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SCIENCE DIRECT
Antiviral Research 69 (2006) 1–8
Antiviral Research
www.elsevier.com/locate/antiviral

Inhibition of drug-resistant HIV-1 by RNA interference ☆

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Received 22 June 2005; accepted 5 October 2005

Abstract

RNA interference is a powerful tool used to inhibit human immunodeficiency virus type 1 (HIV-1) replication in vitro. Almost all HIV-1 genes have been targets for small interfering RNA (siRNA) molecules, and HIV-1 replication can be specifically and successfully inhibited by this technique. RNA interference has been proposed as an alternative strategy to inhibit replication of drug-resistant viruses that emerge during suboptimal antiretroviral therapy for HIV-1. To investigate specific inhibition of drug-resistant HIV-1 by RNA interference, we designed siRNA molecules that recognize codons 181–188 of the reverse transcriptase (RT) gene of wild-type HIV-1 and HIV-1 carrying the M184V mutation, which confers high-level resistance to the RT inhibitor lamivudine. Using viral variants with single point mutations at codon 184, we measured the impact of these mutations on virus replication. We have demonstrated that siRNA targeting either wild-type HIV-1 or M184V variants inhibits replication of the corresponding virus, but does not influence replication of virus with a mismatch in the targeted region. Combining two effective siRNAs did not show synergistic inhibitory effect on HIV-1 replication. However, a combination of lamivudine and siRNA–M184V was very effective in inhibiting replication of both wild-type and variant M184V viruses in mixed infection experiments. Taken together, these results demonstrate that RNA interference might be useful in the treatment of drug-resistant HIV-1 infection.

Keywords: RNA interference; HIV-1; Drug resistance; M184V; Lamivudine

1. Introduction

Over the past two decades, infection with human immunodeficiency virus type 1 (HIV-1) has given rise to a devastating pandemic. Despite improvements in antiretroviral therapy (ART), HIV-1 infection is incurable and a substantial fraction of patients fail therapy and/or experience serious side effects from treatment. Thus, the search for new antiretroviral strategies is one of the major challenges in the fight against HIV-1. Small interfering RNAs (siRNA) are double-stranded RNA-molecules, approximately 21 nucleotides long, that hybridize to a homologous mRNA target and result in degradation of the mRNA (Elbashir et al., 2001; Fire et al., 1998). This post-transcriptional gene silencing process is called RNA interference (RNAi) and is evolutionarily conserved in both plants and eukaryotic cells, as reviewed by Novina and Sharp (2004). Due to the high degree of sequence specificity and efficiency, RNAi is a powerful laboratory tool for inhibiting specific genes and may offer a new therapeutic option for treating certain viral infections. RNAi has been shown to efficiently inhibit HIV-1 replication in both cell lines and primary cells in vitro, as reviewed by Cullen (2002) and Haasnoot et al. (2003), and might therefore provide an effective strategy against drug-resistant virus.

Conventional ART, provided as a combination of several effective antiretroviral drugs, has dramatically decreased the mortality rate of HIV-1-infected patients (Palella et al., 1998). Nevertheless, therapy failure and the development of drug-resistant HIV-1 represents a major problem not only for further treatment but also for subsequent transmission of these viruses (Cheung et al., 2004). Given the sequence specificity of siRNAs (Amarzguioui et al., 2003), RNAi might offer a promising avenue of exploration to specifically control drug-resistant viruses. The combination of RNAi and lamivudine has already been successfully used to inhibit hepatitis B virus replication in vitro (Chen et al., 2003). Current progress in the administration of siRNA in vivo is also encouraging for the application of RNAi as a therapeutic modality (Morrissey et al., 2005; Soutschek et al., 2004).

☆ This work was presented in part at the Annual Meeting of the German Society of Virology, 17–20 March 2004, Tuebingen, Germany (Abstract 536).

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doi:10.1016/j.antiviral.2005.10.001

The M184V mutation in the RT gene of HIV-1 is clinically important and frequently appears during the course of antiretroviral regimens that include lamivudine (Cheung et al., 2004). This amino acid exchange is caused by a single nucleotide substitution and confers high-level resistance to lamivudine (Tisdale et al., 1993). We have investigated the potential of RNAi to specifically inhibit this drug-resistant HIV-1 variant, using siRNAs targeting the M184V mutation. Moreover, we tested the combination of siRNA and lamivudine in cells infected with both wild-type HIV-1 and M184V variants to further explore the range of this therapeutic approach.

