CHAPTER 5
Chapter 5: To design multi-target shRNAs to down regulate HIV-1 genes

RNA interference, RNAi, is considered to be one of the most promising and potent recent addition to arsenal of anti-viral weapons. It was called “Scientific breakthrough of the year (2002)” by the journal Science (Couzin, 2002) and honored with the 2006 Nobel Prize in Physiology or Medicine (Andrew Z. Fire and Craig C. Mello); RNAi is a natural phenomenon of gene silencing by small duplex RNAs. The extreme proficiency and specificity of this process make RNAi highly prominent for anti-viral therapies. A particular benefit over conventional approaches is that RNAi is an innate cellular pathway. Only the introduction of a trigger for its activation is required, which should minimize the side effects. A gene therapy approach involving delivery of RNAi expression cassettes is more appropriate for treatment of chronic infections such as HBV, HCV, and HIV (Grimm and Kay, 2006). Typically, the trigger is a miRNA like sequence (derived from a natural miRNA15 or an artificial short hairpin RNA (shRNA)) under the control of an RNA polymerase II or III promoter. These cassettes are small and thus readily incorporated into any of the established gene therapy vectors, such as lentiviruses and AAVs (adeno-associated viruses). Another general advantage of RNAi as an anti-viral therapy is that triggers with perfect viral sequence complementarity induce target cleavage. This offers the likelihood not only to suppress the pathogen but actually to exterminate it from the host (Dykxhoorn and Lieberman, 2006; Leonard and Schaffer, 2006). Last but not least, RNAi silencing requires a minimal target of only 19–21 nt, which might be sufficient to co-suppress related viral isolates. Various studies support the idea by documenting concurrent inhibition of multiple HIV or flavivirus strains with a single siRNA. (Lee et al., 2005) and (Kumar et al., 2006).

Human immunodeficiency virus (HIV)-1 continues to be a global pandemic of enormous repercussions to humanity. Gene therapy has shown many potential targets. The toxicity associated with anti-HIV-1 drugs, together with the appearance of newly resistant strains to current drugs, drives the continued search for novel strategies to fight HIV-1. RNAi can
be considered as a gene-specific therapeutic option for controlling HIV-1 replication. However, the control of HIV-1 replication has become complex because of the limited success of existing anti-HIV-1 agents and the high speed mutation rate of the HIV-1 genome. Careful assessments are prerequisite for the potential of RNAi as a gene therapy approach for controlling HIV-1 replication.

Either as an alternative or as an addition to anti-retroviral chemotherapy, gene therapy has gained consideration as a possible treatment for acquired immunodeficiency syndrome (AIDS). Several types of RNA gene therapies have been developed and shown to inhibit HIV-1 replication in mammalian cell cultures; these include antisense RNA, catalytic RNA (ribozyme) and high-affinity RNA ligands (aptamers or decoys). Several of these RNA-based approaches have been safety tested in phase I clinical trials, although there are no reports yet of efficacy trials. Nevertheless, in vitro data suggest that their anti-viral efficiencies are variable and can often be overcome by increasing the multiplicity of HIV infectious particles. The limitations have led several investigators to explore the potential of RNA interference (RNAi) as an anti-HIV therapy.

The 9 kb HIV-1 RNA encodes for nine open reading frames, while the prototypic gag, pol, and env genes are essential for replication of these animal retroviruses, the rest six of them are the auxiliary genes. The study of these genes has elucidated the underlying mechanism of HIV-1 replication, its pathogenesis as well as the molecular biology of the eukaryotic cells. These include Tat, Rev, Nef, Vpr, Vif and Vpu.

To tackle the HIV-1, we decided to target the overlapping regions of the genome, that code for 2 or more genes. Moreover, by clustal W analysis between subtype B and C, the region of overlapping portions of genome was considered which was conserved in the two subtypes of HIV-1, emphasizing the importance of the region since it has not undergone change over period of time. Three such regions were identified, as mentioned in the figure 1. These regions are Tat and Rev overlapping region at Exon I, Vif and Vpr...
overlapping region and Tat, Rev and Env region. For the first and second regions, clustal W analysis showed an area that could be targeted which is conserved between subtypes B and C. The third region, that targets three genes is subtype B specific only. The shRNA were designed against these regions and cloned in RNAi Ready-RetroQZs-green vector, and its efficacy was checked by luciferase assays, RT-PCR, flow cytometry and virus challenge assay.
shRNAs targeting overlapping regions of HIV-1

Overlapping sequences of HIV-1 against which the shRNAs are designed

shRNA against overlapping regions of HIV-1:

(A) An shRNA against Tat, Rev, Env of HIV-1 Subtype B;
(B) An shRNA against Tat, Rev of HIV-1 Subtypes B and C; and
(C) An shRNA against Vif, Vpr of HIV-1 Subtypes B and C.

FIGURE 1:

HIV-1 genome and the overlapping genes. The encircled regions show the regions on HIV-1 targeted by the three shRNAs.
Materials and methods

CELL LINE

HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (Biological Industries).

TZM-bl cells (Cat # 8129), previously designated JC53-bl (clone 13) is a HeLa cell line. The parental cell line (JC.53) stably expresses large amounts of CD4 and CCR5. This cell line was generated from JC.53 cells by introducing separate integrated copies of the luciferase and β-galactosidase genes under control of the HIV-1 promoter. These cells are highly sensitive to infection with diverse isolates of HIV-1. (Obtained from NIH AIDS Research & Reagent Program, NIH, MD, USA). TZM-bl cells were maintained in DMEM (90%), 10% FBS, 100 units of Penicillin and 0.1 mg/ml of Streptomycin.

