were used in attempts to inhibit cancer growth. For clinical trials, ribozymes were either expressed intracellularly from vectors or delivered as chemically presynthesized ONs.

The first phase I clinical trials in the mid 1990s were conducted with retroviral vectors that delivered hammerhead and hairpin ribozymes targeting HIV-1 RNA. Lymphocytes or hematopoietic precursor cells that had been isolated from infected individuals or their healthy twins were treated with the ribozyme vectors ex vivo. After selection and expansion, the transduced cells were infused into the bloodstream of the infected patient. The treatment was supposed to increase the resistance of hematopoietic cells against spreading of the virus. Although the procedure was found to be safe and ribozyme expression in transduced cells could be detected, the duration of the protective effect was unsatisfactory. It will therefore be necessary to develop and improve methods to transduce pluripotent hematopoietic stem cells in order to achieve long-term resistance against HIV (Michienzi et al. 2003).

In addition to these studies to treat patients with HIV infections, one clinical trial was initiated with a hammerhead ribozyme targeting HCV. In contrast to the trials described in the preceding paragraph, in which viral vectors were employed to deliver ribozymes, the aforementioned chemically presynthesized ribozyme Heptazyme was used for these tests (Usman and Blatt 2000). Encouraging results of initial clinical studies led to the initiation of a phase II trial with Heptazyme alone and in combination with interferon (IFN) in 2001. The finding that the HCV RNA level in the serum of patients treated with the ribozyme was reduced by only 10%, along with results from toxological studies, led to the decision to stop clinical experimentation of Heptazyme (Peracchi 2004).

Taken together, ribozymes to treat viral infections or cancer were well tolerated in clinical settings, but their therapeutic efficiency was rather low. Recent findings indicate that the minimized ribozyme variants used in these trials lacked sequence elements that are essential for high catalytic activity under physiological conditions (Khvorova et al. 2003a). Since a new technique believed to be much more efficient has emerged with the discovery of RNA interference (RNAi), most approaches to develop RNA-based therapeutics have now been switched to the use of siRNA rather than ribozymes.

4
RNA Interference

RNAi denotes the disruption of RNA molecules induced by double-stranded (ds)RNA of the same sequence. The process was first identified in the nematode *Caenorhabditis elegans* (Fire et al. 1998). Subsequent work elucidated the biochemical basis underlying this mechanism (for a recent review, see Meister and Tuschl 2004): Long dsRNA is cut up by an RNase III-type endonuclease termed Dicer, resulting in 19mer RNA duplexes with symmetrical two-nucleotide overhangs at both 3′-ends, known as "small interfering RNAs" (siRNAs). One of the strands, preferably the AS strand, is subsequently incorporated into a proteinaceous complex called "RNA-induced silencing complex," RISC, whose exact composition is still under investigation. The RNA strand programs RISC to act specifically on RNAs of the complementary sequence. The targeted molecule is bound and cleaved in the center of the target sequence. Afterwards, the damaged RNA molecule is quickly degraded by cellular nucleases, while RISC can go on through several rounds of cleavage (Haley and Zamore 2004). In contrast to RNase H-mediated cleavage induced by AS ONs, this process takes place predominantly in the cytoplasm (Fig. 1).

The overall mechanism of gene silencing mediated by dsRNA is conserved in virtually all eukaryotes. In mammals, however, the presence of dsRNA exceeding 30 bp in the cytoplasm can initiate the innate IFN immune response, resulting in extensive cell death. This unspecific reaction, which has hampered the use of RNAi in mammalian cells, can be avoided if 21mer siRNA molecules are employed (Elbashir et al. 2001). siRNAs were shown to be extremely potent inhibitors of target gene expression (e.g., Grunweller et al. 2003). They have meanwhile been used to knock down a plethora of individual genes in cell culture experiments in order to investigate gene function or to trace molecular pathways (Silva et al. 2004). RNAi has also been applied in mouse models of human disease (summarized in Sioud 2004; and the chapter by M. Sioud, this volume). Comprehensive overviews on the current status and application of RNAi methods are given in numerous recent reviews (e.g., Mittal 2004; Dorsett and Tuschl 2004).

