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Research article

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

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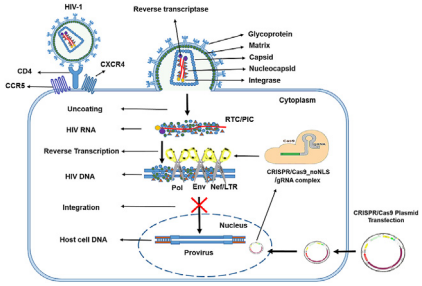
GRAPHICAL ABSTRACT



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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 pro-virus. 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

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virus transduction. Although these results revealed the potential of the CRISPR/Cas9-no NLS nuclease system as a prophylactic strategy against HIV-1 infections, due to inefficient impairments of HIV-1 DNA, further studies are required to enhance its effectiveness and application in clinical practice.

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 [12] may cause several side effects such as cerebral vascular toxicity [13], hepatotoxicity, cardiovascular disorder, chronic inflammation, and metabolic complications [14, 15].

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 [18, 19]. Therefore, the intention of genome editing that can specifically target the provirus has increased and shown promising results [20].

There are four main nucleases for genome editing, including ZFN (Zinc finger nucleases), TALEN (Transcription activator-like effector nuclease), meganucleases, and the CRISPR/Cas9 system. CRISPR/Cas9-nuclease system surpasses other nuclease-based systems due to the simplicity of its design, specificity, and versatility for genome engineering purposes. Meanwhile, unlike the other three methods, which are protein-guided to a specific DNA sequence, the CRISPR/Cas9 system is RNA-guided [21, 22, 23]. The term CRISPR/Cas is derived from clustered regularly interspaced short palindromic repeats (CRISPR) and associated proteins (Cas) found in the Streptococcus pyogenes genome [24]. The CRISPR/Cas system promotes resistance to bacteriophages in bacteria and acts as an acquired immunity against bacterial viruses by creating site-specific DNA double-stranded breaks (DSBs) [25, 26].

The CRISPR/Cas9 system requires two components of Cas9 endonuclease and a transcript fusion of crRNA - Tracer RNA, which acts as a gRNA or sgRNA (single guide RNA) and activates it by binding to the inactive Cas9 complex [27, 28]. In the CRISPR/Cas9 system, sgRNA targets DNA and acts as a scaffold for the Cas9 protein. Specific binding to target DNA and cleavage by Cas9 requires 20 nucleotides of gRNA and three basepairs of PAM (Protospacer Adjacent Motif) located upstream of the target DNA sequence, respectively [29, 30].

CRISPR/Cas9-nuclease genome editing technology has many research and therapeutic applications, including CRISPR/Cas9-guided Chromatin immunoprecipitation (ChIP) [31], activation or inhibition of gene transcription [32], epigenetic modification [33], cancer immunotherapy [34], gene therapy [35], and treatment of latent viral diseases [36, 37].

There are different strategies to target HIV-1 by the CRISPR/Cas9 system as a prophylactic or therapeutic approach. The first prophylactic method to inhibit HIV-1 replication is to target cellular factors involved in virus replication such as CCR5 and CXCR4 coreceptor genes by CRISPR system and prevent the virus from the cell entry [38, 39]. It has been observed that in individuals who homozygously have a deletion mutation of 32 bp in this gene (CCR5Δ32), HIV-1 cannot replicate [40]. Although the simultaneous removal of CCR5 and CXCR4 coreceptor genes as a prophylactic or therapeutic strategy through transplantation of cells edited in *invitro* and *invivo* conditions has been successful [41, 42, 43], the use of this strategy should not violate ethical principles [44].

Upon membrane fusion and entry of HIV-1 into the cytoplasm, the viral RNA in the reverse transcription complex (RTC) is reverse transcribed into DNA and then transferred to the nucleus in the pre-integration complex (PIC) for integration of viral DNA into the host cell chromosomes [45, 46]. Another prophylactic strategy by CRISPR/-Cas9 system inhibits the integration of viral DNA enclosed in a PIC into the cellular genome [45, 47]. According to the studies, reverse transcription of the HIV-1 RNA genome to complementary DNA (cDNA) occurs 2–6 h post-infection (h p.i.) [48], and transferring of PIC from the cytoplasm to the nucleus occurs 10–12.5 h after infection [49]. Also, integration of the HIV-1 cDNA into the host cell genome occurs ~19 h p.i., and virions begin to be released between 30 and 40 h p.i [50].

