Inhibition of HIV-1 replication using the CRISPR/cas9-no NLS system as a prophylactic strategy

Ali Salimi-Jeda a,***, Maryam Esghaei a,*, Hossein keyvani a, Farah Bokharaei-Salim a, Ali Teimoori b, Asghar Abdoli c,**

a Department of Virology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
b Department of Virology, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran
c Department of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran

Graphical Abstract

Abstract

Globally, it is estimated that 43 million people are living with human immunodeficiency virus type 1 (HIV-1), and there are more than 600,000 acquired immunodeficiency syndrome (AIDS)-related deaths in 2020. The only way to increase the life expectancy of these patients right now is to use combination antiretroviral therapy (cART) for the lifetime. Due to the integration of the HIV-1 DNA in lymphocytes, the replication of the virus can only be reduced by using antiretroviral drugs. If the drug is stopped, the virus will replicate and reduce the number of lymphocytes. In recent years, the clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonuclease Cas9-mediated genome editing system has been considered, preventing HIV-1 replication by causing DNA double-stranded breaks (DSBs) or disrupting the integrated virus replication by targeting the provirus. In this study, we utilized the CRISPR/Cas9 without the nuclear localization signal sequence (w/o NLS) system to inhibit the VSV-G-pseudotyped HIV-1 replication by targeting the HIV-1 DNA as a prophylactic method. To this end, we designed a multiplex gRNA (guide RNA) cassette to target the pol, env, and nef/long terminal repeat (nef/LTR) regions of the HIV-1 genome and then cloned it in plasmid expressing no-NLS-Cas9 protein as an all-in-one CRISPR/Cas9 vector. Using HIV-1 pseudovirus transduction into HEK-293T cell lines, our results showed that the CRISPR/Cas9-no NLS system disrupts the pseudotyped HIV-1 DNA and significantly (P-value < 0.0001) decreases the p24 antigen shedding and viral RNA load in cell culture supernatants harvested 48h after transduction.

Keywords:
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* Corresponding authors.
** Corresponding author.
*** Corresponding author.
E-mail addresses: maryam.esghaei@gmail.com (M. Esghaei), a_abdoli@pasteur.ac.ir, asghar.abdoli7@gmail.com (A. Abdoli).

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1. Introduction

Approximately 43 million people are living with HIV, and 1.5 million people acquire HIV-1 per year globally [1, 2].

HIV-1 is a lentivirus belonging to the Retroviridae family with the diploid RNA genome by binding to the CD4 receptor, CC chemokine receptor 5 (CCR5), and C-X-C chemokine receptor type 4 (CXCR4) co-receptors fuses with the immune cells, and releases into the cytoplasm of these target cells. Then, the viral genomic RNA is reverse transcribed into the double-stranded DNA by the viral reverse transcriptase enzyme in the reverse transcription complex/pre-integration complex (RTC/PIC). After nuclear entry, viral DNA integrates into the host cell chromosomes called a provirus. Replication of HIV-1 by reducing the number of immune cells, and the risk of opportunistic microbial infections, eventually, leads to the acquired immunodeficiency syndrome (AIDS) [3, 4].

Among many susceptible cells to HIV-1, CD4+ T cells, dendritic cells, as well as macrophages are the best-described HIV-1 reservoir and the carriers of integrated provirus [5]. The provirus is eligible for replication in latently infected reservoirs but is silenced for transcription [6, 7]. The latent HIV-1 infection is the major obstacle to HIV-1 eradication and allows the HIV-1 to have life-long persistence in the body despite long-term antiretroviral treatments [8].

HIV-1 treatment is more focused on antiretroviral therapy (ART). However, by inhibiting the various stages of the virus lifecycle in immune cells, antiretroviral drugs can reduce virus mortality and increase patients' longevity [9]. Nevertheless, due to the integration of the HIV-1 genome into the host cell's chromosome, antiretroviral drugs cannot completely eradicate the virus from the immune cells; even by stopping antiretroviral drugs, HIV-1 will be multiplied again [10, 11]. On the other hand, increasing life expectancy with the long-term use of antiretroviral drugs for a lifetime in addition to drug resistance may cause antiretroviral drugs to become ineffective [12].

Another strategy to treat HIV-1 in latent infections is reactivating the latent provirus with latency-reversing agents (LRAs) such as methyltransferase inhibitors, histone deacetylase, NF-KB stimulants, TLR agonists, as well as chromatin modulators and then killing the proliferating cells by the immune system called the "shock and kill" process. Despite numerous studies, the non-specificity of these compounds on provirus and the possibility of affecting cellular homeostasis and failure to activate all latent cells, there have been no satisfactory results in HIV-1 cure [16, 17].

