Endoribonuclease-Prepared Short Interfering RNAs Induce Effective and Specific Inhibition of Human Immunodeficiency Virus Type 1 Replication

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Short interfering RNAs (siRNAs) targeting viral or cellular genes can efficiently inhibit human immunodeficiency virus type 1 (HIV-1) replication. Nevertheless, optimal HIV-1 gene silencing by siRNA requires precise complementarity with most of the target sequence. The emergence of mutations in the targeted gene could lead to rapid viral escape from the siRNA. In the present study, Escherichia coli endoribonuclease III (RNase III) or mammalian Dicer was used to cleave double-stranded RNA into endoribonuclease-prepared siRNA (esiRNA). esiRNAs generate a variety of siRNAs which can efficiently and specifically target multiple sites in the cognate RNA. esiRNAs targeting the region encoding the HIV-1 reverse transcriptase (RT) reduced viral replication by 90%. The inhibition was dose dependent and sequence specific because several irrelevant esiRNAs did not inhibit HIV-1 replication. Importantly, esiRNAs obtained from the prototypic RT sequence of the HXB2 strain and from highly mutated RT sequences showed similar degrees of viral inhibition, suggesting that the heterogeneous population of esiRNAs could overcome individual mismatches in the RT sequence. Finally, esiRNAs generated by Dicer cleavage were five times more potent than those generated by bacterial RNase III digestion. These results show that esiRNAs are potent HIV-1 inhibitors. Moreover, sequence targets do not need to be highly conserved to reach a high level of viral replication inhibition.

Double-stranded RNA (dsRNA) can induce the specific degradation of homologous mRNA species, a process termed RNA interference (RNAi) (14). dsRNAs are processed by the RNase Dicer, a member of the RNase III family of dsRNA-specific endonucleases, into ~22-nucleotide fragments that bear 2-nucleotide 3'-end overhangs (2, 16, 50). These short interfering RNAs (siRNAs) are the effector molecules of this evolutionarily conserved mechanism. siRNAs are incorporated into the ~500-kDa RNA-induced silencing complex (RISC) (16, 17, 50). One strand of the siRNA is used to target RISC to homologous mRNAs, which are cleaved and degraded. Transfection of 21-nucleotide siRNAs inhibits the expression of the target gene in a sequence-specific manner (13). siRNAs have become the method of choice for mammalian cell genetics as well as for sequence-specific therapeutic approaches (11, 12, 22, 24, 38, 39, 43). Several studies have reported the use of siRNAs to specifically inhibit human immunodeficiency virus type 1 (HIV-1) replication by targeting viral or cellular genes (4, 8, 9, 20, 29, 30, 33, 34, 36, 37, 40). These results suggest that RNAi represents an important new therapeutic approach for treating HIV-1 infection. However, a major problem of all antiretroviral therapies is the emergence of resistant variants. Recently, we showed that optimal HIV-1 gene silencing by siRNA requires precise complementarity with most of the target sequence and that substitutions at only a few positions at the 5' and 3' ends are partially tolerated (40). Not surprisingly, several studies have shown that HIV-1 promptly escapes previously effective siRNAs (4, 9, 46). Recent work with HIV-1 has also shown that tolerance to target sequence mismatches may depend on the sequence of the siRNA tested (30). This fact, coupled with the enormous genomic heterogeneity of HIV-1 quasispecies, may hinder the efficacy of single defined siRNAs. Coexpression of multiple siRNAs that target conserved RNA sequences could reduce the emergence of single siRNA-resistant viruses, with an effect comparable to that achieved by three- or four-anti-HIV-drug combinations commonly known as highly active antiretroviral treatment. Recently, the use of multiple short hairpin RNAs (shRNAs) against HIV-1 has been shown to delay virus escape (45). Similarly, work with poliovirus has shown that targeting multiple viral sequences with a pool of siRNAs overcomes resistance mechanisms to RNAi and prevents viral escape (15).

In the present study, a mixed population of endoribonuclease-prepared siRNAs (esiRNAs) was generated to inhibit HIV-1 replication. esiRNAs produce a variety of siRNAs, which are able to efficiently and specifically target multiple sites in the cognate RNA. esiRNAs targeting the region encoding the HIV-1 reverse transcriptase (RT) may be a valid option for inhibiting viral replication and overcoming resistance to siRNAs.