In the therapeutic approach, using the CRISPR/Cas9 system in the cell nucleus, a specific sequence of the integrated viral genome is double-strand breaks (DSBs) by Cas9 endonuclease. Following this, the activation of the cell repair systems, including homologous recombination (HR) or non-homologous end-joining (NHEJ), causes InDel (insertion/deletion) mutations [51], which consequently inhibits the HIV-1 replication by incomplete protein expression or inactivation of the promoter [47].

Since the use of the CRISPR/Cas9 system as a therapeutic method to knock out integrated HIV-1 provirus in the host genome has the possibility of off-target effects, and there have also been reports of virus escape from CRISPR/Cas9 editing with a single guide RNA due to the introduced mutations by DNA repair process at the site of DNA-cleavage [52], we intend to use CRISPR/Cas9-no NLS system as a prophylactic approach. In this study, by using the CRISPR/Cas9-no NLS system, we aim to target the three uncovered regions of HIV-1 double-stranded DNA enclosed in the PIC before integration into the cell chromosome. For this purpose, by designing a plasmid vector expressing the Cas9 endonuclease and gRNA cassette for three regions, including the *pol*, *env*, and *nef*/LTR, we targeted the HIV-1 DNA. We then evaluated the replication of HIV-1 by testing the HIV-1 p24 shedding and viral RNA load in a time-dependent manner. Thus, by degrading HIV-1 DNA, it loses the ability to integrate

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 Daronavir 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 Daronavir and Raltegravir at three times of 48 h

