Long-term suppression of HIV-1C virus production in human peripheral blood mononuclear cells by LTR heterochromatization with a short double-stranded RNA

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Objectives: A region in the conserved 5′ long terminal repeat (LTR) promoter of the integrated HIV-1C provirus was identified for effective targeting by a short double-stranded RNA (dsRNA) to cause heterochromatization leading to a long-lasting decrease in viral transcription, replication and subsequent productive infection in human host cells.

Methods: Small interfering RNAs (siRNAs) were transfected into siHa cells containing integrated LTR-luciferase reporter constructs and screened for efficiency of inducing transcriptional gene silencing (TGS). TGS was assessed by a dual luciferase assay and real-time PCR. Chromatin modification at the targeted region was also studied. The efficacy of potent siRNA was then checked for effectiveness in TZM-bl cells and human peripheral blood mononuclear cells (PBMCs) infected with HIV-1C virus. Viral Gag-p24 antigen levels were determined by ELISA.

Results: One HIV-1C LTR-specific siRNA significantly decreased luciferase activity and its mRNA expression with no such effect on HIV-1B LTR. This siRNA-mediated TGS was induced by histone methylation, which leads to heterochromatization of the targeted LTR region. The same siRNA also substantially suppressed viral replication in TZM-bl cells and human PBMCs infected with various HIV-1C clinical isolates for ≥ 3 weeks after a single transfection, even of a strain that had a mismatch in the target region.

Conclusions: We have identified a potent dsRNA that causes long-term suppression of HIV-1C virus production in vitro and ex vivo by heritable epigenetic modification at the targeted C-LTR region. This dsRNA has promising therapeutic potential in HIV-1C infection, the clade responsible for more than half of AIDS cases worldwide.

Keywords: HIV-1, small interfering RNA, transcriptional gene silencing, epigenetics, RNAi

Introduction

Transcription of the HIV-1 provirus genome, and hence viral replication, is regulated by the interaction of viral regulatory proteins and cellular transcription factors with the conserved 5′ long terminal repeat region (5′ LTR) of the provirus, a transcriptional promoter of HIV.1–3 It is reported that the LTRs of different HIV-1 subtypes show significant variation in sequences and transcription factor binding sites, which influence the transcriptional activity of the LTR (Figure 1a and Figure S1, available as Supplementary data at JAC Online).4–6 The HIV-1C LTR has three NF-κB binding sites, whereas only two NF-κB binding sites are present in the majority of other subtypes, such as subtype B LTR. Previous studies have shown that the additional NF-κB binding site within subtype C LTR is associated with increased transcriptional activity and viral replication of HIV-1C.7–9

The RNA interference (RNAi) pathway represents an emerging potential therapeutic approach for the treatment of HIV-1.10 Double-stranded RNA (dsRNA) or small interfering RNA (siRNA), the effector molecule of the RNAi pathway, leads to sequence-specific cleavage of complementary mRNA, which is known as post-transcriptional gene silencing (PTGS).11,12 Some dsRNAs targeting the promoter region of a gene can induce DNA CpG islands and/or histone (H3K9 and H3K27) methylation of the targeted locus, resulting in the silencing of gene expression by a process called transcriptional gene silencing (TGS).13–17 TGS operates via heritable epigenetic modification of the targeted promoter loci and has potential to silence genes for longer duration than PTGS, as the continued presence of the effector siRNA is no longer required, contrary to the situation in PTGS.18 Previously, we demonstrated silencing of the locus control region of human papilloma
Suppression of HIV-1C virus production by dsRNA

While the majority of studies on the effect of siRNA on HIV-1 treatment are focused on subtype B, a predominant clade in the USA and Western Europe accounting for only ~10% of the total worldwide HIV-1 infection, subtype C infection, which predominates in Africa, India and other parts of Asia and accounts for >50% of the worldwide HIV-1 infection, has received less attention.20–22 Recent-ly, siRNA targeting the conserved NF-\( \kappa \)B binding sites of the enhancer in the U3 region of HIV-1B 5′ LTR has been shown to induce TGS and cause the suppression of viral replication.23–25 However, a similar locus for TGS of HIV-1C has not been identified so far.

siRNA-mediated TGS is reported to be a highly sequence-specific phenomenon, where even a two to three nucleotide mismatch in the homology of siRNA and the target sequence is not able to induce effective silencing.23 Sometimes, shifting of the target region even by a single nucleotide can alter the modulatory effect of siRNAs.19,26 Sequence alignment of HIV-1B and HIV-1C 5′ LTRs reveals significant variation in nucleotide sequence and CpG sites in the enhancer and core promoter of the U3 region (Figure S1).4–6 Thus, we hypothesized that siRNA effective against HIV-1B LTR might not be effective against HIV-1C LTR due to sequence variation in the target site. We have identified an siRNA that induces heterochromatization of C-LTR, which leads to the silencing of downstream gene expression and causes suppression of viral replication and marked reduction in the apoptosis of HIV-1C-infected human peripheral blood mononuclear cells (PBMCs). This siRNA is also effective against HIV-1C LTR promoters having a common one-base mismatch in the target site. In future, this dsRNA may serve as a therapeutic approach to suppress clade virus (HPV-16) in cervical carcinoma cell lines by the same mechanism using siRNA.19

