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## ORIGINAL ARTICLE

# Molecular Approach for HIV-1 Replication Inhibition: Assessment of Different siRNAs Targeting Tat and Nef Genes to Effectively Suppress their Expression

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### SUMMARY

***Background:*** **Inhibition of viral genes through siRNA seems to be promising for treatment of complicated viral infections like human immunodeficiency virus (HIV-1). HIV-1 Tat (Trans Activator of Transcription) and Nef (Negative regulatory Factor) proteins are very interesting targets for designing siRNAs.**
***Methods:*** **The effectiveness of suppressing Tat and Nef was investigated using three specific siTATs and three siNEFs. They were used to transfect the developed stable and infected Human Embryonic Kidney cells (HEK293) as an ex-vivo model. Both stable and virus infected HEK293 cells were transfected with each siTAT and siNEF. The inhibitory effect was evaluated using qRT-PCR, western blot analysis, and HIV P24 ELISA.**
***Results:*** **siTAT-100, siTAT-162, and siNEF-136 and at a concentration of 100 nM/mL showed the most inhibitory effect on their target genes.**
***Conclusions:*** **Utilization of more developed molecular inhibition strategies such as RNAi or even a combination of different molecular approaches could be promising to overcome emerging HIV escape mutants.**
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### KEY WORDS

gene silencing, siRNA, stable cell line development, Tat, Nef

### INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is one of the well-known devastating worldwide diseases caused by human immunodeficiency virus-1 (HIV-1) [1,2]. Despite the availability of promising anti-HIV-1 drugs, new molecular approaches such as using RNA interfering (RNAi) or short interfering RNA (siRNA) attempt to specifically inhibit the expression of viral genes [1-8]. HIV-1 trans-activator of transcription (Tat) with molecular weight of 14 KDa is one the most important viral proteins to increase of transcription efficiency by co-operating with cellular transcription machinery. It also promotes the activity of cellular RNA

polymerase II [9-14]. HIV-1 negative factor, with 206 amino acids and approximately 27 Da weight, is an N-myristoylated protein encoded by a single exon that extends into the 3' end of long terminal repeats (LTR) of the viral genome. Nef is also involved in cell apoptosis, down regulation of CD4, MHC- I, and IL-2 expression, inhibition of immunoglobulin class switching, and activation of T-cell [15-21].

Mammalian cell lines, such as human embryonic kidney cells (HEK293), are used to develop stable cell lines with the ability of constitutive expression of a target protein as an essential tool in different studies on structure, function, and inhibition of the expression of proteins of interest [17,18]. The current study was a pilot study aimed at suppressing the expression of HIV-1 Tat and Nef genes using their specific siRNAs, while they were constitutively expressed separately in two developed stable cell lines of HEK293. Also, to complete the assessment, we applied one of the most suppressive siRNA targeting each Tat and Nef to evaluate their inhibition effect on HIV-1 infected HEK293 cells.

## MATERIALS AND METHODS

The sequence of HIV-1 Tat and Nef genes was derived from GenBank with accession numbers: GQ473128.1 and AF324493.2, respectively, and synthesized by Biomatik (Ontario, Canada). The plasmid pEGFP-N1 as a non-viral mammalian expression vector containing CMV promoter and carrying the fluorescent protein gene (EGFP) was purchased from Clonetech Biotechnology (Mountain View, CA, USA). Tat and Nef genes were successfully sub-cloned into pEGFP-N1 separately and confirmed through colony PCR (polymerase chain reaction) using the universal forward and reverse primers (CMV-fwd-primer and EGFP-N-rev-primer) located in multiple cloning site (MCS) of pEGFP-N1.

Human embryonic cells (HEK293) were purchased from NCBI, Pasteur Institute of Iran, and cultured in 5 mL DMEM (Dulbecco's modified eagle medium) high glucose (Gibco™, Thermo Fisher Scientific, USA), 10% fetal bovine serum (FBS) (Gibco™, Thermo Fisher Scientific, USA), 1% L-glutamine, and 1% penicillin/streptomycin (Gibco™, Thermo Fisher Scientific, USA) until reaching a density of 60%. Cell transfection was performed using Turbofect transfection reagent (Thermo Scientific, Lithuania), and transfectants were incubated at 37°C with 5% $CO_2$ for 48 hours. The efficiency of transfection through enhanced green fluorescent protein (eGFP) expression was randomly verified using fluorescent microscopy (data not shown) and flow cytometry (Sysmex, Norderstedt, Germany). To select stably transfected cells, the optimal dose of G418 (1.5 mg/mL) (Sigma- Aldrich) was used for 15 days. Not-transfected cells were used as the negative controls. After two weeks cell viability was verified through cell counting. The mRNA expression of HIV-1 Tat and Nef genes was verified using quantitative real-time (qRT) PCR compared with the housekeeping gene; HPRT (hypoxanthine phosphoribosyl transferase) in stable HEK 293 cells and also after inhibition by transfecting those cells separately with related Tat and Nef specific siRNAs.

