Expert Opinion

Anti-Infectives

Antiviral applications of RNAi for coronavirus

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Until the appearance of severe acute respiratory syndrome (SARS), caused by the SARS coronavirus (SARS-CoV) in early 2003, coronavirus infection was not considered to be serious enough to be controlled by either vaccination or specific antiviral therapy. It is now believed that the availability of antiviral drugs effective against SARS-CoV will be crucial for the control of future SARS outbreaks. Recently, RNA interference has been successfully used as a more specific and efficient method for gene silencing. RNA interference induced by small interfering RNA can inhibit the expression of viral antigens and so provides a new approach to the therapy of pathogenic viruses. This review provides an overview of current information on coronavirus and the application of small interfering RNA in viral therapeutics, with particular reference to SARS-CoV.

Keywords: antiviral agent, coronavirus, severe acute respiratory syndrome, short interfering RNAs

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1. Introduction

Coronaviruses are large, enveloped, positive-stranded RNA viruses. They have a broad host range and are capable of infecting many species, particularly the tissues of the upper respiratory and gastrointestinal tracts, the liver and CNS [1,2]. Coronaviruses, such as human coronavirus (HCoV)-229E and HCoV-OC43, are the major causative agent for 15 – 30% of common colds in humans each year. Colds are usually mild and self limiting, with a clear increase in neutralising antibody titre soon after infection; however, a certain percentage of the population can be reinfected by the same virus. Colds due to coronavirus infection are particularly prevalent in the winter. In fact, outbreaks of two known human coronaviruses, occurring in alternating years, has become a common seasonal event [2]. Coronavirus infections were not previously considered to be serious enough to require treatment by either vaccination or specific antiviral therapy [2]. The situation may have altered since the quasi-pandemic outbreaks during late 2002 and early 2003 of severe acute respiratory syndrome (SARS), caused by a newly identified coronavirus, SARS-CoV [3-8]. The disease has typical influenza-like symptoms such as high fever, myalgia, dyspnea, lymphopenia and lung infiltrates (pneumonia). Then, as it develops further, acute breathing problems that raise the overall mortality to 10% can result. A total of 8,098 probable SARS cases were reported to the WHO between 1 November 2002 and 31 July 2003, of which 774 who died were shown to be SARS-CoV-positive [8,101]. Owing to this abrupt pandemic outbreak, certain empirical measures, such as antibiotics, antiviral agents (ribavirin, oseltamivir and HIV-1 protease inhibitors), corticosteroids, IFNs and normal human immunoglobulin preparations, were applied to treat those suffering from SARS [9-11]. Researchers continue to test all kinds of regimens to treat SARS, including neutralising antibodies, fusion inhibitors, natural products, vaccines and RNA interference (RNAi) [12-14]. A large-scale re-emergence cannot be ruled out even though only sporadic SARS infections have been reported since June 2003. As a result, antiviral drugs with high activity against

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SARS-CoV are in high demand. RNAi is an innate, adaptive defence mechanism triggered by double-stranded RNA (dsRNA). Recently, small interfering RNA (siRNA) has shown promise in the protection from viral invasion, as it can inhibit the expression of viral antigens and accessory genes as well as control the transcription and replication of the viral genome. This review focuses on the current information available on viral gene expression and coronavirus genome replication. Based on this knowledge, RNAi strategies that have been designed to interfere with viral replication in the treatment of SARS-CoV infection are reviewed.

2. Coronaviruses and SARS-CoV

2.1 Taxonomy

Coronaviruses, a genus of the *Coronaviridae* family, are large, enveloped, positive-stranded RNA viruses, and are the causative agent of many infectious diseases in humans and animals [2,15]. The viral envelope is studded with long, petal-shaped spikes, which give the virus its characteristic crown appearance from which it was named [1]. Before the SARS outbreak, coronaviruses were antigenically divided into three groups and viruses within each group were further classified on the basis of host, nucleotide sequence and serological relationships [1]. Members of group I include HCoV-229E, porcine transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), feline infectious peritonitis virus (FIPV), feline enteritis virus (FECoV) and canine coronavirus (CCoV), whereas HCoV-OC43, mouse hepatitis virus (MHV) and bovine coronavirus (BCoV) belong to group II. Group III includes avian infectious bronchitis virus (IBV) and turkey coronavirus (TCoV). Gene analysis of the entire SARS-CoV RNA genome (29,727 nucleotides) [7,16] suggests that SARS-CoV should be included in the *Coronaviridae* family. Although SARS-CoV reacts with antibodies against group I viruses [4], SARS-CoV clearly represents a new group of coronavirus [4,7,16,17].