PLASMID CONSTRUCTS

Three shRNAs and various HIV-1 genes of subtypes B, C were cloned and sub-cloned. The shRNAs were cloned in Retro-QZs-green vector system, while the HIV-1 genes of subtype B, C were cloned in pDS2-red Nl for flow cytometry and in psiCHECK-2 for luciferase assays.

HIV-1 isolate pNL4-3 (GenBankTM accession number AF324493) was used as the template for cloning of Tat B, Rev B, Vif B, , Vpr B, and Env B; and HIV-1 isolate 93IN905 (GenBankTM accession number AF067158) was used a s template for cloning of Tat C, Rev C, Vif C and VprC.

Cloning of Tat, Rev
The two exons of Tat B, C were stitched together as explained previously in chapter 3. The two exons of Rev B and Rev C were amplified and stitched together using the fusion primers (figure 3, panel A).

Tat B was subcloned into pDS2Red-N1 (Cat no#632406, Clontech) and psiCHECK-2 (Cat. No. C8021, Promega) as described in chapter 3 (figure 3, panel A).

Tat C was amplified from 93IN905 using following primers to be cloned in pDS2RedN1.

(for figure 3, panel A).

FORWARD PRIMER:

5’ GGC CTCGAG ATGGAGCCAGTAGATCCTAA 3’

REVERSE PRIMER:

5’ GGC AAGCTT ATCGAATGGATCTGTTTTGT 3’

(XhoI and HindIII in forward and reverse primers)

Tat B, C was also sub-cloned in psiCHECK™-2 vector system using following set of primers.

(for figure 3, panel A).

FORWARD PRIMER: (common for TAT B, C) 5’GGC CTC GAG ATGGAGCCAGTAGATCCTA 3’

REVERSE PRIMER: TAT B 5’ GCC GCGGCCGC CTATTCCTTCGGGCCT 3’

REVERSE PRIMER: TAT C 5’ GCC GCGGCCGC CTAATCGAATGGATCTGT 3’
The two exons of Rev B were amplified from 93IN905 using following primers and stitched together using fusion primer technique as described earlier to be cloned in pDS2Red-N1 (figure 3, panel B).

FORWARD PRIMER: FP1

5' GGC CTC GAG ATGGCAGGAAGAAGCGG 3'

REVERSE FUSION: RP2

5' CTCGGGATTGGGAGGTGGGTTGCTTTGATAGAGAAGCTTG 3'

FORWARD FUSION: FF3

5' CAAGCTTCTCTATCAAAGCAACCCACCTCCCAATCCCGAG 3'

REVERSE PRIMER: RP4

5' GGC AAG CTT CTATTCTTAGTTCTGACTCCAA 3'

(XhoI and NotI in forward and reverse primers, FP1 and RP4)

The two exons of Rev C were amplified from 93IN905 using following primers and stitched together using fusion primer technique as described earlier to be cloned in pDS2Red-N1 (figure 3, panel B).

FORWARD PRIMER: FP1
shRNAs targeting overlapping regions of HIV-1

REVERSE FUSION: RP2

5' CCTGGGTTCCGGGTAAAGGGTTGCTTTGATATAAGATTTTGAT 3'

FORWARD FUSION: FF3

5' ATCAAAATCTTTATATAAAGCAACCCCTTACCCGAACCCAGG 3'

REVERSE PRIMER: RP4

5' GGC AAG CTT AGGCCTTCCCATCCCTT 3'

(XhoI and HindIII in forward and reverse primers, FP1 and RP4)

Rev B, C was also sub-cloned in psiCHECK™-2 vector system (Cat. No. C8021, Promega) using following set of primers (figure 3, panel B).

FORWARD PRIMER: common for REV B, C

5' GGC CTC GAG ATGGCAGGAAGAAGCGG 3'

REVERSE PRIMER: REV B

5' GGC GCGGCCGC CTATTCTTTAGTTCCTGACTCC 3'

REVERSE PRIMER: REV C

5' GGC GCGGCCGC TTAAGGCTTTCCCATCC 3'

(XhoI and NotI in forward and reverse primers)

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Cloning of Env B

The Env B was amplified from pNL4-3 using primers mentioned below to be cloned in DS2Red-N1 (Cat no#632406, Clontech) and psiCHECK-2 (Cat. No. C8021, Promega) (figure 3, panel C).

FORWARD PRIMER: 5′ GCC CTCGAG ATGAGAGTGAAGGAGAAGTATCA 3′

REVERSE PRIMER: 5′ GCC GAATTG TAGCAAAATCCTTTCCAAGC 3′

(Xhol and EcoRI in forward and reverse primers)

Env B was also sub-cloned in psiCHECK™-2 vector system (Cat. No. C8021, Promega) using following set of primers (figure 3, panel C).

FORWARD PRIMER: 5′ GCC CTCGAG ATGAGAGTGAAGGAGAAGTATCA 3′

REVERSE PRIMER: 5′ GCC GCGGCCGC G TAGCAAAATCCTTTCCAAGC 3′

Cloning of Vpr

HIV-1 isolate pNL4-3 (GenBankTM accession number AF324493) and HIV-1 isolate 93IN905 (GenBankTM accession number AF067158) were used as templates to amplify Vpr B and Vpr C respectively (figure 3, panel D). The primers sequences are mentioned below.