2. Materials and methods

2.1. Cells and transfections

HeLa CD4$^+$ cells (kindly provided by David Kabat, University of Oregon, Portland) were maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 170 mM penicillin and 40 mM streptomycin. Twenty-four hours prior to transfection, 10^5 HeLa CD4$^+$ cells were plated in 6-well plates, washed and transfected with siRNA at the indicated concentrations using the calcium phosphate method (Graham and van der Eb, 1973). After 12 h, the cells were carefully washed twice with PBS and fresh medium was added. Lamivudine (kindly provided by Glaxo-SmithKline) was added at a concentration of 10 μM. Cell viability was determined by trypan blue staining (Sigma, Seelze, Germany).

2.2. Synthesis of siRNA

All siRNA molecules were synthesized by Alnylam AG, Kulmbach, Germany, as previously described (Soutschek et al., 2004). Sequences are provided in Fig. 1B. The sequence of siRNA-tat was chosen as previously described (Coburn and Cullen, 2002). siRNA-non targets the firefly luciferase gene and has no sequence similarity to either human or HIV-1 genes (Elbashir et al., 2001).

2.3. Plasmid constructs

The M184V (ATG to GTG transversion in codon 184 of the RT gene) and M184I (ATG to ATA transversion in codon 184 of the RT gene) mutations were introduced in the HIV-1 full-length clone pNL4-3 (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) by site-directed mutagenesis using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and the oligonucleotides tg M184V 5′-GACATAGTTATCTATCAATACGTGGATGATTTGTATGTAGGATCTGAC-3′, tg M184V rc 5′-GTCAGATCCTACATACAAATCATCCACGTATTGATAGATAACTATGTC-3′ (Metzner et al., 2003), tg M184I 5′-CCAGACATAGTTATCTATCAATACATAGATGATTTTGTATGTAGGATCTGAC-3′ and tg M184I rc 5′-GTCAGATCCTACATACAAATCATCTATGTATTGATAGATAACTATGTCTGG-3′ according to the Manufacturer's instructions. The mutations were confirmed by sequencing.

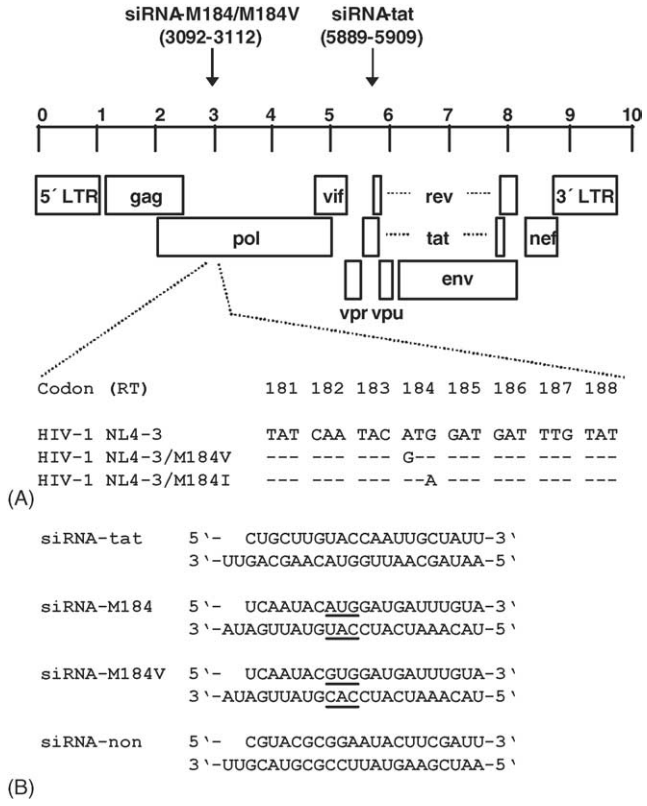

Fig. 1. Scheme of the HIV-1 genome showing positions of siRNAs and sequences of viral variants and siRNAs. (A) The siRNA-targeted regions are depicted by arrows. The nucleotide numbers given in parentheses are based on the reference HIV-1 full-length clone HXB2 (accession number K03455). Nucleotide sequences of codon 181–188 in the RT gene of HIV-1 are shown for the viruses HIV-1 NL4-3, HIV-1 NL4-3_{M184V} and HIV-1 NL4-3_{M184I}. (B) Sequences of chemically synthesized siRNAs. Codon 184 is underlined.

2.4. Viruses and infections

293T cells were transfected with pNL4-3, pNL4-3_{M184V} and pNL4-3_{M184I}, and supernatants were used for infection of CEMx174 cells. Virus stocks were titrated on CEMx174 cells and the 50% cell culture infectious dose ($CCID_{50}$) was calculated using the Spearman–Kaerber formula (Kaerber, 1931). Infection of HeLa CD4$^+$ cells was performed using 0.01 multiplicity of infection (MOI) of each virus stock. Cells were infected at 24 h after transfection with siRNA. Two hours after infection, cells were washed twice with PBS and further cultivated with fresh medium. Supernatants were collected daily after infection and virus production was estimated by an in-house p24 ELISA as previously described (McKeating et al., 1991). In mixing infection experiments using HIV-1 NL4-3 and HIV-1 NL4-3_{M184V}, the ratio of both viral variants were determined at day 4 after infection using quantitative real-time PCR for differential amplification as described elsewhere (Metzner et al., 2003).