2.2 Genome organisation of coronaviruses

The virion of coronavirus is a spherical particle 100 – 120 nm in diameter and contains a capped, polyadenylated, single-stranded, positive-sense genomic RNA, which is 27 – 32 kb in length, and is the largest known RNA virus genome [1,18]. Coronavirus-infected cells contain a characteristic 3′ coterminal and nested mRNA. The mRNA has a capped leader sequence consisting of ~ 70 nucleotides that is derived from the 5′ end of the genome [1,19]. An untranslated region (UTR) of 200 – 400 nucleotides follows this leader sequence. Another UTR at the 3′ end of the RNA genome is followed by a polyA tail of variable length. Both the 3′- and 5′-UTRs are important in RNA replication and transcription, as is the transcription-regulatory sequence (TRS), which is a typical feature of coronaviruses [1]. This short motif is usually near the beginning of each open reading frame (*ORF*) and the 3′ end of the leader sequence (**Figure 1**). In addition, the consensus sequence, 5′-CUAAAC-3′, is found immediately in front of the spike (*S*) protein and membrane (*M*) protein genes and *ORF* 10 [7]. Gene organisation in most coronaviruses follows the same pattern of genes coding for polyproteins 1a and -b, S, M, envelope protein (E) and nucleocapsid protein (N; **Figure 1**) [18,20]. Some group II coronaviruses have a fringe of short spikes consisting of haemagglutinin esterase (HE). Coronavirus genomes also contain a variety of additional ORFs that encode 2 – 4 nonstructural proteins with no known function; these genes are not conserved among coronaviruses.

2.3 Gene expression of coronavirus

Polyproteins 1a and -b are required for viral RNA synthesis and believed to be the only viral proteins that are synthesised directly from the original viral (other viral proteins are translated from subgenomic mRNAs). Once viral RNA synthesis takes place, more product of gene 1 are translated from the newly synthesised genomic RNA. The primary gene products (*ORF 1A* and *-B*), predicted to be ~ 700 – 800 kDa, undergo co- or post-translational processing into various proteins as a result of their own protease activity. The virus-encoded proteases, papain-like cysteine protease (PLpro) and 3C-like cysteine protease (3CLpro), cleave the polypeptide into small polypeptides that are required for replication and transcription [21,22]. RNA-dependent RNA polymerase (RdRp) and helicase are essential components of the replicase complex, which, presumably, contains other viral and cellular proteins. The replicase complex is responsible for transcribing: the full-length negative and positive RNAs; the 3′ coterminal set of nested subgenomic mRNAs; and subgenomic negative RNA strands [1,23,24].

The entry of enveloped coronaviruses usually involves the three steps of attachment, receptor binding and virus-cell fusion, mediated by viral envelope proteins [25]. The S glycoproteins, which make up large, petal-shaped spikes on the surface of the virion, bind to a cellular receptor, promoting fusion of the viral and cellular membranes, and this event probably explains the primary viral tropism [25]. This highly glycosylated protein, with a molecular mass of ~ 150 – 180 kDa, can be divided into three structural domains: a large external N-terminal domain (divided into subdomains S1 and -2); transmembrane domain; and short C-terminal cytoplasmic domain [26]. In many cases, the cell receptors for coronaviruses have been identified. The MHV receptor is a murine biliary glycoprotein belonging to the carcinoembryonic antigen family of the Ig superfamily [27,28]. The cell membrane-bound metalloproteinase, aminopeptidase N (CD13), is probably the receptor for TGEV, HCoV-229E and CCoV [29-31]. CD13 is widely distributed in cells in many tissues, including respiratory, enteric epithelial, neuronal and glial cells. The receptor for SARS-CoV is considered to be angiotensin-converting enzyme 2 (ACE2), required for binding to permissive cells and the S1 subunit [32]. No receptors for type III viruses have yet been identified.