4.1
Designing Efficient siRNA Approaches

4.1.1
Selection of Efficient siRNAs and Suitable Targets

Targeted degradation of an RNA molecule triggered by siRNAs is a multistep process. At least two factors are critical to its efficacy:

1. siRNA structure: The assembly of the RNAi enzyme complex RISC has been shown to be dependent on thermodynamic characteristics of the siRNA

with respect to the relative stability of both ends of the duplex (Khvorova et al. 2003b; Schwarz et al. 2003). Based on these findings, several algorithms have been developed to predict efficient siRNAs (e.g., Reynolds et al. 2004).

2. Accessibility of the target sequence: We and others have observed that even a well-designed siRNA with excellent thermodynamic features may show no silencing activity when the target region is sequestered in stable internal structures (Kretschmer-Kazemi Far and Sczakiel 2003; Schubert et al. 2005b). For example, targeting of the 5′-UTR of picornaviruses has turned out to be particularly inefficient (Schubert et al. 2005a; Phipps et al. 2004; Saleh et al. 2004). This region is known to be highly structured, and it associates with cellular and viral proteins, possibly precluding siRNAs from hybridizing. Similarly, some viral RNA species have been found to be resistant to degradation by RNAi: siRNAs directed against HIV-1, rotavirus, and respiratory syncytial virus each afforded degradation only of the mRNA species of the viruses, but did not interfere with viral genomic RNA of the same sequence (Bitko and Barik 2001; Arias et al. 2004; Hu et al. 2002). This observation is most likely due to extensive association of the viral genomic RNAs with proteins or its localization to compartments inaccessible to RISC.

4.1.2 Delivery of siRNAs

Looking back on 20 years of effort with the delivery of ribozymes and AS ONs, researchers today can benefit from a rich body of experience regarding ON transfer into cells (see above and Seksek and Bollard 2004 for a review). Similar to ribozymes, siRNAs can be delivered as presynthesized dsRNA molecules, or they can be expressed intracellularly from plasmids. Transfection efficiency for presynthesized siRNAs is usually quite high in standard cell lines, but the silencing effect wears off in a matter of days as the intracellular concentration of siRNA decreases due to cell division and degradation by cellular nucleases. In addition, siRNAs have to be modified chemically to increase their nuclease resistance in body fluids.

To achieve longer lasting knockdown, plasmid vectors have been designed that afford continuous expression of short hairpin (sh)RNAs, which are intracellularly processed to give siRNAs (Fig. 4; Mittal 2004). The use of plasmid vectors allows for the selection of stably transfected cells that have been shown to exert gene-silencing activity, even 2 months after vector delivery (Brummelkamp et al. 2002). Delivery of plasmid DNA using standard transfection protocols, however, is often unsatisfactory, particularly when primary cells or stem cells are concerned. More efficient uptake can be achieved by exploiting the natural ability of viruses to infect host cells. Oncoviruses, lentiviruses, adenoviruses, and recombinant adeno-associated viruses are the viral classes most often employed as shuttles for delivery of transgenes into cells. An excel-

