RNAi based gene therapy for HIV-1, from bench to bedside
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8.1 Abstract

RNAi based gene therapy for HIV-1 infection will require multiple small interfering RNAs (siRNAs) to circumvent the selection of viral escape mutants. Various systems expressing multiple siRNAs against HIV-1 have been described. However, many of these multiplexed siRNAs do not function at similar levels. Here we utilize a strategy in which three short hairpin RNA (shRNA) precursors are expressed in a single transcript driven by a U6 Polymerase (Pol) III promoter. All three siRNAs down regulate reporter constructs harboring cognate targets, but with different knockdown efficiencies. When the triple construct was inserted in a lentiviral vector and transduced into CEM T cells, HIV-1 challenge assays resulted initially in potent inhibition, but eventually viral replication occurred. Genotypic analysis confirmed viral escape, but mutations were observed only in the region targeted by the 5’ most proximal hairpin. Thus, even though the middle and 3’ proximal hairpins produced functional siRNAs, the levels were insufficient to inhibit viral replication once mutational escape of the first (5’ proximal) hairpin occurred. Based upon our findings we propose that viral escape analysis provides an effective tool to functionally test the selection pressure imposed by the individual inhibitors in a combinatorial RNA strategy.
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8.2 Introduction

RNAi has been shown to be effective in inhibiting the replication of a number of RNA genome viruses including poliovirus, hepatitis B and C viruses, enteroviruses, coxsackievirus, rhinovirus, influenza virus and HIV-1 [53, 65, 70, 103, 128, 143, 164, 202, 214, 233, 263, 308, 321, 337]. Short hairpin RNAs (shRNAs) are promising candidates for gene therapy approaches for chronic virus infections such as HIV-1 [23, 287, 291, 309]. Incorporation of anti-HIV shRNAs into viral vectors that can transduce hematopoietic cells has been shown to be an effective approach for blocking HIV replication in cell culture [185, 244] and in T lymphocytes derived from hematopoietic stem cells which have matured in a human immune system (HIS) mouse model [17, 23, 291]. The first gene therapy trial using an expressed shRNA for HIV inhibition has recently been initiated at the City of Hope National Medical Center.

Despite initial potent inhibition by many single shRNA inhibitors, the error prone replication mechanisms of RNA genome viruses allows them to readily escape the inhibitory activity of RNAi [41, 70, 113, 222, 253, 290, 298, 308, 318]. To circumvent this, multiplexing strategies involving simultaneous expression of multiple RNAi triggers have been utilized [3, 25, 161, 188, 189, 256, 287, 290]. These strategies include expression of multiple siRNAs from separate Pol III promoters or a combination of Pol II and III promoters [287, 290]. Alternatively, multiplexed siRNAs expressed from a single RNA transcript have also been developed. These include long hairpin RNAs (lhRNAs) that can be processed into two or more siRNAs [25, 161, 250]. A disadvantage of an lhRNA approach is that the processing efficiency of siRNAs from the long precursor decreases from the 3′ overhang towards the loop, creating a gradient of effective inhibitors [32]. Pol II expressed polycistronic miRNA clusters have also been developed [3, 189], in which each hairpin is separately processed via the miRNA processing pathway. In the present study we used constructs expressing a single, three short hairpin containing transcript that is processed into three siRNA species.

While most reports on multiple siRNA expression against HIV-1 argue that the reason for their multiplexed design is the prevention of escape, to our knowledge only one study critically tested this hypothesis. In that study, four shRNAs targeting different HIV-1 regions were expressed from individual promoters (4-shRNA), after prolonged culturing viruses in two out of six cultures started to replicate even in the presence of the 4-shRNA expression [287]. In contrast, five out of six virally challenged cultures showed replication when only a single shRNA was expressed. When these replicating viruses were passaged in cultures of cells expressing each individual shRNA or cells expressing the 4-shRNAs, the virus obtained from the 4-shRNA culture only replicated in the control cells whereas the single shRNA virus was able to replicate in control cells and cells expressing the original single shRNA, but not in cells expressing a different single anti-HIV shRNA. Sequencing of the escape viruses revealed that the 4-shRNA virus was not a mutant escape virus, but replication was caused by viral breakthrough, in contrast to the single shRNA that in fact did harbor mutations in the region complementary to the shRNA derived siRNA.