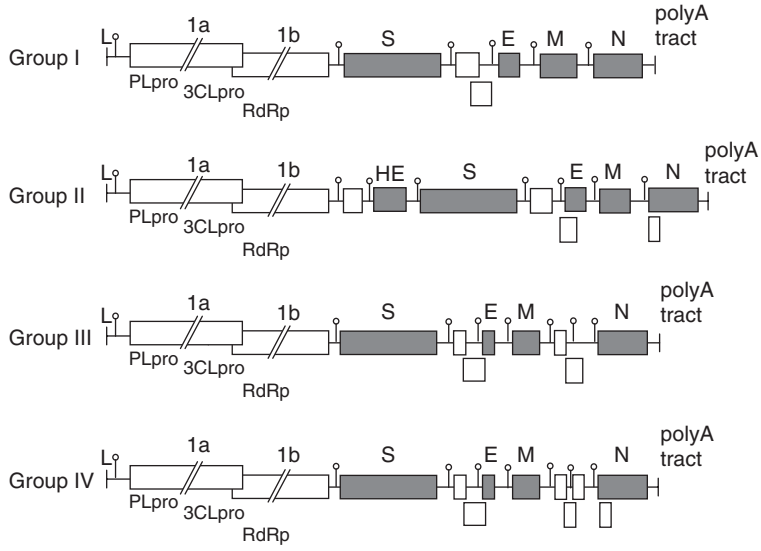

Figure 1. Gene organisation of coronaviruses. Coronaviruses are divided into four classes on the basis of their genome organisation: HCoV-229E belongs to group I; mouse hepatitis virus to group II; avian infectious bronchitis virus to group III; and SARS-CoV represents a new class of coronavirus, group IV. The boxes indicate ORFs and the shaded boxes are coding sequences for structural proteins. The gene structure of coronavirus consists of a cap, a 65 – 98 base L sequence, a TRS, the coding sequence and a polyA tract at the 3′ end. The round-headed pins represent TRS; the TRS for groups I – IV are UCUC/AAACU, UCUAAC, CUU/GAACA and CUAAAC, respectively. PLpro and 3CLpro cleave the polyprotein into small polypeptides. RdRp, an RNA-dependent RNA polymerase, is an essential component of the replicase complex. M is required for virus budding. S (a viral spike glycoprotein) is involved in receptor binding and membrane fusion. E (a small membrane protein) is responsible for coronavirus assembly. Protein N is a virion RNA-associated protein.
CLpro: 3C-like cysteine protease; E: Envelope protein; HCoV: Human coronavirus; L: Leader; M: Membrane protein; N: Nucleocapsid protein; ORF: Open reading frame; PLpro: Papain-like cysteine protease; RdRp: RNA-dependent RNA polymerase; S: Spike protein; SARS: Severe acute respiratory syndrome; TRS: Transcription-regulatory sequence.

The integral membrane proteins, M and E, are the minimum protein units required for virus assembly [33]. M protein is found not only on the viral envelope but also in the viral internal core [34], and associates with the Golgi complex in the cell, thereby dictating the site of virus assembly [35]. The expression of M and E proteins may be sufficient enough to trigger the formation of virus-like particles (VLPs). When S protein is coexpressed with the M and E proteins, it is incorporated into VLPs with (presumably) an authentic conformation that is able to infect cells [33-37]. As they do not contain viral nucleic acid, VLPs are an ideal candidate for vaccine preparation. The final structural protein, N protein, with a molecular mass of 50 – 60 kDa, probably associates with viral RNA to form a long and flexible helical nucleocapsid [34,38]. N protein may also play a role in viral RNA synthesis, as coronavirus RNA polymerase activity is inhibited *in vitro* by the anti-N protein antibody [39].

3. RNA interference

3.1 Introduction

dsRNAs usually play pivotal roles as intrinsic intermediates during the genomic replication of many viruses, but they are rare in eukaryotic cells. Higher eukaryotes respond to dsRNA regardless of whether it is produced by viruses, introduced artificially or formed in immune defence responses. In mammalian cells exposed to dsRNA, the major immune response is an increase in serum IFN levels, which can suppress viral spread by inhibiting viral gene expression and initiating the apoptosis of infected cells [40,41].

In contrast to mammalian cells, many other higher eukaryotes, including plants, nematodes and insects, are not able to mount an IFN response. Nonetheless, RNAi was first described in plants, in which it acts as a natural immune mechanism against viral infection [42-48]. Triggering post-transcriptional gene silencing (PTGS) by siRNA molecules has been observed in a wide variety of species [49-51].

