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Small interfering RNA molecules as potential anti-human rhinovirus agents: in vitro potency, specificity, and mechanism

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Abstract

RNA silencing or interference (RNAi) is a sequence-specific, post-transcriptional process of mRNA degradation. The degradation of target gene mRNA can be induced by short dsRNA molecules (21–25-nt) corresponding to the sequence of the target gene to be silenced. Short dsRNA molecules have been shown to be very effective in inducing RNA silencing in several human cell lines. In this study, we have shown that short dsRNA molecules corresponding to the human rhinovirus-16 (HRV-16) genome induce effective inhibition of the viral replication in cell culture. This inhibition is sequence-specific and dose-dependent. A single or double nucleotide sequence change in an effective dsRNA molecule can significantly reduce the ability of the molecule to induce RNA silencing. Reducing the length of siRNA molecules to 19-nt or shorter abolishes their activity. Therefore, the results of this study demonstrate certain siRNA molecules are inhibitory for the replication of HRV-16 when transfected into human cells; further studies are warranted to explore the potential clinical value of these siRNA molecules as anti-human rhinovirus agents.

Keywords: Rhinovirus; siRNA; RNA silencing; Anti-viral agents

1. Introduction

RNA silencing or interference (RNAi) is a process of post-transcriptional gene silencing, which is initiated by dsRNA molecules (Baulcombe, 1996; Fire et al., 1998; Montgomery et al., 1998). Mechanistically, it is believed that upon introduction into or production of, long dsRNA molecules in the cell, these dsRNA molecules are first processed by an endonuclease, DICER, into 21–25-nt small interfering RNA (siRNA) molecules (Bernstein et al., 2001; Hammond et al., 2000; Hutvagner and Zamore, 2002; Ketting et al., 2001; Knight and Bass, 2001). The resulting siRNA molecules then generate the RNA-induced silencing complexes (RISCs), which in turn degrade the target mRNA molecules in the cell (Bernstein et al., 2001; Hammond et al., 2000; Hutvagner and Zamore, 2002; Ketting et al., 2001; Knight and Bass, 2001). RNAi was initially discovered in plants and subsequently in nematodes (Baulcombe, 1996; Fire et al., 1998; Montgomery et al., 1998). It has been shown to be very effective in the degradation of the cellular mRNAs in mammalian cell culture systems (Elbashir et al., 2001). More recently, it has also been shown to be functional in mice (Hasuwa et al., 2002; Lewis et al., 2002; McCaffrey et al., 2002). More importantly, these studies have demonstrated that short dsRNA molecules (21–25-nt) can efficiently induce RNAi in mammalian cell lines and in mice. Therefore, RNAi appears to represent a novel platform technology for drug discovery.

To explore the utility of this technology for the discovery of novel anti-viral agents, we have designed many different siRNA molecules corresponding to the genome of human rhinovirus (HRV), a member of the picornavirus family that is composed of more than 200 isotypes including hepatitis A virus, and poliovirus (Couch, 2001). HRV is a positive-strand RNA virus and is a major causative agent of the common cold (Couch, 2001). We then tested the ability of these siRNA molecules to induce the inhibition of HRV replication in a cell culture assay and found that many of the siRNA molecules are effective in inducing RNA silencing, resulting in the inhibition of viral replication. The results of this study demonstrate that siRNA molecules derived from the HRV genome are potent inhibitors of HRV-16 replication in the cell.

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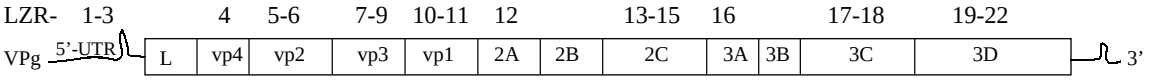

Fig. 1. The locations of siRNA molecules and the organization of the HRV-16 genome. Top panel: The relative locations of the HRV siRNA molecules, LZR-1, LZR-2, LZR-3, LZR-4, LZR-5, LZR-6, LZR-7, LZR-8, LZR-9, LZR-10, LZR-11, LZR-12, LZR-13, LZR-14, LZR-15, LZR-16, LZR-17, LZR-18, LZR-19, LZR-20, LZR-21, and LZR-22 corresponding to the HRV coding region are shown and their respective nucleotide sequences are described in Section 2. Bottom panel: The organization of the HRV-16 genome. The genome-linked protein VPg at the 5′-end, the 5′-untranslated region, the protein coding region, the 3′-untranslated region, and the poly(A) tail.

