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REVIEW ARTICLE

RNA interference for antiviral therapy

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Summary

Silencing gene expression through a process known as RNA interference (RNAi) has been known in the plant world for many years. In recent years, knowledge of the prevalence of RNAi and the mechanism of gene silencing through RNAi has started to unfold. It is now believed that RNAi serves in part as an innate response against invading viral pathogens and, indeed, counter silencing mechanisms aimed at neutralizing RNAi have been found in various viral pathogens. During the past few years, it has been demonstrated that RNAi, induced by specifically designed double-stranded RNA (dsRNA) molecules, can silence gene expression of human viral pathogens both in acute and chronic viral infections. Furthermore, it is now apparent that in *in vitro* and in some *in vivo* models, the prospects for this technology in developing therapeutic applications are robust. However, many key questions and obstacles in the translation of RNAi into a potential therapeutic platform still remain, including the specificity and longevity of the silencing effect, and, most importantly, the delivery of the dsRNA that induces the system. It is expected that for the specific examples in which the delivery issue could be circumvented or resolved, RNAi may hold promise for the development of gene-specific therapeutics. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords acute viral infection; chronic viral infection RNA interference; antiviral therapy; gene therapy

Introduction

The battle against viral infections is ferocious. Since viruses are developing resistance to therapy, novel antiviral therapeutic modalities are in great demand. The currently approved antiviral therapies are based on the use of small molecular weight drugs, utilization of proteins simulating the innate immune response, and the adaptive immune system for both passive and active vaccination [1]. Recently, an antisense drug against viral infection has also been approved, suggesting that newly developed approaches are acceptable. The first drug using antisense technology, Fomivirsen (Vitravene), developed by Isis Pharmaceuticals, was approved for the treatment of cytomegalovirus (CMV) retinitis. In general, small molecular weight drugs are mainly used today for chronic viral disease, although there are exceptions such as the anti-influenza compound, Oseltamivir/Tamiflu, which is used for acute infection. Passive and active vaccination approaches are being developed for the prevention of severe acute diseases (in this case, there are also exceptions, such as life administration of anti-hepatitis B virus (HBV) antibodies to liver transplant patients at risk for HBV infection recurrence). The development and use of specific antiviral drugs and vaccines have been slower than expected and face major challenges. One particular example is the sluggish progress in generating effective drugs against the hepatitis C virus (HCV), even though the genomic sequence of the virus was unfolded over 15 years ago. HIV drug development

also faces major drawbacks. Although HIV replication is efficiently inhibited by the combination of highly active antiretroviral therapy (HAART), long-term complications of HAART include significant morbidity on the one hand, and the generation of multi-drug-resistant HIV strains on the other in a large proportion of patients.

In spite of the comprehensive advancement in understanding the biology of the immune response, translating these findings into rational therapeutic platforms remains slower than expected. The need for preventive and effective vaccines remains as much a requisite today as it was in previous times in the history of mankind. Indeed, at the beginning of the previous century, the world suffered devastating viral infection pandemics such as the one that occurred in 1918 where a quarter of the world population fell ill and the death toll reached over 25 million people from acute influenza infection. Today, the achievement of peak vaccine efficacy to treat influenza stands at 75% among those under 65 years old and just 35% among the elderly. Even today, the annual death toll from acute influenza infection in the USA tops 20,000 in spite of national vaccination programs. Thus, different strategies are currently being suggested in an effort to be prepared for future pandemics [2], and these include the development of escape mutants (antigenic drift) and reassortment of genetic segments of different quasispecies of the same virus or of different viruses (antigenic shift). Presently, human viral pathogens are spreading worldwide, such as the much publicized menacing spread of the avian flu which is reported to have expanded to remote sites of Russia and Kazakhstan from the South-East, posing a major health threat to the entire world.

In an effort to identify a novel class of efficient antiviral therapeutics, numerous technologies are currently being assessed for their antiviral potential. These include antisense oligonucleotides, antisense phosphorodiamidate morpholino oligonucleotides, ribozymes and, in recent times, RNA interference (RNAi). Incidentally, we have been encouraged to learn that siRNA (short interfering RNA) against vascular endothelial growth factor (VEGF) has recently been administered to patients in a clinical study without major side effects (ASGT meeting, 2005). In this review, we will summarize the recent developments in the use of RNAi as an antiviral agent.

RNA interference

RNA interference (RNAi) is a sequence-specific silencing of genes, induced by small molecules of double-stranded RNA (dsRNA). This phenomenon was first observed in plants in the late 1980s, but its molecular mechanism remained unclear until it was discovered in 1998 by Fire *et al.*, in the nematode *C. elegans* [3]. They showed that the presence of a very small quantity of dsRNA led to almost a complete shut-off of the expression of the gene that was homologous to the dsRNA.

The interference process starts with cleavage of the dsRNA that induced it into small RNA duplexes, 21–23 nucleotides (nt) long, called siRNAs (short interfering RNAs) [4,5]. These small dsRNA duplexes have 2 nt overhang at their 3′ end, a 5′-monophosphate and a 3′-hydroxyl group [6]. The enzyme responsible for that first step is Dicer, a dsRNA-specific nuclease that belongs to the RNaseIII family, and acts in an ATP-dependent manner [7]. In the next step, the siRNAs generated by the Dicer are incorporated into the RNA-induced silencing complex (RISC), a multi-component enzymatic complex [5]. The RISC unwinds the siRNA in an ATP-dependent manner, and uses its single-strand form to target the homologous transcript by base-pairing interactions. It then cleaves the mRNA by its endonuclease component in a homology-dependent manner, only in the region corresponding to the sequence of the siRNA. This process leads to degradation of the mRNA [4,8].

siRNA-mediated gene silencing has also been found in lower organisms, such as plants, fungi, worms and flies [9]. It is a conserved mechanism of intracellular antiviral immunity that also protects the host genome from foreign genetic elements such as retroviruses, transposons and retrotransposons. These elements may have deleterious effects on the genomic DNA of the host, and thus their mRNA elimination may represent an earliest form of innate immunity.

RNAi was first suggested to evolve as a natural antiviral defense in plants, especially against RNA viruses [10,11]. In mammalians, RNAi has also been reported to have gene-silencing properties. The RNAi machinery was triggered experimentally by the introduction into the cells of artificially designed dsRNA molecules, 21 nt in length, and the target gene was inhibited in a sequence-specific manner [8]. This effect has become very effective in silencing and knocking-down expression of specific genes in the cells. siRNAs have become the method of choice for mammalian cell genetics as well as for potential sequence-specific therapeutic approaches.

Inhibition of viral infection by RNAi

The inhibitory action of siRNAs has been documented for numerous viruses. It works against RNA viruses with negative- or positive-strand genome polarity, as well as against DNA viruses. The siRNA, as a therapeutic tool, can be targeted against the various phases of the viral life cycle of DNA and RNA viruses including replication, transcription, assembly of new virions, and budding out of the target cells (Figure 1).

