Toward a Durable Anti-HIV Gene Therapy Based on RNA Interference

Ben Berkhout

Laboratory of Experimental Virology, Academic Medical Center, University of Amsterdam, the Netherlands

Basic research in the field of molecular biology led to the discovery of the mechanism of RNA interference (RNAi) in *Caenorhabditis elegans* in 1998. RNAi is now widely appreciated as an important gene control mechanism in mammals, and several RNAi-based gene-silencing applications have already been used in clinical trials. In this review I will discuss RNAi approaches to inhibit the pathogenic human immunodeficiency virus type 1 (HIV-1), which establishes a chronic infection that would most likely require a durable gene therapy approach. Viruses, such as HIV-1, are particularly difficult targets for RNAi attack because they mutate frequently, which allows viral escape by mutation of the RNAi target sequence. Combinatorial RNAi strategies are required to prevent viral escape.

Key words: **HIV-1; RNAi; gene therapy; virus evolution; viral escape; combination therapy; lentiviral vector; HIS mouse**

The gene suppression effects of RNA interference (RNAi) were first noticed about 20 years ago in plant transgenesis studies but only described in mechanistic terms in 1998 by Fire and Mello in *Caenorhabditis elegans*.[1] More recent studies underscored the importance of the RNAi mechanism for regulated gene expression in mammalian cells. The role of RNAi in mammals and humans is the processing of small noncoding microRNAs (miRNAs) that regulate cellular gene expression to control cell differentiation and development.[2] The RNAi pathway can also be induced by artificial substrates, either transiently by transfection of small interfering RNA (siRNA)[3] or stably by intracellular expression of short hairpin RNAs (shRNAs), which are processed by the cellular RNAi machinery into effective siRNAs.[4] Perfect base-pairing complementarity of the siRNA with a target sequence in a messenger RNA (mRNA) results in cleavage of that transcript by the RNA-induced silencing complex (RISC).[5] Since its discovery, RNAi has been widely used in gene knockdown studies, and several reviews have recently been written on the mechanistic details of RNAi action.[6–9]

RNAi has been proposed as a novel therapeutic strategy for the specific inhibition of human pathogenic viruses. For acute virus infections, such as influenza virus A and respiratory syncytial virus (RSV), such a therapy is particularly attractive because local delivery of the siRNA drug to the lungs is feasible.[10,11] Another major advantage of targeting acute virus infections is that a single siRNA administration may be sufficient to reduce the viral load and thereby dampen or avoid the induction of disease symptoms. The virus will subsequently be controlled and eventually cleared by the immune system. Mice treated with siRNAs against influenza virus, RSV, and severe acute respiratory syndrome coronavirus showed reduced virus titers and reduced virus-induced mortality, both with prophylactic siRNAs and in treatment of established infections.[10,12,13]

Address for correspondence: Ben Berkhout, Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center, University of Amsterdam, K3-110, Meibergdreef 15, 1105 AZ Amsterdam, the Netherlands. Voice: +31 20 566 4822; fax +31 20 691 6531. b.berkhout@amc.uva.nl

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Recently, Alnylam Pharmaceuticals (Cambridge, MA, USA) initiated a phase II clinical trial with an siRNA for the treatment of RSV infection. No adverse effects were observed in the initial phase I trial. The safety, tolerability, and antiviral activity of the siRNA will subsequently be tested in adults experimentally infected with RSV. However, recent siRNA results do indicate that the development of safe siRNA-based therapies might be more challenging than generally expected. Alarm bells went off when new results indicated that the preclinical efficacy, as observed in mouse models of macular degeneration, probably results from nonspecific side effects rather than sequence-specific RNAi-mediated gene knockdown.[14] A recent study confirmed that nonspecific siRNA effects on the innate immune responses can be mistaken for sequence-specific therapeutic effects.[15]