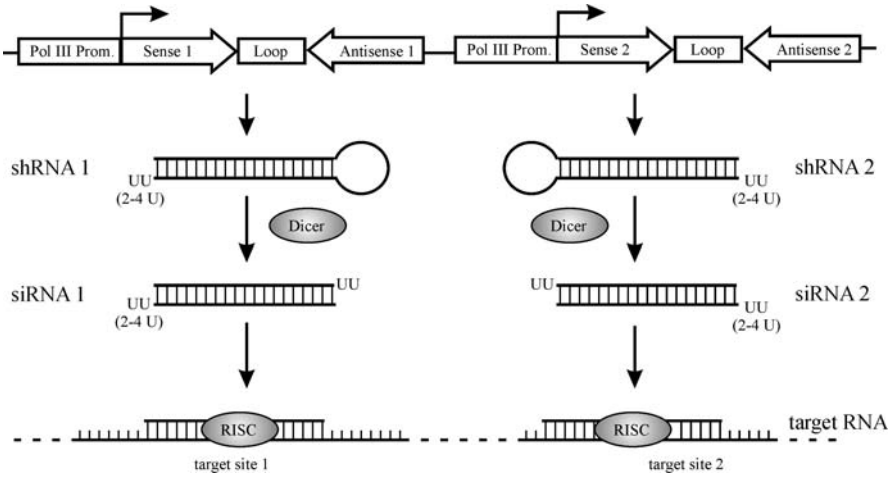

Fig. 4 Vector expression of siRNAs. In the first step, a self-complementary RNA, named short hairpin RNA (shRNA), is expressed under control of a polymerase III promoter. The shRNA is subsequently processed to give an siRNA by the endonuclease Dicer. An siRNA double expression (SiDEx) vector can be employed to express two shRNAs directed against different sites on the same target RNA simultaneously (Schubert et al. 2005a). Thus, mutations in one of the target sites leading to abrogation of silencing can be compensated by the unaltered efficiency of the second siRNA (for further details, see text)

lent comprehensive overview on viral delivery systems is given in the paper by Thomas et al. (2003).

Systemic delivery into mice is usually performed by injecting the siRNA into the tail vein in a large volume of liquid using high pressure, a method termed hydrodynamic tail vein injection (Fig. 5). siRNAs are subsequently found to be enriched in the liver. High-pressure injection, however, is not applicable to humans, and more subtle ways of systemic administration need to be sought. One of the most promising reports yet has come from Alnylam Pharmaceuticals. In their study, an siRNA was conjugated with a cholesterol molecule. This construct had substantially improved pharmacokinetic properties and efficiently reduced plasma levels of endogenous apolipoprotein B in mice after intravenous administration (Soutschek et al. 2004).

4.1.3 Specificity of siRNAs

siRNAs are considered highly specific molecular tools, but their capacity to discriminate against mismatches seems to be dependent on the siRNA sequence and position of the mismatched nucleotides. Unspecific effects of siRNAs have been described in several reports. Semizarov et al. (2003) found induction of genes related to stress and apoptosis at higher siRNA concentrations. Further-

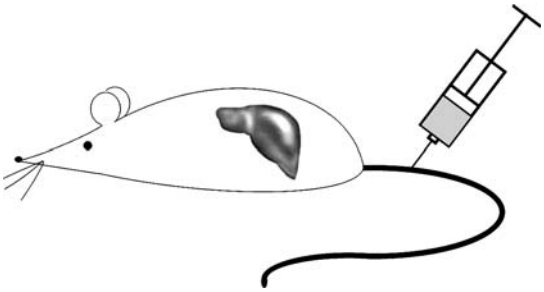

Fig. 5 Hydrodynamic tail vein injection: siRNAs are rapidly injected into the tail vein of mice in a large volume of fluid. The siRNAs accumulate preferentially in the liver

more, off-target effects were detected by expression profiling, and induction of the IFN response by shRNAs or siRNAs has also been reported (Jackson et al. 2003; Bridge et al. 2003; Sledz et al. 2003). Moreover, siRNAs were shown to inhibit translation of partially complementary mRNA molecules by imperfect hybridization in a microRNA-like manner (Doench and Sharp 2004; Scacheri et al. 2004). Most siRNAs will have partial complementarity with one or more human genes and are thus prone to unintended gene silencing. It is therefore advisable to employ only highly efficient siRNAs, so that low doses yield sufficient results and side effects are expected to be minimized. For a further discussion of relevant issues concerning efficiency and specificity of RNAi approaches, see Hannon and Rossi (2004).

4.1.4 Viral Escape

Viruses often elude long-term inhibition by acquiring mutations that render the antiviral compound ineffective. This problem has been observed in RNAi approaches for sustained inhibition of both poliovirus and HIV-1 (Gitlin et al. 2002; Boden et al. 2003; Das et al. 2004). Although the siRNAs employed in these studies inhibited viral propagation initially, virus titers increased again upon prolonged incubation. Analysis of the resistant mutants revealed either a point mutation in the target region or extensive rearrangements of the viral RNA. Several strategies may be useful to prevent the enrichment of escape mutants. One is targeting of conserved sequences that are less likely to yield viable virus after mutation. Another is reducing levels of host cellular genes necessary for viral entry or propagation, which has led to substantial reduction of virus titers (Zhang et al. 2004; Novina et al. 2002). Cellular genes are less prone to mutation and are therefore less likely to allow viral escape of silencing. When pursuing such an approach, however, the risk of side-effects must obviously be addressed thoroughly. In analogy to conventional combination therapy, a third strategy to prevent escape-mutant enrichment involves two or more siRNAs being used