Inhibition of viral transcription by siRNAs

After entering the target cells, the virus has to transcribe its genome. In order to do so, the viruses use the host cell machinery, and many viruses also use their own

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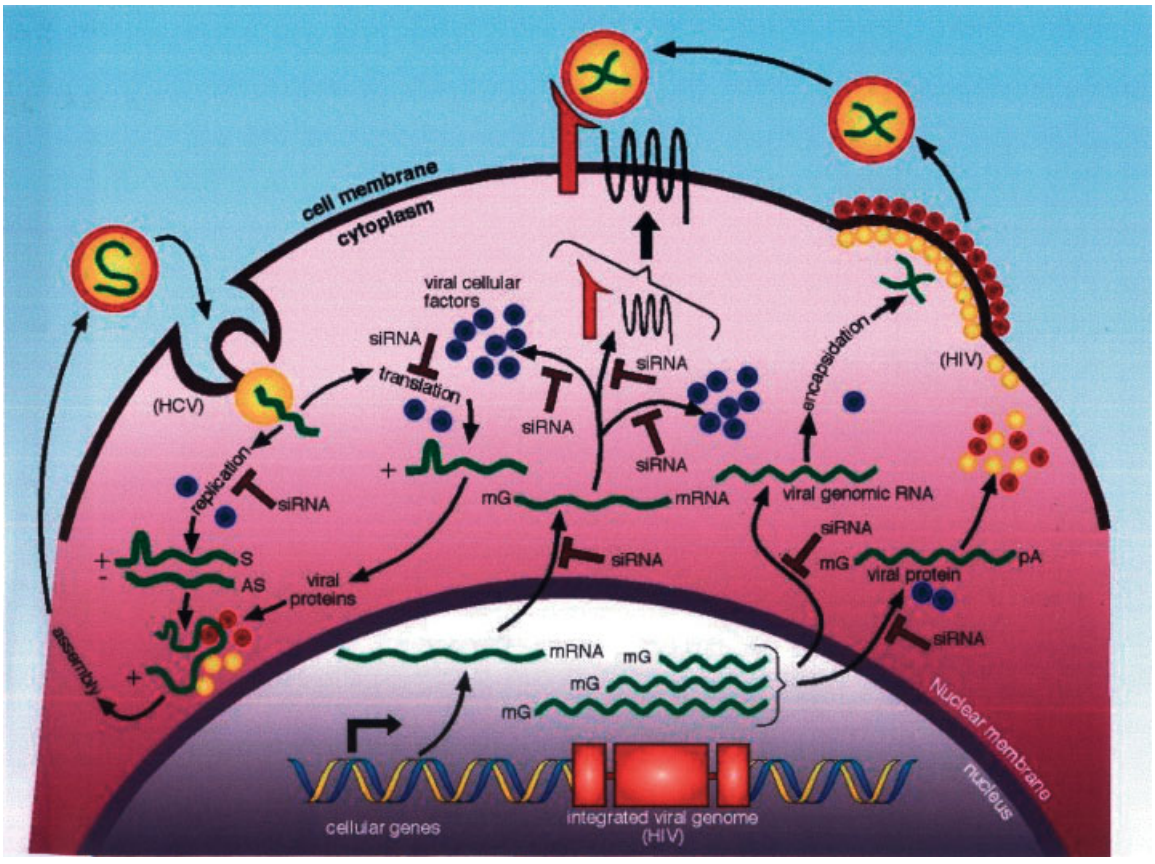

Figure 1. Potential sites that RNAi could be affecting viral infection. This is a simplified pictorial description of the host cells attacked by two types of RNA viruses. The left side of the illustration describes possible targets against an enveloped + strand virus, in this case HCV; the right side shows an integrating RNA virus, in this case HIV

proteins for transcription. Many RNA viruses encode their own transcription factors. For example, the gag, pol and env genes of retroviruses are needed for an efficient transcription. Indeed, siRNAs directed against the gag and env genes of HIV-1 [12,13] or the avian sarcoma leucosis virus [14] significantly reduce their overall transcription. Another example is the DNA human papilloma virus; siRNA directed against the viral transcription factor E6 inhibits viral gene expression and growth in tissue culture [15,16]. Baculoviruses infect many different insect species. Over many years, the *Autographa californica* nucleopolyhedrosis virus has caused severe economic losses in the silk industry. Inhibition of that virus was achieved by a pair of siRNAs that target specifically the viral coded early transcription activator, and the major nucleocapsid [17].

Inhibition of viral replication

One of the main steps in the process of viral infection is DNA and RNA genome replication of the virus. The genome of RNA viruses, especially with plus polarity, serves both as mRNA and as a replication template. Many research groups applied the siRNA method to inhibit replication of viruses i*n vitro* and *in vivo*. In the absence of an efficient cell culture system for growing HCV, the sensitivity of HCV to RNAi was shown in the replicon-based system. An HCV replicon is derived from an HCV consensus genome that was cloned from a viral RNA isolated from an infected human and used to construct subgenomic selectable replicons. Upon transfection of these subgenomic selectable constructs into a cell line, these RNAs were found to replicate to high levels. In several studies, siRNAs were directed against different targets in the virus genome [18–27]. For example, siRNA specific for the 5′ untranslated region (UTR) of the HCV genome, introduced into Huh7 cells carrying the replicon system, inhibits HCV replication by up to 90% [20], as measured by the expression level of the replicon luciferase reporter gene. siRNAs targeting the viral polymerase NS5B region reduced expression of NS5B-Luc chimera in mice [27] or in the replicon system *in vitro*. Other studies that target other regions of the HCV genome reported a significant decline in the level of HCV proteins and the level of both the sense and antisense RNA strands [25]. The siRNA effect shown for HCV is IFN- and cell-cycle-independent [23].

In the hepatitis A virus (HAV) replicon-based system, it has been reported that siRNAs targeting the regions coding for the non-structural proteins of the virus give rise to partial inhibition of HAV replication [28]. In that study, two siRNAs specific for HAV sequences increased rather than inhibited HAV replication. This could be due to

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complex secondary structures of the target region that can limit and reduce the efficiency of the RNAi process [29]. In another study, siRNA targeted to various domains of the HAV internal ribosomal entry site (IRES) induced efficient and sustained suppression of viral genome translation and replication [30].

Poliovirus is a highly cytopathic RNA virus. siRNAs specific to the poliovirus genome inhibited viral replication, as was demonstrated in a poliovirus replicon system. The siRNA effect led to viral genome clearance from the infected cells, without destruction of the cells harboring the virus [31].

Additional examples of inhibition of viral replication by siRNA originated from the study of positive RNA viruses such as dengue (DENV), West Nile (WNV), and severe acute respiratory syndrome (SARS) [32–36]. siRNAs targeting the 3′-UTR sequence of DENV, in a region that is conserved in all the dengue serotypes, reduced viral replication and infection in dendritic cells [32]. siRNAs targeting the SARS-CoV RNA polymerase gene inhibited viral RNA replication, protein synthesis and reduced the viral cytopathic effects on Vero cells [36]. Likewise, expression vectors of siRNAs specific for two different regions of the WNV genome protected 293T cells from WNV infection, and significantly reduced viral RNA replication and virus production [35]. Coxackievirus B3 (CVB3), a member of the Picornaviridae family, is a major cause of many human diseases, such as meningioencephalitis and myocarditis. Synthetic siRNA targeted to the VP1 or to the viral polymerase showed antiviral effects in infected HeLa cells by inducing a significant reduction of viral replication [37]. The foot-and-mouth disease virus (FMDV) replication was inhibited in BHK-21 cells by siRNAs targeting various conserved regions of the FMDV genome [38]. Multiple siRNAs have been used to target multiple conserved viral genes that are essential for virus replication, including a long 5′-non-coding region, a short 3′-non-coding region, the viral protein VPg, the viral polymerase, and the viral capsid protein VP4. The combination of those siRNAs gave rise to a 10–1,000-fold inhibition in virus yield by specific inhibition of viral replication [38]. The antiviral properties of RNAi have not been assessed in comparison for their effectiveness upon targeting the different intracellular stages of the viral life cycle. However, from current reports, we could surmise that targeting viral replication, similar to what has been described in several other types of antiviral methods, would probably be the suggested approach to suppress viral infection. Replication of DNA viruses can be inhibited by targeting their viral mRNA, whereas replication of RNA viruses can be inhibited by targeting either their mRNAs or their viral RNA, as was elegantly demonstrated for HIV [39].

Inhibition of virus assembly and morphogenesis

In the later stages of the virus life cycle, the structural proteins are produced to assemble and form mature virions before egress. Rotavirus causes severe diarrhea in infants and children worldwide. To combat this virus, Dector *et al.*, utilized siRNA directed to the VP4, a viral structural protein that is essential for the attachment of the infecting virus to the cell surface. They showed a significant reduction in the number of viral particles produced in MA104, an infected monkey kidney cell line. Moreover, most of the viral particles that were produced were poorly infectious [40]. However, there are only few reports assessing specifically the potential of the RNAi effect on viral assembly [41]. The antiviral properties of RNAi against viral assembly, a late stage in the intracellular viral life cycle, is expected to be less effective than RNAi in the early steps of the viral life cycle.