2. Materials and methods

2.1. Design and synthesis of siRNA molecules

The siRNA molecules designed consist of 19 nucleotides corresponding to the various genes of HRV-16 (Lee et al., 1995, and also see Fig. 1 and Table 1) and two additional deoxythymidine nucleotides located at the 3′-terminus of each molecule. The GC content of each siRNA molecule is about 30–70% GC. The nucleotide sequences (the sense-strand) of the siRNA molecules designed, LZR-1, LZR-2, LZR-3, LZR-4, LZR-5, LZR-6, LZR-7, LZR-8, LZR-9, LZR-10, LZR-11, LZR-12, LZR-13, LZR-14, LZR-15, LZR-16, LZR-17, LZR-18, LZR-19, LZR-20, LZR-21, and LZR-22 are listed in Table 1. As controls, two siRNA molecules, Neo-1 and Neo-2, corresponding to the neomycin resistance gene (Lohmann et al., 1999) and two siRNA molecules corresponding to the human hepatitis C virus, LZ-16 and LZ-35 (Lohmann et al., 1999) were also designed and synthesized as described above. The nucleotide sequences (the sense-strand) of Neo-1, Neo-2, LZ-16, and LZ-35 are also listed in Table 1. The siRNA molecules were synthesized in vitro by Dharmacon Research, Inc., using 2′-ACE chemistry. The synthesized molecules were then de-protected and de-salted, and annealed.

2.2. siRNA transfection and rhinovirus plaque assay

The synthesized siRNA molecules were transfected into human HeLa cells (gift from R. Rueckert, Univer-

Table 1
Properties of siRNA molecules corresponding to the indicated segments of the HRV-16 genome

siRNA	Nucleotide sequence (sense strand)	Nucleotide location (bp)[a]	Corresponding gene	Inhibition (%)[b]
LZR-1	5′-CGGUAAUCUUGUACGCCAGdTdT-3′	63–81	5′-UTR	5.6 ± 4.7
LZR-2	5′-CCAACAGUAGACCUGGUAGdTdT-3′	299–317	5′-UTR	6.5 ± 2.8
LZR-3	5′-GUGCACACAAUCCAGUGUGdTdT-3′	480–498	5′-UTR	4.7 ± 5.6
LZR-4	5′-GAUGCAGCUUCCAGUGGUGdTdT-3′	728–746	vp4	91.6 ± 0.9
LZR-5	5′-AGGAUGCAACGGCUAUAGAdTdT-3′	966–984	vp2	25.6 ± 0.9
LZR-6	5′-GAGAAACAACCUAGUGAUGdTdT-3′	1319–1337	vp2	49.8 ± 18.8
LZR-7	5′-UCCAUGUGCACUGCCUUGGdTdT-3′	1678–1696	vp3	−0.47 ± 9.4
LZR-8	5′-GGACUGCAAACACUACCUUdTdT-3′	1977–1995	vp3	93 ± 0.38
LZR-9	5′-CCAGACACAUACUCCUCAGdTdT-3′	2153–2171	vp3	70.5 ± 16
LZR-10	5′-UACCACAUCAAAUGCAGCCdTdT-3′	2404–2422	vp1	6.6 ± 7.5
LZR-11	5′-CAGGAGCACCUAUACCAACdTdT-3′	2769–2787	vp1	77.1 ± 6.6
LZR-12	5′-GCCUAGUGACAUGUAUGUGdTdT-3′	3187–3205	2A	3.8 ± 13.2
LZR-13	5′-CUCUUCAGAUCCGCAAACAdTdT-3′	3775–3793	2C	90.2 ± 3.8
LZR-14	5′-CAGAGCAGCUGAUACUAACdTdT-3′	4081–4099	2C	79.9 ± 9.4
LZR-15	5′-AUCACUCACUCUUGGCACCdTdT-3′	4542–4560	2C	48.9 ± 23.5
LZR-16	5′-CGCUCAGUAAGGACACCAGdTdT-3′	4913–4931	3A	79.9 ± 2.8
LZR-17	5′-UACCAACACAUGCUGACCCdTdT-3′	5265–5283	3C	9.4 ± 6.6
LZR-18	5′-GGUGGCAAUGGUAGAGAUGdTdT-3′	5642–5660	3C	61.1 ± 9.4
LZR-19	5′-GAUGGCUUGGAAGCUUUGGdTdT-3′	6017–6035	3D	34.8 ± 0.9
LZR-20	5′-CCUUGAUGUUAGAUGGAGAdTdT-3′	6369–6387	3D	94 ± 0.47
LZR-21	5′-GAAGCCAUAGCCAAGGAAGdTdT-3′	6722–6740	3D	65.8 ± 2.8
LZR-22	5′-CUCCUAAGACAUGAAUGGUdTdT-3′	7055–7073	3D	71.4 ± 3.8
LZ-16	5′-GCGGGAUACAAUAUUUAGCdTdT-3′	4046–4064	4B	0
LZ-35	5′-GGAAACUUGGGGUACCGCCdTdT-3′	7463–7481	5B	0
Neo-1	5′-AUGGAUUGCACGCAGGUUCdTdT-3′	71–89	Neo	0
Neo-2	5′-CUGUCAUCUCACCUUGCUCdTdT-3′	365–383	Neo	0