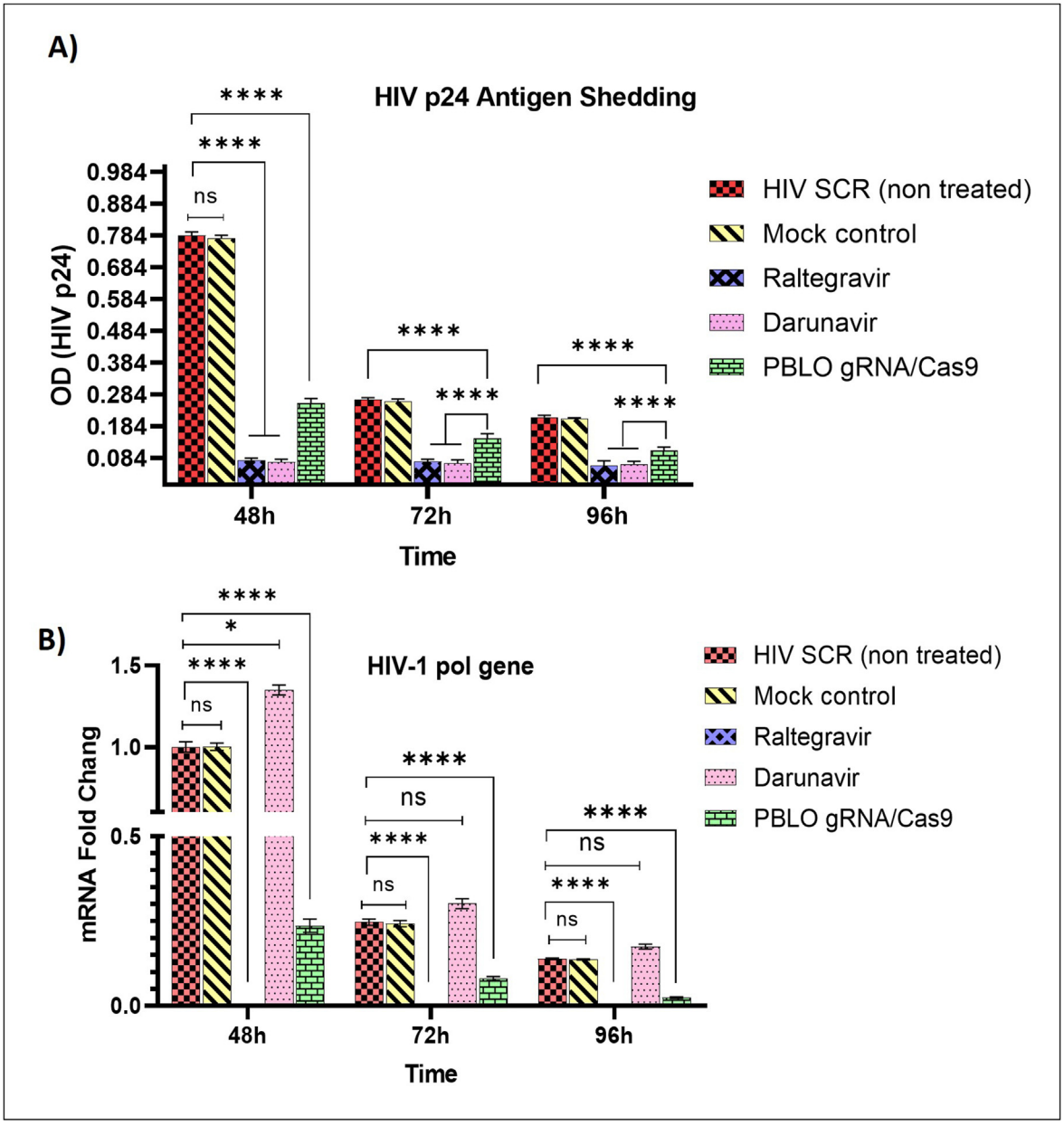

Figure 1. The Effect of multiplex gRNA/Cas9 (without NLS) on HIV-1 **p24 antigen shedding and** HIV-1 **RNA expression. The graph of means of the optical density (OD) for** HIV-1 **p24 antigen of different groups according to the time of sample harvest at 48, 72, and 96 h post-infection (h p.i.) (A).** 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).

(P-Value = 0.97), 72 (P-Value = 0.96), and 96 h p.i. (P-Value = 0.98) (Figure 1A).

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 1.74×10^6 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 pseudotyped 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 LTR1 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 *env3* (from nucleotide 7615–8370), *pol4* (nucleotide 4150–4393), and *nef*/LTR 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 transferring 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, nuclear localization signals (NLS), with importin-α and importin-β [55], some studies have shown that Cas9 protein without NLS can only enter the nucleus in dividing human cells during the nuclear membrane breaks down [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 Lijuan Yin 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 crisper 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 provirus 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 provirus 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 mRNA 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 plasmid 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, CXCR4, 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-no NLS 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 (7×10^5 cell) with 4 mg of psPAX2 (Addgene plasmid # 12260, Contains HIV-1 *gag* and *pol* gene regions) and pMD2.G (Addgene plasmid # 12259, expressing the G protein of the VSV virus) vectors complexed 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 45000 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 2×10^8 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 immune-sorbent assay). According to the Optical Density (OD) of HIV-1 p24 antigen at 72 h after transduction of different MOIs of pseudotyped HIV-1to 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 III (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, DH5α) 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)**	CCAGCTGTGATAAATGTCAGCTA	-	0-1-2-3-4 0-0-6-16-113	135	CRISPOR-Tefor	78
			4	35	off-Spotter	
			5	243		
>NC_001802.1:5771–8341 HIV-1 **env3 (7171–7853)**	CTCAATGACGCTGACGGTACAGG	+	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)**	CCGCCTAGCATTTCATCACGTGG	+	0-1-2-3-4 0-0-0-1-32	33	CRISPOR-Tefor	93
			4	8	off-Spotter	
			5	37		