Figure 1. siRNA target sites in HIV-1C 5′ LTR and screening of six siRNAs identifies an siRNA causing TGS. (a) Schematic representation of the U3 region of HIV-1C and HIV-1B 5′ LTR, highlighting the transcription start site (TSS) and transcription factor binding sites (NF-\( \kappa \)B and SP1). (b) siRNA sequences targeting NF-\( \kappa \)B and SP1 binding sites in the enhancer and core promoter region of C-LTR. (c) Outline of HIV-1 subtype C LTR (S-B253)- and B LTR (K03455)-driven firefly luciferase reporter constructs (C-LTR-luciferase and B-LTR-luciferase) and subtype C and B Tat protein expression cassette with CMV promoter in pGL3-basic-based vector. Dual luciferase assay showing a significant decrease in luciferase activity (firefly/Renilla luciferase) after (d) 48 h (\( P = 0.01 \)) and (e) 72 h (\( P = 0.02 \)) of S4-siRNA transfection in siHa (C-LTR-Luci + Tat) cells. Luciferase activity is expressed as a ratio of firefly to Renilla luciferase normalized to scrambled control siRNA. (f) Real-time PCR showing S4-siRNA-mediated silencing of luciferase mRNA expression was sustained over a period of 3 weeks (\( P < 0.001 \)). Expression ratio was calculated with respect to 18S, PPIA, GAPDH and \( \beta \)-actin by REST software.
C isolate, which predominantly exists in Africa, India and other parts of Asia.

**Methods**

**Ethics statement**

This study was approved by the Ethics Committee of the All India Institute of Medical Sciences (ref. no. A–63/A.12.2006). Blood from healthy donors was drawn after written informed consent.

**Construction of LTR-luciferase reporter and Tat expression vectors**

pcDNA3.1 (Invitrogen, Carlsbad, CA, USA)-based bicistronic LTR reporter vectors expressing secreted alkaline phosphatase (SEAP) and enhanced green fluorescent protein (EGFP) downstream of the HIV-1C LTR (pC-LTR-SEAP-IRESEGFP) or HIV-1B LTR (pB-LTR-SEAP-IRESEGFP) \(^2\) were used to generate the C-LTR- and B-LTR-luciferase (firefly) reporter constructs, respectively. The full-length C-LTR (EF178612) and B-LTR (K03455) of the bicistronic reporter originated, respectively, from the S-B253 clinical isolate and the pHIV-CAT reporter vector (no. 2619, AIDS Research and Reference Reagent Program at NIH). \(^2\) The firefly luciferase gene was retrieved from the pGL3-basic vector (Promega) using Nhel and XbaI restriction sites and directionally cloned between Nhel and XbaI sites of pC-LTR-SIE or pB-LTR-SIE after eliminating the SEAP-IRESEGFP cassette to generate the C-LTR- or B-LTR-luciferase reporter construct.

The pGL3-basic-based subtype-specific CMV-Tat expression vector was generated by isolating the CMV-C-Tat and CMV-B-Tat cassette from pcDNA3.1-based pcDNA C-Tat and B-Tat using MluI and XbaI, and cloned between the same restriction sites of the pGL3-basic vector backbone after removing the firefly luciferase gene. The full-length subtype C and B Tat originated, respectively, from an Indian seropositive donor (BL43/02) and a reference molecular clone pYU2 (catalogue no. M2393, AIDS Research and Reference Reagent Program at NIH). \(^2\) All the recombinant constructs were confirmed by DNA sequencing and restriction digestion.

The LTR bicistronic reporter constructs (pC-LTR-SIE and pB-LTR-SIE) and pcDNA3.1-based Tat expression vectors (pcDNA C-Tat and pcDNA B-Tat) were a gift from Dr. Udaykumar Ranga (Molecular Virology Laboratory, Molecular Biology and Genetics Unit, JNCASR, Bangalore, India).

**Cell culture**

The siHa/LTR luciferase cells and TZM-bl cells used in this study were maintained in Dulbecco’s modified Eagle’s medium (Sigma – Aldrich, Germany) supplemented with 10% fetal calf serum (FCS; BioWest, USA), 3.7 g/L sodium bicarbonate and 10 μg/mL ciprofloxacin and incubated in 5% CO\(_2\) at 37°C.