simultaneously. We have recently described an siRNA double expression vector (SiDEx) containing genes for two different siRNAs both targeted against the RNA-dependent RNA polymerase of coxsackievirus B3 (Schubert et al. 2005a; Fig. 4). Even when an artificial point mutation was introduced into the target gene so that the respective mono-expression vector was no longer capable of silencing, the double expression vector maintained high silencing activity. Although we did not find any deleterious effect of the simultaneous use of two siRNAs, it has been observed that different siRNAs present in one cell may compete for access to the RNAi machinery (Bitko et al. 2005). Thus, the efficiency of one siRNA may be compromised due to the presence of a second species. Another difficulty in preventing viral escape may be the amount of viral particles present in the blood of patients in the state of viremia. In HCV infection, up to 10^7 virions per milliliter may be present in the bloodstream, so that even 90% reduction of the titer may still leave enough virus particles intact to allow escape. The highest efficiency knockdown is therefore even more desirable.

4.2 Antiviral RNAi Approaches

A vast body of studies reporting the employment of RNAi for antiviral purposes in mammalian cells has already been published. The extent of inhibition of viral propagation measured in these studies is dependent on several factors, some of which are choice of cell line, virulence and titer of the virus strain, siRNA delivery method, time between infection and siRNA delivery, and the time and method of readout and detection. It is thus difficult to make quantitative comparisons between the studies or to draw conclusions as to a possible clinical utility of individual compounds from cell-culture experiments. In the following paragraphs, some studies will be discussed that are likely to lead to clinical applications in the nearer future. We are aware of the fact that our selection is biased. Comprehensive overviews of the classes of viruses that have been targeted by RNAi are provided in the papers by Haasnoot et al. (2003) and Ryther et al. (2005).

4.2.1 Respiratory Diseases

Influenza is a major threat for societies worldwide. At least 20 million people died in the catastrophic outbreak of the virus in 1918. Influenza viruses are enveloped, single-stranded RNA viruses whose genome is segmented into eight parts of negative polarity. They are notorious for changing their outward shape due to small mutations in the principal antigens. Thus, the virus may escape protective immunity induced by a previously prevalent viral strain. Vaccination can provide effective protection against influenza virus infection.

In older or immunocompromised people, however, vaccination's efficacy is rather limited. Recently, warnings about the threat of an influenza pandemic have been discussed in a news focus by Enserink (2004).

Ge and colleagues (2003) tested the antiviral effect of 20 siRNAs directed against conserved regions of influenza A virus in cell culture and embryonated chicken eggs. Three of these siRNAs were potent inhibitors of viral propagation. While one siRNA reduced only the level of its specific target mRNA, two others resulted in reduced levels of all viral RNA species. The authors conclude that the targeted proteins have critical roles in viral transcription and replication.

About 1 year later, successful application of the previously identified siRNAs in mice was reported in two studies. Tompkins and coworkers (2004) administered siRNAs by hydrodynamic tail vein injection followed by viral infection 16–24 h later. After 2 days, virus titers in the lungs were 10- to 56-fold lower than in mice receiving an unrelated control siRNA. By day 18 post challenge, 60% of the animals treated with control siRNA had died, whereas mortality was reduced to 10%–20% in siRNA-treated animals. A combination of both siRNAs yielded 100% survival.

In the second study, siRNAs were administered complexed with polyethyleneimine (PEI) by intravenous injection, a method compatible with use in humans (Ge et al. 2004). PEI-complexed nucleic acids were found predominantly in the lungs. Reduction of virus titers 24 h after infection was approximately tenfold, even when siRNAs were administered 5 h after infection. Also, plasmid DNA coding for shRNAs was found to inhibit viral propagation in mice when administered intravenously in complex with PEI or instilled in the lungs.