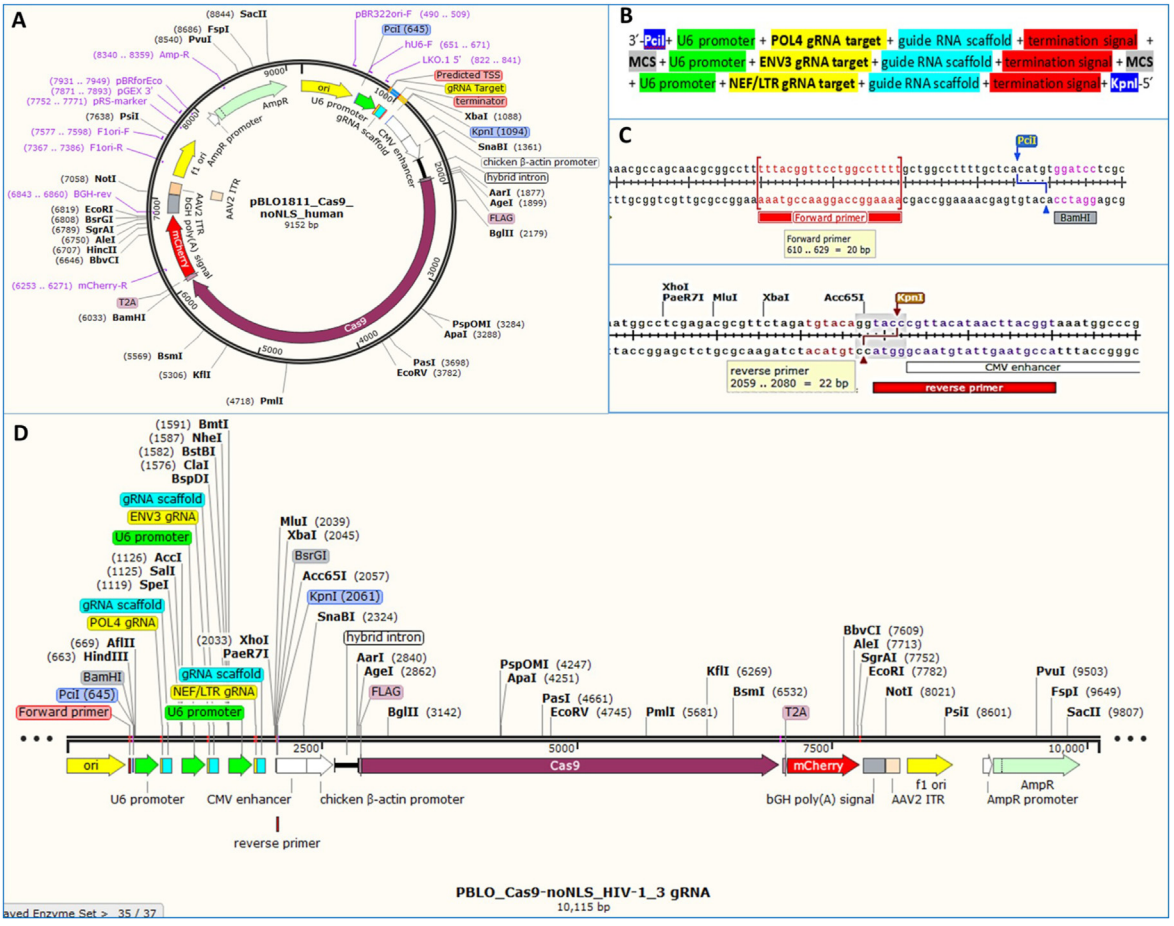

Figure 2. Circular DNA map of PBLO_ noNLS_Cas9 Plasmid (A). Components of the multiplex gRNA cassette (B). Cloning primers (C). Linear DNA map of pBLO_Cas9_noNLS_HIV-1_3gRNA Plasmid (10115 bp) (D). Various segments, including cas9, mCherry, gRNA cassette, and PciI and KpnI restriction enzyme sites have 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