RNAi, which is a powerful technique, has been widely used to silence specific genes in mammalian and human cells [52,53]. However, dsRNAs > 30 bp can be destroyed as they can induce an immune response in mammalian cells and activate dsRNA-dependent kinase and 2′-5′-oligoadenylate synthetase, leading to the induction of IFN expression [54]. Because of their relatively small size, synthetic siRNAs can escape the immune response [55]. As a result, researchers are developing many RNAi-based drugs to prevent and treat diseases such as viral infection, tumours and metabolic disorders [56-60].

3.2 Mechanism

Recent studies indicate that RNAi exerts it effects via a two-step mechanism: an initiation and effector step [61,102]. In the initiation step, long dsRNAs are introduced either directly or via a transgene or virus. Subsequently, the host protein, Dicer, an ATP-dependent ribonuclease and member of the RNAse III family, binds with high affinity to the introduced dsRNA and cleaves it into 21 – 23 nucleotide fragments referred to as siRNA duplexes [62,63]. In the effector step, the siRNA duplexes complex with multinucleases to form the so-called RNA-induced silencing complex (RISC) [64], which then undergoes an ATP-dependent activation step that results in the unwinding of the double-stranded siRNA component to form a single-stranded guide RNA that leads the RISC to other complementary mRNAs. After the mRNA is bound, an unidentified ribonuclease component of RISC cleaves the bound mRNA at the centre of the region complementary to the guide RNA. After this reaction has taken place, RISC is released to seek out other mRNA homologues, at the same time as the cleaved mRNA is degraded by cellular exonucleases [65]. The effectiveness of RNAi has been improved using RNA-directed RNA polymerase, which produces a large number of copies of the triggering RNA [66].

3.3 Selection of siRNA

Specifically targeting siRNAs is very important because slight positional changes in the siRNA relative to the mRNA can have drastic effects on silencing, indicating that the secondary structure of the target mRNA plays a role in siRNA accessibility [67]. However, an exact sequence match in the sense strand of siRNA duplexes may not be necessary, as a single-stranded antisense siRNA has been shown to initiate the cleavage of target RNA [65] and as many as five mismatches in the sense-strand RNA can be tolerated [68]. In contrast, a single basepair mismatch on the antisense strand corresponding to the target RNA can significantly reduce siRNA-mediated message degradation [69]. Genetic mutation in the regions coding for major antigenic proteins may limit the inhibitory effect of siRNA. To circumvent the sequence variation among viral strains, highly conserved siRNA target sequences must be selected. If possible, the target sequences should be chosen from beyond the coding regions, where they may have structural roles [53]. General guidelines for the choice of siRNA sequences have been reported [70-73]: and a G:C bp at the 5′ end of the sense strand, A:T bp and A-T-rich sequence at the 5′ end of the antisense strand, and no G-C stretch > 9 bp are highly recommended. siRNAs can be prepared enzymatically, which has the advantages of improved efficiency and lower costs [74]. In addition, it can provide a large panel of siRNAs, cover a long segment of mRNA and avoid the re-emergence of escaped mutants. Another method is to use RNA polymerase to transcribe siRNAs from short DNA templates in which the encoded siRNAs are downstream of the RNA promoter [75]. A major limitation when using either chemically or enzymatically synthesised siRNAs is that they are unstable and may only have a transient effect; however, the use of chemically protected siRNAs and viral vectors may avoid this problem [76-78].