For chronic infections with HIV-1, hepatitis B virus, and hepatitis C virus, a lifelong supply of siRNAs will probably be required for a sustained antiviral effect. This scenario seems much more difficult than the acute infections also because the persistent viruses will tend to escape from the imposed RNAi inhibition. A recent study did, however, advocate the repeated delivery of exogenous siRNAs as anti-HIV therapy.[16] Virus replication was controlled and the associated loss of CD4-positive T cells was prevented in a mouse model with a humanized immune system. An obvious challenge remains the *in vivo* delivery of these siRNAs to the relevant cell types, and it remains doubtful whether one can achieve and maintain sufficiently high siRNA levels in infected humans to durably control virus replication. Incomplete virus inhibition in certain body compartments will provide the means for subsequent viral escape and therapy failure. Kumar and colleagues[16] argued that siRNA therapy provides the flexibility to change the siRNA cocktail in order to keep pace with the mutating virus. Indeed, frequent adaptation of the siRNA drug regimen seems easier than repeatedly changing the shRNA-encoding viral vector, although the latter adjustment is also feasible. However, this therapy adjustment concept is more complex as a simple change in siRNA regimen will block the virus escape variant but not the original virus. Thus, one should ideally add new siRNAs to the existing siRNA cocktail once virus escape is noticed. This strategy will soon fail because of the increasing siRNA load and the concomitant increase in toxicity, e.g., from saturation of the RNAi machinery. We argued that it is of key importance to prevent viral escape in the first place, which can be achieved by a combinatorial RNAi approach.[17]

A gene therapy that makes cells resistant to HIV-1 because of constitutive shRNA expression provides a more durable approach. Several reviews have been written on antiviral RNAi approaches.[18–25] I will focus in more detail on gene therapeutic strategies against chronic HIV-1 infection.[26,27] In fact, a phase I clinical trial against HIV-AIDS has recently been launched with a viral vector that delivers three inhibitor genes, including an anti-tat/rev shRNA.[28] The use of viral gene therapy vectors obviously carries risks. This includes the induction of unwanted immune responses and—for retroviral and lentiviral vectors—the unknown consequences of random integration of the vector into the human genome. In addition, concerns remain about the toxicity related to shRNA expression and interferon induction by these double-stranded RNA (dsRNA) molecules.[29] That is why safety will be an important parameter in the first clinical studies.

Not all viruses and all segments of a viral RNA genome are equally susceptible to RNAi-mediated inhibition. Proteins bound to the viral RNA may block RNAi action, the viral transcript may adopt an obstructive RNA structure, the viral RNA may be protected inside a virion particle, or the viral RNA may reside in a subcellular compartment that is not surveyed by the RNAi machinery.[18] Nevertheless, early studies indicated that RNAi is very effective in inhibiting HIV-1 replication in stably transduced T-cell lines expressing an antiviral siRNA/shRNA/miRNA.[16–18,27,30–63]

Although theoretically two forms of the HIV-1 RNA provide targets for RNAi-attack, one form seems protected. The "incoming" RNA genome present in infectious virion particles is the first putative target when it is delivered to the cytoplasm of an infected cell. Later in the infection cycle, the de novo synthesized viral transcripts form the second mRNA targets for RNAi attack. Several studies suggested that the incoming RNA genome is a target for RNAi,[44,64–67] but other studies have presented solid evidence against this possibility.[52,68–71] We measured complete protection of the incoming RNA genome and reasoned that the RNAi machinery—that is the RISC complex—is unable to access this RNA genome because it remains encapsulated in a core particle.[70] Coating of the RNA by the HIV-1 nucleocapsid protein may also shield the viral genome from RNAi attack. One could argue that it does not really matter at which step the replication cycle is inhibited, as long as the block is fairly complete.