**Stable transfection**

At day 0, 2 × 10\(^5\) siHa cells/25 cm\(^2\) flask (Corning, NY, USA) were plated. After 24 h, cells were co-transfected with the C-LTR/B-LTR-luciferase reporter construct (C-LTR-Luc/B-LTR-Luc) and the pGL3-basic C-Tat/B-Tat vector in a 1:10 ratio (200-2000 ng) using Lipofectamine 2000 \(^2\) (Invitrogen) according to the manufacturer’s protocol. Transfected cells were cultured under G418 (Sigma – Aldrich) selection pressure, at a concentration of 700 μg/mL during medium replenishment starting from the day after transfection. The LTR reporter- and Tat expression vector-positive colonies were isolated using cloning cylinders (Corning) and expanded under selection pressure.

**siRNA design and synthesis**

Six siRNAs were designed with thymine dinucleotide 3’ overhangs (dTdT) targeting the HIV-1C LTR. Characterization of TF binding sites to C-LTR was done using the Transcription Element Search System, available online at http://www.cbl.iupenn.edu/cgi-bin/tess/tess. One control siRNA with a randomized sequence was also designed that had no target or homology to any other human sequence, as checked by NCBI’s BLAST software. siRNA was synthesized by Eurofins MWG (Germany). The siRNA sequences are given in Table S1 (available as Supplementary data at JAC Online).

**siRNA transfection**

 Cells were plated at 40,000 cells/well in a 24-well plate, 1 × 10\(^3\) cells/well in a 6-well plate, 2 × 10\(^5\) cells/25 cm\(^2\) flask or 1 × 10\(^6\) cells/75 cm\(^2\) flask. After 24 h, cells were transfected using a concentration of 50 nM of specific or control siRNA using Oligofectamine (Invitrogen, USA) according to the manufacturer’s instructions.

**Dual luciferase assays**

These were performed according to the manufacturer’s instructions (Promega, USA) using a luminometer (Sirus, Berthold Detection Systems). siHa cells stably expressing the firefly luciferase gene downstream of the HIV-1C/B-LTR promoter and the C/B-Tat protein downstream of the CMV promoter were co-transfected with siRNA targeted to the LTR region along with a Renilla luciferase vector (pRL-TK vector, Promega) as an internal control. The LTR-driven firefly luciferase activity was normalized to background Renilla luciferase activity. The ratio of luciferase activity (firefly/Renilla) of control dsRNA transfected cells was set to 100%, and relative luciferase activity of other dsRNA transfected cells calculated accordingly.

**Real-time PCR**

RNA was isolated from cells at particular timepoints by using Trizol reagent (Sigma – Aldrich). The RNA was DNase (MBI Fermentas, USA) treated and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). One microgram of RNA was reverse transcribed using random decamers and MMLV-RT (MBI Fermentas). All real-time PCRs were performed on a Rotor-Gene 6000 real-time PCR machine (Corbett Research, Australia) using gene-specific primers. Taq Master Mix with SYTO 9 green fluorescent dye (Invitrogen, USA) was used to amplify and detect the respective DNA during the reaction. The thermal cycling conditions started with 1 min initial denaturation at 95°C, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 30 s. The specificity of the real-time PCR products was confirmed by melt curve analysis and agarose gel electrophoresis. Accurate quantification of gene expression by real-time PCR is possible by averaging the geometric mean of multiple internal control genes and using them as a reference. Therefore, four reference genes, namely 18S, PP2A, GAPDH and β-actin, were used. Relative quantification of the genes was done using the Relative Expression Software Tool (REST). All primer sequences used in the experiment are listed in Table S2 (available as Supplementary data at JAC Online).

**Cell proliferation assay**

Cell proliferation was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer’s instructions.

**Micrococcal nuclease digestion/chromatin accessibility real-time PCR (MNase/CHART-PCR) assay**

The MNase/CHART-PCR assay was carried out as described previously. Confluent cells were scraped and pelleted by centrifugation at 2000 rpm for 10 min at 4°C. Then, 10\(^5\) cells were washed in ice-cold PBS and resuspended in 200 μL of cold NP40 lysis buffer [10 mM NaCl, 10 mM Tris-Cl
Chromatin immunoprecipitation (ChIP) assays
ChIP assays for dimethylated H3K9 and trimethylated H3K27 histone tails were done using an EZ-ChIP™ kit (Millipore). Input DNA and chromatin immunoprecipitated with dimethylated H3K9 antibody (mAbcam1220), trimethylated H3K27 antibody (mAbCam6002) and control mouse IgG antibody (Upstate) were amplified using specific primers for the targeted HIV-1C LTR region on a Rotor-Gene 6000 real-time PCR machine and the heterochromatized centromeric region of chromosome 16 was used as a reference for the analysis. The ratio of undigested (UD) to digested (D) DNA of region of interest (GOI) and positive standard chromosome 16 centromeric region (CHR16) was calculated using the ΔΔCt method, \( \left\{ \frac{\text{Ct value of GOI (UD)} - \text{Ct value of CHR16 (UD)}}{\text{Ct value of GOI (D)} - \text{Ct value of CHR16 (D)}} \right\} \).