3.4 siRNA antiviral strategy for coronavirus

RNAi technology is an ideal tool for inhibiting viral replication in host cells as the siRNA can interact with certain viral genes and silence their expression. It can also be used to probe the steps during the viral cycle, not only for plus-strand RNA viruses (**Figure 2**) but also for many other types of virus [58,79]. Genes encoding vital proteins in reproducing SARS-CoV virions can be chosen for chemotherapeutic intervention (e.g., those coding for S, 3C-like protease [3CLpro], RNA-dependent RNA polymerase and possibly other gene products involved in viral-protein-mediated processes) [81] first demonstrated that siRNA was able to silence the replicase of SARS-CoV (1a region of the genome) and that this approach was effective *in vitro* against SARS-CoV. Wang *et al.* [82] subsequently observed that vector-based siRNAs could inhibit the replication of SARS-CoV, and showed that expression in the plasmid, pSUPER, of siRNAs specifically targeting viral RNA polymerases could block the cytopathic effects of SARS-CoV on Vero cells. These plasmids were also able to block viral replication, as shown by both the titre and levels of viral RNA and protein. Zhang *et al.* [83] reported that siRNA can reduce the cytopathic effects when used to transfect VeroE6 cells immediately before SARS-CoV infection. Moreover, Zhang *et al.* [84] showed that DNA vector-driven siRNA can selectively silence S gene expression in SARS-infected 293T cells, and reported that siRNA targeting the leader sequence decreased both mRNA levels and protein levels of reporter genes in 293T cells. In addition, the steady expression of siRNA in VeroE6 cells decreased the transcription of SARS-CoV gene mRNA. Furthermore, these researchers pointed out that siRNA specific for the leader sequence has a stronger inhibitory effect on SARS-CoV replication than that targeting either the *S* gene or antisense oligodeoxynucleotides [85]. Zheng *et al.* [86] showed that three chemically synthesised siRNA duplexes targeting viral RNA polymerases, and one targeting the *S* gene potently inhibited SARS-CoV infection and replication in fetal rhesus kidney cells (FRhK-4). They observed a prolonged prophylactic effect of siRNA duplexes, with ≤ 90% inhibition of transcription, lasting for ≥ 72 h. Combinations of active siRNA duplexes targeting different regions of the viral genome resulted in ≤ 80% inhibition. The authors [87] focused on four regions in the SARS-CoV genome: the leader sequence; TRS, 3′-UTR; and the S protein-coding sequence. Purpose-designed siRNAs were prepared to test their specificity for the SARS-CoV viral RNAs and examine their inhibitory effects on viral protein expression and replication. The results showed that *S* gene-targeted siRNAs profoundly reduced levels of both