[a] LZR-1 to LZR-22 correspond to the genome of the HRV-16 (Lee et al., 1995), LZ-16 and LZ-35 correspond to the subgenomic replicon of the human hepatitis virus C (Lohmann et al., 1999), and Neo-1 and Neo-2 correspond to the neomycin resistance gene of the subgenomic replicon of the human hepatitis virus C (Lohmann et al., 1999).

[b] The siRNA-mediated inhibition of HRV replication was measured as described in Section 2 using a plaque assay. This experiment was carried out twice each with two replicates. The percentage of inhibition was calculated by using the average number of plaques obtained from the cells that were transfected with LZ-16, LZ-35, Neo-1, and Neo-2, using Oligofectamine, as the control (0% inhibition).

sity of Wisconsin) as described below. HeLa cells, maintained in MEM with Earle's Salts (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) and 1% Pluronic F-68 (Sigma), were first seeded (at 9×10^5 cells/plate) in plates (60 mm, Fischer) containing attachment medium (MEM with Earle's Salts, Gibco BRL) supplemented with 10% newborn calf serum (NCS) (Gibco BRL). The medium was removed from the plates before the cells were infected with 500 μl/plate of a stock of HRV-16 that had been diluted to 200 plaque forming units (PFU)/ml in the attachment medium. The infected cells were incubated at 35 °C for 1 h, allowing viruses to attach and enter the cells. After the incubation, 30 μl of 20 μM siRNA molecules and 340 μl of Opti-MEM serum-free medium (Gibco BRL) were added to one tube, and 8 μl of Oligofectamine (Gibco BRL) and 30 μl of Opti-MEM serum-free medium were added to another tube. Both tubes were kept at room temperature for approximately 5 min and then mixed with each other. The transfectant mixture was incubated at room temperature for 20 min (total volume 408 μl) and then washed twice with Opti-MEM serum-free medium (room temperature). After the wash, the transfectant mixture was added in 408 μl of Opti-MEM serum-free medium. Then, 200 μl of the final mixture was added to each plate along with 800 μl of Opti-MEM (the final concentration of siRNA molecules being 300 nM). The plates were incubated at 35 °C for 4 h and then overlaid with a 1:1 combination of 1.6% SeaPlaque Agarose (BioWhittaker Molecular Applications) and 2× MEM medium [100 ml 10× MEM (Gibco BRL), 10 ml L-glu (Gibco BRL, 25 ml 7.5% sodium bicarbonate (Gibco BRL), 10 ml NEAA, 100× (Gibco BRL), 25 ml NCS (Gibco BRL), 12 ml 1 M $MgSO_4$, and 500 ml water]. The plates were incubated at 35 °C for 3 days. After the incubation, the plates were fixed with 7.5% formaldehyde (Mallinckrodt) and stained with crystal violet (Sigma). The number of plaques formed was counted.