Bisulfite treatment and PCR sequencing
Genomic DNA was isolated from cells transfected with dsRNA using a Puregene™ blood core kit (Qiagen) and 500 ng of bisulfite treated DNA was used for nested PCR using EpiTect Bisulfite Kit (Qiagen, Germany). Nested PCR was done for the amplification of the target region and primers for bisulfite-treated DNA were designed from http://bisearch.enzim.hu. The inner primers were M13 tagged and sequencing was done using universal M13 primers.

Histone deacetylase (HDAC) and DNA methyl transferase (DNMT) inhibition
Inhibition of HDAC or DNMT was done by adding 300 nM trichostatin (TSA) or 5μM 5-aza-2-deoxycytidine (AZA) dissolved in DMSO, to the respective culture medium. No significant change was observed in cell morphology and proliferation under these conditions. Transfection of siRNA was done after 48 h of AZA/TSA treatment of cells or AZA/TSA treatment of cells was done after 72 h of siRNA transfection.

HIV infection and siRNA transfection of TZM-bl cells
TZM-bl cells (3 × 10^5) in a 25 cm^2 flask were infected with the HIV-1C primary isolates (AIIMS 201, 212, 254 and 261) using a TCID<sub>50</sub> 1000 and infection was allowed to establish for 3 days (where TCID<sub>50</sub> stands for 50% tissue culture infective dose). HIV infection was done by mixing of virus stock of the HIV-1C primary isolate with a PHA- and IL-2-stimulated PBMC culture and leaving for 3 days. On day 4 of infection, cells were washed three times with PBS and infected cells were plated at 2 × 10<sup>4</sup> cells/mL in a 6-well plate. Cells were transfected with Sα or control dsRNA (50 nM) using Oligofectamine. No viral p24 was detected in the culture supernatant on the day of transfection (day 0 of transfection). At 0, 4, 7, 14 and 21 days after transfection, culture supernatants were removed and viral Gag-p24 was measured using an HIV-1 p24 ELISA kit (Xpress-Bioscience Life, USA). Every seventh day, uninfected PHA- and IL-2-stimulated PBMCs suspended in fresh medium were then added to one-half volume of culture medium to replenish the dead cells.

Western blot analysis
After siRNA transfection, PBMCs were washed with PBS and lysed with triple lysis buffer containing a protease inhibitor cocktail. Protein lysate was quantified by using the biocinchoninic acid protein assay (BCA Protein Assay Kit, Pierce). Equal amounts of protein were resolved on 5%–12% SDS–PAGE gels and transferred to nitrocellulose membranes. The immunoblots were blocked with 4% BSA for 2 h at room temperature in TBBS. The antibodies used for immunoblotting were anti-β-actin (Cell Signalling Technology, USA) and anti-HIV-1 p24 (sc-69728, Santa Cruz Biotechnology). Specific proteins were detected by using appropriate secondary antibodies labelled with alkaline phosphatase and BCIP/NBT (Promega) as the substrate.

Caspase-3 assay
The caspase-3 activity of HIV-1C-infected PBMCs after 1 week of dsRNA transfection was measured using a caspase-3 assay kit (Promega) according to the manufacturer’s instructions.

Generation of mutant C-LTR-luciferase (M-I, M-II and M-III) reporter constructs and luciferase reporter assay after transient transfection
Different C-LTR-luciferase reporter constructs having mutations within the target sequence of Sα-siRNA were generated using a Phuson Site-Directed Mutagenesis Kit (Thermo Scientific, USA) according to the manufacturer’s instructions. Mutant C-LTR-luciferase reporters M-1 and M-II with single mutations were generated using a wild-type C-LTR-luciferase reporter (C-LTR-luciferase) construct as a template and 5′-phosphorylated mutagenic primers. The C-LTR-luciferase reporter M-I was generated using primers M1 and M2, while the C-LTR-luciferase reporter M-II was generated using primers M3 and M4. Similarly, the C-LTR-luciferase reporter M-III with two mutations was generated by using primers M5 and M6. All mutant primer sequences used in the site-directed mutagenesis are listed in Table S3 (available as Supplementary data at JAC Online).

Luciferase expression was measured from siHa cells co-transfected with siRNA and wild-type C-LTR-luciferase reporter/mutant C-LTR-luciferase reporter (M-I, M-II, M-III) construct along with C-Tat expression vector (CMV-C-Tat) and Renilla luciferase vector. Briefly, siHa cells were plated at 1 × 10<sup>5</sup> cells/well in a 6-well plate (Corning). After 24 h, cells were transfected with 50 nM siRNA and then after 20 h with a mixture of 1 μg of C-LTR-luciferase reporter, 200 ng of C-Tat vector and 10 ng of Renilla vector per well. Both transfections were done in serum-free Opti-MEM media (Invitrogen, USA) using Oligofectamine according to the manufacturer’s instructions. Luciferase activity was measured from transfected siHa cells after 72 h of reporter vector transfection by dual luciferase assay.