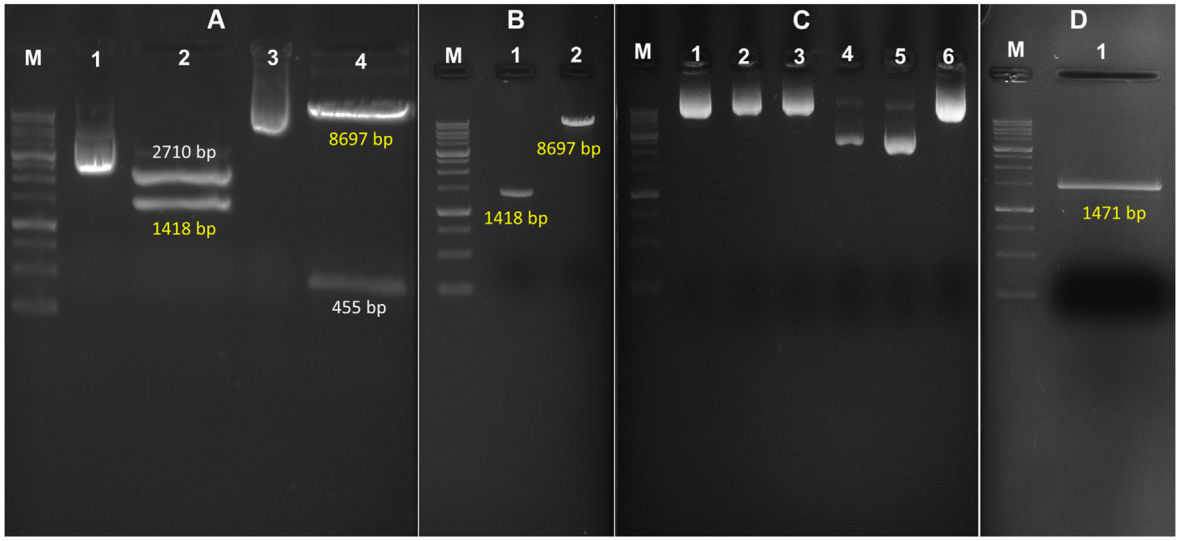

Figure 3. A. The image of Gel electrophoresis of PUC-57 plasmid containing synthesized multiplex gRNA cassette (4128 bp) and PBLO_noNLS_Cas9 plasmid (9152 bp) before digestion by restriction enzymes (Lane 1 and 3) and after digestion (lane 2 and 4). Lane 2 contains the PUC-57 plasmid DNA (2710 bp) DNA fragments and the synthesized multiplex gRNA cassette (1418 bp). Lane 4 also relates to the linear PBLO_noNLS_Cas9 plasmid DNA (8697 bp) and the own gRNA cassette (455 bp) fragment. Lane M is the DNA marker (1Kb DNA Ladder). **B. The gel electrophoresis image of multiplex gRNA cassette and PBLO_noNLS_Cas9 plasmid DNA fragments.** The 1481 bp multiplex gRNA cassette and 8697 bp PBLO_noNLS_Cas9 plasmid DNA were cut from the gel. After DNA extraction, electrophoresis was performed on 1% agarose gel to confirm its extraction and use for the ligation step. Lane M is the DNA marker (1Kb DNA Ladder). **C. Gel electrophoresis image of plasmids is extracted from six colonies selected from LB agar medium.** These plasmids were generated by cloning of multiplex gRNA cassette and PBLO_noNLS_Cas9 plasmid DNA. The plasmids of lanes 4 and 5 have lower bands compared to other plasmids due to incorrect ligation and short DNA length. **D. Gel electrophoresis image of PCR cloning.** Cloning of multiplex gRNA cassette in PBLO_noNLS_Cas9 plasmid was confirmed by 1471 bp amplicon of cloning PCR. Lane M is the DNA marker (1Kb DNA Ladder).