Another way to inhibit HIV-1 replication is to use the small interfering RNA (siRNA) method. RNA interference (RNAi) does not affect the HIV-1 DNA, and just by binding to and cleaving a target RNA sequence, the translation of viral or cellular proteins is downregulated [13], hepatotoxicity, cardiovascular disorder, chronic inflammation, and metabolic complications [14, 15].

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into the host cell's chromosome and, as a result, inhibits or reduces the replication of the virus.

2. Result

2.1. Evaluation of the inhibitory effect of CRISPR/Cas9-no NLS system on pseudotyped HIV-1 replication

2.1.1. ELISA p24 assay

The results showed that the Optical Density (OD) of the pseudotyped HIV-1 p24 antigen was the highest in the supernatant harvested 48 h post-infection (h p.i.). Also, the OD was reduced in the samples harvested at 72 h and 96 h p.i., respectively.

Data analysis was demonstrated by Graphpad prism software using two-way ANOVA and Tukey's multiple comparisons tests and there were no significant differences in p24 antigen shedding among Mock and pseudotyped HIV-1 groups at 48 h (P-Value = 0.81), 72 h (P-Value = 0.95), and 96 h (P-Value = 0.97). Meanwhile, Raltegravir and Darunavir completely (100%) and significantly (P-value <0.0001) were able to inhibit p24 shedding every three times. Furthermore, there was no significant difference between p24 OD in samples treated with Darunavir and Raltegravir at three times of 48 h.

Figure 1. The Effect of multiplex gRNA/Cas9 (without NLS) on HIV-1 p24 antigen shedding and HIV-1 RNA expression. The graph of means of the optical density (OD) for HIV-1 p24 antigen of different groups according to the time of sample harvest at 48, 72, and 96 h post-infection (h p.i.) (A). According to the cut-off value (0.084), the OD < 0.084 was considered negative and ≥0.084 was considered positive. The graphs show that the full maximal inhibitory concentration of Raltegravir and darunavir prevented the HIV-1 p24 antigen shedding. Also, PBLO_noNLS_Cas9_HIV-1_3gRNA plasmid reduced mean p24 OD 75.6% in 48 h, 65.7% in 72h, and 78.1% in 96h (A). The graph of fold changes of different groups based on the time of sample harvest at 48, 72, and 96 h p.i. (B). As shown in the graphs, the full maximal inhibitory concentration of raltegravir completely prevented the virus replication but 100% inhibitory concentration (IC100) of darunavir does not have an inhibitory effect on HIV-1 RNA replication. In the meantime, PBLO_Cas9_noNLS_HIV-1_3gRNA plasmid reduced mean HIV-1 RNA load 4.23 fold (fold change = 0.236), 3.0 fold (fold change = 0.327), and 5.74 fold (fold change = 0.174) at 48 h, 72 h, and 96 h p.i. compared with the control HIV-1 RNA load at 48 h (fold change = 1), respectively. Error bars indicate SD (n = 3; *p < 0.05; ****p < 0.0001).
The results showed that the pBLO_Cas9_noNLS_HIV-1_3gRNA plasmid was able to significantly (P-value < 0.0001) reduce the p24 shedding of the pseudotyped HIV-1 at all three times. For samples pre-treated with CRISPR plasmid and harvested 48 h p.i., the mean HIV-1 p24 protein OD was reduced by 75.6% (4.06 fold). For samples harvested at 72 h and 96 h p.i., the mean OD decreased 65.7% (2.91 fold) and 78.1% (4.56 fold), respectively (Figure 1A).

2.1.2. Real-time PCR

Real-time PCR data, including the cycle threshold (Ct) and copy number of pol gene of pseudotyped HIV-1 and Ct of internal control gene for triplicate samples, were saved in an Excel file. Then ΔCt (Target Ct - internal control Ct), ΔΔCt (ΔCt - reference Ct), and Fold change were calculated, and the data were analyzed using Graphpad prism software with two-way ANOVA and Tukey test. The mean Ct of virus control samples (untreated cells infected with the virus) at 48 h was determined as the reference Ct, and the rest of the samples were compared to this sample (Figure 1B).

The results indicated that the pBLO_Cas9_noNLS_HIV-1_3gRNA CRISPR plasmid significantly reduced the pseudotyped HIV-1 RNA load in all three sample harvesting times (P-value < 0.0001). The results showed that the pre-transfected HEK-293T cells with this CRISPR plasmid were significantly able to (P-value < 0.0001) reduce the HIV-1 RNA titer in samples harvested at 48 h p.i. from 1.74 × 10^5 copy/ml (in non-treated control sample) to 4.39 × 10^5 copy/ml (75%). Also, for samples harvested at 72 h p.i., the viral RNA titer was reduced from 4.67 × 10^5 copy/ml (non-treated control sample) to 1.63 × 10^5 copy/ml (66%), and for 96 h p.i. samples were reduced from 3.27 × 10^5 copy/ml to 6 × 10^4 copies/ml (82%) (Figure 1B).