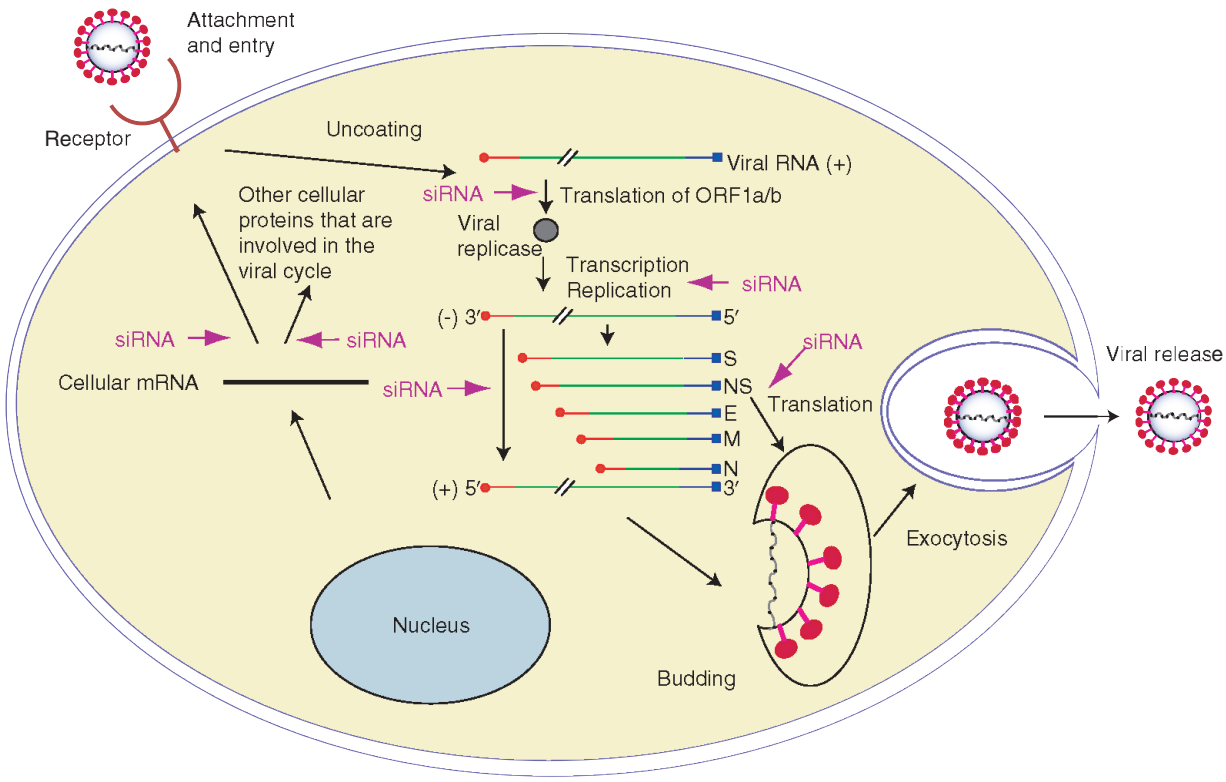

Figure 2. Replication and potential targets. The coronavirus genome is a single-stranded, positive-sense RNA molecule. The life cycle of the virus starts with the interaction between S protein and the cellular receptor. Silencing the virus receptor of the host cell with siRNA may prevent entry of the virus into the cell. S protein is also a good target for RNAi. The RNA genome of the virion is released into the cytoplasm after the virion attaches to the host receptor, and the virus takes advantage of the host translational machinery to translate ORF1a and -1b into polyproteins. Cleavage by virally encoded proteases yields the components that are required to assemble the viral replicase complex, which synthesises full-length, negative-strand RNA. The protease and polymerase involved in viral replication are potential siRNA targets. A discontinuous transcription strategy is taken by the virus during negative-strand synthesis through which a set of nested subgenomic and minus-sense RNAs are formed. The resulting mRNA has a 70 base leader sequence at the 5′ end and a polyA tail at the 3′ end (shown as circles and squares, respectively). Viral mRNAs are then translated to protein (indicated on the right). These negative strands act as templates of the synthesis for their positive counterpart. These subgenomic RNAs are good targets for siRNA silencing. N protein and the newly synthesised genomic RNA associate into a helical nucleocapsid. M, E and S proteins are incorporated into the lipid bilayer of the endoplasmic reticulum and transported to a budding compartment. N protein then binds to M protein, initiating virion assembly. The virus is finally released from the host cell by the fusion of virion-containing vesicles with the plasma membrane. The steps of the viral replication cycle that can be inhibited by RNAi are highlighted with arrows.
E: Envelope protein; M: Membrane protein; N: Nucleocapsid protein; ORF: Open reading frame; RNAi: RNA interference; S: Spike protein.

RNA transcripts and viral antigens, although 3′-UTR-oriented siRNAs were not as effective, the two other siRNAs had no effect.

The siRNAs results mentioned above were obtained in cell culture studies. Recent studies using mouse models have demonstrated that airway infections caused by influenza virus and respiratory syncytial virus can be treated prophylactically by intranasal delivery of siRNAs [88-91]. Li *et al.* [92] administered chemically synthesised siRNA duplexes intranasally in the rhesus macaque SARS model and found a reduction in SARS-CoV infection-induced fever, SARS-CoV viral levels and acute diffuse alveoli damage [93]; accumulated dosages of siRNA 10 – 40 mg/kg did not result in any siRNA-induced toxicity. These results provide strong evidence that these siRNA agents are potent both in the prophylactic and therapeutic treatment of SARS infection as well as lacking toxicity in this nonhuman primate model. These encouraging findings suggest that siRNAs may be applicable against SARS-CoV in man, and that suppression of the viral cycle or expression of viral antigen using RNAi treatment shows promise for the therapy of viral infection.

4. Expert opinion

Conventional drugs and vaccines used for the treatment of viral diseases may have many adverse effects, such as toxicity, cost and resistance, and complicated administration protocols. Considerable information regarding the mechanism and use

of RNAi as a tool for manipulating gene expression and inhibiting viral replication has been obtained in recent years. The marked advantages of siRNA can be attributed to the relative ease of its design, construction and testing, but, in particular, its low cost. More importantly, siRNA is a short length of nucleic acid and believed not to generate an unfavourable immune response when administered to patients, thus making it an ideal candidate for the treatment of viral infection. siRNA normally carries a negative charge under physiological conditions, which makes it difficult for the molecule to traverse the cell membrane, but carriers such as liposomes and many viral vectors and electroporation can efficiently deliver siRNAs into mammalian cells. The current challenge for the success of siRNA-based treatment is how to precisely deliver the siRNAs in an efficient and safe way to the target cells and organs. Considerable effort will be required for a better understanding of virology and the further development of this technique. siRNA-based drugs may soon be deployed to relieve or even eradicate the intractable viral infections that currently afflict mankind.

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