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, 1h 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 were complexed with 0.6 µl of TurboFect and 1 and 1.5 µg of plasmid with 0.8 µl of TurboFact 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 200µL 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 24h 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 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 24h. Forty-eight h after virus transduction, viral p24 antigen shedding was assessed with GB HIV-1 Ag-

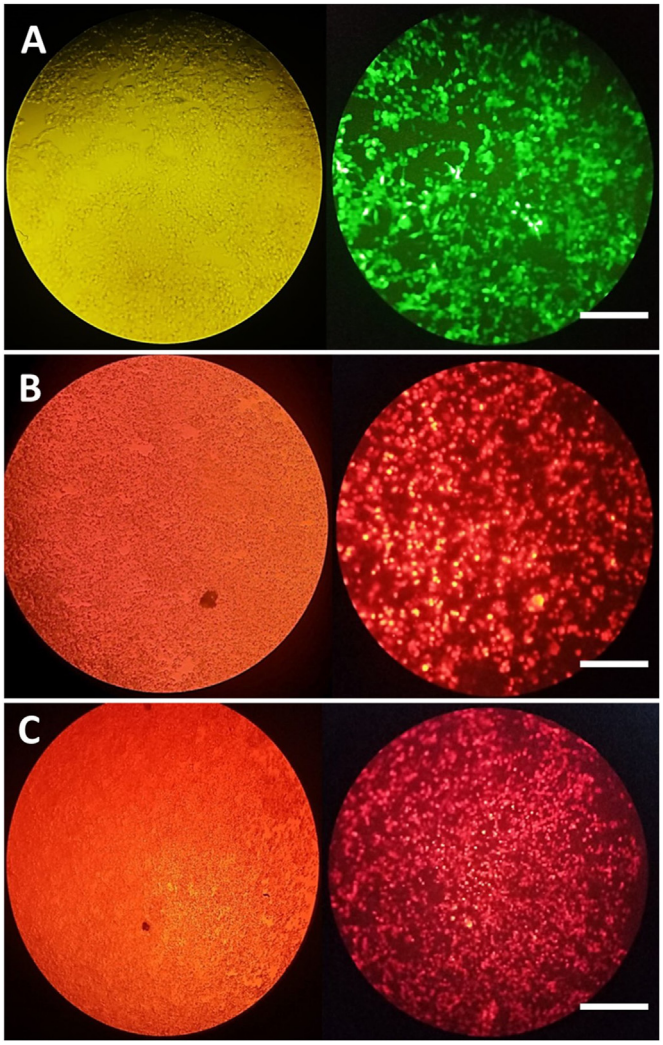

Figure 4. Optimization of HEK-293 T cell transfection with EF1-GFP control plasmid (A), pBLO_Cas9_noNLS Mock plasmid (B), and pBLO_Cas9_noNLS_HIV-1_3 gRNA plasmid (C). Left-hand pictures are the visualization of cells under native microscopic light, and right-hand pictures are the visualization of cells under the fluorescent microscope. This image shows GFP in green and mcharry in red. Images captured at 10X magnification. The scale bar represents 100 μm.

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 CC50, and 2-fold dilutions from 125 to 0.95 nmol of Darunavir (PREZISTA, Leapchem) were employed to evaluate the IC50 as mentioned above.

According to the Optical Density (OD) values obtained at 570 nm for blank, cell control, and serial dilutions of Raltegravir, using Graphpad prism.8 data analysis software, a concentration of 198.1 μM as a 50% cytotoxic concentration (CC50) and the concentration of 15.24 nm were determined as the 50% inhibitory concentration (IC50) of Raltegravir for HEK-293 T cells. For Darunavir, 83.92 μM and 5.53 nM were identified as CC50 and IC50, respectively (Figure 5).

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 Daronavir was added to the related wells and incubated at 37 °C for 3 h, then 4×10^5 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_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

