siRNA-mediated inhibition of the HIV-1 transactivator of transcription

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The HIV-1 tat gene expresses as small regulatory protein plays an essential role in the upregulation of viral transcription and elongation of viral transcripts, and is necessary for viral replication. Because of its importance, anti-viral reagents that specifically target and inhibit tat function, should effectively reduce viral replication. In this study, two anti-tat siRNAs were designed, synthesized, and cloned into the retroviral vector, pSuper.retro.neo+GFP. These siRNA constructs, designated pSRNGsi5860 and pSRNGsi5892 were subsequently analyzed for their ability to down-regulate luciferase activity in a tat-dependent assay. The results indicated that one of these, siRNA5892 reduced luicerase expression in a dose-dependent manner.

Introduction

Infection by the Human Immunodeficiency virus (HIV-1) leads to a gradual loss of immune surveillance due to infection and loss of the CD4+ T_H lymphocyte population [1-3]. The resulting failure of the immune system, concomitant with the rise of opportunistic infections, defines the Acquired Immune Deficiency Syndrome [4]. The current treatment for HIV-1 infection is termed HAART or Highly Active Anti-Retroviral Treatment and is designed to reduce the viral load by targeting two important viral functions: reverse transcription (RT) and virus capsid maturation. Although very effective at reducing the viral load, this treatment is not curative. Importantly, viral resistance to these drugs has already developed [5]. Therefore, it is important that research continue into new ways to inhibit viral replication. Among the possible targets for new anti-viral inhibitors are a number of non-enzymatic functions encoded by the HIV genome to control the overall infection process. Among these genes are *nef, rev, tat, vif, vpr*, and *vpu* [6].

Tat, or trans-activator of transcription, is a viral regulatory gene required for HIV gene expression [7]. The tat protein functions by binding to the stem loop of the trans-activation response region (TAR), which is transcribed within the first 60 nucleotides of all HIV mRNAs [8]. The *tat*/TAR interaction promotes the binding of several host transcription factors, including Cyclin T and CDK9 [9]. Once recruited to the transcription complex, CDK9 hyperphosphorylates the C-terminal domain of RNA Polymerase II, increasing its processivity and allowing efficient elongation of viral transcripts [10]. Because *tat* plays a major role in the viral replication process, anti-viral reagents targeting its function should inhibit HIV replication.

One such group of reagents takes advantage of RNA interference. RNAi is a naturally occurring cellular process that is initiated by small, double-stranded RNA molecules that trigger degradation of homologous mRNAs and lead to gene silencing [11-13]. The RNAi pathway is initiated by endogenous or exogenous double-stranded RNAs that are acted upon by Dicer, a member of the RNase III family of proteins [14]. Dicer cleaves double-stranded RNAs into small, ~22 bp siRNA fragments, one strand of which associates with the Argonaute protein [15]. The siRNA fragment, in association with Argonaute, binds to the target mRNA via Watson-Crick base pairing and initiates formation of the RISC complex [16], which carries out silencing through a number of mechanisms including target RNA degradation. It has been shown that siRNAs can be introduced into cells exogenously and have been used to specifically silence genes in cell culture and *in vivo* [17-19]. There are several advantages to the use of siRNAs to silence gene expression including (i) silencing can be mediated by relatively low levels of siRNA expression in target cells, (ii) silencing is target mRNA specific, (iii) siRNAs are stable in the cell, and (iv) siRNAs are not likely to induce interferon responses [20, 21].

This paper describes the design, cloning, and initial testing of two siRNAs targeted to HIV-1 *tat* mRNA. These siRNA constructs, designated si5860 and si5892 to denote their target sequence within the HIV-1 genome, were cloned into the retroviral vector, pSuper.retro.neo+GFP. This unique vector was chosen because it was developed to specifically express siRNAs from the RNA Polymerase III H1 promoter. Initial testing of these reagents in a transient *tat*-dependent luciferase assay indicated that the activity of one, si5892, acted in a dose dependent manner to decrease HIV *tat* function.