Inhibition of the viral life cycle by targeting cellular genes

In many RNA viruses, there is emergence of quasispecies that contain point mutations in the siRNA's target sequences leading to evasion from inhibition by siRNA. Using a pool of siRNAs to simultaneously target multiple sites in the viral genome can prevent the emergence of these resistant viruses [42,43]. Another approach that may partially solve this problem is targeting cellular factors rather than viral genes. During their life cycle, viruses apply cell membrane receptors for penetration, and cellular transcription factors for viral replication, harnessing very efficiently the cellular transcription and translation machinery for their life cycle. Targeting those cellular genes may be another strategy for inhibition of viruses. And indeed, Zhang *et al.*, for example, succeeded in suppressing the replication of HCV in the replicon system by the expression of siRNAs against cellular cofactors that are needed for viral replication, the polypyrimidine tract-binding protein (PTB) or eIF2Bg [44]. Inhibition of the PTB alone by siRNAs resulted in an efficient decrease in the levels of HCV proteins as well as HCV RNA replication in Huh7 cells harboring the HCV replicon [45]. In another study, siRNA against cellular RNA helicase p68 reduced HCV negative strand replication [46]. The E7 protein of human papillomaviruses (HPVs) contributes to oncogenesis. E7 was found to specifically activate the transcription of the cellular transcription factor E2F2 in an *in vitro* system of differentiating human keratinocytes. While suppression of E2F2 levels through the use of siRNA decreased HPV replication, this loss did not affect cell proliferation. Thus, E2F2 is a potential target for antiviral therapies [47].

The human cyclin T1 (hCycT1) is a cellular factor essential for transcription of messenger and genomic RNAs from the long terminal repeat (LTR) promoter of the HIV-1 provirus. siRNA directed against hCycT1 could effectively suppress HIV-1 replication without any induction of apoptotic cell death [48]. In previous studies, downregulation of other cellular factors, such as CD4, CXCR4, CCR5, NF-kB, P-TEFb, cyclophilin A, DC-SIGN, SPT-5 and PARP-1, successfully inhibited HIV-1

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replication [49–64]. However, since many of these molecules are essential for cellular processes (CD4, e.g., is a cell-surface molecule important for adaptive immune response), not all of them can serve as a practical target for HIV gene therapy.

To conclude, siRNAs can be used for inhibition of both RNA and DNA viral infections from the early stage of viral attachment to the cell to the late stage of viral assembly. siRNAs can be targeted directly to the viral genes involved in the viral life cycle, or against cellular genes which are used by the viruses. In each case, the best strategy for viral inhibition needs to be assessed according to the virus type and its unique life cycle. Interestingly, a combination therapy of two siRNAs, targeting different viral sequences, each with inhibitory function, did not have an enhanced effect. This was also found by other groups, assessing the RNAi effect in other human and non-human pathogens. These repeated observations could be related to the early saturation of the RNAi cellular machinery. However, this issue will need further investigation, which could lead to improvement of the efficacy of RNAi against viral infections.

RNA interference against acute viral infection

In some specific cases of acute viral infections, in particular those cases which could pose a major threat to the health care system, several hurdles remain to be overcome for the development of vaccines and specific small drugs. However, in most cases, the viral agent causing the acute severe endemic, possibly pandemic disease, could be rapidly identified and sequenced, as was the case during the recent outbreak of SARS. In these cases, the development of siRNAs targeted against various regions of the viral genome could lead to a quick development of a therapy against the acute viral infection. The production, delivery, dose, and modes of administration of siRNAs could be tailor-designed for any group of targets. Suffice to say, it is imperative that the timetable for the generation of a new siRNA-based RNA silencing drug could be shorter, so that a therapeutic platform against many specific infectious agents with pandemic potential could be forthcoming. Obviously, it must be remembered that additional important factors need to be implemented for the development of an antiviral drug, such as *in vitro* and *in vivo* models, although these requirements are essential for any other type of therapeutic modality to be produced and tested. One recent report nicely exemplified the fact that synthetic siRNA could be generated against a single (respiratory syncytial virus, RSV) or a double infection (with parainfluenza virus, PIV), and rapidly tested *in vitro*, as a sufficiently predictive tool for an *in vivo* effect [65]. In this report, the siRNAs against both RSV and PIV were administered nasally with profound antiviral preventive and therapeutic effects without inducing interferon production. As mentioned in previous sections, siRNA is very effective against other life-threatening pandemic threats such as influenza infections [66]. However, it must be remembered that we are probably just at the beginning of experimental assessments to determine the potential antiviral effects of siRNA.

The antiviral effects of short hairpin RNAs (shRNA) or siRNAs were also assessed in other viral infections where there is practically no available therapy (shRNAs are short hairpin RNAs expressed by plasmid and viral vector systems and are subsequently processed to siRNAs by the cellular machinery). siRNA targeting the protease 2A region of the most common viral agent causing myocarditis, Coxsackievirus B3, was found to be effective in inhibiting viral replication *in vitro* [67]. In addition, this same group also showed that the antisense siRNA strand is critical for the RNAi effect and that single nucleotide mutations at the central or 5′ regions are detrimental for the antiviral effect. siRNA was also effective against SARS caused by the newly discovered coronavirus in a preventive model *in vitro* [68]. Currently, since there is no available effective specific therapy against SARS, RNAi could be developed for this serious infection. In other cases of severe human infections by viral pathogens, there are fewer promising results than for the antiviral potential against viral replication by inducing RNAi. siRNA was also assessed against WNV infection. In one report, while the investigators failed to show an antiviral effect in active replicating cells [69], they showed prevention of infection in a previous report. Another group assessed the potential effect of siRNA against WNV infection *in vivo*. Again, only a preventive mode of therapy was found partially effective against viral replication and disease outcome in mice [70]. From the reports on the use of siRNA against human viral pathogens causing acute disease, we could learn that for each specific pathogen infecting a specific cell lineage or tissue, we would probably need to perform an in-depth assessment, with proper *in vitro* and *in vivo* models, and develop specific delivery systems. Although the road towards RNAi development could be visible for some of its destinations and the traveling speed could be changing, the target time remains unknown and unpredictable. An interesting new approach of preventing viral infection was reported by the group of Judy Lieberman [71]. In an effort to suggest a method of prevention of HSV2 sexual transmission, intravaginal installation of siRNAs targeting two HSV-2 genes protected mice from a lethal HSV-2 challenge without inducing interferon-responsive genes. This encouraging result once again proves that combining a realistic method of gene delivery with a specific genetic drug payload for a specific disease could result in a beneficial gene therapy outcome.

In most acute viral infections, the host overcomes the invading pathogen through a robust innate and adaptive immune response. However, in those cases where the virus causes severe disease as a result of a significant cytopathic effect, which could be related to a high multiplicity of infection (MOI), low immunogenicity, high replication capacity or direct toxic effects, or a

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combination of all, could result in organ failure or even death. In these cases, RNAi could significantly support, or even enhance, the antiviral effects for a short period of time and this could be achieved by the administration of siRNAs. Characterizing the specific viral pathogen, even in a pandemic situation, could enhance the rational design of a sequence-specific siRNA that can be used as therapy. This situation is very different from cases of chronic viral infections of RNA viruses, in that there could be quasispecies; in addition, the effect of the RNAi should be prolonged and generated through an expression system rather than through a synthetic siRNA administered once or only a few times.

RNA interference against chronic viral infection

Human chronic viral infections such as HBV, HCV and HIV are a worldwide threat. For HCV and HIV infections, there are no available vaccines, and, in addition, in both the prospect of vaccine development is not encouraging. Furthermore, current therapeutics for both of these infections are suboptimal. For these viral infections, numerous gene-based approaches have been developed. Although there are effective vaccines against HBV infection, chronic infection is still a major therapeutic challenge.