The data showed that full maximal inhibitory concentration of Raltegravir completely (100%) inhibited HIV-1 replication (P-value < 0.0001). Also, the pre- transfected HIV-1 RNA titer in darunavir treated samples that were harvested at 48 h p.i. was significantly (P = 0.0295) 1.35 fold higher than the HIV-1 RNA load in the non-treated samples. However, for 72 h (P = 0.4166) and 96 h (P = 0.2079) samples, the increased titer was not significant (Figure 1B).

3. Discussion

Increasing the life expectancy of HIV-infected patients requires the lifetime use of antiretroviral drugs that may result in many side effects. For this reason, the tendency to CRISPR/Cas9-mediated genome editing technology has particular importance to inhibit the integration of HIV-1 DNA or complete elimination of the provirus from infected cells [53]. In using the CRISPR/Cas9 system as a prophylactic method, selecting the appropriate gene region to target HIV-1 DNA prior to integrating it into the host cell chromosome is crucial due to the confining of HIV-1 DNA in the PIC.

In the study by Dheeraj et al. on the resistance of HIV-1 DNA to DNase-I in infected cells by the Nuclease Protection Assay, it has been shown that about 8.5 h after HIV-1 infection, the LTR regions of 5′ end of HIV-1 cDNA (about 6% of the virus genome) are covered by pre-integration complex (PIC) (including CA, MA, Vpr, IN, and RT proteins) and is protected from access to DNase I. Ten h after infection, about 90% of the virus DNA is protected, but env (from nucleotide 7615–8370), pol4 (nucleotide 4150–4393), and nefLTR region (nucleotide 8936–9435) are still available. This study also showed that the PIC is found in the cytoplasm 5–10 h p.i., and transfering of PIC from the cytoplasm to the nucleus occurs 10–12.5 h p.i. [49]. However, a more recent study showed that the HIV-1 RNA reverse transcription occurred in the nucleus [54].

Therefore, in the present study, after designing an efficient gRNA with low off-target for these regions, a multiplex gRNA cassette is developed and cloned in a Cas9-no NLS expressing plasmid to target the HIV-1 DNA before integration in the host cell chromosome as a prophylactic approach.

Although the molecular mechanism of nuclear import of proteins relies on the interaction of small peptide motifs present in protein cargos, the recent study showed that the HIV-1 RNA reverse transcription occurred in the nucleus [56, 57]. In most studies, HIV-1 provirus DNA has been targeted in the nucleus using multiplex gRNA cassette for LTR [47], LTR with pol and tat/rev genes [58], and gag with LTR [59]. To the best of the authors’ knowledge, there are few studies on using CRISPR/Cas9-no NLS to target HIV-1 and prevent viral DNA integration.

Although CRISPR/Cas9 pretreated cells targeting provirus have been shown resistant to new HIV-1 infection, there was little knowledge whether the CRISPR/Cas9 system prevents the virus integration in the nucleus or causes breaks LTR in the cytoplasm. Liao et al.’s study showed the constant expression of gRNA for LTR, gag, pol, env regions, and Cas9-NLS protein in engineered HEK-293 cells by targeting the HIV-1 provirus in the nucleus which decreased the production of the virus to be 18%–72% depending on the used gRNA. This study also showed that CRISPR/Cas9-NLS system effectively degraded the non-integrated viruses and prevented HIV-1 DNA integration [47]. In a similar study by Liu et al. using the HIV-infected HEK-293 cells and the vector expressing Cas9-without NLS and gRNAs (for LTR, gag, pol, and env regions), the HIV-1 production was reduced by 44%–74% depending on the used gRNA [60]. However, in these studies, the cells could stably express Cas9 and gRNA through genetic engineering or by viral vectors and did not have low transfection problems.

Studies have shown that targeting HIV-1 proviral DNA with single gRNA/Cas9 rapidly and consistently escapes the caspase system due to mutations around the Cas9/gRNA cleavage site that are critical for NHEJ DNA repair [52]. Most studies on inhibiting the HIV-1 replication by CRISPR/Cas9 system have focused on HIV-1 provirus degradation and shown that by using a pair of sgRNA (Cas9/dual gRNA) for different regions of one or more genes, the efficiency of provirus DNA cleavage increases. In these studies, one gRNA targeted the LTR region, and the other gRNAs targeted gag or env genes [59, 61, 62]. In addition, Lebbink, R. J. et al. [61], Gang Wang et al. [62], and Ophinni Y et al. [63] studies showed that using multiplex gRNAs (all in one vector) to target essential genes can be efficiently surmounted to HIV-1 escape mutations.