FOR VPR B

FORWARD PRIMER: 5′ GCC CTCGAG ATGGAACAAGCCCCAGAA 3′
REVERSE PRIMER: 5’ GGC GGATCC GG GGCTACTGGCTCCATTTC 3’

FOR VPR C

FORWARD PRIMER: 5’ GGC CTCGAG ATGGAACAATCCCCAGAAG 3’

REVERSE PRIMER: 5’ GGC GGATCC GG GGATCTACTGGCTCCATTTC 3’

The forward and reverse primers have XhoI and BamHI, respectively.

Vpr B, C was also sub-cloned in psiCHECK™-2 vector system (Cat. No. C8021, Promega) using following set of primers (figure 3, panel D).

VPR B

FORWARD PRIMER: 5’ GGC CTCGAG ATGGAACAAGCCCCAGAA 3’

REVERSE PRIMER: 5’ GGC GCGGCCGC GG GGATCTACTGGCTCCATTTC 3’

VPR C

FORWARD PRIMER: 5’ GGC CTCGAG ATGGAACAATCCCCAGAAG 3’

REVERSE PRIMER: 5’ GGC GCGGCCGC GG GGATCTACTGGCTCCATTTC 3’

Cloning of Vif

HIV-1 isolate pNL4-3 (GenBank™ accession number AF324493) and HIV-1 isolate 93IN905 (GenBank™ accession number AF067158) were used as templates to amplify Vif B and Vif C respectively (figure 3, panel E). After ClustalW analysis for Vif Band Vif C,
the regions common between both subtypes were selected to amplify the genes. The primers sequences are mentioned below.

FORWARD PRIMER: 5’ GGC CTCGAG ATGGAAAACAGATGGCAGGT 3’

REVERSE PRIMER: 5’ GGC GGATCC GG GTGCCATTCA TGTATGG 3’

The forward and reverse primers have XhoI and BamHI, respectively.

Vif B, C was also sub-cloned in psiCHECK™-2 vector system (Cat. No. C8021, Promega) using following set of primers (figure 3, panel E).

FORWARD PRIMER: 5’ GGC CTCGAG ATGGAAAACAGATGGCAGGT 3’

REVERSE PRIMER: 5’ GGC GC TAGC GC GG GTCCATT CA TGTATGG 3’
Cloning of shRNA and target genes

1. shRNA AGAINST TAT/REV/ENV:

```
5' GGATCC GAACCACTCCAATCCCG TCCAGAGA CGGGATGGG AGTGGGCTC TTTTTT GAATTC 3'
3' CTTAGG CGGGTTGGAGGGTTAGGCC AAGTTCTC GCCCTAACCCTCACCAGAAAAAA CTTAG 5'
```

2. shRNA AGAINST TAT AND REV (EXON I) SUB TYPE B, C:

```
5' GGATCC GAATGGCAGGAGAGGCCTGA TCCAGAGA TCGGCTTCTTTCCGCACT TTTTTTTT GAATTC 3'
3' CTTAGG CTACGGCTCTTCCTCCTCTCTCTCA AAGTTCTC AGGCGGAGAGGCGGAGAGGCTTAAG 5'
```

3. shRNA AGAINST VIF AND VPR SUBTYPE B, C:

```
5' GGATCC CCATACAATGGAATGGACAC TCCAGAGA GTGTCCATTCATTGTATGG TTTTTTTT GAATTC 3'
3' CTTAGG GTATGTTACTTACCTGTG GAGTTCTC CACAGGTAAGTAACATACCA AAAAAAAA CTTAAG 5'
```

**FIGURE 2:**

Cloning of the three shRNAs into RNAi-Ready-RetroQZSgreen.
The shRNA were designed against various regions of HIV-1. 1) Tat/Rev/Env of subtype B, 2) Tat/Rev of subtype B, C; and 3) Vif/Vpr of subtype B, C are the three regions targeted by shRNA. The shRNAs were cloned in RNAi-Ready-RetroQZSgreen (figure 2).

**Cloning of shRNA against Tat/Rev/Env**

The cloning of the Tat/Rev/Env shRNA is described in chapter 3 (figure 3, panel F).

**Cloning of Tat/Rev shRNA**

The sense and non-sense strands of Tat/Rev shRNA were synthesized to be cloned in RNAi-ready-RetroQZs-green vector (figure 3, panel F). The templates were generated with sticky ends of EcoRI and BamHI. The phosphorylated oligos were ordered from Sigma. This protocol is used to add a phosphate group to the 5' end of a single or double stranded DNA molecule. The following two templates were synthesized to clone the Tat/Rev shRNA. The two templates were annealed together by heating at 92°C for 2 minutes, and then rapidly cooled to 4°C for 30 minutes. The annealed templates were cloned in EcoRI-BamHI digested RetroQZs-green vector using T4-DNA ligase.