Inhibition of HBV by RNAi

The RNAi was used to inhibit replication of DNA viruses. HBV replication was inhibited *in vitro* and *in vivo* by RNAi by us and by others [72–76]. siRNAs targeted to different regions of the HBV surface antigen gene robustly inhibited viral gene expression and replication both *in vitro* and *in vivo* [72]. Due to the overlapping gene structure of the HBV genome, targeting a region in the open reading frame (ORF) of the X gene which is shared by all the viral transcripts resulted in a significant reduction of up to 90% in all viral transcripts and proteins and in a dramatic reduction of ~95% in viral replication [75]. The X gene of HBV was also recently assessed as a target for RNAi *in vivo* [77]. Using two HBV mice models as a naked DNA approach with the hydrodynamic method or expressed from an adenovector, the Pol III U6 promoter encoded short shRNAs targeting conserved sequences of the HBx ORF. The anti-HBV effect was significant without stimulating the interferon system. It is also known that HBx plays an important role in hepatocarcinogenesis. siRNA against HBx was also used to test its effect against hepatocellular carcinoma cell lines which express HBx sequences [78]. This group demonstrated a significant reduction in cell proliferation, cell growth, anchorage-independent growth in soft agar, and tumor development in nude mice following the expression of siRNA against HBx. A recent report suggested that the inhibitory effect of RNAi on HBV expression is stronger than that of Lamivudine *in vitro* [79]. We could further speculate that a combination of RNAi and nucleoside analogs might encounter a synergistic effect, although this is yet to be determined.

Inhibition of HCV by RNAi

The most challenging part of RNAi approaches for chronic viral infections is to design the best delivery method that would facilitate the targeting of the specific organ/cells with the appropriate expression system, for durable intracellular levels of gene-silencing effect. This also applies when designing an RNAi approach for HCV infection, as well as for other chronic viral infections. The studies assessing the effect of RNAi against HCV were mostly restricted to *in vitro* replicon systems, as discussed above. Alternative *in vivo* systems were also adopted by some investigators, with reporting proteins used to assess the antiviral effect [80]. In early studies using the *in vitro* HCV replicon system, it was shown that a synthetic siRNA targeting the 5′ core region of HCV inhibited viral proteins and significantly suppressed viral replication for at least 8 days [21]. At about the same time, a different group showed, also in the replicon system, that siRNA against the NS5B region (viral polymerase) is most effective in suppressing HCV replication [19]. This group had also transfected the HuH7 replicon cells with a vector expressing complementary strands of siRNA, again targeting the NS5B region, under the control of two separate H1 promoters (pCEP4, Invitrogen). In this case, the suppression effect on HCV replication lasted over 3 weeks. Another group targeted similar HCV regions, NS3 and NS5B [26]; they introduced shRNAs targeted against these two genes into Huh7-replicon cells. The delivery systems that were used in their study were plasmids or lentiviral vectors harboring shRNAs against NS3 or NS5B, expressed from the U6 promoter. In both cases, they observed similar effects, i.e., suppression of HCV proteins and viral replication. However, shRNA against the 5′-UTR of HCV resulted in very low levels of inhibition of HCV replication.

As mentioned earlier, viral replication is dependent on numerous cellular factors. Targeting these viral replication/gene expression cofactors is a potential target for inhibition of viral replication. However, this approach should always be balanced against the potential of generating side effects which could overshadow the beneficial antiviral outcome. A recent study assessed this approach *in vitro*, targeting two HCV replication cofactors: proteasome α-subunit 7 and Hu antigen R. shRNA targeted against these two genes that were expressed from an expression vector transfected into HuH7 HCV replicon cells showed a modest reduction of HCV transcription [81]. However, this modest inhibition on the one hand, and the potential role of both proteins in normal cellular function on the other, might suggest that it is advisable to abstain from such approaches, if possible.

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An interesting study *in vivo* targeting the HCV IRES, translating a luciferase reporter protein, revealed that the *in vitro* synthesized shRNA, administered systemically by the hydrodynamic method, encountered a sustained antiviral effect lasting over 4 days compared to synthetic siRNA [80].

Inhibition of HIV by RNAi

The use of RNAi against HIV infection was reported by a number of groups (Table 1). Although the results of these studies suggest that HIV could be targeted by RNAi, there are major obstacles in translating this therapeutic approach into the clinical setting. Most reports have used sequences from laboratory HIV strains. Viruses with mutations at the RNAi recognition site produced an escape mutant after a long-term RNAi pressure. Targeting relatively conserved HIV sequences could improve the efficacy of the RNAi effect. A recent study looked at the protective effect of shRNAs targeting the *rev, gag*, and *vif* sequences of a panel of HIV clades [82]. This study showed that targeting the *vif* HIV region had a significant inhibition effect on HIV replication. However, the long-term use of any specific siRNA or shRNA against HIV could probably induce the generation of escape mutants containing nucleotide substitutions at or near the target sites. Furthermore, the escape from RNAi-mediated inhibition could also signify the emergence of mutations that change the HIV RNA secondary structures [83]. All of these data emphasize the significance of HIV evolution during RNAi pressure and its potential impact on the use of RNAi against HIV. The virus also harbors a specific mechanism that evades the nucleic-acid-based innate immunity of human cells against HIV. The genome of HIV contains a plethora of dsRNA regions capable of being processed into siRNAs targeting the viral genome to suppression [84]. However, the virus has evolved by a counter process, rendering itself resistant to RNAi through the Tat protein, altering the Dicer effect on viral sequences, and abrogating the host cell innate immunity against HIV infection. Interestingly, on the other hand, it is possible that HIV does apply the cellular RNAi machinery for regulation of its own gene expression. The HIV *nef* region encodes a microRNA, miR-N367, which can block nef expression [85]. Later, it was shown by the same group that miR-N367 targets the HIV LTR promoter region, downregulating viral transcription; this might be a mechanism by which the virus regulates its own transcription [86].

Circumventing the high mutation rate of HIV genomic targets

Single anti-HIV therapy is ineffective against viral replication and gene expression due to the high mutation rate of the virus. One option of overcoming this major obstacle is to generate therapeutics against highly conserved viral genomic regions. A recent report showed that it is possible to clone shRNAs against the conserved regions of the HIV genome into HIV vectors, and to suppress HIV infection upon targeting the gag, pol, int and vpu sequences [87]. However, although this approach could be applied for prevention of infection, cessation of an ongoing HIV replication is prone to failure due to the high mutation rate of the virus. An alternative strategy could be to target essential cellular determinants for HIV infection. The early step of HIV infection would be attaching to the viral cellular receptor. During the progressive stage of the disease, most HIV isolates use the chemokine receptor CXCR4 for viral attachment and penetration into the host cells. Patients with mutations of the CXCR4 receptor are less prone to HIV infection and are healthy by any significant measure. This finding was the rationale used to develop an anti-HIV approach by targeting CXCR4 expression and inhibiting viral fusion with the cellular membrane [54]. It is expected that such an approach will pose a major obstacle to viral evolution and prevent infection. Other groups have also adopted this strategy to render cells resistant to HIV infection [52,53,58–60]. The HIV regulatory protein, rev, is essential for viral life cycle in a number of ways including splicing, translation and *trans*-activation. With regard to rev *trans*-activation properties, it needs to interact with the hypusine-containing protein eIF-5A. The eIF-5A rev cofactor is activated following a catalytic step performed by the human deoxyhypusine synthase (DHS). A recent study has suggested that RNA interference inhibiting DHS blocked HIV replication [88]. Again, as with other drugs in development against HIV infection, we are confronted with the following major questions: what are the potential side effects from such an approach, and what would be the therapeutic dose window? Indeed, studies aimed at unfolding these issues are crucial before entering any clinical studies. Other groups have also suggested the targeting of other cellular factors essential for the HIV life cycle, including PARP-1 [55], the elongation transcription factor P-TEFb [56], cyclophilin A, DC-SIGN [61,63], the human elongation transcription factor SPT5 [64], and human cyclin T1 [89]. The road towards the development of an efficient therapeutic modality using the anti-HIV RNA interference strategy is bumpy, due to potential side effects; moreover, significant strides will have to be made towards harnessing clearcut approaches as described, as well as designing additional rational studies through wet and dry investigations [89].