MATERIALS AND METHODS

Generation of the esiRNA libraries. DNA for in vitro transcription was generated by PCR using two oligonucleotides with the T7 promoter appended to the 5' ends. The T7 promoter-containing PCR primers were used either in separate PCRs

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 RESULTS

 Escherichia coli RNAse III-generated esiRNAs targeting the HIV-1 RT efficiently inhibit viral replication. To test whether enzymatically prepared siRNAs have antiviral activity, we generated esiRNAs by bacterial RNAse III digestion of a 0.7-kb HIV-1 RT dsRNA (esiRTHIV) (Fig. 1). The antiviral potency of esiRTHIV was assessed in U87-CD4 cells with cotransfecting an HIV-1 HXB2 infectious plasmid clone and different concentrations of esiRTHIV. As shown in Fig. 1A, HIV-1 replication was markedly inhibited by transfection of an esiRNA directed against the RT coding region. In particular, a 100 nM concentration of esiRTHIV suppressed viral replication at day 5 by nearly 90% compared with the control, demonstrating effective silencing activity. Cotransfection of different amounts of esiRTHIV demonstrated that the inhibition was dose dependent (Fig. 1A and C). The same inhibitory effect was obtained with an independently prepared batch of esiRNA (data not shown). As a control for specificity of inhibition, the anti-viral activity of unrelated esiRNAs, namely, esiLuc and esiHCV, was assessed. Cells treated with either esiLuc or esiHCV did not exhibit an inhibitory effect on HIV-1 replication (Fig. 1C). These results demonstrated that HIV-1 replication can be efficiently inhibited in a dose-dependent manner by transfection with esiRTHIV and that this inhibition is sequence specific.

 esiRTHIV was analyzed for its ability to inhibit infectious HIV-1. To this end, U87-CD4 cells were transfected with the concentration of esiRTHIV indicated below (Fig. 1D) and then infected with the wild-type HIV-1 HXB2 strain 24 h after transfection. As shown in Fig. 1D, these results paralleled those obtained with the cotransfected infectious viral plasmid. However, viral replication inhibition was lower than that obtained in cells cotransfected with the infectious HIV-1 clone. Recent findings indicate that the incoming HIV-1 RNA genome is not targeted by RNAi (47). Overall, these results demonstrated efficient inhibition of HIV-1 replication with esiRTHIV preparations.

 esiRNAs generated from highly mutated RTs inhibit wild-type HIV-1 replication. Our previous finding that a single mutation in the viral genome can facilitate escape from siRNA
control prompted us to test whether the heterogeneous population of esiRNAs could efficiently inhibit the replication of viruses with mismatches in the target region. In other words, we questioned whether an esiRNA might overcome the limitations imposed by mutations in a region susceptible to nucleotide variation during antiretroviral therapy. To this end, the potency of esiRNA preparations generated from two subtype B viruses carrying several drug resistance-encoding substitutions within the RT target sequence was evaluated (Fig. 2A). These two viruses, D and G (7), differ by 3.5% and 5%, re-
FIG. 2. esiRNAs generated from highly mutated RTs inhibit wild-type HIV-1 replication. (A) U87-CD4 cells were mock transfected or transfected with a 100 nM concentration of the esiRTHIV, esiRTHIVD, or esiRTHIVG preparation. Cells were cotransfected with an HXB2 infectious clone. At days 1, 2, 3, 4, and 5 after transfection, viral replication was monitored by determining the p24 antigen level in the culture supernatant. (B) U87-CD4 cells were mock transfected or transfected with 100 nM esiRTHIV subtype B, siRTHIV subtype C, siRTHIV subtype A1, siRTHIV subtype J, esiRTHIVD, or esiRTHIVG preparations. Cells were cotransfected with an HXB2 infectious clone. Five days posttransfection, the supernatants of transfected cells were analyzed for p24 antigen production. (C) U87-CD4 cells were mock transfected or transfected with 100 nM esiRTHIV subtype B, siRTHIV subtype C, siRTHIV subtype A1, siRTHIV subtype J, esiRTHIVD, or esiRTHIVG preparations. Cells were cotransfected with an HIV-2 (ROD strain) infectious clone. Five days posttransfection, the supernatants of infected cells were assayed for p24 antigen. (D) U87-CD4 cells were mock transfected or transfected with different concentrations (150, 75, or 50 nM) of esiRTROD or esiRTHXB2 preparation and the ROD or HXB2 infectious clone. Values represent the means ± standard deviations from at least three independent experiments.
spectively, from the wild-type HXB2 RT target nucleotide sequence. Similar to the results described above, these two esiRNA preparations, esiRTHIVdicer and esiRTHIVG, efficiently inhibited the replication of the wild-type HXB2 isolate (Fig. 2A). This finding indicates that some nucleotide variation within a population of siRNA sequences obtained by digestion of dsRNA does not measurably affect the antiviral activity exerted by the esiRNA preparation. To test this hypothesis...(Continued...
further, three additional esiRNAs were generated from different HIV-1 group M subtypes (A1, C, and J) (Fig. 2B) and tested against the wild-type HXB2 strain. The RT coding region of these three esiRNA preparations differed 10 to 12% in nucleotide sequence from the HXB2 strain. Notably, the replication of the wild-type HXB2 isolate could be inhibited by each of these three esiRNAs preparations (Fig. 2B). Moreover, the inhibition was sequence specific because HIV-2 ROD, which shares only 50% nucleotide identity with HIV-1 HXB2 in the targeted RT region, was not affected by any of the five esiRNA preparations tested (Fig. 2C). Instead, HIV-2 ROD replication was efficiently inhibited in a dose-dependent manner by its cognate esiRT HIV-2 preparation (Fig. 2D). These findings show that treatment with esiRNAs efficiently inhibits HIV-1 replication and that the heterogeneous population of esiRNAs circumvented potential mismatches within the target sequence.