Briefly, cellular RNA was extracted using High Pure RNA Isolation Kit (Roche, Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. cDNA was synthesized using cDNA synthesis kit, (Yekta Tajhiz Azma YT4500, Iran) in a 20 µL reaction mixture according to the manufacturer's instruction. RT-PCR was performed using the specific primers of Tat-forward: 5'-CAAAAGGCTTAGGCATCTCC-3', Tat-reverse: 5'-CTCCACCTTCTCCTTCGATT-3', Nef-forward: 5'-CACTGACCTTTGGATGGTGCT-3', Nef-reverse: 5'-CGGCTGTCAAACCTCCACTC-3', HPRT-forward: 5'- GCTATAAATTCTTTGCTGACCTGCTG-3', and HPRT-reverse: 5'-AATTACTTTTATGTCCCCTGTTGACTGG-3', employing the Tli RNaseH Plus kit, (TaKaRa, USA), according to the manufacturer's instruction. The amplification reactions were performed as follows: initial activation at 95°C for 30 seconds and then 40 cycles of 95°C for 10 seconds and 62°C for 20 seconds followed by an increasing temperature program from 50°C to 99°C every second to verify the product integrity of each gene by DNA melting curve analysis.

Western blot analysis was carried out to evaluate the expression of Tat and Nef proteins in stable cells and after their inhibition by specific siRNAs. A confirmed expressed HCV core+1 protein was used as the positive control. Specific primary anti-HIV-1 Nef antibody with a dilution of 1:10000 (ab42358, Abcam, Cambridge, UK) and rabbit anti-mouse-horseradish peroxidase (HRP) (ab6728, Abcam, Cambridge, UK) as the secondary antibody with a dilution of 1:4,000 were used to identify Nef. To verify Tat, polyclonal anti-HIV-1 Tat antibody (Biotin labeled) (ab43015, Abcam, Cambridge, UK) with a dilution of 1:2,000 and streptavidin horse radish peroxidase (HRP) (ab7403, Abcam, Cambridge, UK) with a dilution of 1:200 as the secondary antibody were used. The bands of proteins were visualized by adding 3,3′,5,5′-tetramethylbenzidine (TMB) chromogenic substrate (Roche Diagnostics, Mannheim, Germany). Three siRNAs targeting Tat gene and three siRNAs targeting Nef gene were designed: siTAT-100 and siTAT-162 (100, 162, and 198 nucleotides from the initial codon of Tat gene) containing sense siTAT-100: CCAAGUUUGUUUCAUAACAAA, anti-sense siTAT-100: UGUUAUGAAACAAACUUGGCA, sense siTAT-162: GCGACGAAGAGCUCCUGAA, anti-sense siTAT-162: UUCAGGAGCUCUUCGUCGC, sense siTAT-198: AGGCCAUCUAUCAAAGCAA, and anti-sense siTAT-198: UUGCUUUGAUAGAUGGCCU. For the Nef gene, siNEF-124, siNEF-286, and siNEF-136 (124, 286, and 136 nucleotides from the initial codon of Nef gene) with sense siNEF-124:

GCAAUCACAAGUAGCAAUA, anti-sense siNEF-124: UAUUGCUACUUGUGAUUGC, sense siNEF-286: GGACUGGAAGGGCUAAUUCA, anti-sense siNEF-286: UGAAUUAGCCCUUCCAGUCC, sense siNEF-136: CAAUACAGCAGCUAACAAUGC, and anti-sense siNEF-136:
AUUGUUAGCUGCUGUAUUGCU, were designed using BLOCK-iT™ RNAi Designer. In addition, a negative scrambled control, NC-siRNA (sense: GCCGAU CUAAUCAGAGUUA, and anti-sense: UAACUCUGA UUAGAUCGGC), was designed and labeled with TAMRA at the 3′ end of the sense strand. All siRNAs were synthesized by Bioneer (South Korea). Each siRNA at three concentrations of 50, 100, and 150 μM/mL was used in triplicate to transfect HEK293 stable cells separately, using transfection reagent Lipofectamine RNAiMAX in Opti-MEM medium following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). After 48 hours, evaluation of inhibitory effect of each specific siRNA on HIV-1 replication by measuring Tat and Nef expression, was carried out by qRT-PCR and western blot as described previously.
According to the results of si-TATs and si-NEFs on suppressing their target genes in stable HEK293 cells, those siRNAs for each gene at concentration of 100 μM/mL were chosen to assess their inhibitory effects on Tat and Nef expression in single cycle replication (SCR) mutant of HIV-1 infected HEK293 cells as an ex-vivo model [24,25]. This HIV-1 mutant is constructed from three plasmids, psPAX2, pMD2.G, and pmzNL4-3, and contains a mutation into the pol sequence (deletions in RT and IN genes) and has a national patent at the Pasteur Institute of Iran [24,25]. Actually, SCR virions are assembled successfully in their first host cells (for example HEK293) containing functional envelope glycoproteins (ENV) and infect new host cells; however, they are not able to complete viral genome replication in these new infected cells [24-26]. The SCR mutant HIV-1 was produced in HEK293 cells as described previously [24-26]. The resultant VSVG pseudo-viruses were harvested at 24, 48, and 72 hours after infection and evaluated by P24 ELISA test (HIV p24 ELISA Capture P24 ELISA, BioMerieux, Marcy l'Etoile France) [24-28]. They were stored at -80°C to be used for infecting HEK293 cells. Cells were separately transfected with each siTAT (100, 162, 198) and siNEF (124, 136, 286) at a concentration of 100 μM/mL and their inhibitory effect was investigated using P24 ELISA test (HIV-1 p24 ELISA Kit, ab218268, Abcam, Cambridge, UK) according to the manufacturers' instructions.

**Statistical analysis**

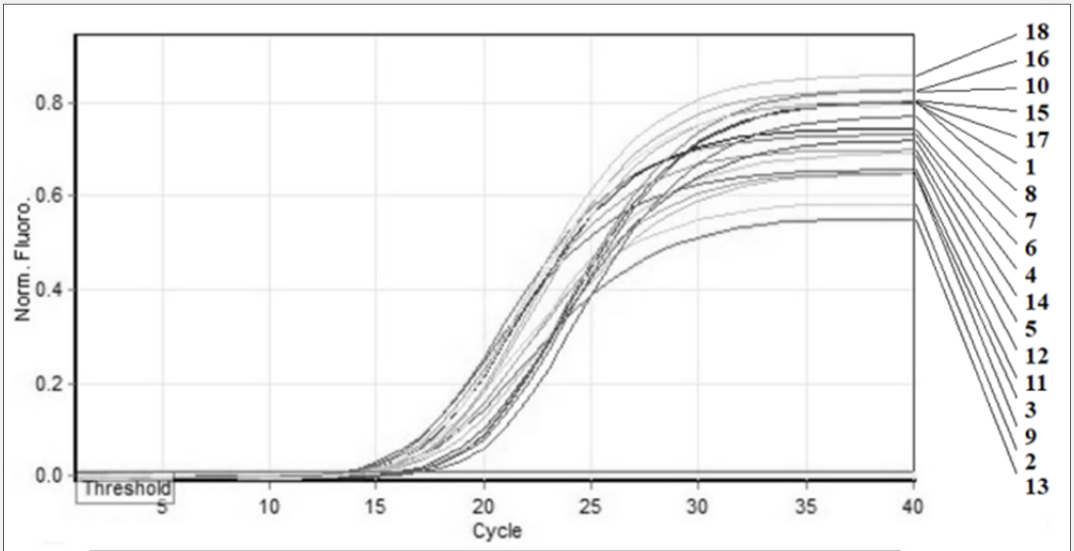
Statistical analysis was conducted using the one-way ANOVA or paired *t*-test. p-value $\leq 0.05$ was considered as the level of significance. In addition, to evaluate each Tat and Nef gene silencing, ΔΔCt was calculated according to the related Cts for each gene obtained from qRT-PCR.