Studies have demonstrated that using lentiviral-based CRISPR-Cas9 and several gRNA targets for different regions of HIV-1 proviral DNA has reduced HIV-1 replication by more than 96% [59]. Thus, the use of viral vectors to express Cas9 and multiple gRNA targets can be more successful in targeting HIV-1 proviral DNA [63, 64, 65]. Some studies have suggested the concomitant use of CRISPR/Cas9 and antiretroviral drugs or RNA interference (RNAi) [66].

Although RNAi through therapeutic strategy can reduce the expression of viral miRNA or down-regulation of cellular receptors [19], studies on the use of RNAi as a prophylactic method have not been functional. Westerhout et al.’s study has shown that using the RNAi system could not target the incoming HIV-1 RNA genome that has not yet been a reverse transcription to DNA and entered the nucleus [67]. This inability to target the virus's genomic RNA upon viral entry before reverse transcription of the RNA genome may be due to the inaccessibility of viral RNA to the RNAi machinery because of being enclosed in RTC/PIC and its short presence in the cytoplasm.

Although, in the present study, by designing a plasmid expressing three gRNAs and no-NLS Cas9 protein, the pseudotyped HIV-1 p24 protein shedding was reduced by 4.06 fold (75.6%) and pol gene expression compared to the control group, the low efficacy of the CRISPR/Cas9-no NLS system and the inability to completely inhibit virus replication can be attributed to the incomplete transfection of the pseudotyped into HEK-293 cells, the high MOI of the used HIV-1, and little time to target HIV-1 DNA in the cytoplasm or nucleus before the viral DNA integration in the...
chromosome. The efficiency of this study requires further confirmatory studies using TZM-bl cell lines, the engineered HeLa cells constantly expressing CD4, CXC4, and CCR5 [68], and viral vectors in lymphocyte cells that may have promising results for the prevention of HIV-1 infection. Also, whole-genome sequencing had to be used to confirm the viral DNA cleavage by each gRNA, which is one of the limitations of this study. Furthermore, developing the therapeutic strategy to assess the effectiveness of the CRISPR/Cas9 system on HIV-1 infected cells could help evaluate the accumulation of no-NLS Cas9 in the nucleus, double-stranded breaks in provirus DNA, mutations in the cleavage sites, and the off-target effects.

As an approach to the future, the CRISPR/Cas9-no NLS system may be applied in clinical practice as a prophylactic or even therapeutic method to cleave the HIV-1 DNA and prevent the cell to cell transfer of the virus. Also, it will dispel the limitation of pro-and post-exposure prophylaxis and the possibility of drug resistance.

4. Conclusion

In conclusion, although the CRISPR/Cas9-no NLS system using a plasmid vector expressing three gRNA and no-NLS Cas9 protein can impair the pseudotyped HIV-1 DNA with low efficiency and preempt a productive infection, and regarding a small window of opportunity for prophylactic targeting before HIV-1 DNA integration into the host cell chromosome, further experiments are needed to be performed to increase the efficiency of this system and applied in the clinical practice.

5. Material and methods

5.1. Cell culture

Human embryonic kidney (HEK) cell line 293 T was obtained from the cell bank of Pasteur Institute of Iran and cultured in high-glucose Dulbecco's Modified Eagle's Media (DMEM) medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen/Strep), in a humidified incubator with 5% CO2 at 37 °C. Cells were maintained with a 2% FBS medium for virus isolation.

5.2. VSV-G-pseudotyped HIV-1 construction and MOI determination

The pseudotyped SCR (single cycle replication) HIV-1 stocks were generated by transfection of HEK-293T cells (7 x 10^5 cell) with 4 μg of pSfPAX2 (Addgene plasmid # 12260, Contains HIV-1 regions) and pMD2.G (Addgene plasmid # 12259, expressing the G protein) vector complexes with 20 μl of TurboFect Transfection Reagent in 6-well plate (250 μl plasmid-Turbofect complex in DMEM medium for each well). The medium was replaced 24 h after transfection for three days with new 2% FBS, 1% Pen/Strep-containing DMEM, and the supernatants were collected each day. Then, the collected medium was filtered (0.45 mM) and centrifuged at 15000 r.p.m. for 2 h. After the removal of the supernatant, viral sediments were dissolved in 1 ml and titrated by Real-time PCR. HIV-1 pseudovirus titer was calculated as 2 x 10^6 Copy/ml using a Real-time PCR assay. Meanwhile, to determine the appropriate multiplicity of infection (MOI) of pseudotyped HIV-1, four MOIs (2, 5, 10, and 20) were transduced into HEK-293T cells for 48 h, and the p24 antigen shedding was examined using ELISA (Enzyme-linked immuno-sorbent assay). According to the Optical Density (OD) of HIV-1 p24 antigen at 72 h after transduction of different MOIs of pseudotyped HIV-1 to HEK-293T cells, the MOI10 was selected for the assessment of the inhibitory effect of the CRISPR/Cas9 system.