Statistical analysis
All dual luciferase assay experiments were performed in triplicate and repeated three times. The DNA methylation, p24 ELISA, caspase-3,
MNase and ChIP assay experiments were repeated at least twice. The data are presented as the mean ± SD. Significance in the differences was determined using Student’s t-test. P < 0.05 was taken as significant and P < 0.001 as highly significant.

Sequence data
GenBank accession numbers of LTR of the HIV-1C isolates are as follows: KC197029 (AIIMS 201), KC197030 (AIIMS 212), KC197032 (AIIMS 254) and KC197033 (AIIMS 261).

Results
Screening of siRNAs targeting the HIV-1C LTR promoter for down-regulation of downstream coding sequences
Six siRNAs were synthesized that targeted two regions within the enhancer and core promoter region of the U3 promoter of HIV-1C LTR, each region encompassing one CpG site. Of these, three siRNAs (siRNA-1 (S1), siRNA-2 (S2) and siRNA-3 (S3)), shifted in position by a single nucleotide, were homologous to the NF-κB-II and -III binding sites while the other three siRNAs (siRNA-4 (S4), siRNA-5 (S5) and siRNA-6 (S6)), also differing in position by a single nucleotide, targeted the NF-κB-I and SP1-III binding sites (Figure 1b).

siHa cells, stably expressing the firefly luciferase gene downstream of the HIV-1C LTR promoter (C-LTR-Luci) and subtype C-Tat protein downstream of the CMV promoter (CMV-C-Tat) (Figure 1c), were transfected with different siRNAs (Figure 1b) and screened by the dual luciferase assay at different timepoints. siHa (C-LTR-Luci+ Tat) reporter cells stably expressing the Tat gene were confirmed by RT–PCR and HIV-1C LTR-driven luciferase gene expression in response to Tat was checked by luciferase assay (Figure S2, available as Supplementary data at JAC Online). S4-siRNA was found to decrease luciferase activity maximally by 50% (P = 0.01) and 60% (P = 0.02) at 48 and 72 h post-transfection, respectively, in comparison with control dsRNA (Figure 1d and e). S4-siRNA-mediated silencing was sustained over a period of 3 weeks (Figure 1f). S2-siRNA, which targeted a region in C-LTR homologous to a region that was successfully targeted in B-LTR by Proma-siRNA, did not have any significant effect on C-LTR (Figure 1d and e). The reduction in reporter gene expression in the presence of Tat is an indicator of the efficacy of the siRNA in conditions resembling human infection.

S4-siRNA does not induce cytotoxicity or an interferon response
A cell proliferation assay was performed to assess whether transfection of S4-siRNA resulted in non-specific cytotoxic effects. No significant change was observed in the rate of proliferation of reporter cells transfected with either S4-siRNA (P > 0.4) or control siRNA (P > 0.5) in comparison with mock-transfected cells (Figure S3a, available as Supplementary data at JAC Online).

It is reported that transfection of siRNA can induce sequence non-specific off-target effects by triggering an interferon response in eukaryotic cells either by activation of the PKR/RNase pathway or by activation of the endosomal Toll-like receptor (TLR) pathway.15,16 An interferon response can be confirmed by 50–500-fold up-regulation of 2’5’-oligoadenylate synthetase-1 (OAS1), a well-known interferon response marker.35 No significant increase was observed in OAS1 mRNA levels after S4-siRNA or control siRNA transfection in comparison with mock transfection at different timepoints (P > 0.05) (Figure S3b, available as Supplementary data at JAC Online).

S4-siRNA-mediated TGS is specific to HIV-1C
siHa cells containing integrated (C-LTR-Luci + Tat/B-LTR-Luci + Tat) constructs were used to check the specificity of S4-siRNA. After transfection, S4-siRNA showed a significant (P < 0.05) decrease in the luciferase activity of C-LTR-Luci + Tat cells after 3 days but no significant change was observed in B-LTR-Luci + Tat cells (Figure 2a). This specific down-regulation of the luciferase gene was also confirmed at the mRNA level (P < 0.001) after S4-siRNA transfection (Figure 2b).

In a previous study, an siRNA (PromA) targeting the NF-κB sequences of HIV-1B LTR (Figure 2c) was demonstrated to induce TGS.23,24 Here, we observed that Proma-siRNA significantly (P < 0.05) reduced the luciferase activity of B-LTR-Luci + Tat cells but no significant decrease was observed in C-LTR-Luci + Tat cells (Figure 2a). This was also confirmed at the luciferase mRNA level (Figure 2b).

We also checked the efficacy of another siRNA (B-siRNA), targeting the B-LTR, which corresponds to the target region of S4-siRNA in C-LTR (Figure 2c). B-siRNA did not show any significant decrease in the luciferase activity and luciferase mRNA level in C-LTR as well as B-LTR reporter cells in the presence of the Tat protein (Figure 2a and b).