2.5. Statistical analysis

Differences in the inhibitory effects of different siRNAs on virus replication were tested for statistical significance by using Student's *t* test. A *p*-value of <0.05 was considered as a significant difference between two groups.

3. Results

3.1. Design and synthesis of siRNA specific for HIV-1

To investigate specific inhibition of drug-resistant HIV-1 by RNAi, we designed different siRNAs targeting codons 181–188 of the RT gene of either wild-type HIV-1 NL4-3 or HIV-1 NL4-3_{M184V}. In addition, siRNA recognizing HIV-1 *tat* (Coburn and Cullen, 2002) and siRNA-non (Elbashir et al., 2001) were used as positive and negative controls, respectively. A BLAST search did not reveal any sequence similarity between these siRNAs and any human genes. The positions of these siRNAs in the HIV-1 genome are shown in Fig. 1A. An alignment of the sequences spanning codons 181–188 of the RT gene shows the differences between HIV-1-NL4-3 and the viral variants HIV-1 NL4-3_{M184V} and HIV-1 NL4-3_{M184I} (Fig. 1A).

3.2. Replication kinetics of viral variants in HeLa CD4$^+$ cells and lack of inhibition by control siRNA-non

To investigate whether HIV-1 NL4-3 and the viral variants HIV-1 NL4-3_{M184V} and HIV-1 NL4-3_{M184I} replicate with similar kinetics, we infected HeLa CD4$^+$ cells with each virus and measured HIV-1 p24 antigen production daily for 4 days. No differences in p24 production were observed, indicating that these mutations do not significantly affect virus replication in HeLa CD4$^+$ cells (data not shown). The impact of siRNA-non used as negative control on virus replication was tested by transfection of HeLa CD4$^+$ cells with either transfection reagent alone (mock) or with 100 nM siRNA-non. Subsequently, the cells were infected with either HIV-1 NL4-3, HIV-1 NL4-3_{M184V} or HIV-1 NL4-3_{M184I} at an MOI of 0.01. We observed similar replication kinetics for each of the viruses showing that siRNA-non has no inhibitory or enhancing effect on virus replication (Fig. 2A). Calcium phosphate reagent is not usually used as transfection reagent for siRNA. However, FACS analyses indicate higher transfection efficiencies with anti-CD4 siRNA in HeLa CD4$^+$ cells using calcium phosphate compared with transfection reagents based on liposomes (data not shown).

3.3. Specific inhibition of HIV-1 NL4-3 and HIV-1 NL4-3_{M184V} by siRNA

The M184V mutation in the RT gene of HIV-1 confers high-level drug resistance to lamivudine. To investigate specific inhibition of drug-resistant HIV-1 NL4-3_{M184V}, we designed siRNAs that target codons 181–188 of the RT gene of either wild-type HIV-1 NL4-3 or HIV-1 NL4-3_{M184V}. HeLa CD4$^+$ cells were transfected with 100 nM siRNA. Subsequently, cells were infected with either HIV-1 NL4-3, HIV-1 NL4-3_{M184V} or HIV-1 NL4-3_{M184I} and virus replication was monitored by p24 antigen ELISA. In mock-transfected cell cultures, we observed an increase in p24 antigen on day 3 accompanied by the appearance of cytopathic effects. By day 4, p24 values reached a peak and there were clearly discernible differences in the effects of each siRNA (Fig. 2A). In the absence of any inhibition, the cytopathic effects were such that no further increase in HIV-1 p24 antigen was measurable after day 4. Therefore, in each experiment we calculated the effects of siRNA on virus replication on day 4 after infection. All p24 values were normalized to the siRNA-non. Virus replication in mock-transfected HeLa CD4$^+$ cells was neither inhibited nor enhanced (Figs. 2–4).