Specific points for consideration upon designing a gene therapy approach utilizing RNAi

Availability of the Dicer machinery

During the course of differentiation, the expression of proteins involved in RNA interference decreases [90].

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Table 1. The effect of RNA interference against viral infection

Virus	RNAi expression system	Delivery method	Model system	Effect	Ref.
RNA viruses non-integrating					
Severe acute respiratory syndrome – coronavirus (CoV- SARS)	siRNA expression plasmid (pSUPER) siRNA expression plasmid (pSilence1-U6; pBS/U6) Synthetic siRNA	Transfection (Lipofectamine) Transfection (Lipofectamine; CalPhos) Injection intratracheally into mouse lung Intranasally in monkey model	Infected Vero cells *in vitro* 293 cells, Infected Vero cells Mouse model Rhesus macaque SARS model	Message and titer Inhibition, knockdown to ~20% Inhibition of viral N-gene expression Diminished viral level Reduced infection-induced symptoms	[36,154–156]
Foot-and-mouth disease virus (FMDV)	siRNA expression plasmid (pCDNA3/U6 promoter) In vitro transcribed In Dicer siRNA Generation kit	Transfection (Lipofectamine) Subcutaneous injection in the neck	BHK-21 cells Suckling mice model	Inhibits VP1 expression and FMDV replication	[38,157]
Influenza A virus	siRNA expression plasmid (U6 promoter) Synthetic siRNA lentiviral vector expressing siRNA from U6 promoter	*In vivo* i.v. delivery by polyethylenimine (PEI), a cationic polymer mixed with siRNA/vector Intranasal delivery	Mouse animal model	Preventive and therapeutic effects	[35,134,135]
Hepatitis A virus (HAV)	Synthetic siRNA	Transfection (Lipofection TransMessenger)	*In vitro* in HuH7 cells	Inhibition of replication and gene expression	[28,30]
Coxackievirus B3	Synthetic siRNA	Transfection (Oligofectamine)	In vitro in Permissive HeLa cells	Reduction of viral Replication	[37]
Hepatitis C virus (HCV)	Endoribonuclease-prepared siRNAs (esiRNAs) siRNA expression vector (MoMuLV-based vector, pBABE/puro) with H1 promoter *In vitro* transcribed siRNAs Synthetic siRNA siRNA expression vector (U6 promoter)	Transfection (Oligo-Fectamine) Retroviral transduction Transfection (Lipofectamine)	*In vitro* against the HCV replicon in Huh7 cells HepG2 cells transiently transfected with HCV proteins expression plasmid Hep5A cells	Inhibition of viral replication Simultaneously targeting multiple sites may prevent generation of escape mutants Reduction in viral gene expression	[18,22,24]
Human rhinovirus	Synthetic siRNA	Transfection (Oligofectamine)	*In vitro* in HeLa cells	Suppression of HRV-16 replication	[158]
Hepatitis delta virus (HDV)	siRNA expression plasmid (pSilencer)	Transfection (Lipofectamine)	*In vitro* in HuH7 cells	siRNA against mRNA were also effective against HDV replication	[159]

Enterovirus 71	Synthetic siRNAs Targeted to the 3′UTR, 2C, 3C and 3D regions	Transfection (Lipofectamine)	*In vitro* rhabdo-myosarcoma cells	Reduction in viral replication, gene expression and plaque formation	[160]
Retroviruses					
Porcine endogenous retroviruses (PERV)	Synthetic siRNAs siRNA expression plasmid (pSUPER)	Transfection (Lipofection: GeneEraser; TransFast)	*In vitro* infected 293 cells	Significant suppression of replication	[161]
HIV	Utilization of the human miR-30 pre-microRNA: siRNA expression plasmid (U6 promoter) siRNA expression plasmid (pSUPER, H1 promoter) Lentiviral vectors, H1 promoter	Transfection (Lipofectamine) Lipofection Transduction	*In vitro* 293 cells *In vitro* in monocytes-derived macrophages	Effective reduction of HIV-1 p24 antigen Protection of cells from HIV infection Inhibition of viral gene expression	[49,53,59,106,107,119,162–165]
DNA viruses					
JC virus	Synthetic siRNA	Transfection (Lipofectamine)	*In vitro*: SV40-transformed human fetal glial cells	Significant inhibition of JCV production	[166]
EBV	siRNA expression plasmid (pSUPER)	Transfection (Lipofectamine)	*In vitro*: NPC cell line	EBV lytic cycle effectively blocked	[167]
Human papilloma virus (HPV)	Synthetic siRNA	Transfection (Fugene)	*In vitro* differentiating keratinocytes	Reduction in HPV copy number, suppression of viral replication	[16,47]
Hepatitis B virus (HBV)	Synthetic siRNA	Transfection *in vitro* (Lipofectamine) Hydrodynamic tail vein injection *in vivo*	HepG2, 2215 cells and SCID mice	Significant short-term antiviral effect	[72–76,109,168,169]
Herpes simplex virus (HSV)	siRNAs against glycoprotein E generated *in vitro* by T7 polymerase	Transfection (Lipofection complexing)	*In vitro* model of infection of human keratinocytes	Reduction in cell-to-cell spread	[170]

The cellular level of Dicer could be crucial for gene therapy approaches while utilizing the RNAi machinery in targeted cells. Recent data suggest that, although the expression of Dicer and other proteins that participate in digesting long dsRNAs into 21–25 nt, e.g., eIF2C1 $\sim$ 4, decreases in differentiated cells, they retain a sufficient amount of enzymatic activity to induce RNAi. However, when designing and planning any specific approach using siRNA for gene therapy, it is advisable to assess the Dicer activity in the targeted tissue if the expected siRNA is unmet.

Inhibition of RNAi by viral suppressor genes

Since RNA silencing acts as an antiviral defense mechanism in plants [91], insects [92] and other eukaryotes, including mammalian cells [93], it is not surprising that viruses have developed strategies to interfere with this effect. Many plant DNA and RNA viruses have developed proteins that function as suppressors of RNA silencing [94–98]. Since the silencing suppression reduces the antiviral effects, the viruses can accumulate in their target cells and reach higher titers. Comparing those suppressor genes did not reveal any sequence homology between them. The RNA silencing suppressors can act upon the various sequences of the RNAi machinery in several ways such as inhibition of siRNA processing, inhibition of the incorporation step into the RISC, or preventing the action of its effector molecules. Although the mechanisms of inhibition of silencing are not fully understood, there are several suppressor genes that their targets have identified. The p19 protein from the tombusvirus was shown to bind specifically to the siRNAs, and thus may inhibit the incorporation step of the siRNAs into the silencing effector complexes [98]. The HC-Pro protein, expressed by polyviruses, acts by targeting the RISC [99,100]. Another example is the mosaic virus 2b protein (Cmv2b). This nucleus-localized RNAi suppressor protein inhibits the activity of the spreading signal of the RNAi, and inhibits DNA methylation processes in the nucleus that control the silencing pathways [97,101]. The coat protein of the turnip crinkle virus (TCV) strongly suppresses the RNA silencing process at an early initiation step, probably by interference with the function of the Dicer cleavage reaction [96].

Since the RNAi machinery is conserved in mammals, it appears possible that, similar to plant viruses, viruses that infect invertebrates and vertebrates, including human viruses, have developed strategies to suppress RNA silencing. And indeed, there is evidence that such inhibitors are not limited to plant viruses. In insects, the flock house virus (FHV) is a target of RNA silencing. It has been shown that in Drosophila and mosquito cultured cells, the FHV-encoded protein, B2, inhibited RNA silencing, but the mechanism is still unknown. This protein also inhibited RNAi in transgenic plants, suggesting that the RNA silencing pathway is conserved in plants and animals [92,102,103]. Recent evidence also indicates that human virus genes have the ability to inhibit the RNAi pathway. Both the influenza virus NS1 protein and the vaccinia virus E3L protein can inhibit RNAi in Drosophila S2 cultured cells [102]. The adenovirus encoded inhibitor of RNAi is functional in mammalian cells. The human adenovirus inhibits RNAi in the later stages of infection by suppressing the activity of Dicer and the RISC. The virus-associated RNAs, VA RNAI and VA RNAII, bind directly to Dicer and function as competitive substrates, squelching it, and the resulting siRNAs are incorporated into the active RISC [104,105]. The antiviral therapeutic potential of RNAi would require identifying possible viral suppressors of the RNAi machinery and designing strategies to inhibit their expression.