In the present study we tested a construct that expresses three anti-HIV shRNAs in
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a single transcript expressed from the U6 snRNA Pol III promoter. All three shRNAs trigger individual target knockdown in a dual luciferase assay, demonstrating processing of all three shRNAs into mature, functional siRNAs. This triple shRNA construct was inserted in the backbone of an HIV lentiviral vector and transduced into CD4-positive T lymphocytes. When cells harboring this construct were challenged with HIV-1, there was long term inhibition of HIV replication. However viral replication did emerge with delayed kinetics, and when the viral populations were analyzed we observed mutations only in the target site for the 5’ most proximal shRNA derived siRNA. Importantly, virus that harbored a mutation in the site targeted by this siRNA was able to replicate in the context of the triple shRNA expression vector. These results are consistent with the relative potencies of the three processed hairpins. Thus, analyses of escape virus in combination drug strategies can provide important information about which member(s) of the combination are the most effective inhibitors of HIV replication.

8.3 Material and methods

Plasmid construction
psiCHECK luciferase reporters were constructed by inserting the double stranded target sequences into the multiple cloning site of the psiCHECK-2 plasmid (Promega, CA, USA) and the detailed cloning methodology has been previously described [256, 260]. The shRNA encoding sequences in the constructs are shA (sense—loop—antisense): GCGGAGACAGCGACGAAGAGC—TTTGTGTAG—GCTCTTCGTC-GCTGTCTCCGC, shB: GCCTGTGCCTCTTCAGCTACC—TTTGTGTAG—GG-TAGCTGAAGAGGCACAGGC, shC: CATCTCCTATGGCAGGAAGAA—TTTGTGTAG—TTCTTCTGCCCATAGGAGATG.

The 3shABC was constructed by PCR amplification and subsequently cloned into pBluescript II SK(+). The U6+1 promoter functioned as a template for the production of the short hairpin at the 5′ position. A BamHI restriction site was included on the 5′ end and an XhoI site (for ligation to the SalI sequence of the next hairpin) on the 3′ end of the PCR product. The 5′ hairpin was introduced using the BamHI and XhoI restriction enzymes. After introduction of each hairpin construct, the correct size and sequence were verified using restriction and DNA sequence analyses. The 5′ end of the hairpins for the middle and 3′ positions contain a SalI sequence, which is compatible with the XhoI sequence on the 3′ end of the 5′ and middle hairpin and results in loss of both restriction sites upon ligation. The middle hairpin contained a KpnI site after the XhoI site to facilitate introduction into the pBSK backbone. The 3′ end of the 3′ hairpin contained a string of six Ts to create a Pol III terminator followed by XbaI and KpnI sites. The following primers were used: U6forward: ATAAGAATGCGGCCGCCCCGGGGATCC-AAGGT, shAreverse: CATTGAGGTACCCTCGAGGTGTGCTCTTCGTC-GCTGTCTCCGCCTACACAAAGCGGAGACAGCGACGAAGAGCGGTGTTTCGTCCT-TCCACAAGA, shBforward: TCAATGGTCGAGCCTGTGCCTCTTCAGCTACC-
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TTTGTGTAGGG, shB reverse: TCAATGGGTACCCTCCTGCTGTGCCTTCA-GCTACCCCTACACAAAGG, shC forward: TCAATGGTCGACACCAGCTCCTCTAT-GCCAGGAAAGATTTTGTTAGT, shC reverse: TCAATGGGTACCTCTTAGAAA-AAAAGCATCTCCTATGGCAGGAAGAACTACACAAA. The Not1 sites on the 5′ end and XbaI site on the 3′ end of the constructs facilitated cloning into the pHIV-7-GFP vector.