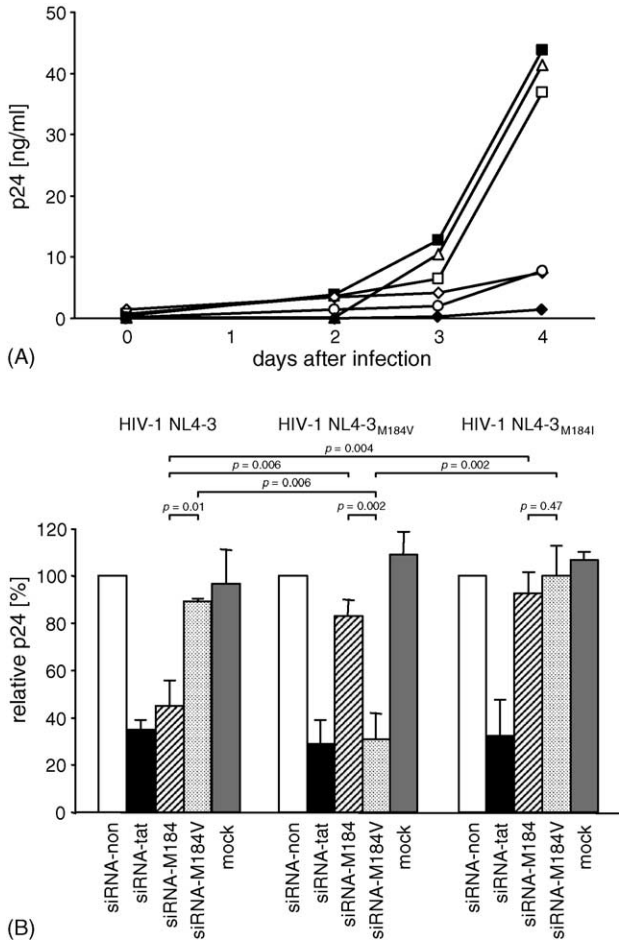

Fig. 2. Inhibition of HIV-1 replication in HeLa CD4$^+$ cells by different siRNAs. HeLa CD4$^+$ cells were transfected with 100 nM of indicated synthetic siRNAs. Mock-transfected cells were used as a control. Twenty-four hours after transfection the cells were infected either with HIV-1 NL4-3 or the viral variants HIV-1 NL4-3_{M184V} or HIV-1 NL4-3_{M184I}. On days 0, 2, 3 and 4 after infection, virus supernatants were collected and assayed for viral p24 antigen by ELISA. (A) Replication of HIV-1 NL4-3_{M184V} influenced by different synthetic siRNAs: siRNA-tat (◊), siRNA–M184 (□), siRNA–M184V (○), and siRNA-non (△). Virus replication in mock-transfected cells is shown in black squares and the negative control is indicated in black diamonds. (B) Impact on virus replication by different siRNAs on day 4 after infection. Values of p24 antigen were normalized to the siRNA-non. Shown are means and standard deviations of three independent experiments. *p*-values below 0.05 indicate the effects of siRNA–M184 and siRNA–M184V on replication of the different viral variants.

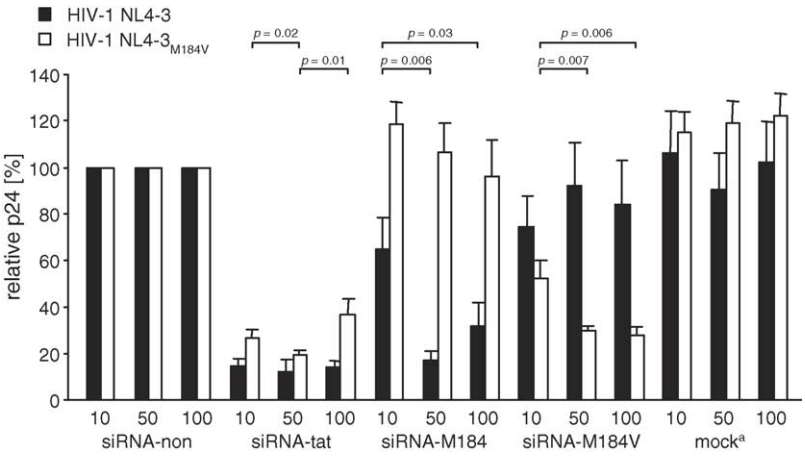

Fig. 3. Impact of different concentrations of siRNAs on inhibition of replication of HIV-1 NL4-3 and HIV-1 NL4-3_{M184V} in HeLa CD4$^+$ cells. HeLa CD4$^+$ cells were transfected with indicated concentration of synthetic siRNAs. Mock-transfected cells were used as control. Twenty-four hours after transfection the cells were infected with either HIV-1 NL4-3 (black bars) or HIV-1 NL4-3_{M184V} (white bars). Virus replication was monitored by p24 antigen ELISA. Values of p24 antigen were normalized to the siRNA-non. Data represent the means and standard deviations of triplicates. *p*-values below 0.05 are indicated for each effective siRNA. [a]Values obtained from mock transfections and subsequent infection with HIV-1 were separately calculated based on 10, 50, and 100 nM siRNA-non.