2.3. Quantitation of viral RNA in the cell

HeLa cells grown in 6-well plates to a density of approximately 1.5×10^6 cells/well in MEM (Gibco BRL) supplemented with 10% NCS (Gibco BRL) were infected with HRV-16 at a multiplicity of infection of 2. The viruses were allowed to attach to the cells at 35 °C for 1 h. Infected cells were washed twice with warm Opti-MEM (Gibco BRL) and then treated with or without Actinomycin D (4 μg/ml, Sigma) for 2 h at 35 °C. The cells that had been treated with or without Actinomycin D were then transfected with siRNA molecules (300 nM) as described above in the presence or absence of 4 μg/ml Actinomycin D for 2 h at 35 °C. After the transfection, ^{3}H-uridine (NEN) (50 μCi/well) was added to the transfection mixtures that were incubated for an additional 3 h. Cells were again washed with warm Opti-MEM followed by the addition of cell lysis buffer. The resulting preparations were subjected to RNA extraction using the QiaRNeasy assay (Qiagen) according to the supplied kit protocols. The RNA collected was then counted using a scintillation counter (LS6000-IC, Beckman).

3. Results and discussion

3.1. The siRNA molecules derived from the HRV genome induced potent inhibition of viral replication in the cell

To test if the siRNA molecules corresponding to the HRV-16 genome could induce RNA silencing, which results in the inhibition of viral replication, we designed siRNA molecules corresponding to the various parts of the viral genome and also control siRNA molecules corresponding to the neomycin resistance gene and the genome of the human hepatitis virus C, and tested their ability to induce RNA silencing in a HRV plaque assay. When infected cells were treated with Oligofectamine, a transfection agent, but not siRNA molecules, a large, expected number of plaques were formed (on average approximately 100 plaques/plate). Similar results were obtained when infected cells were transfected without or with the control siRNA molecules, Neo-1, Neo-2, LZ-16, and LZ-35 (Table 1). Together, these results indicate that neither Oligofectamine nor the control siRNA molecules tested significantly affected HRV replication in the cell as measured by plaque formation. However, a significant reduction in plaque formation was observed when infected cells were transfected with a number of siRNA molecules corresponding to the HRV-16 genome (Table 1). For example, when the HRV-16-infected cells were transfected with LZR-4, LZR-6, LZR-8, LZR-9, LZR-11, LZR-13, LZR-14, LZR-15, LZR-16, LZR-18, LZR-20, LZR-21, or LZR-22, a significant decrease in viral plaque formation was observed (Table 1). On the other hand, when the HRV infected cells were transfected with LZR-1, LZR-2, LZR-3, LZR-7, LZR-10, LZR-12, LZR-17, or LZR-19, a low level of or no inhibition of viral plaque formation was observed (Table 1). These results show that more than 50% of the siRNA molecules designed corresponding to the HRV-16 genome induced RNA silencing in the cell, which in turn specifically and significantly inhibited viral replication. Therefore, these results clearly demonstrate the potential of using these RNA molecules for the treatment of HRV infection. It is not clear as to why the other 50% of the molecules designed failed to induce RNA silencing. It is possible that certain regions of the viral genome are not accessible for siRNA molecules to interact with and subsequently elicit RNA silencing. For example, all three siRNA molecules, LZR-1, LZR-2, and LZR-3, corresponding to the 5′-untranslated region, failed to induce RNA silencing. It is well established that this region is heavily involved in RNA–protein and RNA–RNA interactions (Couch, 2001; Huang et al., 2001; Rohll et al., 1994).

3.2. The inhibition of HRV replication in the cell by siRNA molecules is sequence-specific and dose-dependent

As described above, many siRNA molecules corresponding to the HRV-16 genome exhibited a significant inhibition of viral replication in the cell. To establish that this inhibition is sequence-specific, several siRNA molecules were tested as controls. The control siRNA molecules corresponding to the neomycin resistance gene (Neo-1 and Neo-2), and the HCV genome (LZ-16 and LZ-35) have been shown to induce potent inhibition of the HCV replicon replication in a human liver cell line (unpublished results). However, these HCV specific siRNA molecules did not induce any significant inhibition of viral replication when the HRV-16 infected cells were treated with these siRNA molecules under the identical conditions as described above (see also Table 1). We then introduced a series of nucleotide changes into LZR-4, a potent siRNA molecule that induces a significant inhibition of HRV-16 replication. These nucleotide changes resulted in mismatches between the siRNA molecule and the HRV-16 genome, as shown in Fig. 2. We then tested the resulting molecules for their ability to induce the inhibition of HRV-16 replication as described in Section 2. As shown in Fig. 2, nucleotide sequence changes at either the 3′- or 5′-end of the molecule (LZR-4-5 and LZR-4-6) did not significantly decrease the ability of the molecule to induce inhibition of viral replication. However, changes in the central part of the molecule (LZR-4-1, LZR-4-2, LZR-4-3, and LZR-4-4) significantly hindered this ability. Together, the results of this study demonstrate that the inhibition of the HRV replication in the cell by siRNA molecules is clearly sequence-specific and that the central part of the siRNA molecules appears to be critical for activity.