Recently, intranasal administration of siRNAs to the lungs has been reported by two groups (Bitko et al. 2005; Zhang et al. 2005). The siRNAs were directed against different regions of respiratory syncytial virus (RSV), a major respiratory pathogen of the family Paramyxoviridae that contains nonsegmented (−) strand RNA genomes (Barik 2004). When 5 nmol of an efficient siRNA was administered intranasally with or without transfection agents, viral titers were reduced in the lungs of mice by up to three orders of magnitude. In addition, siRNA-treated mice showed no signs of respiratory distress during up to 6 weeks of observation. When used against an ongoing infection, the antiviral effect decreased, but remained clearly visible even when siRNAs were given 4 days after viral challenge.

The siRNA applied by Zhang and coworkers (2005) was directed against the viral NS1 gene that antagonizes IFN-β signaling in the host. shRNA-expressing plasmids complexed with nanochitosan, a natural, biodegradable polymer, were delivered 2 days before viral inoculation. NS1 expression in the lungs was diminished 18 h later, whereas other viral genes remained unaffected. This observation lends further proof to the finding discussed above, namely that the viral genomic RNA may be inaccessible to RNAi. IFN-β levels in the lung tissue of mice were significantly increased in animals that received anti-NS1 shRNA before infection. Treatment with siRNA up to 3 days after infection still

reduced virus titer in the lungs. Moreover, mice treated with NS1 shRNA 2 days after infection showed a substantial decrease in lung inflammation compared to control mice.

4.2.2 Viral Hepatitis

HBV is a partially double-stranded DNA virus of the Hepadnaviridae family that primarily infects the liver. Although efficient vaccines are available, an estimated one million people die every year of HBV-related diseases, and the number of carriers lays around 350 million. Chronic infection can give rise to liver cirrhosis and hepatocellular carcinoma. After having entered the cell, the viral genome is transported to the nucleus, where four RNA species are transcribed and exported to the cytoplasm. One 3.5-kb transcript, referred to as pregenomic RNA, also serves as a template for reverse transcription. RNAi cannot target the viral circular dsDNA that resides in cell nuclei of chronically infected patients, but interference with one or more of the mRNA species has been reported repeatedly. Replication of HBV is not supported by most animals. Therefore, replication-competent plasmids have been developed that mimic most of the steps of the viral life cycle in cell culture and small animals.

In what was one of the first reports on functional antiviral RNAi in mammals, McCaffrey and colleagues (2003) co-injected shRNA expression vectors and a plasmid containing an HBV replicon into the tail vein of mice, using hydrodynamic transfection. After 7 days, the livers of the animals were probed for replicated genomic viral DNA and RNA transcripts by Northern and Southern blotting. The shRNAs significantly reduced the level of HBV RNA and DNA. Also, the amount of HBV surface antigen HBsAg in serum was decreased by up to 85% using the most effective of the shRNAs under investigation. In addition, however, two unexplained observations were reported: (1) An shRNA directed against a site present only in the longest of the four mRNAs was found to reduce not only the level of the targeted mRNA, but also levels of the other viral transcripts. (2) Treatment with any shRNA including an unrelated control shifted the balance of single-stranded and double-stranded genomic DNA towards the single-stranded form. These data raise the possibility that shRNAs may have sequence-independent antiviral effects whose mechanisms are unknown as yet.

Chemically synthesized siRNAs were employed by Klein and coworkers (2003) for tail vein injection together with the replication-competent HBV plasmid. Both siRNAs inhibited expression of their target proteins in the liver of mice. While the effect of one of the siRNAs was sustained for at least 11 days, inhibition by the second siRNA ceased around day 5.

Uprichard and coworkers (2005) succeeded in clearing a preexisting HBV gene expression from the liver of mice. Antigen levels in the serum, RNA expression, and the presence of replicative DNA intermediates were all significantly reduced in transgenic mice containing an HBV-plasmid. shRNA-expressing

vectors were administered by hydrodynamic tail vein injection of recombinant adenoviruses. The antiviral effect was sustained for at least 26 days. Interestingly, in this study the level of 3.5-kb RNA was somewhat less reduced than the level of the 2.1-kb transcripts, although both species contained the target sites. The reason for this observation lends itself to some speculation.