TGS of the HIV-1C LTR promoter involves H3K9 and H3K27 methylation and heterochromatization of the targeted locus
The 5’ LTR of HIV-1 is flanked by two nucleosomes: nuc-0 at the 5’ end in the U3 region spans nt 40–200 and nuc-1 that is juxtaposed to the transcriptional start site encompassing nt 465–610 in the R-U5 region (Figure 3a).16–38 The chromatin state of the targeted region was checked by MNase/CHART-PCR assay.30 After transfection of siHa (C-LTR-Luci + Tat) reporter cells with S4-siRNA, there was a significant reduction in the accessibility of the targeted LTR region to MNase digestion in comparison with mock- and B-siRNA-transfected cells (Figure 3b). This suggested that the repression of transcriptional activity of C-LTR by S4-siRNA was associated with heterochromatization of the targeted locus.

Furthermore, epigenetic chromatin modifications such as dimethylation of H3K9 (H3K9me2) and trimethylation of H3K27 (H3K27me3) of the histone tail, the marks of silent chromatin, were investigated at the targeted region. ChIP assay showed 3.5- and 4.5-fold enrichment of H3K9me2 and H3K27me3, respectively (P < 0.001 and P < 0.01), at the targeted LTR region in siHa (C-LTR-Luci + Tat) reporter cells after S4-siRNA transfection in comparison with control siRNA-transfected cells, but no significant enrichment was observed in mock- and B-siRNA-transfected cells (Figure 3c). Bisulfite treatment of genomic DNA of siHa (C-LTR-Luci + Tat) cells after single transfection of S4-siRNA followed by PCR and sequencing did not show methylation of cytosine at the targeted and nearby CpG sites at day 4 and even at day 10 of
transfection (Figures S4 and S5, available as Supplementary data at JAC Online).

After treatment of siHa (C-LTR-Luci + Tat) reporter cells with TSA (HDAC inhibitor) or AZA (DNMT inhibitor) for 48 h before S4-siRNA transfection, AZA-treated cells still showed a significant ($P < 0.05$) decrease in luciferase activity, while cells treated with TSA showed no change in luciferase activity ($P = 0.32$) (Figure 4a). The cells treated with only DMSO (the solvent for TSA and AZA) followed by S4-siRNA transfection showed a significant decrease ($P < 0.001$) in luciferase activity, indicating that the DMSO solvent alone did not affect TGS (Figure 4a). In another experiment, reactivation of HIV-1C LTR promoter activity ($P = 0.07$) was observed in siHa (C-LTR-Luci + Tat) reporter cells after 6 days of S4-siRNA transfection when treated with TSA after 3 days of S4-siRNA transfection, but cells treated with AZA did not show up-regulation of luciferase activity ($P = 0.01$) or reactivation of the heterochromatized HIV-1C LTR promoter (Figure S6, available as Supplementary data at JAC Online).

**Figure 2.** S4-siRNA is specific to HIV-1C LTR. (a) After S4-siRNA transfection, there was a significant ($P < 0.05$) decrease in luciferase activity of C-LTR-Luci + Tat siHa cells after 3 days but not in B-LTR-Luci + Tat siHa cells post-transfection, while PromA-siRNA was only active on the B-LTR construct. B-siRNA targeting the B-LTR region corresponding to the S4-siRNA target region in C-LTR was also not effective in HIV-1B LTR. (b) siRNA-specific effect on HIV-1C LTR- and HIV-1B LTR-driven luciferase gene expression at the mRNA level. (c) Sequence alignment of C-LTR (S-B253) and B-LTR (HXB2) and comparison of the target sequences of different siRNAs. CpG dinucleotides, the susceptible sites of methylation, are in bold. Asterisks indicate mismatches and - symbols represent gaps.

**Decrease in virus production in the HIV-1C-infected TZM-bl cell line on treatment with S4-siRNA**

TZM-bl cells expressing CD4, CXCR4 and CCR5 were used to study the effect of S4-siRNA-mediated TGS on HIV-1C viral production. TZM-bl cells were infected with different HIV-1C clinical isolates (AIIMS 201, 212, 254 and 261; AIIMS 212 with a nucleotide difference in the target region (Figure 4b and Figure S7, available as Supplementary data at JAC Online)) and transfected once with siRNAs as described in the Methods section. Viral p24 antigen levels were determined in the culture supernatants at different timepoints over 2 weeks as an indicator of viral infection. There was a 50%–60% reduction in the p24 level observed on day 4 of S4-siRNA transfection (day 8 post-infection) in comparison with control siRNA-transfected cell cultures in all four HIV-1C isolates. On day 7 of transfection (day 11 post-infection), 70%–80% suppression of productive infection was observed and was sustained for ≥14 days of transfection (day 18 post-infection) (Figure 4c). It was also observed that the S4-siRNA
can also tolerate a single-base mismatch in the target sequence of the AIIMS 212 strain (Figure 4c) and suppress viral production in the mutated target strain (Figure 4c).