In addition, we tested the effects of siRNA length on their ability to induce inhibition of viral replication. When the length of the siRNA molecule was reduced from 21 to 19-nt or shorter, the siRNA molecules appeared to lose their ability to induce inhibition of viral replication (data not shown). Interestingly, siRNA molecules with a length of 22–26-nt did not exhibit an inhibition higher than that of the original 21-nt siRNA molecule (data not shown). Therefore, the optimal length for siRNA molecules appears to be 21-nt.

To establish whether the inhibition of HRV-16 replication is dose-dependent, HeLa cells were infected with the virus and transfected with the siRNA molecules LZR-4, LZR-8, LZR-13, and LZR-20 at different concentrations, and the inhibition of the viral plaque formation was measured. As shown in Fig. 3, the inhibition of the viral plaque formation by the molecules increased linearly with the increase of their concentrations up to about 30 nM. LZR-4, LZR-8, LZR-13, and LZR-20 showed IC_{50} values of 0.93, 1.18, 0.79, and 0.73 nM, respectively. Interestingly, when these siRNA molecules were subjected to a similar analysis in which the molecules were mixed with different amounts of a control siRNA molecule to yield a final concentration of 300 nM, a similar dose-dependent inhibition curve was obtained, except that the IC_{50} values determined under these conditions were about two- to four-fold higher than those determined in the absence of the control siRNA molecule (data not shown). These results suggest that the presence of the control siRNA molecule inhibited the transfection efficiency or the ability of the HRV siRNA molecules to bind to the RISC complex, or both.

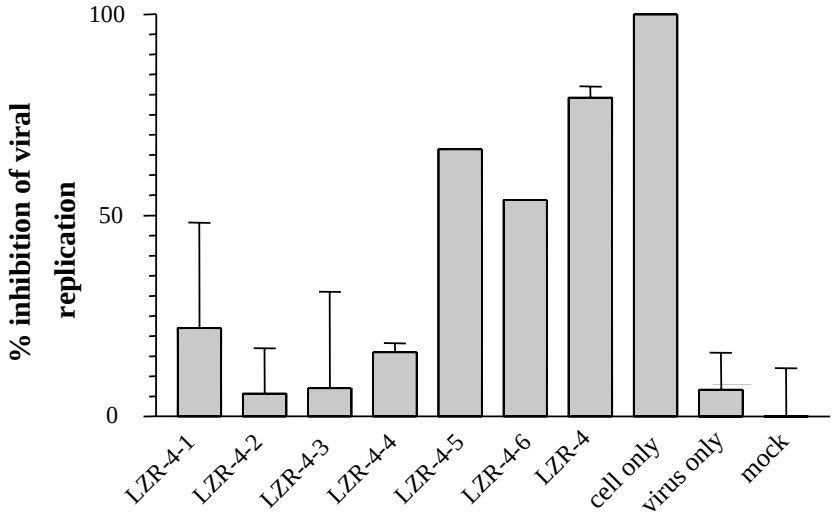

Fig. 2. Effects of nucleotide sequence changes in LZR-4 on its ability to mediate the inhibition of HRV-16 replication in the cell. To determine the effects of nucleotide sequence changes in LZR-4 (5′-GAUGCAGCUUCCAGUGGUG-3′) on its ability to mediate the inhibition of HRV replication, the following nucleotide sequence changes (in bold) were introduced, which resulted in the generation of the siRNA molecules, LZR-4-1, LZR-4-2, LZR-4-3, LZR-4-4, LZR-4-5, and LZR-4-6 with the following nucleotide sequences: 5′-GAUGCAGCU**G**CCAGUGGUG-3′, 5′-GAUGCAGCU**GA**CAGUGGUG-3′, 5′-GAUGCAGC**AGA**CAGUGGUG-3′, 5′-GAUGCAGC**AGAG**AGUGGUG-3′, 5′-GA**A**GCAGCUUCCAGUGGUG-3′, and 5′-GAUGCAGCUUCCAGUG**C**UG-3′, respectively. The resulting siRNA molecules were transfected into the HRV-infected HeLa cells and their ability to induce the inhibition of HRV replication was determined by the plaque assay as described in Section 2. This experiment was performed twice each with four replicates.