## RESULTS

HEK293 cells were separately transfected with two recombinant vectors, pEGFP-N1-tat and pEGFP-N1-nef, and the results of transfections were analysed using fluorescent microscopy and flow cytometry after 48 hours. Transfected HEK293 cells were evaluated for at least two weeks and no significant loss in cell viability was detected.
The inhibitory effect of siRNAs targeting each Tat and Nef was evaluated in stable transfected cells by qRT-PCR and Western blot. In qRT-PCR analysis three different concentrations of each siRNA (50, 100, and 150 μM/mL) were analysed and compared with non-treated cells and scrambled siRNA as negative controls. Also, HPRT expression as the reference gene in siRNA treated and non-treated cells was depicted in Figure 1. The most effective siTAT was identified for siTAT-162 at 100 μM/mL; thus, this concentration was used for further analysis. For the Nef gene, siNEF-136 showed the highest silencing effect on the Nef expression at 100 μM/mL. ΔΔCt for siTAT-162 was calculated at 11%, and for siNEF-136, ΔΔCt was 12% in comparison with HPRT expression. These results showed that both siTAT-162 and siNEF-136 could impair the expression of their target genes significantly.
Using western blot analysis, specific bands of Tat+ eGFP and Nef+eGFP with a molecular weight of approximately 41 and 50 KDa were detected in HEK293 cells that were successfully propagated to the stable cell lines, constitutively expressed Tat and Nef proteins in conjunction with eGFP (molecular weight of 27 KDa). There were no specific bands of Tat and Nef detectable after transfection of related stable cells with siTAT-162 and siNEF-136 and also with siTAT-162 and siNEF-136 simultaneously. The results were shown in Figure 2.
The replication of SCR-HIV virus was inhibited significantly in SCR infected HEK293 cells after being transfected with siTAT-100 and siTAT-162 at a concentration of 100 μM/mL in comparison with siTAT-198 ($p < 0.05$). The results showed that only siNEF-136 at a concentration of 100 μM/mL could supress the replication of SCR virions ($p < 0.05$). Also, SCR-HIV infected cells were transfected with siTAT-162 and siNEF-136 simultaneously (both at a concentration of 50 μM/mL) and the results revealed significant inhibition of virus replication ($p < 0.05$) (Figure 3).

## DISCUSSION

The sequence-specific gene silencing mechanism using RNAi is used as a potent replication inhibitor of many viruses such as HIV-1, and hepatitis B and C viruses (HBV and HCV) [3,24]. Designing siRNAs is the most critical step for specific gene silencing as any mismatches, especially in the antisense strand, could impair the interaction between siRNA and its target RNA [7,8,




| No. | Colour | Name | Ct | |
|---|---|---|---|---|
| 1 | | a | 17.08 | siTAT-100 |
| 2 | | b | 16.21 | siNEF-124 |
| 3 | | c | 13.63 | non-traeted cells as negative control |
| 4 | | d | 13.54 | scrambled siRNA (negative control) |
| 5 | | e | 13.67 | HPRT gene expression in treated cells |
| 6 | | f | 13.92 | HPRT gene expression in non-treated cells |
| 7 | | a | 17.92 | siTAT-162 |
| 8 | | b | 17.32 | siNEF-136 |
| 9 | | c | 14.21 | non-traeted cells as negative control |
| 10 | | d | 14.32 | scrambled siRNA (negative control) |
| 11 | | e | 14.69 | HPRT gene expression in treated cells |
| 12 | | f | 14.81 | HPRT gene expression in non-treated cells |
| 13 | | a | 15.82 | siTAT-198 |
| 14 | | b | 16.63 | siNEF-286 |
| 15 | | c | 14.45 | non-traeted cells as negative control |
| 16 | | d | 14.53 | scrambled siRNA (negative control) |
| 17 | | e | 14.08 | HPRT gene expression in treated cells |
| 18 | | f | 14.58 | HPRT gene expression in non-treated cells |

**Figure 1. The qRT-PCR analysis of three siRNAs targeting HIV-1 Tat and three siRNAs targeting HIV-1 Nef genes.**

**a1, a7, and a13: cells treated with siTAT-100, siTAT-162, and siTAT-198, respectively. b2, b8, and b14: cells treated with siNEF-124, siNEF-136, and siNEF-286, respectively. c3, c9, and c15 show the results of non-treated cells (negative controls); d4, d10, and d16: cells treated with scrambled siRNA (negative controls); e5, e11, and e17: the HPRT gene expression in treated cells; f6, f12, and f18: the HPRT gene expression in non-treated cells. ΔΔCt was calculated for Tat and Nef separately using the formula: ΔΔCt = ΔCt (test) -ΔCt (calibrator).**