5.3. Identification of HIV-1 target genes and bioinformatic design of multiplex gRNA cassette

We have targeted three parts of the HIV-1 genome sequence (Human immunodeficiency virus 1, complete genome, 9181 bp ss-RNA, NCBI Reference Sequence: NC_001802.1), including rev response element (RRE) in the env coding region (nucleotide 7615–8370), pol (nucleotide 4150–4393) and the nef/LTR region (nucleotide 8936–9435) that, according to the Dheeraj et al.'s study, these regions are uncoated by pre-integration complex and may be targeted by the CRISPR/Cas9 system before integration in the host cell chromosome [49]. The sequences were identified by nucleotide BLAST and then one gRNA with high efficiency and low off-target was designed using gRNA design software for each region (Table 1). We utilized the CRISPOR-Tefor (v4.99) [69,70] and Off-Spotter (RRID: SCR_015739) [71] gRNA designing and off-target prediction softwares to design an efficient gRNA with low off-target.

In this study, using SnapGene software, we designed one multiplex gRNA cassette containing three sequentially gRNA cassettes (All in one). In this cassette, a U6 Type 3 RNA polymerase Ill (Pol III) for the expression of small RNAs) promoter sequence was placed before each gRNA target. After these, the scaffold RNA guide and termination signal sequences were placed. The gRNA sequences were initiated with a G residue as a U6 promoter transcription start site (TSS) [72]. Also, multiple cloning site (MCS) sequences were placed between each cassette (Figure 2B).

In this study, pBLO1811_Cas9_noNLS_human plasmid was purchased from Addgene (Addgene, plasmid number 74490) (Figure 2A) and since the Cas9-noNLS lacks the nuclear localization signal sequence (NLS), it was used to construct CRISPR/Cas9-no NLS plasmid vector (Figure 2D) to target the HIV-1 DNA before integration into the cell chromosome [73]. Restriction enzyme recognition sites PciI and KpnI were used at the end of 5' and 3' sequences of multiplex gRNA cassette, respectively, to clone the gRNA multiplex cassette sequence instead of the gRNA cassette contained in the plasmid itself.

Cloning primers were also designed for the 5' and 3' ends of multiplex gRNA cassette to be used after cloning the gRNA cassette in plasmid pBLO1811_Cas9_noNLS_human by PCR to confirm cloning and sequencing of the gRNA cassette (Figure 2C).

5.4. Cloning of the multiplex gRNA cassette in the plasmid vector

A multiplex gRNA cassette was synthesized by Proteogenix and obtained in PUC57 plasmid. The pBLO1811_Cas9_noNLS_human Plasmid was also purchased from Addgene.

First, after the process of Escherichia coli (E.coli, DH5a) competence (by calcium chloride method) and transformation of plasmids to the competent bacteria separately (by heat shock method) [74], the bacteria were cultured overnight in LB (Luria-Bertani) agar medium containing ampicillin antibiotic (100 μg/ml) at 37 °C. After that, one of the colonies was cultured in a tube containing LB broth medium with ampicillin antibiotic. The next day, the plasmids were extracted by FavorPrepTM Plasmid Extraction Maxi Kit, and Plasmid DNA concentration was measured by nanodrop.

In the next step, the plasmids were digested by PciI and KpnI restriction enzymes, and the digestion products were electrophoresed in 1% agarose gel (Figure 3A). DNA fragments of the multiplex gRNA cassette (1418 bp) and the plasmid pBLO1811_Cas9_noNLS_human (8697 bp), from which the prior gRNA cassette sequence (455 bp) was digested, were cut and extracted from the gel (Figure 3B) and ligated each other by T4 DNA ligase. The resulting all-in-one CRISPR/Cas9 plasmid vector (pBLO_Cas9_noNLS_HIV-1_3 gRNA) was transformed into bacteria and cultured overnight in LB agar medium. Six colonies were removed and cultured in LB broth medium overnight, then plasmids were extracted (Figure 3C), and DNA concentrations were measured. The plasmids were electrophoresed on a 1% agarose gel and based on the band that was created, one of the plasmids was selected to continue the study. Plasmid pBLO_Cas9_noNLS_HIV-1_3 gRNA was digested to confirm cloning by restriction enzymes, and the reaction product was electrophoresed on a 1% agarose gel. The DNA fragment was cut and extracted from the agarose gel. Then PCR cloning was performed using a Biotech rabbit Pfu PCR Master Mix kit.
Table 1. Designing gRNAs Target sequence for use in the multiplex gRNA cassette. Targets, the number of mismatches, the number of off-targets, the tools used for gRNA design, and efficacy Scores are determined for each gRNA target. Off-target risk is determined by the number of Mismatches and their location. The lower the number of mismatches and the farther away from the PAM area, the greater the risk.