Several theoretical criteria can be formulated to identify optimal target sites for RNAi attack on the 9-kb HIV-1 RNA genome: i) It may be beneficial to select target sequences in the early spliced mRNAs encoding the early proteins Tat, Rev, and Nef. An early block of viral gene expression will seriously hamper the expression of the late structural proteins and virion assembly. ii) It may be a good idea to select targets that are present in all subgenomic HIV-1 RNAs. For instance, sequences in the untranslated leader and the 3′-terminal nef gene qualify for this because they are present in all spliced RNA forms and obviously also in the full-length genomic RNA. iii) The HIV-1 genome may be targeted to induce transcriptional gene silencing (TGS) of the viral long terminal repeat (LTR) promoter.[72–74] The molecular mechanism of TGS requires further description, but transcriptional silencing of HIV-1 remains an attractive way to establish durable silencing. Epigenetic promoter DNA modifications through TGS could be an interesting method of achieving a more robust and especially a more prolonged gene silencing effect. iv) It may be advisable to select targets that are not part of a stable RNA structure, as will be discussed in the next paragraph. v) Viruses and their hosts have co-evolved for millions of years, and viruses have developed mechanisms to neutralize RNAi action. Viral proteins or RNA molecules may frustrate an RNAi attack by acting as RNAi suppressor or by binding to the target sequence.[75–79] Although the presence of a viral RNAi suppressor protein may seem problematic, promising results have been reported against suppressor-encoding viruses, such as hepatitis C virus and HIV-1, in gene therapy settings.[17,80] Specifically targeting the viral RNAs that encode such suppressor functions could be considered. Thus, a better understanding of the natural virus–RNAi interactions will be important for future fine tuning of RNAi-based therapies. vi) Last but not least, an important consideration for target selection is the sequence variation in natural HIV-1 isolates, which can be analyzed using the available sequence databases. The goal would be to design a therapy that is active against as many HIV-1 strains as possible. The idea to attack highly conserved viral sequences is also relevant for preventing viral escape, as natural sequence variation provides an obvious indication of the allowed sequence variation and thus the likelihood of viral escape. Within a well-conserved target sequence, many of the possible RNAi-resistance mutations will not be selected because they impose a high cost in terms of viral replication capacity. Indeed, silent codon changes were preferentially selected for targets that encode important protein domains, thus visualizing the restricted area of sequence space that is available for HIV-1 evolution in these domains.[37,81,82] Despite these theoretical considerations, it remains important to screen a set of RNAi inhibitor candidates and to select the most potent ones.

Several studies described the selection of RNAi escape mutants when a single inhibitor was used.[30,33,67,83,84] Different classes of escape mutations were observed: i) The

majority of HIV-1 escape mutants acquired a single point mutation within the target sequence. These results underscore the strict sequence specificity of the RNAi mechanism. On the other hand, there is also some evidence that prolonged RNAi inhibition can select for multiple point mutations, indicating that a single mutation may not provide complete resistance.[33,34] ii) Mutations outside the target sequence that result in an RNA structural change have also been observed as an exotic escape route. Further studies revealed that the efficiency of RNAi attack is largely determined by the availability of an accessible, that is unpaired, $3'$ end of the target sequence.[34,85] iii) Partial or complete deletion of the target sequence forms another escape route for nonessential viral genome segments. Deletions were described for a shRNA directed against the nonessential nef gene, which allows the virus more freedom in the selection of escape variants.[33] iv) A more indirect viral escape route was recently proposed upon targeting of the TAR hairpin motif that is involved in HIV-1 transcriptional activation by the viral Tat protein.[86] Viral escape coincided with multiplication of the number of binding sites for the Sp1 transcription factor in the viral LTR promoter. Thus, transcriptional upregulation seems to overcome the imposed RNAi inhibition. However, we argued that this adaptation reflects a general improvement of virus replication and not a specific escape from RNAi attack.[87] A very similar Sp1 duplication has been reported in HIV-1 evolution studies without any RNAi pressure.[88]

The clinical success of highly active anti-retroviral therapy is based on the combination of multiple anti-retroviral drugs. A similar strategy to counteract viral escape is to use multiple potent shRNA inhibitors.[19,22,26,58,89–91] In a large screen, we identified 21 potent shRNAs that target highly conserved HIV-1 sequences, which enabled us to design such a combinatorial gene therapy.[36] First, the combined expression of multiple shRNAs resulted in additive inhibition compared to the individual inhibitors. Second and most importantly, viral escape will be more difficult in a combination therapy because HIV-1 has to acquire resistance mutations in all targets at about the same time. The number and type of mutations (e.g., easy transitions versus difficult transversions) do similarly dictate the evolution of drug-resistant HIV-1 variants.[92,93] Alternatively, one could include "second-generation" shRNAs that specifically target popular viral escape mutants.[58]