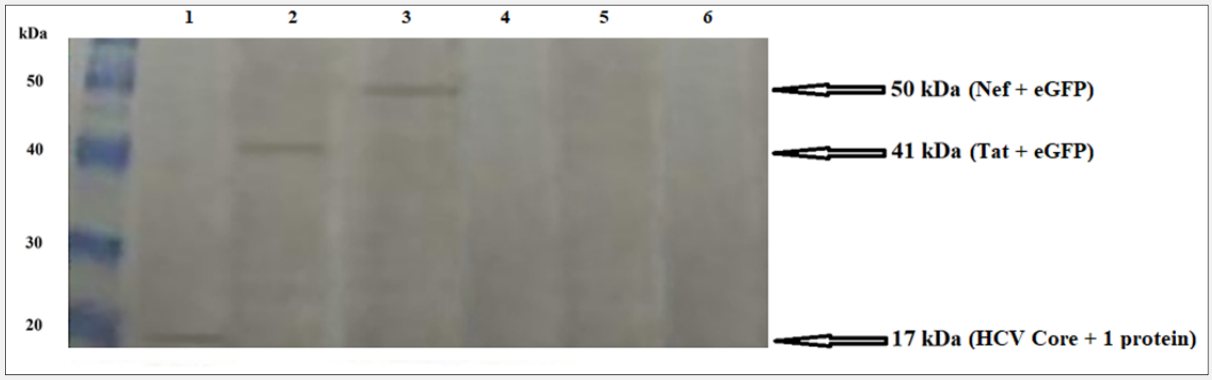



**Figure 2. Western blot analysis to assess HIV-1 Tat and Nef expression.**

**Left to right: Protein ladder, Lane 1: HCV Core+1 protein as a positive control with a molecular weight of 17 KDa identified using anti-His antibody (Abcam, UK). Lane 2: Tat protein + eGFP (27 kDa) with approximately 41 KDa expressed by stable HEK293 cells after being transfected with scrambled siRNA as a negative control and detected using specific primary antibody: polyclonal anti-HIV-1 Tat antibody (Biotin) (ab43015, Abcam, UK) at a dilution of 1:2,000 and streptavidin (HRP) (ab7403, Abcam, UK) at a dilution of 1:200 as the secondary antibody. Lane 3: Nef protein + eGFP with approximately 50 KDa expressed by stable HEK293 cells after transfection with scrambled siRNA as a negative control detected using specific primary anti-HIV-1 Nef antibody at a dilution of 1:10,000 (ab42358, Abcam, UK) and rabbit anti-mouse-HRP (ab6728, Abcam, UK) as the secondary antibody at a dilution of 1:4,000. Lanes 4 and 5: inhibition of Tat and Nef expression in stable HEK293 cells after transfection with siTAT-162 and siNEF-136, respectively. Lane 6: inhibition of Tat and Nef expression in stable HEK293 cells by transfecting with siTAT-162 and siNEF-136 simultaneously.**