| Target          | gRNA Target                  | Strand | Number of Mismatch and their location | Number of potential off-target | Online tool | Efficacy Score |
|-----------------|------------------------------|--------|--------------------------------------|-------------------------------|-------------|----------------|
| >NC_001802.1:1655–4639 HIV-1 pol4 (3361–3606) | CCAGCTGTGATAATGTCAGCTA     | -      | 0-1-2-3-4 0-0-6-16-133                | 135                           | CRISPOR-Tefor | 78             |
|                 |                              |        | 4                                    | 35                            | off-Spotter          |                |
|                 |                              |        | 5                                    | 243                           |              |                |
| >NC_001802.1:5771–8341 HIV-1 env3 (7171–7853) | CTTAATGACGCTGACGGTAGTACAGG | +      | 0-1-2-3-4 0-0-0-3-17                  | 20                            | CRISPOR-Tefor | 96             |
|                 |                              |        | 4                                    | 3                             | off-Spotter          |                |
|                 |                              |        | 5                                    | 32                            |              |                |
| >NC_001802.1:8343–8963 HIV-1 nef/LTR3 (8343–8963) | CCGCTAGCATTTCATACCTGAGG    | +      | 0-1-2-3-4 0-0-0-1-32                  | 33                            | CRISPOR-Tefor | 93             |
|                 |                              |        | 4                                    | 8                             | off-Spotter          |                |
|                 |                              |        | 5                                    | 37                            |              |                |

Figure 2. Circular DNA map of PBLO_noNLS_Cas9 Plasmid (A). Components of the multiplex gRNA cassette (B). Cloning primers (C). Linear DNA map of pBLO_Cas9_noNLS_HIV-1_3gRNA Plasmid (10115 bp) (D). Various segments, including cas9, mCherry, gRNA cassette, and PciI and KpnI restriction enzyme sites have been specified in these plasmids (A and D).

(Figure 3D) and cloning was confirmed by 1471 bp amplicon size. The analysis of two-way Sanger sequencing (Applied Biosystems genetic analyzer) results of this DNA fragment using Chromas software and NCBI nucleotide BLAST confirmed the similarity between the sequence of this fragment and that of the synthesized multiplex gRNA cassette.

The MOCK plasmid (empty gRNA) was also prepared as a control. To produce Mock plasmid, after digestion of pBLO1811_Cas9_noNLS_human by restriction enzymes and cutting pBLO_Cas9_noNLS fragment from agarose gel and DNA purification, the two ends of plasmid DNA sequences were ligated with each other by T4 ligase enzyme. Then MOCK plasmids were amplified, extracted, and validated as mentioned. The
concentration of CRISPR vector and Mock plasmids were calculated and used to optimize the plasmid transfection into HEK-293T cells.

5.5. Optimization of HEK-293T cell transfection with pBLO_Cas9_noNLS_HIV-1_3 gRNA plasmid

Turbofect transfection reagent was used for HEK-293T cell transfection. HEK-293T cells were seeded in a 96-well plate 24 h before transfection in a total volume of 200 μL DMEM/well, supplemented with 10% FBS and 1% Pen/Strep. The next day, 1 h before transfection, the medium was replaced with 100 μL of new 2% FBS, 1% Pen/Strep-containing DMEM, and then TurboFect/plasmid complex was prepared in different concentrations. To this end, in the volume of 20 μL of DMEM (without FBS and Pen/Strep)/well, 0.25 and 0.5 μg of plasmid was prepared with 0.6 μL of TurboFect and 1 and 1.5 μg of plasmid with 0.8 μL of TurboFect and then were incubated for 20 min at room temperature. The complexes were then added to the cells and incubated at 37°C for 24 h. The next day, the medium was replaced with 200 μL of new 2% FBS, 1% Pen/Strep and incubated for another 24 h at 37°C. Finally, 48 h after transfection, the cells were evaluated for cell viability analysis by MTT, and the images of live cells were captured under an Olympus IX51 inverted microscope.

For the MTT assay, the medium of each well was replaced with 100 μL of Gibco™ DMEM, High Glucose, HEPES, No Phenol Red culture media supplemented with 10 μL of the 12 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution. After 3 h incubation at 37°C, the supernatant was replaced with 50 μL of DMSO and incubated for 10 min at 37°C. Absorbance was measured at 570 nm employing Synergy H1 Hybrid Multi-Mode Reader (BioTek), and the cell viability percentage was calculated utilizing GraphPad Prism 8.