Cell lines and cell culture
HEK-293 and HEK-293T cells were obtained from ATCC and grown in DMEM (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% fetal calf serum (Irvine Scientific), 1 mM L-glutamine and sodium pyruvate. The human T cell line CEM was maintained in RPMI1640 medium supplemented with 10% FBS. CEM stable cell lines were generated as described previously[183].

Dual luciferase assays
Down regulation of the target mRNAs was monitored by dual luciferase assays using the psiCHECK system as previously described [3, 25]. HEK-293 cells were cultured in a 24-well plate at 50-70% confluency. Transfections were carried out using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturers instructions. In each well, 50 ng psiCHECK and 100 ng of shRNA construct were transfected. After 48 hours of incubation supernatants were removed and cells were lysed with the lysis buffer provided with the Dual-Luciferase Reporter Assay System (Promega, CA, USA). Luciferase expression levels were measured using the dual-luciferase reporter assay kit (Promega) in a luminometer (Veritas, Turner Biosystems), as described by the manufacturer. Target-specific Renilla luciferase expression was normalized to firefly luciferase. The average expression ratio for control samples was set to 100%, and relative expression levels for other samples were calculated accordingly.

HIV replication assay and CA-p24 assays
A total of 1 × 10⁶ transduced CEM T cells were infected with HIV-1 strain IIIB at an MOI of 0.01. After overnight incubation, cells were washed three times with Hanks balanced salts solution and cultured in RPMI 1640 with 10% FBS. Every week, culture supernatants were collected for HIV-1 CA-p24 analyses (HIV-1 CA-p24 antigen ELISA kit, Beckman Coulter Inc., Brea, CA, USA) and cells were passaged in fresh media.

Detection of escape mutants
Viral RNA was extracted from the culture supernatant using a QIAamp viral RNA mini kit (Qiagen, CA, USA) according to the manufacturers instructions. Viral RNA was PCR amplified using primers flanking the target site, and the PCR product was sequenced. Primers: for target region A and C forward primer: 5’ ACTAGAGCC-CTGGAAGCAT 3’ and reverse primer: 5’ ACTACTAAGGCTACTATTGTT 3’ and for the target region B forward primer: 5’ TAGGCAGGGATATTCCACCA 3’ and reverse primer: 5’ ATTGTAGGAGATTCCACCA 3’. For the 3shRNA escape mutants,
multiple clonal mutation variants were identified via cloning the cDNA into the TOPO TA vector (Invitrogen, Carlsbad, CA, USA). Individual clones were DNA amplified with the T7 and M13R primers from the TOPO TA cloning kit and sequenced using the M13R primer.

8.4 Results

8.4.1 The transient dual luciferase assay shows functionality of each siRNA in the multiplexed construct.

One approach for minimizing HIV-1 escape mutants in response to the pressure of RNAi is to simultaneously target multiple sequences in the HIV-1 genome [290]. Therefore we selected three shRNAs targeting HIV-1 in the tat, rev and env genes. The shRNA targets are tat/rev overlapping exons for A, and rev/env overlapping exons for B and a unique tat exon for C. (Figure 8.1A). We generated the 3shABC construct in which all three shRNAs are transcribed from a single U6 RNA polymerase III promoter (Figure 8.1B).

To measure the functionality of 3shABC versus U6 driven individual shRNAs, we used a dual luciferase reporter system harboring targets for the individual shRNAs within the 3′ UTR of the Renilla luciferase construct and a separately expressed Luciferase reporter that serves as a transfection control. Both luciferase genes are expressed from the same psiCHECK plasmid. Each of the single Pol III expressed shRNAs elicited a greater than ninety percent inhibition of their respective targets (Figure 8.1C). No inhibitor activity was observed for reporters with non-matching targets, demonstrating the sequence specificity of the individual shRNAs. The triple hairpin 3shABC triggered knockdown of all three reporters, although the relative knockdown of targets B and C was somewhat reduced in comparison to the individual shRNAs.