To determine whether siRNA–M184 and siRNA–M184V are specific for their distinct targets, HeLa CD4$^+$ cells were transfected with each siRNA and infected with either wild-type HIV-1 NL4-3 or HIV-1 NL4-3 carrying the M184V or M184I mutation. Replication of all viruses was inhibited with similar efficiencies of approximately 65–70% by control siRNA-tat (Fig. 2B). siRNA–M184 inhibited replication of HIV-1 NL4-3 to the same extent as siRNA-tat but had little effect on replication of HIV-1 NL4-3_{M184V}. Conversely, siRNA–M184V inhibited replication of HIV-1 NL4-3_{M184V} but did not significantly inhibit replication of wild-type HIV-1 NL4-3. Replication of HIV-1 NL4-3_{M184I} was unaffected by either siRNA–M184 or siRNA–M184V (Fig. 2B).

3.4. Dose-dependent inhibition of HIV-1 replication by siRNAs

To investigate dose-dependent inhibition, HeLa CD4$^+$ cells were transfected with 10, 50 and 100 nM of each siRNA. Subsequently, the cells were infected with either HIV-1 NL4-3 or HIV-1 NL4-3_{M184V} and virus replication was monitored by p24 antigen ELISA. Control siRNA-tat showed approximately 80% inhibition of HIV-1 NL4-3 and 60–80% inhibition of HIV-1 NL4-3_{M184V} at concentrations of 10–100 nM, with maximal inhibition occurring at 50 nM (Fig. 3). The inhibitory effect using 10 or 100 nM siRNA-tat was significantly lower ($p = 0.02$ and 0.01, respectively; Fig. 3). By comparison, only weak inhi-

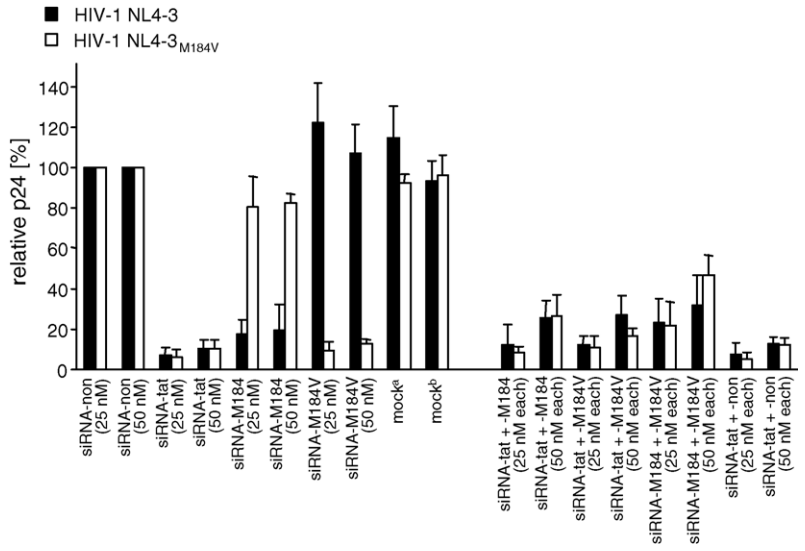

Fig. 4. Inhibition of HIV-1 replication by different mixtures of siRNAs. HeLa CD4$^+$ cells were transfected with indicated concentrations of synthetic siRNAs alone or in combination. Twenty-four hours after transfection the cells were infected with either HIV-1 NL4-3 or HIV-1 NL4-3_{M184V}. HIV-1 production was measured by p24 antigen ELISA and values were normalized to siRNA-non. Shown are means and standard deviations of four independent experiments. Values obtained from mock transfections and subsequent infection with HIV-1 were separately calculated based on 25 ([a]) and 50 ([b]) nM siRNA-non.

bition of virus replication (approximately 25%) was observed using 10 nM siRNA–M184 or siRNA–M184V, whereas maximum inhibition (approximately 80%) was observed using 50 nM concentrations. Increasing the siRNA concentration to 100 nM did not enhance the inhibitory activity. At all concentrations, neither siRNA–M184 nor siRNA–M184V inhibited replication of the viral variant not perfectly matching the sequence of the respective siRNA. Trypan blue staining of HeLa CD4$^+$ cells transfected with siRNA at concentrations up to 200 nM did not reveal any differences in cell viability depending on the concentration of siRNA used (data not shown).