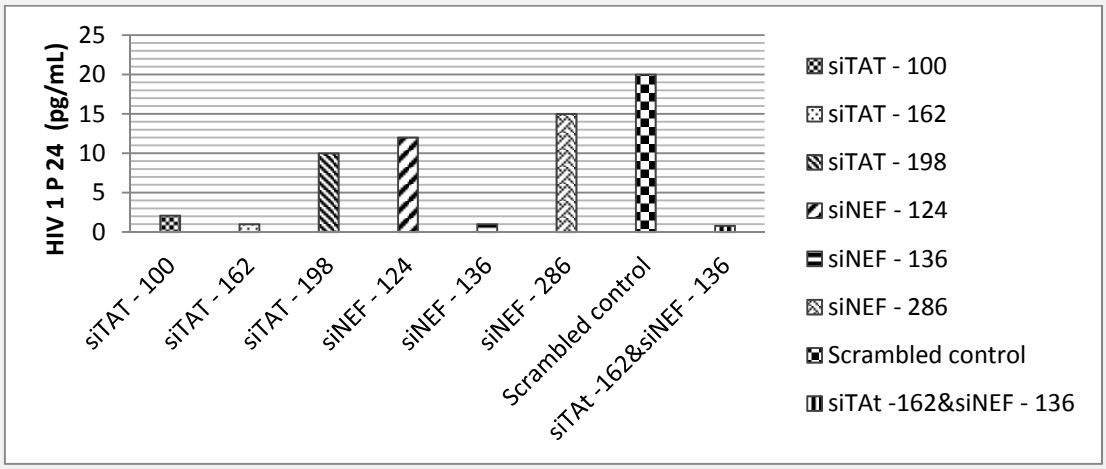



**Figure 3. Replication inhibition assay.**

**Assessment of P24 antigen after three siTAT-100, siTAT-162, siTAT-198 and three siNEF-124, siNEF-136, and siNEF-286 at a concentration of 100 µM/mL were used to separately transfect HEK293 infected with single cycle replicating HIV-1 (SCR). Also, siTAT-162 and siNEF-136 each at a concentration of 50 µM/mL were used simultaneously to transfect SCR-HIV infected HEK293 cells. The inhibitory effect of each siTAT and each siNEF, and the simultaneously used siTAT-162 and siNEF-136 was measured by p24 concentration. To compare the inhibitory results, one-way ANOVA was used and the $p < 0.05$ was considered as significant. The results revealed that siTAT-100, siTAT-162, and siNEF-136 could suppress their target genes significantly. By using siTAT-162 and siNEF-136 simultaneously the inhibitory effect on SCR-HIV replication was higher than was observed in using siTAT-162 and siNEF-136 separately but the difference was not significant.**

28-30]. To overcome viral escape, some investigators used the combination of siRNAs against different viral and cellular targets along with improved designing for the highly conserved region of the target of interest [28, 31]. Developing stable cell lines, such as HEK293 which express the gene(s) of interest constitutively, is one of the accessible tools for evaluating of inhibitory effect of siRNAs [7,28,32,35]. Using non-viral vectors known as transient transfections is a favourite transfection method especially when the main goal is non-constitutive expression of a protein of interest. However, during transient transfection, epigenetic mechanisms could affect transfection outcomes [23,35,37]. HIV-1 is one of the most common health problems worldwide which makes it a very attractive target to manipulate gene investigations [5,6,8,28,34-37]. In this study, the specific suppression of two HIV-1 regulatory genes, Tat and Nef, was considered separately using three different siTAT and three siNEF for each gene. Although HIV is highly mutable virus and there are many reports of viral escape from different types of its inhibitors such as antiviral drugs and different molecular approaches, this problem might be overcome by precise design of a combination of RNA inhibitors targeting different viral genes using improved siRNA designing programmes [2-6,28-30,37]. In this study, six siRNAs were designed and each of the three targeting Tat and Nef genes. The inhibitory effect of each structure was evaluated on both HEK293 cells developed to constitutively express Tat and Nef separately and HEK293 cells which were infected with this special mutant of SCR-HIV [24,25]. Cells were transfected with each siRNA targeting Tat and Nef separately using Lipofectamine RNAiMAX, which is a well-known transfection reagent [6-8,28-30, 37]. The inhibitory effect was evaluated by qRT-PCR, western blot and HIV P24 ELISA. In SCR-HIV infected HEK293 cells all three siTAT and all three siNEF could decrease virus replication. However, their effectiveness was not the same. The siTAT-162 and siNEF-136 at a concentration of 100 μM/mL showed the highest silencing effect on their target genes in stable HEK293 cells and, also, in SCR HIV infected HEK293 cells. siTAT-100, siTAT-162, and siNEF-136 at a concentration of 100 μM/mL showed the best results in replication inhibition in SCR-HIV infected HEK293 cells. The difference between the efficiency of siTAT-100 in inhibition of HIV replication in infected HEK 293 cells in comparison with its suppression effect on its target gene in stable transfected HEK293 cells expressing Tat protein, suggested that evaluation of siRNAs on the HIV replicating system as an ex-vivo system could be more reliable and accurate. However, in this study an especial mutant of HIV-1 with single cycle replication ability was used and as a result the possibility of emerging escape mutants was very low. These results revealed that siTAT-162 and siNEF-136 could be the most suppressing siRNAs on their target genes and as a result on virus replication as they were evaluated in both stable cells developed for constitutively expressing HIV-1 Tat and Nef proteins and in SCR HIV infected cells. Also, according to the results, by using siTAT-162 and siNEF-136 simultaneously the inhibitory effect on SCR-HIV replication was higher than that observed using siTAT-162 and siNEF-136 separately but the difference was not significant.

## CONCLUSION

HIV-1 is a highly mutation prone virus. Emerging of escape mutants against siRNAs, targeting different HIV genes, is the main obstacle for their application in *in vivo* [2-6,22,23]. However, development in designing the more effective strategies for specific gene silencing or editing such as siRNAs or CRISPR/Cas9 and perhaps their combination could be promising to overcome this [38].

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**Declaration of Interest:**
The authors who have taken part in this study declare that they have nothing to disclose regarding any conflict of interest with respect to this manuscript.

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