**TEMPLATE 1:**

5' GATCCGATGGCAGGAAGAAGCGGATTCAAGAGATCCGCTTC TT C CTGCCATCG IIII I IIIG

**TEMPLATE 2:**

5' AATTCAAAAAAAAGATGGCAGGAAGAAGCGGATCTCCTGAATCCGCTTCTTCTGCCCATCG 3'
Cloning of Vif/Vpr shRNA

As described above, the sense and non-sense strands of Vif/Vpr shRNA were synthesized to be cloned in RNAi-ready-RetroQZs-green vector (figure 3, panel F). Both the templates were generated with sticky ends of EcoRI and BamHI. The phosphorylated oligos were annealed together by heating at 92°C for 2 minutes, and then rapidly cooled to 4°C for 30 minutes. The annealed templates were cloned in EcoRI-BamHI digested RetroQZs-green vector using T4-DNA ligase.

Template 1:

5' GATCCCATACAATGAATGGACACTTCAAGAGAGTGTCATTCATGTATGGGTTTTTTTTT 3'

Template 2:

5' AATTCAAAAAAACATACAATGAATGGACACTCTCTTGAAAGTGTCATTCATGTATGGG 3'
FIGURE 3:

Cloning of the Tat, Rev, Env, Vif and Vpr of HIV-1 in pDS2Red-N1 and psiCHECK-2; and cloning of Tat/Rev/Ev, Tat/Rev and Vif/Vpr shRNA in RNAiReady-RetroQZSgreen.
Methods

INTRACELLULAR REDUCTION OF THE TARGET RNA

The intracellular decrease in Tat/Rev/Env/Vif/Vpr-specific RNA of subtype B and/or C in HEK293T cells that were co-transfected with 500ng of expression vector encoding the gene and 1μg shRNA cloned in RNAi-Ready pSIREN-RetroQ-ZsGreen was determined by reverse transcriptase (RT) PCR techniques. Total RNA was isolated using Trizol reagent (Gibco BRL) following the manufacturer’s directions. The RNA was treated with DNase 1 (RQ1, Promega Biotech.) for 45 min at 37°C. Equal amounts of RNA were taken and subjected to RT-PCR. The control RNA (509 bases), human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH), was amplified using the following set of primers:

Forward: 5’-ACCACCATGGAGAAGGCTGG-3’; and

Reverse: 5’-CTCAGTGTAGCCCAGGATGC-3’.

LUCIFERASE ASSAY

1.5x10^5 HEK293T cells were transfected with 100ng of Tat B in psiCHECK™-2 vector along with 250ng shRNA against HIV-1 genes. 24 hours after transfection, cell lysate was prepared using Passive lysis buffer (Promega) and luciferase activity was measured by luminometer.

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2.5x10^5 of HEK293T cells were seeded in six-well plate format 24hrs prior to transfection. The HIV-1 genes cloned in pDs2Red2-N1 (500ng), and shRNA cloned in RetroQZSgreen (1μg) were transfected with JetPRIME transfection reagent (PolyPlus) in 1:2 ratio (1 μg DNA: 2 μl JetPRIME). 48 hrs post-transfection, samples were prepared for FACS analysis.
5x10^5 cells were acquired by BD FACSAria (BD Biosciences) for analysis which was done by FlowJo software.

**β-GALACTOSIDASE ASSAY**

After 48 hours of transfection, cells were harvested and lysate was prepared using Reporter Lysis buffer (Promega). Bradford assay was performed to determine the protein concentration of each lysate. 75 µg of each lysate was used for the assay in a total reaction volume of 300 µl, containing 3µl of 1M Mg^{2+} buffer containing 14M β-mercapto-ethanol and 66µl O-nitrophenyl-β-D galactopyranoside (4mg/ml). The volume was made upto 300µl with 0.1 M phosphate buffer (pH 7.5). The reaction tubes were incubated at 37°C for 2 hrs and the absorbance was read at 420 nm.

**Results**

1. **shRNA against Vif/Vpr subtypes B and C**

The vif and vpr genes of HIV-1 overlap with each other. Between Vif and Vpr, 61 nucleotides are common. ClustalW analysis was done to compare the conserved regions of HIV-1 subtype B (pNL4-3, AF324493) and subtype C (93IN905, AF067158), and from the overlapping 61 bases, a region was identified against which the shRNA can be designed. The shRNA designed against Vif/vpr common region shall be targeting 2 HIV-1 genes across 2 subtypes. Hence, this one shRNA should down-regulate the expression of 4 genes. As shown in figure 4, the clustal W analysis is shown for HIV-1 subtype B, C vif and vpr, and the highlighted regions marks the area against which the shRNA is designed.

**Cloning of shRNA; and vif and vpr B, C**

The shRNA against vif/vpr was cloned into RNAi-ready Retro QZs-green vector. The vif and vpr B, C genes were amplified from pNL4-3 and 93IN905, respectively. These genes were then cloned and sub-cloned in to pDS2RedN1 and psiCHECK-2 vector systems, for reverse
shRNAs targeting overlapping regions of HIV-1

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transcriptase-PCR and flow cytometry analysis with the former; and luciferase assays with the latter.