esiRNAs generated by Dicer more efficiently inhibit HIV-1 replication. To establish an alternative method for generating esiRNA libraries, we explored the ability of the enzyme Dicer to substitute for the bacterial RNase III (Fig. 3). To our knowledge, there is no information regarding the efficacy of esiRNA preparations generated by Dicer compared with those obtained with bacterial RNase III. It was shown previously that recombiant Dicer efficiently converts large dsRNAs to siRNAs suitable for gene silencing (21, 35). These findings prompted us to use Dicer to generate esiRNA libraries from the same target HIV-1 RT sequence employed in the bacterial RNase III experiments. As expected, esiRTHIV preparations generated by Dicer (esiRTHIVdicer) elicited an effective silencing activity and were able to suppress HIV-1 replication (Fig. 3A). Importantly, lower concentrations of esiRTHIVdicer were able to reach the same level of silencing activity obtained with higher concentrations of esiRTHIV. Again, the observed silencing activity was specific because an esiRTHIV-2dicer preparation could not suppress HIV-1 replication (Fig. 3C). A comparison of the silencing efficiencies of esiRTHIV, esiRTHIVdicer, and a previously described siRNA (40) that effectively targets the HIV-1 RT region demonstrated that the esiRTHIVdicer preparation was five times more potent than esiRTHIV (Fig. 3D). Nevertheless, higher concentrations of esiRTHIV were necessary to reach the level of inhibition obtained with an effective siRNA (Fig. 3D). These results indicate the usefulness of Dicer-generated esiRNA preparations for inhibiting HIV-1 replication.

DISCUSSION

With the development of new drugs and treatment strategies, therapeutic options for HIV continue to expand. siRNA provides a robust method for specifically inhibiting the expression of targeted cellular or viral genes, and it shows promise as a novel and broadly applicable approach to antiviral therapy. However, clinical applications of RNAi face several challenges, most notably the potential for viral escape (4, 9, 40). The present study investigated the silencing effects of esiRNAs targeted to a 0.7-kb genomic region encoding the HIV-1 RT. Taken together, our results show that treatment with esiRNAs provides an efficient approach for specific inhibition of HIV-1 replication.

Several studies have demonstrated the efficacy of specific siRNAs or shRNAs in inhibiting HIV-1 replication (4, 8, 9, 20, 27, 29, 30, 33, 34, 36, 37, 40, 45). However, some advantages of esiRNAs over siRNAs or shRNAs can be drawn from the present study. First, it seems extremely unlikely that a viral genome could accumulate enough point mutations to escape the antiviral activity of esiRNAs. Indeed, our results demonstrate that esiRNAs differing by >10% in the nucleotide target sequence can be used to efficiently block viral replication. Interestingly, it was noted recently that esiRNAs targeting a 1-kb sequence in the poliovirus RNA genome can prevent the generation of escape mutants (15). The results obtained here with HIV-2, which shares only 50% nucleotide identity with HIV-1 in the target sequence, suggest that resistance may be attained by variants approaching 50% nucleotide nonidentity in the target sequence. Second, the use of pooled siRNAs that target the same transcript can reduce off-target effects while maintaining efficient silencing of the specific target gene (6). Because the concentration of each siRNA in the mixture is relatively low and each siRNA has the same target but different off-targets, the use of pooled siRNAs may minimize the effects on unintended targets. Off-target effects for individual siRNAs have challenged the reliability of RNAi data (3, 5, 18, 19, 31, 41). Third, esiRNA is an efficient, specific, and adaptable tool that can be used to study different aspects of virus biology. As suggested previously (15), esiRNA may be used even when the exact sequence of the viral genome is unknown because the approach requires only specific PCR oligonucleotides for the amplification of the desired target sequence.

An interesting finding of the present study is the efficacy of esiRNA preparations generated by Dicer. Recently, siRNAs 25 to 30 nucleotides in length were found to be 100-fold more potent than the corresponding conventional 21-mer siRNAs (23, 42). The enhanced potency of the longer duplexes is attributed to the fact that they are substrates of the Dicer endonuclease, directly linking the production of siRNAs to incorporation into RISC. Similarly, the present results indicate that siRNAs generated by Dicer from long dsRNA can have enhanced efficacy for RNAi. As we have previously suggested, it may be interesting to test whether a significant increase in the potency of siRNAs weakens the ability of HIV-1 to escape RNAi inhibition (40). In conclusion, these findings show that esiRNAs are potent viral inhibitors and that sequence targets do not need to be highly conserved among different viral strains to reach high levels of viral replication inhibition.

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