Development of resistance to RNAi

One major drawback for most antiviral approaches is the development of resistance. This is most apparent in cases where the fidelity of the viral polymerase is low, especially in viruses with an RNA genome. To overcome this hurdle, most antiviral therapeutic protocols harness a strategy that uses multiple drugs targeting different viral proteins or steps in its life cycle. One example where such resistance has been developed *in vitro* is in the case of RNAi against the Nef gene of HIV [106,107]. To overcome this problem, it may be advisable to utilize a multi-targeted RNAi approach possibly in combination with additional antiviral modalities. Although targeting multiple genomic sites has probably no advantage with regard to the direct antiviral effect, it could repress the development of resistance. The initial steps towards focusing into this avenue have already been established. The group that initially described the siRNA effect *in vitro* in the replicon system against HCV [19] have shown that, following several rounds of treatment with the same siRNA against HCV, the replicon became resistant to that specific siRNA, developing a point mutation at the target site. However, the replicon was still sensitive to a siRNA targeting a different HCV region. In addition, the development of a resistant replicon was limited by the use of a combination of two siRNAs targeting simultaneously different HCV genomic regions [108]. A similar approach has also been suggested by other groups [109].

Off-target effects

The off-target effect of RNAi is the silencing of non-targeted genes containing partial sequence identity to the siRNA. In experiments conducted on specificity of siRNA in cultured human cells, Jackson *et al.*, have demonstrated that siRNA can cause direct downregulation of unintended targets containing as few as 11 contiguous bp complementarities [110]. To increase siRNA specificity, there are many siRNA design programs that employ various sequence alignment algorithms; however, maximum

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complementarity, by itself, is not enough for accurate prediction of off-targeting. A study done in Dharmacon Inc., identified a significant association between off-targeting and exact complementarity between the seed region of the siRNA (bases 2–7) and their off-targeted genes. This pattern has been recognized in miRNA-mediated gene silencing, thus suggesting that siRNA off-targeting may operate by a mechanism similar to that of miRNA targeting (A. Birmingham, personal communication). Until the off-target mechanism of siRNA is understood, this issue can encounter deleterious side effects on the use of RNAi.

Activation of the innate immune response

The role of interferon signaling in RNAi has given rise to a series of conflicting reports. Although most studies suggest that there is very little non-specific effect of siRNA, others have shown that the Jak-Stat pathway is activated following siRNA transfection. This effect is mediated by dsRNA-dependent protein kinase (PKR) [111]. While we expect that this issue will foster additional debates, at this point, it would be important to impart cautious interpretations upon describing RNAi effects. In addition, recent studies have suggested that although siRNA does not activate the intracellular interferon machinery in mammalian cells upon entrance or *in situ* propagation inside the cells, if they are shorter than 30-nt dsRNA, there is a non-specific innate immune response depicted by cytokine production [112]. Furthermore, this effect is dependent on the Toll-like receptor 3 (TLR3) that senses dsRNA and serves as its receptor. TLR3 is located intracellularly on the endosome membrane and signals through NFκB nuclear translocation for the production of inflammatory cytokines. Incubation of immune cells with siRNA induces the activation of cells [113]. All of these effects could be dependent on the concentration of siRNA. Recent works identified putative immunostimulatory motifs within siRNAs, and showed that even a slight change of these motifs did not significantly hamper the RNAi process [114]. This research provides a basis for the design of synthetic siRNAs that avoids activation of the innate immune response, and helps to minimize immunotoxicity.

Vector design

The group of Reuven Agami was the first to report a new vector system, called pSUPER, which directs the synthesis of siRNAs in mammalian cells. They used the poly III H1-RNA gene promoter to express shRNAs that specifically down-regulated gene expression, resulting in functional inactivation of the targeted genes [115]. Recent reports showed that the expression of shRNA from the H1 or the U6 pol III promoters in a HIV-based vector induces the expression of interferon-stimulated genes (ISGs). This effect is dependent on the presence of an AA dinucleotide near the transcription starting site. Preserving a C/G sequence at positions $-1/+1$ prevents this effect [116].

In some cases, the expression from the U6 promoter is relatively low. The enhancer of the CMV immediate-early promoter enhances the U6 promoter activity [117]. Others have also tested various promoters [118] reporting some beneficial effects on expression with the modified tRNA(met)-derived (MTD) promoter, upon expressing shRNA against HIV-1 compared to U6 or H1 promoters [119]. It may happen that for each specific application, we would need to compare numerous regulatory elements to achieve the desired RNAi effect. In some systems, it may be important to tightly control the expression of the shRNA. Although most controlled systems do not reach the desired stringency *in vivo*, some reports have suggested the use of specific systems. One such method is the tet-on-off expression technology [120]. Again, each investigator should specifically assess the potential of this inducible system in a specific tissue culture or animal model. The tet-on-off systems were developed for naked DNA transfection systems or incorporated into viral vectors like the lentiviral vector [121]. An additional long debate in the literature questions the choice of the loop structure to apply for the design of shRNA. One specific strategy was to adopt the natural loop structure of microRNA [118]. This was also used for shRNA against HCV [80].

Delivery of RNAi

The major challenge in RNAi gene therapy is to transform the *in vitro* robust effects of siRNA into an *in vivo* gene-silencing method. In other words, what would be the preferred delivery system to use in animals and later on in humans? As for gene therapy in general, and the specific aims of delivering RNAi platforms, we need to tailor-design the tools to be used to the sought objective. This includes targeting the tissue, adjusting the desired level of expression (high level of siRNA could induce non-specific silencing [122]), the longevity, and the specific maladies that we wish to treat. This is a complex situation, especially since our major barrier is the lack of a simple, non-immunogenic, targeted delivery system without side effects. However, in spite of all of these hurdles, we would like to discuss the potential available methods to deliver synthetic siRNA.

The most straightforward method of using siRNA *in vivo* is by administering synthetic siRNA. Upon coinjecting siRNA and its target being expressed from a plasmid vector, we could achieve knockdown of expression in the liver following a hydrodynamic injection. In our laboratory, we could show that HBV expression out of an HBV head-to-tail plasmid that supports viral replication in the liver of mice could effectively be silenced transiently with synthetic siRNA [72]. This effect was dose-dependent. However, the kinetics of this effect revealed that the silencing was transient as was also shown by other groups using a similar approach [27,123–125].

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The effect subsides after 48 to 72 h and is probably completely lost after 7 days. The silencing effect of siRNA following systemic administration of duplex RNA could have been hampered by various factors, thus imposing some logistic hurdles upon translating this approach into the clinical setting (additional examples of RNAi against viral infections are depicted in Table 1). Although duplex RNA is quite stable in serum [124], and more stable than ssDNA or ssRNA, a high serum concentration could reduce stability. The introduction of phosphorothioate linkages could enhance stability in the serum [124]. Others have used chemically modified siRNA with the complete absence of 2′-OH residues on the sense and antisense strands of the dsRNA, including 2′-fluoro, 2′-*O*-methyl and 2′-deoxy sugars. These chemical modifications of siRNA were produced in order to enhance its stability and effect [126]. Not all modifications have resulted in beneficial silencing effects [127–129]; a short review of these modifications was reported by Paroo and Corey [130]. One particular interesting report [131] shows that a specific siRNA with a combined chemical modification had a significant and a relatively sustained effect *in vitro* and *in vivo* as compared to non-modified siRNA. However, although siRNA is relatively stable in the serum, there are disadvantages of using synthetic siRNA: (1) The effect of synthetic siRNA is transient; in order to impose a long-term effect, repeated administrations would be needed, and this might still be true for the chemically modified siRNAs. (2) The production of synthetic siRNA is expensive, making repeated administrations for long-term effect very costly. (3) It is very complicated to target synthetic siRNA to a specific cell or tissue. However, in specific cases, the use of siRNA directly administered into the target tissue could encounter a significant effect. In a recent report by Dorn *et al.*, they used siRNA against the pain-related cation-channel $P2X_3$, by intrathecal injection of phosphorothioated (PS) siRNA in a rat model of neuropathic pain [132]. Although they did not compare the non-PS-modified versus the PS-modified siRNA, they have clearly shown a significant effect of siRNA in relieving chronic pain. Furthermore, the effect was superior to the comparable $P2X_3$ antisense oligonucleotides. One specific case where such an approach could be translated into an applicable clinical therapeutic modality is in post-herpetic neuralgia which could follow Varicella–Zoster infection. However, since chronic pain is a condition that is generally expected to last for months, this type of treatment would need to be readministered several times. A recent report took advantage of the knowledge generated regarding the stabilization of siRNA by chemical modifications, and protection of the modified siRNA with pegylated liposomes upon delivery. They assessed the antiviral effect of siRNA against HBV *in vitro* and *in vivo*. Interestingly, the modified siRNA had induced less non-specific cytokine secretion combined with an effective and anti-HBV effect of up to 6 weeks after repeated weekly administrations [133].