**FIGURE 4:**

Clustal W analysis of Vif B and Vif C; Vpr B and Vpr C. The highlighted region is the targeted region of the gene by shRNA.
Reduction of intracellular target RNA with multi-target shRNA

HEK293T cells were transfected with Vif B in pDS2RedN1 (500ng) along with 500ng of Vif/Vpr shRNA in RNAi-ready-RetroQZs-green. Similarly, Vif C/Vpr B/Vpr C in pDS2RedN1 was transfected with the shRNA in similar doses as mentioned earlier. The intracellular decrease in RNA levels of each target was measured. In figure 5, there are 4 gels pictures in as many panels. Panel 1, 2, 3, 4 depict gel pictures for Vpr B, Vpr C, Vif B and Vif C, respectively. In panel 1, Lane 1 depicts RT-PCR from well where no transfection was done. It did not show any amplification, as expected. Robust amplification was seen where Vpr B encoding plasmid was added. When wild type shRNA was added, >90% reduction of target RNA is seen, while no change is seen on addition of unrelated shRNA. Similarly, in panels 2, 3, 4, the Vpr C, Vif B and Vif C encoding plasmids gave robust expression of target RNA. On addition of wild type shRNA, ~90%, >95% and ~90 % reduction of Vpr C, Vif B and Vif C expression was seen. The unrelated shRNA did not show any reduction of the target RNA, signifying that the shRNA is target specific. To ensure uniform transfection efficiency, values were normalized against the control reporter gene (pSV-β-gal, Promega). Since the levels of housekeeping gene (huGAPDH) remained essentially unchanged in all the lanes, it is clear that these are specific effects caused by various treatments. The results showed are representative of 3 experiments.
**FIGURE 5:**

Intracellular reduction of target RNA analyzed by Reverse transcriptase PCR.

**Panel A:** RT-PCR analysis for Vpr B on addition of Vif/Vpr shRNA.

**Panel B:** RT-PCR analysis for Vpr C on addition of Vif/Vpr shRNA.

**Panel C:** RT-PCR analysis for Vif B on addition of Vif/Vpr shRNA.

**Panel D:** RT-PCR analysis for Vif C on addition of Vif/Vpr shRNA.
Intracellular down regulation of Vif B/Vif C/Vpr B/Vpr C-RFP by shRNA construct checked by flow cytometry

The Vif B, C and Vpr B, C were cloned in pDS2Red-N1 so that RFP fusion protein can be made which can be easily analyzed by flow cytometry. The Vif B/Vif C/Vpr B/Vpr C-RFP (500ng) and Vif/Vpr shRNA (500ng) were transfected in HEK293T cells. The presence of wild type shRNA was able to down-regulate the expression of Vpr B, Vpr C, Vif B and Vif C by ~80%, >95%, ~80% and 75%, respectively (figure 6). The unrelated shRNA did not show any nonspecific reduction of RFP fusion protein. These are representative of three independent experiments.

The fluorescent microscopy analysis also supported that flow cytometry data. The four panels are for fluorescent microscopy pictures of Vif B, Vif C, Vpr B and Vpr C (figure 7). In each panel, picture A is of cells in bright field, while picture B is of RFP-fusion protein controls. Pictures E, F are shRNA-GFP wild type and unrelated, respectively. Picture G shows expression of RFP-fusion protein on addition of shRNA wild type, while picture H shows expression of RFP-fusion protein on addition of unrelated shRNA. While reduction of RFP-fusion protein can be seen in picture G in all four panels, no significant reduction can be seen in picture H across 4 panels.
FIGURE 6:

Flow cytometry analysis of the downregulation of Vif-B, C; Vpr-B, C in pDS2Red-N1 by Vif/Vpr shRNA.
FIGURE 7:

Fluorescent microscopy pictures of cells transfected with HIV genes in pDS2Red-N1 and shRNA in RNAi-Ready-RetroQZSgreen.

**PANEL A:** Fluorescent microscopy pictures for Vpr B.

**PANEL B:** Fluorescent microscopy pictures for Vpr C.

**PANEL C:** Fluorescent microscopy pictures for Vif B.

**PANEL D:** Fluorescent microscopy pictures for Vif C.
Reduction of Vif B/Vif C/Vpr B/Vpr C cloned in psiCHECK-2 with shRNA

The Vif B/Vif C/Vpr B/Vpr C was cloned in psiCHECK-2 (100ng) along with Vif/Vpr shRNA (250ng) in a 24-well format. The HIV-1 genes were cloned in frame with the renilla luciferase reporter gene and the luciferase readings were measured with and without shRNA construct (figure 8). Cells with no transfection and shRNA vector alone (controls) (lane 1, 2) showed no luciferase activity. Robust luciferase activity was seen with psiCHECK.Vif B/Vif C/Vpr B/Vpr C, and the reading was used to compare with addition of shRNAs, wild type and unrelated (Lane 3). The wild construct showed ~80%-90% reduction in luciferase activity as compared with the control shRNA. The unrelated shRNA failed to show any reduction in luciferase activity as compared with the control. These values were normalized against the control firefly reporter luciferase gene activity present in the psiCHECK-2 and are representative of 5 independent experiments.
FIGURE 8:

Luciferase assay to check the efficacy of the Vif/Vpr shRNA against Vif and Vpr of HIV-1 B and C.
2. **shRNA against Tat/Rev subtypes B and C**

The Tat and Rev genes of HIV-1 overlap with each other at both exons I and II. ClustalW analysis was done to compare the conserved regions of HIV-1 subtype B (pNL4-3, AF324493) and subtype C (93IN905, AF067158), a region was identified which is common between HIV-1 Tat and Rev of subtypes B and C against which the shRNA was designed. This way, the shRNA designed against Tat/Rev common region shall be targeting 2 HIV-1 genes across 2 subtypes, thereby down-regulating 4 genes. As shown in figure 9, the clustal W analysis is shown for HIV-1 Tat, Rev B and C; and the highlighted region marks the area against which the shRNA is designed. The shRNA is designed at region that marks the middle of exon I for Tat B, C; and beginning of Rev B, C.