3.5. Inhibitory effects on HIV-1 replication by co-transfection of two siRNAs

To test whether there is a synergistic effect of more than one siRNA used in combination, we transfected HeLa CD4$^+$ cells with mixtures of two distinct siRNAs at a 1:1 ratio and at total concentrations of either 50 or 100 nM. Each siRNA was also tested separately at concentrations of 25 and 50 nM for comparison. Inhibition of HIV-1 NL4-3 or HIV-1 NL4-3$_{M184V}$ replication was not enhanced using two effective siRNAs that recognize distinct viral RNA target sequences (siRNA-tat plus either siRNA–M184 or siRNA–M184V). Combinations in which only one siRNA is effective against the virus and the other is not (e.g. siRNA-tat plus siRNA–M184V in context with HIV-1 NL4-3) did not reduce the overall efficiency of inhibition significantly and suggests that the presence of an ineffective siRNA (either siRNA–M184V, siRNA–M184 or siRNA-non) has no negative impact on the efficiency of siRNA-tat (Fig. 4). Next, we tested a combination of siRNA–M184 and siRNA–M184V, which target wild-type and mutant HIV-1, respectively, both within the same region of the viral RNA. HeLa CD4$^+$ cells were co-transfected with these siRNAs and evaluated for inhibition of HIV-1 NL4-3 and HIV-1 NL4-3$_{M184V}$. The inhibitory effect of the combination of 25 nM of both siRNA–M184 and siRNA–M184V was slightly, but not significantly, decreased, as compared to the inhibitory effect of each siRNA alone (Fig. 4). This effect was more pronounced when total siRNA concentrations were increased from 50 to 100 nM. Although it was not significant in regard to the inhibition of HIV-1 NL4-3 replication, the inhibitory effect on HIV-1 NL4-3$_{M184V}$ replication was significantly reduced ($p = 0.001$).

3.6. Inhibition of HIV-1 replication by a combination of lamivudine and siRNA–M184V

Next, we transfected HeLa CD4$^+$ cells with siRNA–M184V and added lamivudine at a concentration of 10 μM. Subsequently, the cells were infected with HIV-1 NL4-3 and HIV-1 NL4-3$_{M184V}$ separately and in combination. Replication of HIV-1 NL4-3 was inhibited by >95% by lamivudine whereas replication of HIV-1 NL4-3$_{M184V}$ was not affected by lamivudine (Fig. 5A). In mixing infection experiments using 0.005 MOI of both HIV-1 NL4-3 and HIV-1 NL4-3$_{M184V}$, we observed no significant inhibition with either siRNA–M184V or lamivudine alone. We examined the outgrowing virus population in mixing infection experiments by quantitative real-time PCR for differential amplification. This assay allows simultaneous detection of both the wild type and drug-resistant variants in the same sample, enabling direct quantification with discriminatory abilities of 0.1% for M184 and 0.2% for M184V as minor population (Metzner et al., 2003). The mixture of both viruses used for infection contained 29.0 ± 4.6% HIV-1 NL4-3$_{M184V}$. HIV-1 NL4-3 represented the major variant in supernatants from HeLa CD4$^+$ cells treated only with siRNA–M184V. In cell cultures treated with lamivudine, HIV-1 NL4-3$_{M184V}$ was the outgrowing virus population (Fig. 5B). However, the combination of siRNA–M184V and lamivudine resulted in potent inhibition of all virus replication. The small amount of virus still replicating under