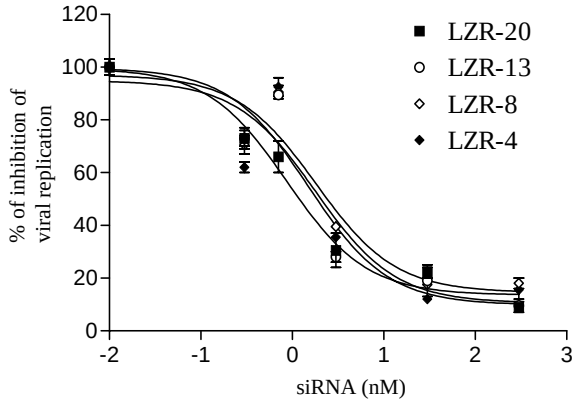

Fig. 3. Dose-dependent inhibition of HRV-16 replication in the cell mediated by the viral specific siRNA molecules. The siRNA molecules, LZR-4 (◆), LZR-8 (◇), LZR-13 (○), and LZR-20 (■) were transfected into the HRV infected cells at various concentrations (0.003–300 nM) and their ability to induce the inhibition of viral replication was assessed by plaque formation as described in Section 2. This experiment was performed twice each with two replicates.

3.3. The HRV RNA level in the cell was significantly reduced when treated with the HRV siRNA molecules

The hallmark of RNAi is the degradation of cellular mRNAs, resulting in the reduction of mRNA level in the cell. To examine whether the inhibition of the HRV-16 replication is due to the reduction of the viral RNA levels in the cell, HeLa cells were treated with or without Actinomycin D, infected with the virus in the presence or absence of this inhibitor, respectively, and then transfected with the siRNA molecules, LZR-4, LZR-13, and LZR-20 as described in Section 2. The incorporation of ^{3}H-labeled nucleoside into RNA was measured. We used this labeling method to quantify viral RNA levels in the cell for the following reasons. This method, as compared with those of Northern blotting or quantitative PCR is quick, simple, quantitative, and does not require the generation of DNA or RNA probes. In addition, using Northern blotting or quantitative PCR method may require the generation of different probes for testing different siRNA molecules, as the initiation sites of viral RNA cleavage mediated by different siRNA molecules may be different. Finally, it was not known how stable the degraded viral RNA molecules would be in the cell. Therefore, this labeling method was employed for the detection of viral RNA degradation.

By using this labeling method, the amount of the ^{3}H-labeled RNA isolated from the cells treated with Actinomycin D alone or Actinomycin D plus the control Neo-1 siRNA molecule was measured (Fig. 4). Similarly, the amounts of the ^{3}H-labeled RNA isolated from the cells treated with Actinomycin D and the HRV specific siRNA molecules, LZR-13 and LZR-20 were measured (Fig. 4). As shown in Fig. 4, the RNA levels in the cells treated with the siRNA molecules LZR-13 and LZR-20 were significantly lower than those in the control cells. As expected, the