**Cloning of shRNA; and tat and rev B, C**

The shRNA against Tat/Rev was cloned into RNAi-ready Retro QZs-green vector. The Tat and Rev B, C genes were amplified from pNL4-3 and 93IN905, respectively. These genes were then clone and sub-cloned in to pDS2RedN1 and psiCHECK-2 vector systems, for Reverse transcriptase-PCR and flow cytometry analysis with the former, and luciferase assays with the latter.
FIGURE 9:

Clustal W analysis of pNL4-3 and 93IN905 from the beginning of exon 1 of Tat. The red arrows show the start codons for Tat and Rev. The underlined region is the targeted region of the gene by shRNA. The bold region is the target region for tat/Rev/Env region that shows no similarity between HIV-1 subtype B and C.
Reduction of intracellular target RNA with multi-target shRNA

HEK293T cells were transfected with Tat B in pDS2RedN1 (500ng) along with 500ng of Tat/Rev shRNA in RNAi-ready-RetroQZs-green. Similarly, Tat C/Rev B/Rev C in pDS2RedN1 was transfected with the shRNA in similar doses as mentioned earlier. The intracellular decrease in RNA levels of each target was measured as shown in figure 10. Lane 1 depicts RT-PCR from well where no transfection was done. It did not show any amplification, as expected. Robust amplification was seen where Tat B, Tat C, Rev B, Rev C encoding plasmid was added (lanes 2, 5, 8, 11). When wild type shRNA was added, >85% reduction of target RNA of Tat B and C is seen, while >90% reduction of target RNA of Rev B and C is observed! The unrelated shRNA did not show any reduction of the target RNA, signifying that the shRNA is target specific. To ensure uniform transfection efficiency, values were normalized against the control reporter gene (pSV-β-gal, Promega). Since the levels of housekeeping gene (huGAPDH) remained essentially unchanged in all the lanes, it is clear that these are specific effects caused by various treatments. The results showed are representative of 3 experiments.
FIGURE 10:

Intracellular reduction of target RNA analyzed by Reverse transcriptase PCR.

Lane 1: control cells

Lanes 2-4: RT-PCR analysis for Tat B on addition of Tat/Rev shRNA.

Lanes 5-7: RT-PCR analysis for Tat C on addition of Tat/Rev shRNA.

Lanes 8-10: RT-PCR analysis for Rev B on addition of Tat/Rev shRNA.

Lanes 11-13: RT-PCR analysis for Rev C on addition of Tat/Rev shRNA.
Intracellular down regulation of Tat B/Tat C/Rev B/Rev C-RFP by shRNA construct checked by flow cytometry

The Tat B, C and Rev B, C were cloned in pDS2RedN1 so that RFP fusion protein can be made which can be easily analyzed by flow cytometry. The Tat B/Tat C/Rev B/Rev C-RFP (500ng) and Tat/Rev shRNA (500ng) were transfected in HEK293T cells. The presence of wild type shRNA was able to down-regulate the expression of Tat B/Tat C/Rev B/Rev C by ~90%, ~80%, ~85% and ~80%, respectively (figure 11). The unrelated shRNA did not show any nonspecific reduction of RFP fusion protein. These are representative of three independent experiments.
Flow cytometry analysis of the downregulation of Tat-B, C; Rev-B, C in pDS2Red-N1 by Tat/Rev shRNA.

FIGURE 11:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Downregulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tat B</td>
<td>~90%</td>
</tr>
<tr>
<td>Tat C</td>
<td>~80%</td>
</tr>
<tr>
<td>Rev B</td>
<td>~84%</td>
</tr>
<tr>
<td>Rev C</td>
<td>~78%</td>
</tr>
</tbody>
</table>
The fluorescent microscopy analysis also supported that flow cytometry data. The four panels are for fluorescent microscopy pictures of Tat B, Tat C, Rev B and Rev C (figure 12). In each panel, picture A is of cells in bright field, while picture B is of RFP-fusion protein controls. Pictures E, F are shRNA-GFP wild type and unrelated, respectively. Picture G shows expression of RFP-fusion protein on addition of shRNA wild type, while picture H shows expression of RFP-fusion protein on addition of unrelated shRNA. While reduction of RFP-fusion protein can be seen in picture G in all four panels, no significant reduction can be seen in picture H across 4 panels.
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FIGURE 12:

Fluorescent microscopy pictures of cells transfected with HIV genes in pDS2Red-N1 and shRNA in RNAi-Ready-RetroQZSgreen.

**PANEL A:** Fluorescent microscopy pictures for Tat B.

**PANEL B:** Fluorescent microscopy pictures for Tat C.

**PANEL C:** Fluorescent microscopy pictures for Rev B.

**PANEL D:** Fluorescent microscopy pictures for Rev C.
Reduction of Tat B/Tat C/Rev B/Rev C cloned in psiCHECK-2 with shRNA

The Tat B/Tat C/Rev B/Rev C was cloned in psiCHECK-2 (100ng) along with Tat/Rev shRNA (250ng) in a 24-well format. The HIV-1 genes were cloned in frame with the renilla luciferase reporter gene and the luciferase readings were measured with and without shRNA construct (figure 13). Cells with no transfection and shRNA vector alone (controls) (lane 1, 2) showed no luciferase activity. Robust luciferase activity was seen with psiCHECK. Tat B/Tat C/Rev B/Rev C, and the reading was used to compare with addition of shRNAs, wild type and unrelated (Lane 3). The wild construct showed ~80%-90% reduction in luciferase activity as compared with the control shRNA. The unrelated shRNA failed to show any reduction in luciferase activity as compared with the control. These values were normalized against the control firefly reporter luciferase gene activity present in the psiCHECK-2 and are representative of 5 independent experiments.
FIGURE 13:

Luciferase assay to check the efficacy of the Tat/Rev shRNA against Tat and Rev of HIV-1 B and C.
3. **shRNA against Tat/Rev/Env region of HIV-1 Subtype B**

The exon II of Tat/Rev overlaps with Env region as well. The beginning of the exon was targeted by a shRNA. Three HIV-1 Subtype B genes are down-regulated by this shRNA; Tat, Rev and Env. The three genes were amplified and cloned from pNL4-3. The cloning strategies for Tat B and Rev B have been discussed before. The genes were cloned and sub-cloned in to pDS2REDN1 and psiCHECK-2 vectors. And the shRNA was cloned into RNAi-ready-RetroQZs-green. The clustal W analysis is shown in figure 14.

**FIGURE 14:**

Clustal W analysis of Env B, Tat B Exon 2 and Rev B Exon 2.
Reduction of intracellular target RNA with multi-target shRNA

HEK293T cells were transfected with Tat B/Rev B/Env B in pDS2RedN1 (500ng) along with 500ng of Tat/Rev shRNA in RNAi-ready-RetroQZs-green. The intracellular decrease in RNA levels of each target was measured as shown in figure 15, panels A, B and C. Lane 1 of each panel depicts RT-PCR from well where no transfection was done. It did not show any amplification, as expected. Robust amplification was seen in panels A, B, C where Tat B, Rev B, Env B encoding plasmid were added (lane 2). When wild type shRNA was added, ~70-75% reduction of target RNA is observed! The unrelated shRNA did not show any reduction of the target RNA, signifying that the shRNA is target specific. To ensure uniform transfection efficiency, values were normalized against the control reporter gene (pSV-β-gal, Promega). Since the levels of housekeeping gene (huGAPDH) remained essentially unchanged in all the lanes, it is clear that these are specific effects caused by various treatments. The results showed are representative of 3 experiments.
FIGURE 15:

Intracellular reduction of target RNA analyzed by Reverse transcriptase PCR.

Panel A: RT-PCR analysis for Tat B on addition of Tat/Rev/Env shRNA.

Panel B: RT-PCR analysis for Rev B on addition of Tat/Rev/Env shRNA.

Panel C: RT-PCR analysis for Env B on addition of Tat/Rev/Env shRNA.
Reduction of Tat B/Rev B/Env B cloned in psiCHECK-2 with shRNA

The Tat B/Rev B/Env B was cloned in psiCHECK-2 (100ng) along with tat/rev/env shRNA (250ng) in a 24-well format. The HIV-1 genes were cloned in frame with the renilla luciferase reporter gene and the luciferase readings were measured with and without shRNA construct (figure 16). Cells with no transfection and shRNA vector alone (controls) (lane 1, 2) showed no luciferase activity. Robust luciferase activity was seen with psiCHECK. Tat B/Rev B/Env B and the reading were used to compare with addition of shRNAs, wild type and unrelated (Lane 3). The wild construct showed ~80%-85% reduction in luciferase activity as compared with the control shRNA. The unrelated shRNA failed to show any reduction in luciferase activity as compared with the control. These values were normalized against the control firefly reporter luciferase gene activity present in the psiCHECK-2 and are representative of 5 independent experiments.
FIGURE 16:

Luciferase assay to check the efficacy of the Tat/Rev/Env shRNA against Tat, Rev and Env of HIV-1 B.
Intracellular down regulation of Tat B/Rev B/Env B-RFP by shRNA construct checked by flow cytometry

The Tat B/Rev B/Env B was cloned in pDS2RedN1 so that RFP fusion protein can be made which can be easily analyzed by flow cytometry. The Tat B/Rev B/Env B-RFP (500ng) and Tat/Rev/Env shRNA (500ng) were transfected in HEK293T cells. The presence of wild type shRNA was able to down-regulate the expression of Tat B/Rev B/Env B by ~80 %, >85% and 75%, respectively (figure 17). The unrelated shRNA did not show any nonspecific reduction of RFP fusion protein. These are representative of three independent experiments.
FIGURE 17:

Flow cytometry analysis of the downregulation of Tat B, Rev B and Env B in pDS2Red-N1 by Tat/Rev/Env shRNA.
The fluorescent microscopy analysis also supported the flow cytometry data. The three panels are for fluorescent microscopy pictures of Tat B/Rev B/Env B (figure 18). In each panel, picture A is of cells in bright field, while picture B is of RFP-fusion protein controls. Pictures E, F are shRNA-GFP wild type and unrelated, respectively. Picture G shows expression of RFP-fusion protein on addition of shRNA wild type, while picture H shows expression of RFP-fusion protein on addition of unrelated shRNA. While reduction of RFP-fusion protein can be seen in picture G in all three panels, no significant reduction can be seen in picture H across three panels.
FIGURE 18:

Fluorescent microscopy pictures of cells transfected with HIV genes in pDS2Red-N1 and shRNA in RNAi-Ready-RetroQZSgreen.

PANEL A: Fluorescent microscopy pictures for Tat B.