8.4.2 Mutations in the 5′ shRNA target region results in viral replication in 3shABC expressing cells.

Since all three shRNAs expressed from 3shABC were able to down regulate their respective targets, albeit to varying extents, we used this construct to evaluate its antiviral efficacy against HIV-1. To do this, we inserted some of the constructs into our pHIV-7-GFP lentiviral vector backbone [185] and stably transduced CEM T cells (Figure 8.2A). GFP positive cells were sorted and subsequently infected with HIV-1. At different time points, viral CA-p24 antigen levels were measured with a representative experiment shown in Figure 8.2B. The shA, shB and the 3shABC vectors all gave initial potent inhibition of viral replication. At day 14 the shA cells gave rise to increased HIV replication and at day 21 the shB as well as the 3shABC cells started to produce virus.
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Figure 8.1: Three shRNA expression system and silencing efficacy. (A) Targeted regions of shA, shB and shC in the HIV-1 genome. (B) The construct 3shABC expresses shA, shB, and shC as a single transcript from the polymerase III promoter U6. (C) The reporter vector psiCHECK, with target A, B or C inserted within the 3' UTR of the Renilla luciferase gene and the independently expressed firefly luciferase gene, was cotransfected with the constructs of interest. Inhibitory efficiencies of the single shA, shB, shC and 3shABC were determined at 48 hours post-transfection. Data are expressed as a ratio of Renilla to firefly luciferase normalized to an empty vector control (n =3).
After initial viral inhibition of HIV by the 3shABC construct, replication eventually takes place. This could be the result of exponential viral replication overwhelming the siRNA mechanism, accumulation of viral mutants resistant to RNAi or a combination of both. To determine which of these mechanisms led to viral replication, we analyzed viral sequences in the supernatants from the first HIV challenges of shA, shB and 3shABC cell lines. Sequence analyses of the individual targets for shA and shB showed point mutations exclusive to the viral targets for the cognate shRNA. Somewhat surprisingly, for 3shABC only mutations in the target sequence for the 5’ shA inhibitor (tat/rev common exon) were identified (Table 8.1). These results suggested that the 5’ hairpin, shA, was largely responsible for the viral inhibition observed with the 3shABC construct.

**Figure 8.2:** Initial potent inhibition of the multiplexed siRNAs, however viral escape mutants emerge. (A) The shABC expression system was inserted in the pHIV-7-GFP lentiviral vector. This vector and a vector expressing a single shA or shB hairpin were transduced into CEM T cells. (B) Stably transduced cells were challenged with with HIV at an MOI of 0.01 and CA-p24 was measured once a week. Note, shB and 3shABC have a similar replication pattern, and overlap in the graph.

To validate that virus with mutations in the shA target alone can replicate in 3shABC expressing cells, supernatants from viral escape cultures shA or shB were used to re-infect control and 3shABC transduced cells. Virus with point mutations in the target sequences for shA and shB both were able to replicate in control cells, but in 3shABC expressing cells only the virus harboring a point mutation in the shA target region was able to replicate (Figure 8.3). These results demonstrated that mutations...
Table 8.1: Viral escape mutants.

<table>
<thead>
<tr>
<th>Target</th>
<th>WT-3shA/B</th>
<th>WT-3shB</th>
<th>WT-3shABC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target A</td>
<td>gcggagacagcgacgaagagc</td>
<td>wt→shA</td>
<td>wt→shB</td>
</tr>
<tr>
<td></td>
<td>c a t c t c t a t c t a t c t a t c t</td>
<td>wt→shA</td>
<td>wt→shB</td>
</tr>
<tr>
<td>Target B</td>
<td>gcctgtgcctcttcagctacc</td>
<td>wt→shA</td>
<td>wt→shB</td>
</tr>
<tr>
<td></td>
<td>c a t c t c t a t c t a t c t a t c t</td>
<td>wt→shA</td>
<td>wt→shB</td>
</tr>
<tr>
<td>Target C</td>
<td>catctcctatggcaggaagaa</td>
<td>wt→shA</td>
<td>wt→shB</td>
</tr>
<tr>
<td></td>
<td>c a t c t c t a t c t a t c t a t c t</td>
<td>wt→shA</td>
<td>wt→shB</td>
</tr>
</tbody>
</table>