Non-viral delivery of siRNA and shRNA expression systems

In an effort to enhance and prolong the effect of siRNA, various approaches have been undertaken using non-viral reagents. Tailoring the specific delivery tactic and method is essential when designing a specific therapeutic strategy. One example is the use of siRNA targeting the influenza virus genome. This malady can cause moderate to severe illness, can affect millions of people each year, and could be life-threatening. For the gene therapist, this means that the genetic therapy effect could be designed for a short window of time. In this case, the use of synthetic siRNA with an enhanced transduction is an appropriate approach. In a study by Ge *et al.*, the systemic and intratracheal delivery of polyethylenimine (PEI), a cationic polymer, promoting siRNA delivery in mice, is beneficial for prophylaxis and therapy of the influenza virus infection [134]. PEI was developed for *in vitro* and *in vivo* local and systemic gene delivery and PEI-mediated gene delivery/transduction into the lung following systemic and intratracheal administrations. Some investigators reported safety problems in specific cases upon the use of PEI in animals. However, plasmid DNA mixed with PEI administered into the human bladder was safe (A. Hochberg, personal communication). Tompkins *et al.*, also assessed the effect of siRNA against the same pathogen, the influenza virus [135]. This group used a different therapeutic regimen. They used a preventive measure by administering naked siRNA systemically, i.e., intravenously, before infection, and at the time of infection, they administered the siRNA/Oligofectamine™ (a lipid carrier from Invitrogen) intranasally. In this model, the undertaken therapeutic approach prevented death of animals. However, this study was limited to asking the general question of siRNA effect against influenza virus infection rather than comparing different siRNA delivery systems. Thus, we cannot draw any conclusions that would suggest a preference for any specific delivery method in this disease model system. Future animal studies would be required to determine the preferred delivery method. In addition to the lipid carriers, traditionally developed for *in vitro* and *in vivo* delivery of DNA and now for siRNA, alternative approaches have also been developed. One specific interesting report by Minakuchi *et al.* describes the use of atelocollagen (AT) [136]. AT is prepared from type I collagen from calf dermis. This is a low immunogenic product that is already available in clinics for various indications like promoting wound healing. The same investigators showed that AT enhanced DNA delivery and supported prolonged expression. AT mediated the delivery of siRNA *in vitro* and was found *in vivo* to encounter a significant advantage over the siRNA/liposome complex in inhibiting tumor growth in mice. Recently, more sophisticated delivery methods of siRNA have also been developed to treat brain tumors [137]. The obtained results could be important for those interested in developing siRNA therapeutics for viral encephalitis. The model that they used *in vivo* to assess

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their delivery and RNAi effects was an immunodeficient mouse with an inoculated intracranial human U87 glioma tumor that was dependent on EGF signaling for growth. One of the major barriers for macromolecules to travel to diseased tissue in the brain is the blood-brain barrier (BBB). Passing the BBB is a major challenge faced for the development of any pharmacological compound with high molecular weight. Pegylated (polyethylene glycol) immunoliposomes (85 nm size liposomes designed with monoclonal antibodies over its outer surface) were able to support transvascular delivery of plasmid DNA and to target and transduce specific cells in the brain. This group conjugated the PEGylated liposomes with two monoclonal antibodies, one against the mouse transferrin receptor to enable BBB crossing, and the second against the insulin receptor to enhance cellular uptake. The generated PEGylated immunoliposomes encapsidated a plasmid payload that was designed to express the siRNA against the EGF receptor in the transduced cell. The siRNA complex of PEGylated immunoliposomes showed an enhanced antitumor effect by prolonging survival of animals. The ability to treat brain tumors with a systemic approach rather than by stereotactic injection is a major achievement. Recently, it has been reported that recombinant HBV capsids can be used as efficient vehicles for oligonucleotide delivery; they can encapsulate the oligonucleotides *in vitro*, and mediate their delivery into cells very efficiently. The process is not cell-type-specific. This method may be useful for *in vivo* systems for HBV-infected individuals or in other diseases provided that the immunogenicity of the viral capsids can be decreased; until then, it can be used in cell culture and in *ex vivo* systems [138].

Viral delivery of siRNA and shRNA expression systems

The potential advantage of viral-mediated siRNA delivery encouraged numerous groups to clone expression cassettes in transgenes and to encapsidate these into viral particles. Each type of viral vector holds specific properties. These viral vector characteristics should be those that determine which viral delivery system needs to be applied for the specific therapeutic target. The adenovector [139], and in particular the Ad-gutless vector, hold major promise for liver-directed systemic delivery. In cases where short-term silencing effect is warranted, the non-gutless vector could then be applied; however, for prolonged silencing effects, the gutless vector might be more beneficial. In the gutless vector, there are practically no restrictions as to the size of the sequences to be incorporated and these could include marker genes, regulatory controlled cassettes or matrix-controlled regions for prolonged expression. Controlling the expression of an siRNA, specifically in tumor cells, could also be designed in a conditionally replicating adenovirus (CRAd). CRAds are designed to replicate and specifically kill tumor cells without harming normal cells. Carette *et al*. [140] applied CRAd, which is dependent on Rb deficiency for replication, to test its potential in silencing expression by shRNAs, a marker gene *in vitro* in tumor cells. They showed that the silencing effect of a marker gene is dependent on CRAd replication. The combination of the CRAd antitumor effect with a siRNA against a tumor-dependent growth gene should be assessed in the future; this possibility was not considered by this group, whether *in vitro* or *in vivo*. These issues would need further studies in an effort to assess and enhance their therapeutic potential.

The major advantage of the retroviral delivery method is the potential to incorporate the payload transgene they 'carry' into the host cell genome. The integration site could not be specifically targeted and this could cause side effects. Integration that occurs near cellular protooncogenes can lead to their aberrant expression from the viral LTR, or alternatively this could cause the disruption of a tumor suppresser gene expression. In the past 2 years, numerous groups have reported on various retroviral deliveries [141], including lentiviral systems to express siRNAs against viral pathogens and tumor cells. Ralf Bartenschlager and associates reported the use of the Moloney murine leukemia virus (Mo-MuLV)-based vector (pBABE) as a delivery system for siRNA targeting HCV [22]. In their publication, they also assessed a unique RNAi approach against HCV infection. This was done in an effort to overcome the low fidelity of the viral polymerase, establishing a state of quasispecies by generating endoribonuclease-prepared siRNA to simultaneously target multiple sites of the viral genome in order to prevent escape. As for the retroviral delivery approach, this group designed their siRNA mainly against the viral IRES sequences. Their readout system to assess the silencing effect involved tissue culture cells transfected with the subgenomic HCV replicon. This replicon harbors the HCV IRES upstream of the luciferase gene and the neomycin resistance region, and an additional non-HCV IRES upstream of the viral non-structural sequences. The presence of the luciferase gene enables the determination of HCV IRES activity *in vitro*. This system enables the assessment of the effect of siRNA against the HCV IRES as well as against the viral non-structural genome. The siRNA in the retrovirus was expressed from the H1 promoter. They designed numerous siRNAs in the vector and found that a specific region in the HCV IRES, near the beginning of the viral coding nucleotides, was the most sensitive to RNAi effect. Although this is an interesting approach with clearcut results, significant developmental steps and modifications are required in order to translate this modality into the clinical setting. However, the results described in this report again suggest that the RNAi encounters a therapeutic potential for chronic viral infection. Similar observations were reported by other groups ([18,24], see Table 1).