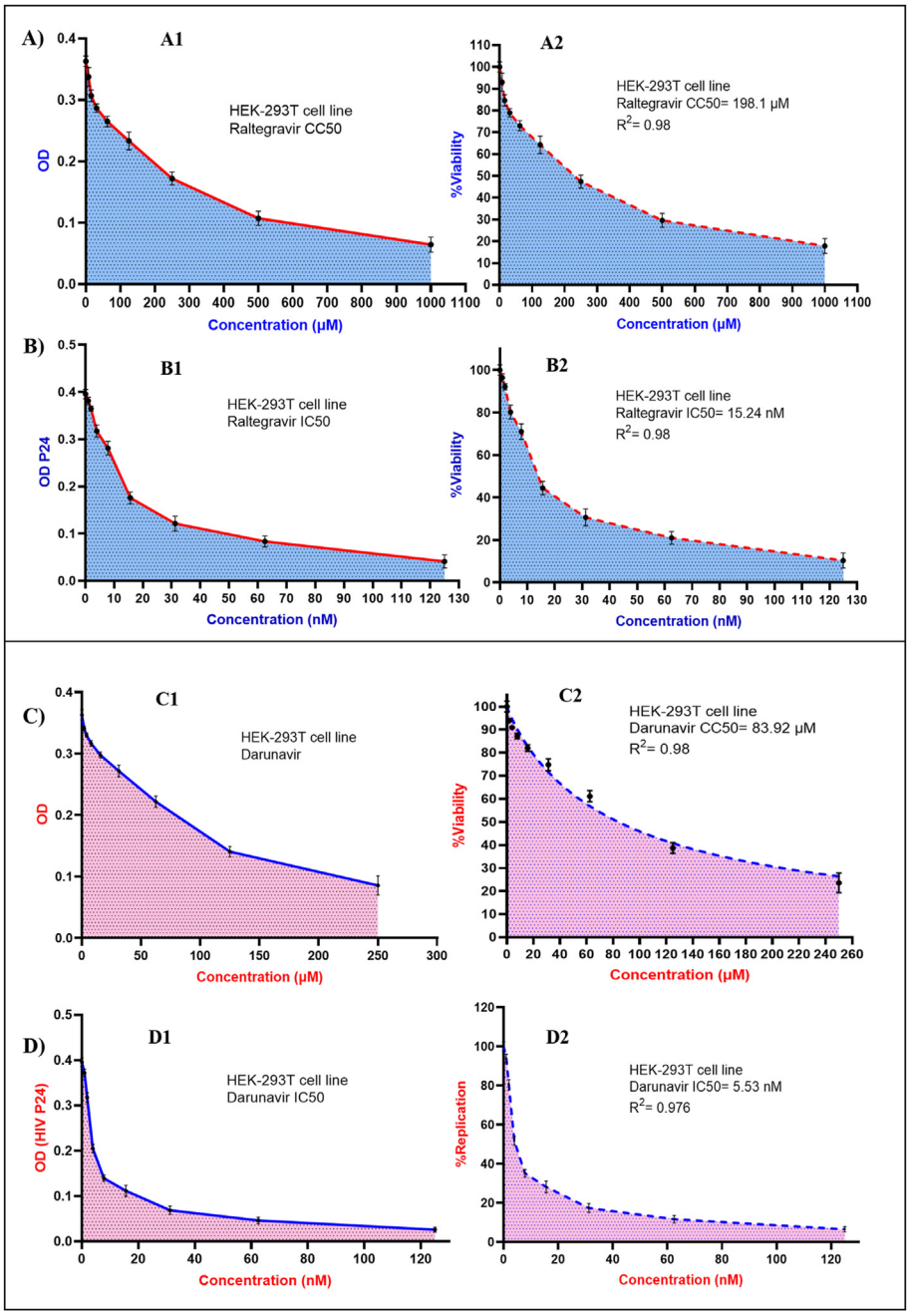

Figure 5. The evaluation of Raltegravir and Darunavir CC50 and IC50 using HEK-293T cell and pseudotyped HIV-1, respectively. CC50 is the cytotoxic concentration of the drug that caused the reduction of viable cells by 50%, and IC50 is the concentration of the drug that resulted in 50% inhibition in HIV-1 infection. Data represent the mean ± SD of three independent experiments performed in triplicate. Optical Density (OD) curve of two-fold dilution of Raltegravir (A1) and Darunavir (C1) in HEK-293T cells. HEK-293T cell viability curve of two-fold dilution of Raltegravir (A2) and Darunavir (C2). CC50 of Raltegravir in HEK-293T cells was determined to be 198.1 μM, and for Darunavir, was calculated 83.92 μM. Optical Density (OD) curve of HIV-1 p24 antigen for two-fold dilution of Raltegravir (B1) and Darunavir (D1) in HEK-293T cells. pseudotyped HIV-1 replication curve at two-fold dilution of Raltegravir (B2) and Darunavir (D2) in HEK-293T cells. The 50% inhibitory concentration (IC50) of HIV-1 was determined 15.24 nM and 5.53 nM for Raltegravir and Darunavir, respectively.