Multiple mutant viruses, but also wild-type could be detected for some of the targets. After replication was observed the genotype of the viral escape mutants in the target region A, B and C. Multiple target sites for shA, shB and shRNA transduced cells was analyzed in the target region A, B and C.
in shA target site alone are sufficient to allow replication in 3shABC transduced cells. Interestingly, subsequent sequence analyses of the virus upon the start of replication showed the shB mutant virus had also acquired mutations in target A when passaged in the 3shABC cells (Table 8.2). Thus while multiple siRNAs are produced, albeit at different levels, only the siRNA that gives the most potent inhibition resulted in selection of viral resistance mutants.

Figure 8.3: Infection with mutants of shA and shB demonstrate viral replication when mutations in the target region of the 5’ shRNA are present. Control and 3shABC cells were infected with mutants generated in shA and shB transduced cell lines. Both mutants replicate effectively in non-transduced control cells within 7 days (data not shown) whereas only the virus harboring a mutation in the target site for shA was able to replicate in the 3shRNA transduced cells.

8.5 Discussion

One of the hurdles in the development of anti-HIV-1 RNAi therapeutics is developing strategies for mitigating or avoiding viral resistance. For RNAi, theoretically this can be accomplished by the use of multiple shRNAs. It has been demonstrated that HIV-1 escape can be prevented if the virus must acquire multiple mutations in critical sequences to become resistant [32, 163, 300]. RNAi based gene therapy for HIV-1 infection will certainly require combinations of small interfering RNAs (siRNAs) or other classes of inhibitors to circumvent viral escape mutants. Various systems expressing multiple siRNAs against HIV-1 have been described [25, 188, 189, 252, 256, 287, 315]. Here we utilized a strategy in which three consecutive hairpins are expressed from a single U6 Pol III promoter. The possible advantage of this approach is that all three shRNAs would be transcribed at the same level. While all three siRNAs are able to down regulate reporter constructs harboring cognate targets, not all siRNAs elicited equal target knockdown. When the triple construct
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Table 8.2: Viral target genotypes after passage of shA and shB mutants on 3shABC cells. Viral genotypes were analyzed for target A, B and C after viral replication occurred.

<table>
<thead>
<tr>
<th>Target A</th>
<th>3shABC</th>
<th>shAmut</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td>gcggagacagcgacgaagagc</td>
<td>shAmut→control</td>
<td>shBmut→control</td>
<td>shAmut→3shABC</td>
</tr>
<tr>
<td>gcctgtgcctcttcagctacc</td>
<td>shAmut→control</td>
<td>shBmut→control</td>
<td>shAmut→3shABC</td>
</tr>
<tr>
<td>catctcctatggcaggaagaa</td>
<td>shAmut→control</td>
<td>shBmut→control</td>
<td>shAmut→3shABC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Target B</th>
<th>3shABC</th>
<th>shAmut</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td>gcggagacagcgacgaagagc</td>
<td>shAmut→control</td>
<td>shBmut→control</td>
<td>shAmut→3shABC</td>
</tr>
<tr>
<td>gcctgtgcctcttcagctacc</td>
<td>shAmut→control</td>
<td>shBmut→control</td>
<td>shAmut→3shABC</td>
</tr>
<tr>
<td>catctcctatggcaggaagaa</td>
<td>shAmut→control</td>
<td>shBmut→control</td>
<td>shAmut→3shABC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Target C</th>
<th>3shABC</th>
<th>shAmut</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td>gcggagacagcgacgaagagc</td>
<td>shAmut→control</td>
<td>shBmut→control</td>
<td>shAmut→3shABC</td>
</tr>
<tr>
<td>gcctgtgcctcttcagctacc</td>
<td>shAmut→control</td>
<td>shBmut→control</td>
<td>shAmut→3shABC</td>
</tr>
<tr>
<td>catctcctatggcaggaagaa</td>
<td>shAmut→control</td>
<td>shBmut→control</td>
<td>shAmut→3shABC</td>
</tr>
</tbody>
</table>
was inserted in a lentiviral vector and transduced into CEM T cells, HIV-1 challenge assays resulted in an initial potent inhibition of viral replication, but after long-term challenge viral replication occurred. Genotypic escape analysis demonstrated that viral escape mutants emerged. The emergent viral variants were only mutant in the region targeted by the 5' hairpin, which is consistent with this being the most potent shRNA in the dual luciferase assays. Thus, even though the middle and 3' hairpins produced functional siRNAs, they were not potent enough to inhibit viral replication once the virus had gained a mutation in the target sequence of the best siRNA. Northern blot analyses of the processed hairpins revealed that the most 5' shRNA was present at higher levels than the middle or 3' shRNA derived siRNAs (see Chapter 7) which is consistent with the relative potencies of the three hairpins.