**Inhibition of HIV-1C replication in infected human PBMCs in primary culture by S4-siRNA**

The efficacy of S4-siRNA-mediated TGS of HIV-1C virus in suppressing viral replication in an ex vivo system was further checked in human PBMC primary culture after infection with HIV-1C primary isolates from different patients. PHA- and IL-2-stimulated PBMCs were infected with four different HIV-1C clinical isolates and transfected once with siRNAs as described in the Methods section. Viral p24 levels were determined in culture supernatants at different timepoints over 3 weeks as a measure of viral replication. There was a 60%–70% reduction in the p24 level observed on day 4 of S4-dsRNA transfection (day 7 post-infection) in comparison with the control siRNA-transfected culture in all four HIV-1C isolates. At day 7 of transfection (day 10 post-infection), almost 90% suppression of productive infection was observed and this suppression was sustained for ≥21 days of transfection (24 days post-infection) (Figure 5a). Western blot analysis also showed that HIV-1-specific Gag-p24 was markedly decreased in PBMC culture treated with S4-siRNA compared with control siRNA-transfected culture at day 7 of transfection (day 10 post-infection) (Figure 5b). We did not observe an siRNA-induced interferon response by the PKR/RNaseL pathway or by TLR activation in HIV-1C-infected PBMCs after transfection (Figure S7).

**Reduction in caspase activity in infected PBMCs after treatment with S4-siRNA**

The pathogenesis of HIV-1 is associated with enhanced apoptosis of HIV-infected CD4 T cells and bystander uninfected T cells. Therefore, caspase-3 activity, a marker of apoptosis and cell-associated HIV infection, was monitored in HIV-infected PBMC culture after transfection. After S4-siRNA transfection, a significant reduction (P=0.004) in caspase-3 activity was observed in HIV-1C-infected PBMCs in comparison with control siRNA-transfected cells on day 7 of transfection (day 10 post-infection), while control
siRNA-transfected cells showed significant up-regulation ($P = 0.004$) of caspase-3 activity compared with uninfected cells. This further demonstrates the protective effect of S4-siRNA in HIV-1C infection in PBMC culture (Figure 5c).

**Analysis of mismatch or mutation tolerance in the target sequence of S4-siRNA**

It was observed that S4-siRNA suppressed the viral production of the AIIMS 212 strain, which had one mismatch in the target sequence (Figures 4c and 5a). After analysis of the variation of the target sequence in different HIV-1C strains in the GenBank (NCBI) database (Figure S8, available as Supplementary data at JAC Online), we checked the efficacy of our S4-siRNA in down-regulating C-LTR having two of the common single-base mismatches and also a target with double-base mismatches. Three C-LTR-luciferase reporter constructs with mutation in the target site were used along with a wild-type C-LTR-luciferase reporter construct and siRNA as described in the Methods section. We observed that S4-siRNA significantly reduced the luciferase activity of the
C-LTR-luciferase wild-type reporter by 70% ($P = 0.002$) in comparison with control siRNA (Figure 6b). In mutant C-LTR reporter constructs, S4-siRNA showed down-regulation of luciferase activity by 60% ($P = 0.01$) in the C-LTR-luciferase M-I reporter and almost 40% ($P = 0.03$) in the C-LTR-luciferase M-II reporter (Figure 6b). However, we did not observe any significant reduction in the luciferase activity of the C-LTR-luciferase M-III reporter ($P = 0.87$) that had two mismatches in the target site (Figure 6b).

**Discussion**

Highly active antiretroviral therapy has been proven to reduce viral replication and emergence of drug-resistant variants of HIV-1, but escape variants still continue to surface after prolonged treatment. Suppression of HIV-1 replication by siRNA-mediated PTGS of structural and accessory genes of HIV-1 has been attempted, but the effect was transient. TGS, on the other hand, by causing heterochromatization of the promoter, can result in the suppression of all viral transcripts even when the effector siRNA has been degraded in the cell. TGS may also have promising application against quasispecies virus in chronic HIV infection and would be effective because of the conserved 5′ LTR promoter.

TGS of HIV-1 by dsRNA targeting the conserved region of 5′ LTR would be an ideal approach to suppress viral transcription and thereby its productive infection. The 5′ LTR of HIV-1 is the common promoter for all viral genes, so inhibition of viral
transcription should reduce the chances for the generation of mutations and, therefore, the emergence of escape variants, which limits the effectiveness of the PTGS approach. There are reports demonstrating prolonged TGS of HIV-1B in the LTR reporter system as well as in the HIV-1 susceptible cell line by an siRNA targeting the NF-κB binding sites of the HIV-1B 5′ LTR. We have identified one potent dsRNA (S4-siRNA) that could induce TGS in the HIV-1C LTR-luciferase reporter system for a longer period of 21 days (Figure 1d–f) without causing cytotoxicity. dsRNA can elicit an interferon response by the PKR/RNaseL and/or TLR pathway to cause a very high increase in OAS1. No increase in OAS1 was noted in our siHa-luciferase reporter cells as well as in the HIV-1 susceptible cell line by an siRNA targeting the NF-κB binding sites of the HIV-1B 5′ LTR.