Another major hepatic pathogen is HCV (see above), which also cannot easily be grown in cell culture. For this reason, plasmid replicons containing subgenomic fragments or the whole genome of HCV are used. To show the general applicability of antiviral RNAi approaches in mice, McCaffrey and colleagues (2002) used high-pressure tail vein injection to deliver a plasmid expressing a fusion construct of luciferase and the viral protein NS5B to the livers of mice. When siRNAs or shRNA-expressing plasmids were cotransfected with the reporter plasmid, 80% and 90% reduction of luciferase expression over the whole body of the animals were measured, respectively. Several groups have reported on siRNAs directed against different regions on the mRNA of HCV that reduced viral RNA and protein levels (for a listing, see Radhakrishnan et al. 2004; Randall and Rice 2004). Randall and colleagues (2003) found a greater than 98% inhibition of RNA levels on transfection of an efficient siRNA. This decrease may have its cause either in equal reduction of HCV RNA in all cells, or in complete destruction of viral RNA in most of the cells. Using immunofluorescence techniques, the authors found that indeed almost all cells were completely clear of the HCV NS5A protein. Few cells, however, fluoresced brightly, indicating that no detectable reduction in HCV levels had taken place. To achieve sustained inhibition, stable transfection of shRNA expression vectors has been performed. Wilson and colleagues (2004) found a 70% reduction of HCV-gene expression when cells were challenged with the replicon that had been stably transfected with shRNA 3 weeks earlier. Zhang and coworkers (2004) used adenoviral delivery to knock down cellular targets indispensable for HCV replication and achieved significant reductions in HCV-expressing cells.

4.2.3 HIV

The greatest body of work regarding antiviral RNAi approaches has been performed on HIV. As outlined above, efforts have already been taken to inhibit the spread of this devastating virus by means of gene therapy employing ribozymes. RNAi has proved significantly more potent than ribozymes in the reduction of virus titers. Therefore, employing shRNA-expressing vectors for gene therapy in settings that have been validated with ribozymes may boost the efficiency of the approach.

Numerous siRNAs and shRNAs have been directed against different regions on HIV RNA that successfully suppressed viral gene expression and replication (see, e.g., the comprehensive list given in Haasnoot et al. 2003; Hannon and

Rossi 2004). Early on, the question was raised which step of the viral life cycle siRNAs would interfere with. Jacque and colleagues (2002) tested a number of presynthesized siRNAs and plasmid-expressed shRNAs against the viral vif and nef genes as well as the long terminal repeat (LTR). These siRNAs suppressed the production of viral particles from an infectious HIV clone 20- to 50-fold. The authors also found a dramatic inhibition of viral genomic RNA, complementary (c)DNA, and integrated DNA. From these studies, it can be concluded that the siRNAs employed in this case inhibited the viral life cycle at an early stage. Somewhat different results, however, were presented by Hu and coworkers (2002) who found no reduction of viral cDNA in cells that had been transfected with potent siRNAs against viral gag and pol RNA. Coburn and Cullen (2002) used siRNAs against tat and rev and noticed a slight decrease in proviral DNA production that does not seem to be sufficient to explain the massive drop in viral gene expression brought about by the siRNAs. If and to what extent incoming viral RNA is accessible to RNAi is thus still under discussion.

Viral escape has been shown to be a major problem when trying to inhibit HIV-1 replication (see above). Several groups have therefore turned to silencing cellular genes necessary for viral propagation (Arteaga et al. 2003). Qin and colleagues (2003) transduced T lymphocytes from uninfected individuals with a lentiviral vector coding for an siRNA against CCR5, the viral coreceptor. A tenfold downregulation of the expression of the receptor was observed that led to a 3- to 7-fold protection against viral challenge. CCR5 may be a suitable target for interference with HIV-1 entry because it is not essential for normal immune function.

An elegant method of targeted delivery of a transgene coding for an siRNA is to isolate hematopoietic progenitor cells that give rise to the hematopoietic cells capable of being infected by the virus. These cells are then transduced with the transgene of choice and reinfused into the patient. Banerjea and coworkers (2003) have used a lentiviral vector to introduce an anti-rev siRNA into primary $CD34^+$ hematopoietic progenitor cells. The cells were subsequently expanded in SCID-hu thy/liv grafts. After 60 days, the mice were sacrificed and thymocytes were collected. In one animal, 3% of the cells were positive for the transgene, whereas another animal showed 53% positivity. The in vivo-derived thymocytes that were positive for the transgene were subsequently enriched and challenged with HIV-1. siRNA-expressing thymocytes showed significant protection against the viral challenge as long as 10 days post infection.