The use of 0.5 μg of pBLO_Cas9_noNLS_HIV-1_3 gRNA plasmid and 0.6 μL turbofect in volume 20 μL shows the highest percentage of viability (99%), high transfection efficiency, and a high percentage of Cas9 and mCharry protein expression in cells (Figure 4).

5.6. The 50% cytotoxic concentration (CC50) and 50% inhibitory concentration (IC50) of raltegravir (integrase inhibitor) and darunavir (protease inhibitor)

The day before the test, 2 × 10^4 HEK-293T cells were cultured in a 96-well plate in 200 μL DMEM/well, supplemented with 10% FBS and 1% pen/strep, and incubated for 24 h at 37°C. After 24 h to determine the raltegravir CC50, a 2mM concentration of raltegravir was prepared by adding dimethyl sulfoxide (DMSO) and after sterilization with a 0.22 μm filter, the triplicate of serial 2-fold dilutions (from 1000 to 7.81 μmol) was made in a volume of 200 μL DMEM/well, supplemented with 10% FBS and 1% penicillin-streptomycin. Afterward, the medium of each well was replaced with 200 μL of drug dilutions and then incubated for 24 h at 37°C. The MTT assay was conducted the next day, and the Cell viability (CC50) was determined for Raltegravir using GraphPad Prism 8.

To determine the IC50 of Raltegravir for pseudotyped HIV-1, serial 2-fold dilutions (from 125 to 0.95 nmol) were made, and similar to what was stated above, the medium of each 80–90% confluent cell/well (in triplicate) was replaced with 200 μL of drug dilutions and then incubated for 3 h at 37°C. After 3 h 4 × 10^5 virus particle (MOI 10) was seeded on cells and incubated for 24 h at 37°C. The next day, the medium of each well was removed, the cells were washed three times with DMDM, and then 200 μL of DMEM containing 10% FBS and 1% pen-strep was added to each well and incubated at 37°C for 24 h. Forty-eight h after virus transduction, viral p24 antigen shedding was assessed with GB HIV-1 Ag-
5.7. The assessment of the effectiveness of CRISPR/cas9-no NLS system on pseudotyped HIV-1 replication as a prophylactic agent

On the first day, in a 96-well plate, 2 × 10^4 HEK-293T cells/well were cultured in 200 μl of DMEM medium supplemented with 10% FBS and 1% antibiotics (in triplicate) and incubated for 24 h at 37 °C.

On day 2, pBLO_Cas9_noNLS_HIV-1_3 gRNA and MOCK plasmids were transfected into cells. For this purpose, 1 h before transfection, the medium was replaced with 100 μl of DMEM supplemented with 5% FBS and 1% of Pen/strep. Then, the turbofect/plasmid complex was prepared in 20 μl/well DMEM without FBS and Pen/strep by combining 0.5 μg of plasmid and 0.6 μl of turbofect and was gently added to the cells.

On the third day (48 h after cell culture), the supernatants were replaced with 100 μl of DMEM supplemented with 2% FBS and 1% Pen/strap. For the standard anti-retroviral controls, the medium containing 250 nM (Full Maximal Inhibitory Concentration) of Raltegravir and Darunavir was added to the related wells and incubated at 37 °C for 3 h, then 4 × 10^6 pseudotyped HIV-1 was added to each well except cell control wells and incubated at 37 °C for 24 h.

In this study, we used Raltegravir (Integrase inhibitor) and Darunavir (Protease inhibitor) as a control for evaluation of the inhibitory effect of the CRISPR/Cas9-no NLS system. Because the nature and mechanism of action of antiretroviral drugs are different from the CRISPR/Cas9 system and the IC50 for the CRISPR system cannot be determined, in the present study, we used the 100% inhibitory dose of darunavir and raltegravir as a control in-vitro.

The culture medium was removed on the fourth day, and the cells were washed twice with DMEM medium. Then 300 μl of DMEM supplemented with 2% FBS and 1% Pen/strep was added to each well and incubated at 37 °C for 24 h.

On the fifth day (48 h after the virus transduction), the medium of each well was collected into a separate microtube and stored at -70 °C to perform ELISA and Real-time PCR tests. Then 300 μl of DMEM supplemented with 2% FBS and 1% Pen/strep was added to each well and incubated at 37 °C for 24 h.

On the sixth day (72 h after the virus transduction), as on the fifth day, the medium was stored at -70 °C and a new DMEM medium was replaced.

On the seventh day (96 h after virus transduction), first the medium of each well was collected into a separate microtube and stored at -70 °C. Afterward, the cells were detached using Trypsin-EDTA (0.25%) (Sigma-Aldrich), washed twice with phosphate-buffered saline (PBS) and finally suspended in 1 ml PBS. Ultimately, the expressions of mCherry and Cas9 were subsequently analyzed using a Cy Flow cytometer.