We have previously demonstrated that a similar phenomenon occurs during TGS of the E6 and E7 integrated oncogenes of HPV-16. We have also found that the previously reported PromA-siRNA, which caused efficient TGS in HIV-1B virus by heterochromatization and DNA methylation, failed to do so in HIV-1C virus. The target region of PromA-siRNA in HIV-1C LTR has three mismatches and absence of the CpG site, while the S4-siRNA target sequence in HIV-1B LTR has four mismatches and absence of the CpG site (Figure 2c). This is the most likely explanation for the siRNA-specific TGS of HIV-1C and HIV-1B, because TGS is a highly sequence-specific phenomenon and we have demonstrated that a shift of the target site by one or more bases causes a dramatic reduction of the observed effect. This indicates the same region in these two clades is differently amenable towards TGS by dsRNA. S4-siRNA-mediated TGS was associated with the heterochromatization of the targeted promoter region, which led to a closed chromatin structure. This heterochromatization was measured by MNase accessibility, which showed reduced accessibility of MNase after S4-siRNA transfection (Figure 3b). The heterochromatin structure induced by S4-siRNA was directed by epigenetic modification, i.e. H3K27 trimethylation and H3K9 dimethylation, around the targeted region (Figure 3c). Mock, control and B-siRNA-transfected siHa reporter cells did not show histone methylation and decreased chromatin accessibility. Treatment with an HDAC inhibitor, TSA, both before and after S4-siRNA transfection prevented and reversed the TGS effect, respectively. No DNA methylation was observed at the targeted promoter region on days 4 and 10 post-transfection, and inhibition of DNMT by AZA had no effect on TGS. Taken together, these data suggest that S4-siRNA-mediated TGS can occur independent of DNA methylation. We have previously demonstrated that a similar phenomenon occurs during TGS of the E6 and E7 integrated oncogenes of HPV-16.

While TGS of HIV-1B by siRNA has been demonstrated in primary human CD4+ T cells in the natural history of disease, HIV can infect various types of PBMCs. We therefore checked the effect of S4-siRNA in both an in vitro and an ex vivo system using TZM-bl cells and human PBMCs after infecting them with various clinical HIV-1C isolates. We found that S4-siRNA targeting the HIV-1C LTR...
LTR promoter profoundly suppresses viral transcription and productive HIV-1C infection in TZM-bl cells (Figure 4c) and also in primary human PBMCs, which was sustained for ≥21 days after single transfection (Figure 5a). The suppression of HIV-1C infection in PBMCs is also accompanied by reduced cellular p24 level and caspase-3 activity, which are associated with the pathogenesis of HIV-1 (Figure 5b and c). The ability of the S4-siRNA to protect HIV-1C-infected PBMCs from apoptotic death also demonstrates its effectiveness against a range of primary clade C isolates. An HIV-1 clade C isolate (AIIMS 212) with a single nucleotide difference in the target sequence at the penultimate position (A→T) was also effectively neutralized (Figures 4b and c and 5a). Analysis of different clade C strains (Figure S8) showed that this target region is invariant in ~50% of cases, while there was only a single base difference in the rest. Since live viruses with such mutations were not available to us, except the AIIMS 212 strain, we mutated a base in the target region by site-directed mutagenesis at sites that were found to be commonly mutated in the HIV-1C database. It was observed that S4-siRNA can tolerate single-base mismatch at the first position of the 5′ end of the S4-siRNA target is also tolerated, while two mutations in the target sequence are not. The ability of the S4-siRNA to tolerate single-base mismatch at the 5′ or 3′ end along with the observed position specificity enhances its ability for potential therapy of HIV-1C infection and indicates it can be effective in HIV quasispecies that arise during chronic infection.

In summary, we have identified a potent dsRNA that was confirmed to establish heterochromatization of the transcriptionally active integrated HIV-1C 5′ LTR promoter, leading to the downregulation of viral gene expression and replication for ≥21 days after single transfection in ex vivo experiments. This heritable chromatin remodelling, viral gene silencing and long-term reduction in productive viral infection in a range of primary clade C viral isolates (one with a nucleotide difference at the target region), as induced by the dsRNA, strengthens the prospect of its usage as an alternate or combinatorial therapeutic approach with classical drugs for the treatment of HIV-1C-infected patients in the future.

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Transparency declarations
None to declare.

Supplementary data
Figures S1 – 8 and Tables S1 – 3 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References
expression results in real-time PCR.