While many of the multiplexed siRNA constructs against HIV-1 are tested for viral inhibition, only one study examined multiplexed constructs for the prevention of resistant mutation in the virus [287]. Interestingly, although no viral escape mutants were observed in that study with the 4-shRNA construct, viral replication did occur for some of the tested cultures [287]. It is possible that competition of the multiplexed shRNAs with each other for entry into RISC reduces their effective concentrations and allows viral replication in the presence of RNAi. While in the case of competition a reduced efficacy and therefore an early observed replication pattern would be expected, the initial long term potent inhibition followed by virus replication in only a few cultures suggest that another mechanism underlies this observed replication. It has been described that the incoming genome is not targeted by RNAi, thus allowing the slow accumulation of virus in the cell [319]. This intracellular viral accumulation could potentially result in concentrations of viral RNA that exceed the amount of RISC complexes harboring anti-viral siRNAs, allowing the virus to replicate even in the presence of potent siRNAs. Thus virus accumulation followed by viral breakthrough is most likely the underlying cause of viral replication in the 4-shRNA cells.

However, saturation of the RNAi pathway and competition of siRNAs and miRNAs upon the expression of multiple highly expressed shRNAs in a single cell is a matter of attention. Saturation has been described in mammalian cells, as a consequence of high levels of shRNA expression [44, 58, 110, 204, 291, 306]. The use of RNAi as an anti-viral therapy will require a thorough understanding of specific dosage requirements and careful design of expression systems and vectors. Producing sufficient siRNAs to maintain antiviral function, while avoiding toxicity, is clearly a challenging problem. A goal of our shared promoter approach is to produce several highly potent shRNAs at comparable levels while avoiding cell toxicity from adverse competition with endogenous miRNAs. However, our results suggest that high levels of each shRNA may be required to gain entry into RISC for effective target down-regulation. This could ultimately be counter productive in that the too highly expressed shRNAs can compete with cellular miRNAs and with themselves for RISC entry as we have previously reported [58]. One method of avoiding the possible competition by multiple siRNAs is mixing a single shRNA with other types of viral inhibitory agents [185, 298].
Many of the described multiplexed anti-HIV siRNA systems do not express siRNAs at equivalent levels. We would therefore argue that thorough, long term testing of multiplexed siRNA constructs for their prevention of viral escape should be carried out for every combination. By analyzing the sequences of virus that escapes suppression with time it is possible to determine whether or not the replication is caused by escape via mutation or viral breakthrough. In addition, infecting stable cell lines expressing the multiplexed construct with molecular clones that each have mutations in one of the target regions, or the use of escape viruses obtained from single expressed shRNAs cultures will demonstrate whether or not all the shRNAs are providing optimal inhibition. And this 3shABC example and the suggested methods could be useful for other researchers that aim to develop expression methods with multiplexed siRNA to prevent viral escape.

8.6 Acknowledgements

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