Because of the significant experience obtained with anti-HIV ribozymes and the high relevance and urgent need for new and cheap HIV-therapeutics, human clinical trials employing the RNAi approach against viruses are expected to begin soon (Hannon and Rossi 2004).

4.2.4
Coxsackievirus B3

In our own research, we focus on the siRNA-induced inhibition of CBV-3, which is a major myocardial pathogen that causes severe heart muscle infections (see Sect. 2.2). In our opinion, CBV-3 is particularly well suited to be targeted by siRNAs, since the virus has a cytoplasmic lifecycle. Complete virus clearance by RNAi can be expected only for this type of virus, because viral DNA genomes or DNA intermediates of retroviruses are not susceptible to RISC-mediated cleavage (Fig. 1). Initial experiments with siRNAs directed against the 5′-UTR of CBV-3 were unsuccessful, most likely due to the stable secondary structure of this region of the viral genome. Subsequent efforts to target the 3D RNA-dependent RNA polymerase with siRNAs, however, led to a concentration-dependent inhibition of the virus. A plaque assay revealed a reduction of virus propagation by one $\log_{10}$ step (Schubert et al. 2005a). Another aspect supports the idea to use RNAi approaches against coxsackieviruses. In a clinical phase II trial, Kühl et al. (2003) demonstrated that IFN-β treatment eliminates cardiotropic viruses and improves left ventricular function. The effect of siRNAs is more specific than that of IFN-β, but the observed induction of the IFN response by siRNAs (Sledz et al. 2003), an undesired side effect in most cases, may prove beneficial in this application.

4.3
From Bench to Bedside

In evaluating the state of development that RNAi techniques have achieved, we must ask: How long will it take until the first drug is approved that acts by means of RNAi? Enthusiasm about this novel and highly efficient technique and skepticism about its applicability are in balance for the time being. Expectations may be somewhat limited, as 20 years of research on the medical application of AS molecules has led to no more than a single approved AS drug of minor commercial significance and one approved aptamer.

A number of companies like Alnylam Pharmaceuticals, Benitec, International Therapeutics, CytRx Corporation, Sirna Therapeutics, and Nucleonics are developing siRNAs for the treatment of viral infections such as HIV, HCV, and HBV, according to the companies' respective Web sites. Two companies, Sirna Therapeutics and Acuity Pharmaceuticals, have recently initiated phase I clinical studies of siRNAs interfering with vascularization for treatment of age-related macular degeneration. Results from these studies would be the first clinical data available for siRNAs and may allow better-founded predictions on the future of RNAi in therapeutic approaches.

5 Conclusions

ONs are a valuable alternative to low molecular weight compounds for the treatment of viral infections. AS ONs and ribozymes have been used for many years to inhibit virus replication. Some of the most advanced molecules have even been evaluated in clinical trials, and the only approved antisense drug to date is an antiviral AS ON. Despite being well tolerated in the doses employed, AS ONs and ribozymes frequently failed to provide convincing evidence for their therapeutic benefit. The more recently developed RNAi technology, however, is likely to overcome most of the problems of the aforementioned approaches due to its higher efficiency. Only three and a half years after the initial description of gene silencing by siRNAs in mammalian cells, the first clinical trials based on the principle of RNAi have commenced. Furthermore, siRNAs against cancer and viral targets can be expected to enter the clinic soon. One of the major problems of long-term inhibition of viruses is their ability to escape any kind of treatment due to their high mutation rate. It will therefore be advantageous to select sequences of the virus that are highly conserved. In addition, several distinct regions of the virus should be targeted simultaneously with either siRNA double expression (SiDEx) vectors (Schubert et al. 2005a) or by combining different types of antiviral agents, e.g., ribozymes and siRNAs (Michienzi et al. 2003). After the principle establishment of ONs as antiviral agents, these techniques will offer the opportunity to rapidly develop inhibitors of newly discovered viruses or variants of well-known viruses.

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