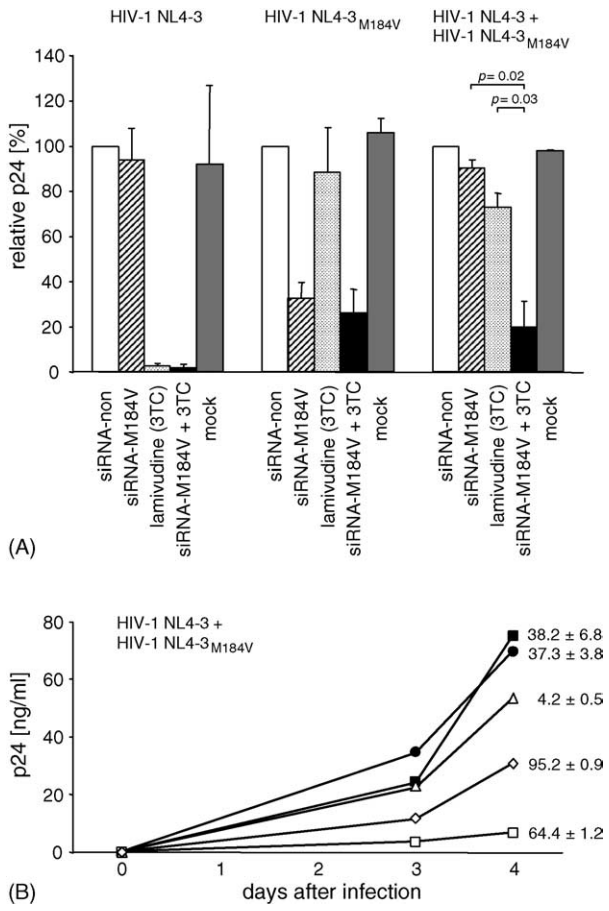

Fig. 5. Inhibition of HIV-1 replication by combination of lamivudine and siRNA–M184V. HeLa CD4$^+$ cells were transfected with 100 nM siRNA, lamivudine was added at a final concentration of 10 μM, and 24 h later the cells were infected with HIV-1 NL4-3 and HIV-1 NL4-3$_{M184V}$ separately or in combination. (A) Values of p24 antigen were normalized to the siRNA-non. Shown are means and standard deviations of two independent experiments. *p*-values below 0.05 are given in regard to the effects of siRNA–M184V, lamivudine and both substances on the inhibition of virus replication in HeLa CD4$^+$ cells infected with both viral variants. (B) Replication of HIV-1 NL4-3 and HIV-1 NL4-3$_{M184V}$ influenced by different synthetic siRNAs and/or lamivudine: lamivudine (◊), siRNA–M184V (△), lamivudine + siRNA–M184 (□), and siRNA-non (●). Virus replication in mock-transfected cells is shown in black squares. The ratio of HIV-1 NL4-3 to HIV-1 NL4-3$_{M184V}$ was determined in the supernatants of cell cultures on day 4 after infection using quantitative real-time PCR for differential amplification. Percentages of HIV-1 NL4-3$_{M184V}$ are given.

those conditions was mainly composed of HIV-1 NL4-3_{M184V} (Fig. 5B).

4. Discussion

In the last two decades, the rate of new HIV-1 infections has dramatically increased in many parts of the world. Despite the success of antiretroviral therapy in decreasing AIDS-related morbidity and mortality, HIV-1 is still incurable and a substantial fraction of HIV-1-infected patients experience therapy failure accompanied by the emergence of drug-resistant viruses, as reviewed by Cheung et al. (2004). New therapeutic strategies are needed against HIV-1 and especially against drug-resistant HIV-1 variants. Recently, RNAi-based treatments of viral infections (Poliovirus, HBV, HCV, Coxsackievirus, and HIV-1) and cancer in vitro have been reported by many groups, suggesting potentially promising therapeutic applications for siRNA, as reviewed by Cullen (2002) and Haasnoot et al. (2003).

In the present study, we investigated inhibition of drug-resistant HIV-1 by taking advantage of the sequence specifity of RNAi. High-level HIV-1 resistance to the commonly used RT inhibitor lamivudine can be achieved by a single point mutation in the codon 184 of the viral RT gene. Initially, a M184I mutation can appear early in the development of drug resistance to lamivudine; however, this mutation is rapidly replaced by the M184V mutation (Frost et al., 2000). Viruses carrying the M184I or the M184V mutation are conferring high-level resistance to lamivudine (i.e., 100- to 1000-fold increase in IC_{50}). No other mutations have been found to confer this magnitude of resistance to lamivudine. Moreover, the M184I mutation has a lower replication capacity compared with viruses carrying the M184V mutation (Frost et al., 2000). Therefore, we selected M184V-carrying viruses as a target for siRNA.

Codon 184 is within the highly conserved YMDD motif of the HIV-1 RT gene making it a desirable target for specific siRNA. To minimize the influence of additional mutations associated with drug resistance, we designed the sequence of siRNA–M184V to exclude the first two nucleotides of codon 181. In this way the mutation Y181C, which confers resistance to non-nucleoside RT inhibitors (NNRTI), would not impair the function of siRNA–M184V. Also of concern, the two first nucleotides of codon 188 form the 5′-end of siRNA–M184V and if present, a Y188L mutation might impair the efficiency of siRNA–M184V. This mutation is associated with resistance to NNRTIs; however, it is observed less frequently than the Y181C mutation (Cheung et al., 2004). In addition, this would only involve the first nucleotide of the antisense strand of the siRNA and mutations at the ends of siRNA have little or no impact on function compared with mutations in the center of the siRNA sequence (Amarzguioui et al., 2003; Pusch et al., 2003).

We evaluated different siRNA molecules transfected into HeLa $CD4^+$ cells and followed by infection with either HIV-1 NL4-3, HIV-1 NL4-3_{M184V} or HIV-1 NL4-3_{M184I}. HIV-1 variants carrying the M184I or M184V mutation have been reported to be less replication competent compared with wild-type HIV-1 (Wainberg et al., 1996); however, we did not observe an impairment in replication of these variants in HeLa $CD4^+$ cells. This finding is consistent with a study showing that these viral variants replicate less efficiently in primary cells but replicate with similar kinetics in cell lines (Back et al., 1996).