We previously estimated the chance of viral escape for a therapy with a single versus multiple shRNA inhibitors.[26,58] We made the assumption that escape by means of a deletion is not an option for the virus, which seems appropriate when essential viral genes are targeted. Only point mutations were allowed to occur, and a single point mutation can, in theory, make the virus (partially) insensitive to RNAi. The error rate of the reverse transcriptase of HIV-1 is 3×10^{-5}, [94] and the chance of viral escape for a 19-nucleotide target in a single infection is $19 \times (3 \times 10^{-5}) = 5.7 \times 10^{-4}$. Studies in the field of drug resistance indicate that an untreated HIV-infected individual has a virus population size of 10^4 to 10^5. This means that several potential escape variants will already be present for each shRNA before the start of therapy, and the emergence of drug-resistant variants seems inevitable when a single shRNA is used. When multiple shRNAs (N) are used simultaneously, the likelihood of selecting an escape variant drops exponentially with the number of inhibitors $(5.7 \times 10^{-4})^N$. If we assume that there is already resistance to at least one of the shRNAs, the chance of a resistant variant emerging is $(5.7 \times 10^{-4})^{N-1}$. For instance, if four shRNAs are used simultaneously, the chance of escape is 1.9×10^{-10}. This chance seems remote given the average viral load in a patient. However, it cannot formally be excluded that multi-shRNA-resistant mutants can evolve *in vivo*. In *in vitro* cell culture infection experiments, we observed delayed viral escape with two shRNAs and no viral escape with four shRNAs.[17] These findings may guide the future development of a durable multi-shRNA-based gene therapy. However, the *in vivo* situation is

likely to be more complex. Virus replication may continue in cells that were not modified by the gene therapy, although these cells will eventually be removed by the immune system upon virus infection and the presentation of viral epitopes on the cell surface. Once partially resistant virus variants appear in such unmodified cell reservoirs, it seems fairly easy for HIV-1 to merge the resistance mutations by means of recombination.

The many cellular factors that support the virus replication cycle can also be targeted. This alternative RNAi strategy seems attractive because the genetic barrier for viral escape may be significantly higher. For instance, HIV-1 adaptation to another cellular co-factor may be impossible when no alternative cellular functions are available. We stress that this concept has not yet been proven experimentally, and the effects may vary widely for different cellular targets. Silencing of several protein co-factors has been tested: the CD4 receptor and co-receptors CCR5 and CXCR4,[41,95] integration factors (such as BAF1 [barrier to auto-integration factor-1], Emerin, and LEDGF [lens epithelium-derived growth factor]/p75),[96–98] transcriptional factors (such as nuclear factor [NF]-κB, P21-activated kinase 1 [PAK-1], and cyclin T1),[45,68,99] or furin, which is involved in envelope protein maturation.[99] Silencing of cellular co-factors is not restricted to protein-coding mRNAs because cellular miRNAs may also play an instrumental role in the viral replication cycle, as recently demonstrated for hepatitis C virus.[62,100–107] The new molecular insight in the virus-RNAi interactions present new therapeutic options.[18,108] The miRNAs that specifically suppress pathogenic viruses could be used to design miRNA mimics as a new class of antivirals. Alternatively, the cellular miRNAs that act as viral co-factors or the virally-encoded miRNAs could be blocked and inactivated by antisense inhibitors.

The attack on cellular functions is obviously not without danger. Host gene targets need to be carefully selected because their knockdown may be detrimental to the cell and the host. For instance, CD4 knockdown is not desirable in a therapeutic setting because this cell-surface receptor plays essential roles in the immune system. The CCR5 receptor is an obvious and attractive target. HIV-1-infected people that carry a defective CCR5 gene, CCR5-Δ32, show delayed disease progression, and people homozygous for CCR5-Δ32 are healthy and largely protected from HIV-1 infection.[109,110] Even partial silencing of CCR5 is expected to provide a therapeutic benefit for HIV-infected patients. Proof of principle was obtained in nonhuman primates that received blood stem cells treated with an SIV lentiviral vector expressing an shRNA against CCR5.[111] CCR5 expression was reduced and T cells from these primates were less susceptible to SIV infection compared to the appropriate control cells. The primates exhibited normal hematopoietic reconstitution, an important indication that the treatment is safe. The potential of such an anti-CCR5 gene therapy is further supported by the intriguing cure of an HIV-infected patient, who had leukemia in addition to AIDS, with a special bone marrow transplant.[133] This condition warranted the high risk of a blood stem cell transplant and a matching donor was identified with the CCR5-inactivating mutation. A standard regimen of drugs and radiation was administered prior to the transplantation to kill the patient's bone marrow and immune cells, and anti-retroviral treatment was stopped during transfusion. Surprisingly, standard tests have not detected HIV-1 in his blood for more than 600 days post transfusion. These results form an indirect proof of principle for a CCR5-targeting gene therapy approach, but one should realize that such a high risk treatment (10–30% of bone marrow transplant recipients do not survive) is unthinkable for the millions of HIV-infected individuals.