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

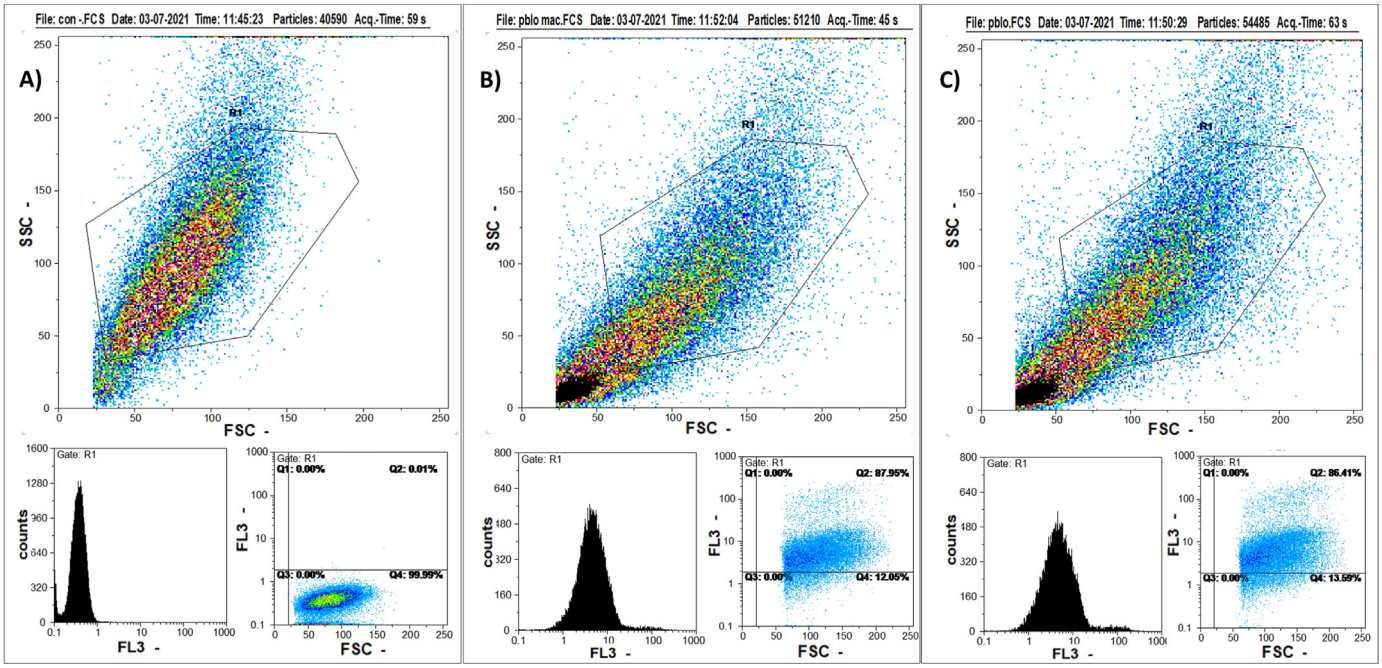

Figure 6. Representative flow cytometry cytogram and histograms of mCherry expression in HEK-293T control cell (A) HEK-293T cells containing MOCK control plasmid (B) and HEK-293T cells containing PBLO_noNLS_Cas9_HIV-1_gRNA plasmid (C) in FL3 channel. The percentage of mCherry positive cells containing PBLO_noNLS_Cas9_HIV-1_3gRNA plasmid was 87.95% and in cells containing plasmid Mock was 86.41%.

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 the 20s, 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.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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