PANEL B: Fluorescent microscopy pictures for Rev B.

PANEL C: Fluorescent microscopy pictures for Env B.

PANEL D: Fluorescent microscopy pictures for Rev C.
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Virus challenge

We employed p89.6 infectious HIV-1 DNA clone and used TZMbl reporter cell line to carry out the virus challenge experiment. The β-gal activity in this assay is a direct measure of the extent of replication of HIV-1. The extent of virus replication in cells transfected with various DNA constructs for 48 hours is shown in figure 19. Since we are targeting the co-receptor HIV-1 uses to gain entry into the cell, any reduction in the co-receptor will result in decrease of β-gal activity. The shRNA that were designed against the various overlapping regions of HIV-1 were taken in combination along with the p89.6 to check out their efficiencies in inhibiting the virus.

100ng of p89.6 was taken along with 250ng of shRNA (either individually or in combination) in a 24-well format. The β-gal activity in TZMbl cells without the antiviral constructs was taken up as control and the effect of wild type and unrelated shRNA was compared with it. The Tat/Rev/Env shRNA showed ~70% inhibition of the virus replication, while Vif/Vpr and Tat/Rev shRNA showed ~65% inhibition. The combination of Tat/Rev/Env+Vif/Vpr, Vif/Vpr+Tat/Rev and Tat/Rev/Env+Tat/Rev showed ~75-85% inhibition of HIV-1 replication. However, the combination of all three shRNAs, however, showed the most protection from HIV-1 challenge. These are representative of three independent experiments.
FIGURE 19:

Virus challenge assay to check the efficacy of the shRNAs against HIV-1 infection.
Discussion

It is essential to target multiple HIV genes as well as the host factors essential for HIV infectivity for an effective antiviral therapy. With this concept it is essential to identify structural, regulatory and auxiliary genes of HIV that can be targeted to develop efficient and novel antiviral approaches. Also the prevalence of various subtypes and recombinant viruses circulating in different geographical regions of the world warrants the development of short catalytic nucleic acid based approaches which would target more than one genetic subtype.

The shRNA designed against the overlapping regions of the HIV-1 genome down-regulate more than 2 genes with a single antiviral component. Analysis of clustal W helped us identify the regions that are common between subtype B and subtype C which constituted the target sites. Hence the shRNA targeting the overlapping regions will down-regulate the genes from other subtype of virus as well. Though we considered the subtype B and C of HIV-1, more analysis of various other subtypes of HIV-1 may point out sequence homology or similarity between the target sequences, which means the same shRNA may be able to down-regulate the expression of the HIV-1 genes of various subtypes.

The three shRNAs designed to target the Tat/Rev exon I, Vif/Vpr and Tat/Rev exonII/Env showed very promising results. The Tat/Rev shRNA targeting the exon I was able to down-regulate the expression of Tat and Rev of subtypes B and C by ~80-90%. The Vif/Vpr shRNA, which was also designed to target the subtype B and C of HIV-1 showed down-regulation of the genes between 80-95%. The third shRNA, targeting that Tat/Rev exon II and the overlapping env B region, showed down-regulation of the three genes by ~75-85%. The efficacy of the three shRNAs showed protection against HIV-1 challenge. In TZM-bl cells, the p89.6 clone of HIV-1 was employed and the three shRNAs were tested against it individually and in combinations. The Tat/Rev, Vif/Vpr and Tat/Rev/Env shRNA
shRNAs targeting overlapping regions of HIV-1

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individually showed >65% protection against HIV-1 challenge. The combination of two shRNAs showed slightly more protection against HIV-1 challenge; about 80% protection was seen. The combination three shRNAs were the most successful in providing protection against HIV-1 challenge as >95% protection was achieved. It is noteworthy to mention that the amount of shRNA in various combinations was same as shRNA were taken individually. The unrelated shRNA failed to provide any protection, emphasizing the sequence specificity of the shRNA.

Introduction of shRNAs specific for HIV-1 into mammalian cells could lead to viral RNA degradation and inhibition of HIV-1 gene expression and replication during different stages of the viral life cycle. Early transcripts such as HIV-1 rev and tat are good targets for shRNAs since the Tat and Rev proteins encoded by these RNAs are essential for subsequent expression of HIV-1 structural genes (Gag, Pol, and Env) and for the synthesis of full length viral genomic RNA. Since we are targeting the Tat/Rev region of exon II (which also covers the Env region), the env gene also gets down regulated. The cloning of the three shRNAs in a cassette in a lentiviral system can become a very potent tool in gene therapy and provide long term protection against HIV-1 challenge. The combination of the shRNA with TAR and RRE decoys can become a highly potent weapon against HIV-1, because after the shRNAs have degraded the tat and rev transcripts, the tat and Rev Proteins made after translation can be sequestered by the TAR and RRE decoys. Thereby the regulatory proteins would not be available to do their natural function that is responsible for the survival of the HIV-1 in host cells.

Targeting the multiples regions is very vital if the menace of HIV-1 is to be tackled. Also, the increasing rate at which the HIV-1 is evolving and recombining with its various subtypes, generating new recombinant viruses, calls for a new approach in gene therapy. The new approaches should be able to target the various subtypes of HIV-1 as well, which are equally hazardous and threatening. The rates at which the new recombinants are appearing are evolving. Hence, the new approaches should be devised in a way that the
upcoming threat of recombinant virus, which might be even more treacherous, can be tackled effectively.
Reference List