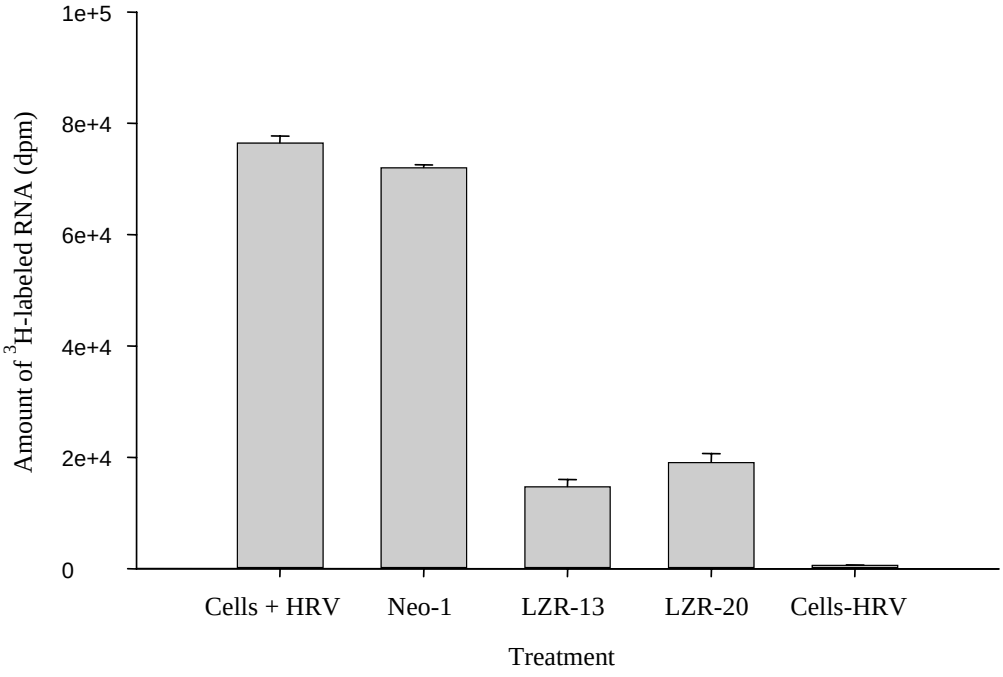

Fig. 4. siRNA-mediated inhibition of HRV-16 replication in the cell. HeLa cells were infected without or with HRV and treated without or with Actinomycin D as described in Section 2. The cells were then transfected without or with siRNA molecules. After the transfection, ^{3}H-uridine was added to the growth medium as described before. After the incubation, RNA was isolated and quantified as described in Section 2. The following treatments were carried out: cells were not infected with HRV-16 (cells − HRV) or infected with the virus and treated with Oligofectamine (cells + HRV) alone or with the control siRNA Neo-1 (Neo-1) or the HRV-16 specific siRNA molecules (LZR-13 or LZR-20).

amount of the labeled RNA in the Actinomycin D-treated uninfected cells was very low. Thus, these results indicate that the viral RNA was degraded in the cell, most probably resulting from siRNA-mediated RNA silencing. In the absence of Actinomycin D, there was no significant difference in the RNA levels in the cells that were treated with or without the siRNA or control siRNA molecules (data not shown). This result is expected as in the absence of Actinomycin D, host RNA synthesis was not inhibited and thereby the majority of the label was probably incorporated into the host RNA, rather than just the viral RNA.

In summary, the results of this study have shown that several short dsRNA molecules corresponding to the HRV genome are potent, specific inducers of RNA silencing in the cell, which results in the efficient inhibition of viral replication. Similar results have been obtained recently with other viruses (Bitko and Barik, 2002; Gitlin et al., 2002; Jacque et al., 2002; Novina et al., 2002; Randall et al., 2003; Seo et al., 2003). In light of these findings, the potential of using short dsRNA molecules as anti-viral agents clearly should be further explored. In addition, short dsRNA molecules do not appear to induce immune responses in the cells (Elbashir et al., 2001). Therefore, the use of siRNA molecules as anti-viral agents may offer better specificity, safety profile and potency as compared with other anti-viral agents on the market (Crumpacker, 2001). However, the use of dsRNA molecules as anti-viral therapeutics may require extensive chemical modifications for better stability. In addition, since HRV is one of many RNA viruses that does not have proof-reading capabilities during replication, the virus mutates frequently (Couch, 2001). The nature of the frequent mutation by the virus also imposes a significant limitation on the use of dsRNA molecules. However, the use of multiple siRNA molecules corresponding to different regions of the viral genome may at least partially circumvent the problem.

Finally, it is also challenging to design siRNA molecules, whose sequences are conserved in all isolates or in the major viral serotypes. Once again, the use of multiple siRNA molecules corresponding to different viral isolates should be contemplated. The 3′- and 5′-untranslated regions appear to be the most conserved regions and should therefore be envisaged for designing siRNA molecules that can be used to target multiple viral isolates. However, as discussed earlier, these regions may not be ideal for this purpose due to their extensive interactions with cellular proteins and nucleic acids. Clearly, they should be explored more extensively as these regions may lead to the identification of the siRNA molecules that are conserved in the major serotypes, if not all, viruses, which, therefore, might be useful as broad-spectrum anti-human rhinoviral agents.

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