Presently, most groups who use retroviral vectors to express siRNA apply lentiviral vectors. However, it must be kept in mind that the clinical experience with this vector is very limited. Numerous papers were recently published describing differently designed lentiviral vectors to meet

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different needs of siRNA expression. These include systems which support the control [121] or conditioning [142,143] of siRNA expression. Veerle Baekelandt and associates have designed a study to assess the potential use of lentiviruses in delivering siRNA into brain tissue. In their recent report [144], they constructed a lentiviral vector with siRNA against the marker gene eGFP. Upon simultaneous administration into the brain tissue by stereotactic injection of the lentivirus expressing the eGFP and the lentiviral vector expressing the siRNA against the same gene, they were able to show almost complete knockdown of eGFP expression. In two additional experiments in which the siRNA lentivirus was administered before or after the marker gene, they were also able to show significant silencing of expression. Interestingly, they claimed, albeit without showing the data, that this effect persisted for 6 months. However, the issue of delivery is again a major barrier. Stereotactic administrations are possible, but alternative approaches of systemic delivery or intra-organ spreading of the vector would be beneficial. Lentiviruses hold major promise in gene therapy. Once the issue of integration and production is overcome, we expect that the lentiviral-based vector will be integrated into the clinical setting.

The AAV vector is currently perceived as a relatively safe vector since it supports long-term expression in most tissues from an episome. Beverly Davidson and associates reported an interesting study on the suppression of polyglutamine-induced neurodegeneration in a mouse model of spinocerebellar ataxia (SCA1), a disease of the polyglutamine-expansion group which also includes Huntington chorea [145]. They expressed the siRNA under a modified CMV promoter due to its enhanced expression/silencing effect compared to the pol III promoter, in their hands. In addition, they revealed that incorporating the miR23 loop (10-nt loop sequence) into the siRNA expression cassette enhanced the silencing effect, resulting in improved suppression of the ataxin protein levels [118]. However, this effect was only apparent in the pol III expression vector. How to select the best loop in any specific case is still an open question, and, presently, this is a matter of empiric assessment. In their mouse model, they injected the AAV vector expressing the shRNA against the mutant SCA1 message directly into the brain tissue. This treatment showed long-term therapeutic effect on motor coordination as well as a histological improvement by reducing intranuclear inclusions. Although this represents a step forward from previous studies with similar models that used antisense and ribozymes as therapeutic agents, we are still far from the clinic. The direct administration of a viral vector into brain tissue is a significant drawback for the current delivery systems. The potential side effects of AAV administration into the brain may soon be revealed once results of clinical studies using the AAV for direct brain administration in Parkinson and Alzheimer disease studies are available [146]. One specific point of importance should be mentioned on the issue of designing loop sequences stated previously. A number of investigators have assessed this matter as related to the effectiveness of silencing. We must bear in mind that for each specific case, there is a need to develop a specific structure to improve the silencing effect [147].

Chemically modified oligonucleotides (oligos) vs. si/shRNA as antiviral drugs

The jury is still out as to when an antisense approach should be adopted against viral infections or when an si/shRNA strategy should be applied. Although antisense oligos were discovered more than 25 years ago [148] their role as antiviral tools in the clinic is still in progress. Early studies with antisense oligos have shown promising antiviral potency. However, later reports determined that such approaches encounter significant problems. Although comprehensive stringent comparison studies *in vitro* and *in vivo* between antisense oligos and siRNA were not performed, we would like to stress a few practical points which characterize each group of compounds, in particular those which are important for those investigators interested in designing an antiviral strategy. Antisense oligos with a high phosphorothioate content, which are early-generation antisense, interact directly and non-specifically with proteins, potentially interfering with their function [149]. However, recently developed 2nd and 3rd generation RNAs – like oligos – have improved significantly the binding affinities of antisense oligos, as well as their nuclease and non-specific protein-binding characteristics. The new generations include 2′-O-methyl (2OM) and the additional version 2′-O-methoxyethyl (2OME), both encountering improved nuclease resistance, binding affinity and reduced non-specific binding affinity. Recently, additional novel chemically modified versions of antisense oligos were developed including the locked nucleic acids (LNA) [150], anhydrohexitol nucleic acids (HNAs), peptide nucleic acids (PNAs), morpholino nucleic acids (MNAs), and other uncharged oligos. All these novel chemicals have been tested and proven to hold significant antisense properties, with antiviral effects in models *in vitro* and *in vivo*. Their effects are due to the high binding affinity and nuclease degradation resistance, without significant RNaseH activity. To overcome this hurdle, a chimeric version of oligos was generated, the gapmers, containing a core region of a phosphodiester/phosphorothioate flanked on both sides with modified oligo backbones. These gapmers also hold the RNaseH activity of traditional antisenses. When designing any specific experiment aimed to assess an antisense effect, it is important to select the best fitted oligo by testing 10 to 20 different sequences, and compare the selected oligo with appropriate controls containing scrambled, mismatched and irrelevant antisense oligos.

Although a significant amount of investigations would be needed to confirm which specific antiviral nucleic acid approaches would be best fitted for a specific disease therapy, by comparing antisense or ribozymes and siRNA,

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some general guidelines can be phrased: (1) For acute viral infections, the preferred type of RNA therapy could be a synthetic designed antisense or siRNA. (2) For chronic viral disease targets, a preferred treatment would be such that continuously generates an antiviral drug. This could include an expression vector for any type of nucleic-acid-based drug, including antisense, ribozyme or shRNA. (3) For diseases of simple accessible organs, such as ocular cavity, oral cavity, vagina and epidermis, synthetic RNA-based drugs might be beneficial. The need for repeated administration might be simple even in chronic disease states in cases of accessible organs. (4) For chronic multi-organ, or internal organ, involved in viral infections, e.g., chronic viral hepatitis B or C, intracellular continuous expression of high-level non-toxic, effective therapy is desirable. In this case, an expression system delivered into infected cells is warranted. Designed shRNA based on up-to-date criteria of shRNA [151–153] might be the preferred gene therapy based treatment. (5) Special attention must be given as to the specific type of virus to be targeted; whether a DNA or a RNA agent. Furthermore, it is important to know where, inside the cellular compartments, should the RNA-based drug be concentrated, e.g., cytoplasmatic vs. nuclear. For RNA viruses, such as HCV, which replicate in the cytoplasm, an antisense approach could be suggested, although it should be kept in mind that the RNaseH effect is preferentially restricted to the nuclear compartment of the cell. However, for chronic hepatitis B virus infection, in which there is a nuclear reservoir of the virus in the form of super-coiled species, a vector which will express its shRNA payload in the nuclear compartment is preferred. Numerous new expression systems were recently suggested to encounter improved silencing properties [147]. Most of these methods are waiting to be assessed as for their truly beneficial properties as antiviral reagents.

Conclusions

In our modern world, viral infections still pose a major threat to mankind. Since viruses are developing resistance to the current available therapies, there is an ongoing battle between the viruses and our ability to develop novel strategies to fight them. *In vitro* and *in vivo* experiments carried out so far conceivably demonstrate the effectiveness of RNAi in inhibiting many viruses that cause severe health and economical problems. Even though *in vivo* experiments in larger animals as well as developing efficient delivery methods have to be done before applying RNAi in humans, this fascinating phenomenon will undoubtedly continue to provide new and exciting data regarding its mechanism of action and therapeutic applications in the years to come.

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