In our experiments, HIV-1 NL4-3, HIV-1 NL4-3_{M184V} and HIV-1 NL4-3_{M184I} were each inhibited by a control siRNA targeting the HIV-1 tat gene (siRNA-tat). This siRNA-tat has been used by others to inhibit replication of HIV-1 (Boden et al., 2003b; Coburn and Cullen, 2002). As a negative control, we used siRNA-non, a previously described sequence targeting the firefly luciferase gene with no sequence similarity to either human or HIV-1 genes (Elbashir et al., 2001). As expected, none of the HIV-1 variants were inhibited by siRNA-non. siRNA–M184, which targets the wild-type HIV-1, inhibited HIV-1 NL4-3 but not variant viruses carrying the M184V or M184I mutations. By comparison, siRNA–M184V only inhibited replication of HIV-1 NL4-3_{M184V}. Taken together, these results show that siRNAs differing at only one position are very specific for their cognate targets. This is consistent with observations that mismatches in the center of the siRNA and/or the target region can reduce or abolish the activity of RNAi (Amarzguioui et al., 2003; Gitlin et al., 2005; Pusch et al., 2003; Schubert et al., 2005).

siRNA-tat was similarly effective at inhibiting HIV-1 NL4-3 replication when used at concentrations of 10, 50 and 100 nM. However, we observed a dose-dependent effect when using siRNA–M184 and siRNA–M184V in which 10 nM was significantly less efficient at inhibiting virus replication than either 50 or 100 nM. This was also seen when using siRNA-tat to inhibit HIV-1 NL4-3_{M184V} replication. Possible explanations for this observation include secondary structures in the viral RNA that may prevent efficient hybridization of siRNA to its target sequence (Westerhout et al., 2005), or differences in siRNA transfection efficiencies. This dose-related phenomenon has also been reported using siRNAs that target CXCR4 (Ji et al., 2003). Hundred nanomolar was significantly less efficient than 10 or 50 nM only when using siRNA-tat for the inhibition of HIV-1 NL4-3_{M184V}. Currently, we have no explanation for this observation. Thus, optimal concentrations appear to vary according to the siRNA molecule, making it necessary to evaluate each siRNA separately.

Due to the high specificity of siRNA and the diversity of HIV-1, siRNA-resistant viruses can emerge rapidly (Boden et al., 2003a; Das et al., 2004). For siRNA to be effective as a therapy it will be necessary to combine several effective siRNAs or, alternatively, to add siRNAs to currently used antiretroviral drugs to avoid rapid development of escape variants. Therefore, we tested the inhibitory effect of mixtures of two siRNAs. We observed neither an enhancement of virus replication nor a decrease in virus inhibition using two effective siRNAs. To date, the advantages of using mixtures of effective siRNAs remains controversial. Some groups observed greater inhibition (Ji et al., 2003; Liang et al., 2005), while others did not show any synergistic effects (Dave and Pomerantz, 2004; Lee et al., 2003). Mixtures of one effective and one ineffective siRNA showed varying results. When both siRNAs targeted different regions of HIV-1 or when the ineffective siRNA was not related to any target (siRNA-non), the ineffective siRNA did not affect the efficiency of the effective siRNA. However, the combina-

tion of siRNA–M184 and siRNA–M184V, both hybridizing to the same target region within the HIV-1 mRNA, impaired the inhibitory potency compared with each effective siRNA alone. This is probably due to spatial hindrance of the effective siRNA by the ineffective molecule.

To test another therapeutic strategy, we used siRNA–M184V and lamivudine in combination to inhibit HIV-1 replication. We found that siRNA–M184V efficiently inhibits replication of the lamivudine-resistant HIV-1 carrying the M184V mutation, and lamivudine is a very potent inhibitor of wild-type HIV-1. The combination of RNAi and lamivudine has been shown to synergistically inhibit replication of hepatitis B virus (Chen et al., 2003). Here we show this combination also efficiently inhibits HIV-1 replication when both wild-type and M184V viral variants are present. This finding further supports the potential of this therapeutic approach to specifically target drug-resistant HIV-1.

We have shown that siRNA–M184 and siRNA–M184V are highly effective in inhibiting HIV-1 NL4-3 and HIV-1 NL4-3_{M184V}, respectively. Both siRNAs, which differ by a single nucleotide, specifically discriminate between wild-type HIV-1 NL4-3 and lamivudine-resistant HIV-1 NL4-3_{M184V}. Combination of the siRNAs that we evaluated did not enhance inhibition of virus replication as compared with each siRNA alone. However, the combination of siRNA with lamivudine inhibited both wild-type (NL4-3_{M184}) and drug-resistant HIV-1 (NL4-3_{M184V}). This result shows promise as a new treatment paradigm for HIV-1-infected patients who experience therapy failure accompanied by the appearance of drug-resistant viruses.

Acknowledgements

We thank Bernhard Fleckenstein for his constant support. We are very grateful to Ruth Connor and Daniel Boden for critical reading of the manuscript and helpful suggestions. pNL4-3 was kindly provided by the AIDS Research and Reference Reagent Program Division of AIDS, NIAID, NIH (M. Martin), HeLa $CD4^+$ cells were kindly provided by David Kabat, University of Oregon, Portland and lamivudine was kindly provided by GlaxoSmithKline.

This study was supported by the Wilhelm Sander Stiftung (grant no 2002.079.1) and the Deutsche Forschungsgemeinschaft, SFB 466 and Graduiertenkolleg 1071.

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