A gene therapy should ideally reach the many different cell types that are infected by HIV-1. Because CD4-positive T cells constitute the major cell population implicated in HIV infection and progression to AIDS, making these

cells resistant to HIV-1 should be a key aspect of any anti-HIV gene therapy. Two strategies have been proposed to make T cells resistant to HIV-1.[6,26,112,113] The first strategy is to isolate peripheral blood mononuclear cells from a patient's blood and to purify the CD4-positive T cells. These cells are subsequently treated *ex vivo* with a viral vector with the anti-HIV gene that provides resistance against HIV-1. The transduced cells are subsequently engrafted back into the patient where they will survive and improve the immunity of the patient. These T cells will have a limited life span, such that repeated infusions may be necessary. The second and more complicated, but hopefully also more durable, therapeutic scenario focuses on the hematopoietic blood stem cells. Blood stem cells continuously populate the myeloid and lymphoid cell lineages in the periphery. Engraftment of genetically modified but autologous blood stem cells will result not only in a steady production of new T cells but also monocytes, macrophages, and dendritic cells. The targeting of the CD34-positive stem cells with a single gene therapy treatment may suffice for a sustained therapeutic effect, although there remain many unknowns that should be addressed in pilot clinical studies. In HIV-infected individuals, the resistant cells will preferentially survive over unprotected cells, which are either killed directly by the virus or removed by the immune system upon recognition of viral epitopes on the cell surface. This stem cell gene therapy may lead to partial reconstitution of the immune system, although it is currently difficult to specify this repair in quantitative and temporal terms.

The lentiviral vector seems ideally suited for a durable gene transfer because it stably integrates in the genome of the target cell, producing a constant supply of antiviral RNAi molecules. The lentiviral vector is also very efficient in transduction of the CD34-positive blood stem cells or the CD4-positive T cells, the major target cells for an HIV–AIDS therapy. However, this vector system is largely based on HIV-1 sequences, and expression of antiviral shRNAs by the lentiviral vector may cause unwanted complications during vector production in the packaging cell. Indeed, targeting of the gag-pol and rev mRNAs of the packaging system can reduce the transduction titer.[114,115] We systematically addressed all possible routes by which the shRNAs can interfere with lentiviral vector production.[116] Both lentivirus production (capsid titer) and the transduction titer were reduced with shRNAs against the gag-pol mRNA. However, this problem could simply be avoided with a human codon-optimized gag-pol version that is not recognized by the shRNAs. Targeting of the rev mRNA in the packaging cell did not affect lentivirus production, probably because only a limited amount of Rev protein is needed to support its nuclear RNA export function. RNAi attack on remaining HIV-1 sequences in the vector genome is a serious possibility, which can be avoided by selecting shRNAs for which the target is absent in the lentiviral vector. Alternatively, one could actually modify the target sequence in the lentiviral vector without affecting important vector motifs. It may in fact be surprising that shRNA-encoding lentiviral vectors can be produced because the shRNA-encoding sequence in the vector genome should be attacked by the shRNA that is also expressed in the packaging cell. It turns out that the vector RNA genome is protected from this self-targeting because of the stable hairpin structure that masks the target sequence from recognition by the RNAi machinery.[34,85]

Other specific problems may be encountered in combinatorial approaches that use the lentiviral vector system. For instance, we reported deletion of shRNA cassettes when the same promoter element is used for their expression. The lentiviral vector was found to recombine at the repeat sequences during the transduction process, resulting in deletion of one or multiple expression cassettes.[36,117] Cells that express only a single inhibitor may form the breeding ground for partially resistant virus variants that can subsequently recombine to acquire multi-shRNA resistance. To

avoid recombination-mediated deletion, multiple shRNAs should be expressed from different promoter elements. Indeed, vector genome stability was improved when four shRNAs were expressed from four different promoters.[36] Alternatively, new shRNA designs may avoid the use of multiple promoter elements. For instance, one could stack multiple shRNA modules on top of each other in a single transcript driven by a single promoter element. We designed such anti-HIV molecules, the so-called extended shRNAs or e-shRNAs, but this approach requires a careful design of the extended hairpin molecule.[50,118] One could also generate a single miRNA-like transcript that encodes multiple antiviral miRNAs/siRNAs.[51] The incorporation of miRNA sequences, e.g., Drosha cleavage sites, within the context of the lentiviral RNA vector genome may also have a negative impact on lentiviral vector production and transduction. Further studies are needed to optimize vector systems, in particular for the more complicated combinatorial RNAi approaches.