Because the mCherry protein expression gene in the pBLO_Cas9_noNLS Cas9 plasmid binds to the Cas9 protein gene through the self-cleaving T2A peptide sequence, expression of this protein acts as a proxy for Cas9 expression. As seen in the picture, the mCherry protein expression percentage in cells transfected with pBLO_Cas9_noNLS HIV-1_3 gRNA plasmid and in cells treated with MOCK plasmid was determined as 86.41% and 87.95%, respectively (Figure 6).

In the next step, the stored samples were assessed for HIV-1 p24 antigen shedding by ELISA and HIV-1 RNA load tested by Real-time PCR assay.

5.8. HIV-1 p24 antigen detection

HIV-1 p24 is the capsid protein encapsulating the genomic complex [75]. ELISA test was performed according to the manufacturer's instruction of GB HIV-1 Ag-Ab COMB kit. The amount of 100 μl of specimens was added to each well except the well for blank. The plate was sealed with slip and incubated at 37 °C for 60 min and then the contents

Ab COMB ELISA kit. The 50% inhibitory concentration (IC50) of Raltegravir was calculated with GraphPad Prism. 8.

Also, Darunavir (DRV) was used as a standard control to assess the effectiveness of the CRISPR/Cas9-no NLS system in inhibiting HIV-1 replication. For this purpose, serial 2-fold dilutions from 250 to 1.95 μmol were used to assess the IC50, and 2-fold dilutions from 125 to 0.95 nmol of Darunavir (PREZISTA, Leapchem) were employed to evaluate the CC50, and IC50, respectively (Figure 5).

Figure 4. Optimization of HEK-293 T cell transfection with EF1-GFP control plasmid (A), pBLO_Cas9_noNLS Mock plasmid (B), and pBLO_Cas9_noNLS_HIV-1_3 gRNA plasmid (C). Left-hand pictures are the visualization of cells under native microscopic light, and right-hand pictures are the visualization of cells under the fluorescent microscope. This image shows GFP in green and mCherry in red. Images captured at 10X magnification. The scale bar represents 100 μm.
of the wells were discarded and washed for five cycles. In the next step, 200 μl of conjugate-1 was added to the wells, and the plate was incubated for 30 min at 37°C. The contents of the wells were discarded and washed three times. Two hundred μl of conjugate-2 was added, and after incubation for 30 min at 37°C, wells were washed five times. Fifty μl of TMB substrate Soln A and then 50 μl of TMB substrate Soln B were added to the wells and mixed carefully. The plate was sealed with a black cover and incubated for 30 min at room temperature. Finally, by adding 100 μl of stop solution to all wells, the wells’ absorption was measured with a Synergy H1 Hybrid Multi-Mode Reader (BioTek) at a wavelength of 450 nm.

5.9. Real time PCR

The Real-time PCR test was performed with the HIV-1 Real-TM Quant Dx/Sacace kit. According to the manufacturer's protocol, ten microliters of internal control (HIV-IC-L), a recombinant RNA-containing structure, were added to the sample microtubes. The primers and probes used in this kit are designed for the HIV-1 polymerase target (pol) gene region, which is present in the pseudotyped HIV-1 gene sequence. RNA extraction was performed with a High Pure Viral Nucleic Acid Kit (Roche). Two-hundred μl of the sample was used for RNA extraction according to the procedure. Fifty μl of extracted RNA from samples was added into 0.2
ml vial containing lyophilized amplification reagents and transferred in a Rotor-GeneTM 6000/Q thermocycler. Real-time PCR was performed according to the temperature profile, including one cycle, 50°C for 30 min; 1 cycle, 95°C for 15 min; 5 cycles, 95°C for 20s, 52°C for 30s, and 72°C for 30s; 40 cycles, 95°C for 30s, 52°C for 40s, and 72°C for 30 s. After real-time PCR assay, target Ct and internal control Ct were obtained for all specimens and the copy numbers of the pseudotyped HIV-1 were determined.

5.10. Statistical tests

Statistical analysis was performed by GraphPad Prism 8 software. Reported values are shown as mean and standard deviation (Mean ± SD). A two-way ANOVA and Tukey’s multiple comparisons test were adopted to compare the obtained values. The difference between the mean values in the two different groups was considered statistically significant when the P-value was less than 0.05.

Declarations

Author contribution statement

Ali Salimi-Jeda: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Maryam Esghaei; Asghar Abdoli: Conceived and designed the experiments; Analyzed and interpreted the data.
Hossein Keyvani; Farah Bokharaei-Salim; Ali Teimoori: Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supp. material/referenced in article.

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