Important issues in relation to safety and efficacy need to be addressed in appropriate animal models before clinical trials can be considered. Several potential side effects have been reported for shRNAs. For instance, the *in vivo* overexpression of shRNAs resulted in the death of mice due to saturation of the RNAi machinery.[119] The observed fatal side effects were primarily ascribed to saturation of Exportin-5, leading to interference with nuclear export of miRNA precursors and miRNA function. This result demonstrates, in a rather dramatic manner, that shRNA overdosing is dangerous; however, the mice that received a moderate RNAi dose were fine. There are also indications that shRNA expression can induce the interferon pathway. The original paradigm is that only dsRNA molecules that are larger than 30 base pairs can induce this pathway, but small dsRNA were shown to evoke this reaction.[120–124] This interferon induction is dose dependent, and some sequence motifs have been implicated.[122,124] Finally, RNAi could also induce off-target effects in which siRNAs silence partially complementary transcripts through an miRNA-like mechanism. Such an effect requires base-pairing complementarity between the siRNA seed region and the 3′ untranslated region of a target gene.[125–127] Any siRNA molecule is likely to have such limited complementarity with a number of cellular genes, and the number of potential off-target genes will increase significantly in a combinatorial setting. Systems for inducible or tissue-specific shRNA expression may control such off-target effects.

To avoid high expression levels that may induce unwanted side effects, we used the multi-shRNA lentiviral vector at a low multiplicity of infection to obtain cells with a single vector copy.[17] Potent HIV-1 inhibition was achieved in this single copy context, and the inhibition remained stable for at least 100 days. The growth rate of transduced cell lines and primary T cells was not affected, and the interferon and dsRNA-dependent protein kinase systems were not induced. These potential side effects remain a genuine concern for the development of a multiple shRNA approach against HIV-1, and the potential risks should be properly assessed in preclinical evaluations. The first *in vivo* studies on therapy efficacy and safety were performed in a mouse model by Akkina and colleagues.[28,114] Recently, a new humanized mouse model was developed that is ideally suited for such preclinical assessment. This humanized Rag2$^{-/-}\gamma_c{}^{-/-}$ mouse sustains long-term multilineage human hematopoiesis and is capable of mounting immune responses.[128] We engrafted human CD34-positive cells after *ex vivo* transduction with a lentiviral vector that expresses an shRNA against the HIV-1 nef gene.[129] The shRNA expression did not affect the development of the CD34-positive stem cells into various mature leukocyte subsets, including CD4-positive T cells. HIV-1 replication was tested *ex vivo*, demonstrating sequence-specific inhibition, which forms an important proof of concept in the clinical development of an anti-HIV RNAi-based gene therapy. These results indicate that the procedure is safe for single shRNA expression vectors. The next step in

the preclinical evaluation is to challenge these mice with HIV-1, which normally results in viremia and depletion of the CD4-positive cells, some of the main features of human HIV-1 infection.[130–132]

Antiviral RNAi strategies seem more efficient than other nucleic acid-based antiviral approaches, probably because they tap in on an existing cell mechanism. Several antiviral RNAi applications are currently being tested in clinical trials, ranging from a transient siRNA therapy for RSV infection to a gene therapy for HIV-infected individuals. Because we are still in the early days of development, it seems obvious that there is ample room for further improvement of the activity and the specificity of the RNAi inducers and the means of delivery. For antiviral therapy of chronic infections, the evolution of RNAi-resistant virus variants remains a serious problem. A combinatorial RNAi approach seems able to prevent viral escape, but further fine tuning of such RNAi-based therapies is warranted. The coming years are likely to see an increasing range of clinical applications, given the immense interest in RNAi as a therapeutic. The development of treatments for infections with pathogenic viruses will probably be among the first successes.

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Conflicts of Interest

The author declares no conflicts of interest.

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