Materials and Methods

Oligonucleotides and cloning

The *tat* RNA sequence of the HIV-1 genomic clone NL4-3 (Accession number M19921) was analysed for the presence of suitable 19 nucleotide siRNA sites. This analysis identified two such sequences located at nucleotides 5860-5878 and 5892-5910 (Fig. 1A). These sequences were used to generate two siRNA constructs designated si5860 and si5892 (Fig. 1B). Two terminal restriction sites, BglII and HindIII were added, and the two complementary strands of each siRNA construct were synthesized (si5860 Upper 5' GAT CCC CTG G AA GCA

TCC AG GA AGT CTT CAA GAG AGA CTT CCT GGA TGC TT C CAT TTT TA 3', si5860 Lower 5' AGC TTA AAA ATG GAA GCA TCC AGG AAG TCT CTC TTG AAG ACT TCC TGG ATG CTT CCA GGG 3', si5892 Upper 5'GAT CCC CTT GTA CCA ATT GCT ATT GTT TCA AGA GAA CAA TAG CAA TTG GTA CAA TTT TTA 3' and si5892 Lower 5'AGC TTA AAA ATT GTA CCA ATT GCT ATT GTT CTC TTG AAA CAA TAG CAA TTG GTA CAA GGG 3').

A. HIV-1 Tat Exon One

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5830        5840        5850        5860
atggagccag tagatcctag actagagccc tggaagcatc

5870        5880        5890        5900
caggaagtca gcctaaaact gcttgtacca attgctattg

5910        5920        5930        5940
taaaaagtgt tgctttcatt gccaagtttg tttcatgaca

5950        5960        5970        5980
aaagccttag gcatctccta tggcaggaag aagcggagac

5990        6000        6010        6020
agcgacgaag agctcatcag aacagtcaga ctcatcaagc

6030        6040
ttctctatca aagcag
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B. Anti-tat si5860 Design

Sense sequence Hairpin Antisense sequence

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TGGAAGCATCCAGGAAGTCttcaagagaGACTTCCTGGATGCTTCCA
||||||||||||||||||||||||||||||||||||||||||||||
ACCTTCGTAGGTCCTTCAGaagttctctCTGAAGGACCTACGAAGGT
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Figure 1. HIV-1 *tat* sequence and siRNA design. (A) The HIV-1 NL4-3 *tat* exon 1 provirus sequence was analysed for potential siRNA target sites. This analysis revealed two regions (5860-5878 and 5892-5910) that were used to create siRNA constructs. (B) Using a published model for siRNA design (Oligoengine), each 19 nucleotide target sequence was used to produce a double-stranded DNA construct. For this the *tat* target sequence (sense) and its inverted complementary sequence (antisense) were connected by a short hairpin sequence. BglII and HindIII sites (not shown) were added to each end of the construct to facilitate cloning into the pSuper.retro.neo+GFP vector. The siRNA shown is targeted to the *tat* RNA sequence at 5860. A second siRNA, si5892 was similarly designed. The sequence located at 6010-6028 was scrambled and used to generate the control siRNA construct, pSRNGsiCtrl.

Each upper and lower oligonucleotide set was combined in 50 µl Annealing Buffer (100mM NaCl, 50mM HEPES buffer (pH 7.4)) and cooled slowly from 90˚C to 4˚C. Each of the resulting siRNA constructs was cloned into the BglII and HindIII sites of the retroviral vector pSuper.retro.neo+GFP (Oligoengine), which has been shown to effectively express siRNAs from an internal RNA Polymerase III H1 promoter [22]. In preparation for cloning each siRNA, the pSuper plasmid was digested with BglII and HindIII. The digested vector fragment was subsequently isolated by gel electrophoresis. Ligation reactions were carried out using T4 DNA Ligase (Promega) at 4ºC overnight. The ligated plasmids were transformed into SURE competent cells (Stratagene) and the resulting colonies were analysed for siRNA presence. For this, miniprep DNA [23] was screened by PCR using a plasmid-specific primer pair that framed the cloning site. Positive clones were indicated by the presence of a 499 bp fragment. Correct siRNA insertion into the resulting plasmids, pSRNGsi5860 and pSRNGsi5892, was verified by sequencing.

Cell lines and transfections

293T cells were grown in standard medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin. The cells were maintained in a humidified 37°C incubator with 5% CO_2. All transfections were done by calcium phosphate precipitation based on the method of Chen and Okayama [24]. Briefly, 293T cells were plated to 50-70% confluency 24 hours prior to transfection. The cells were transiently transfected with plasmid DNA in 125 mM $CaCl_2$, and 2X BBS (280 mM NaCl/ 1.5 mM Na_2HPO_4/ 50 mM N-,N-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid) (pH 7.05)). The plasmid mixture was incubated at room temperature for approximately 10 minutes, and the entire volume was added to the culture medium. The medium was replaced 24 hours post-transfection, and the cells were incubated for a further 24 hours. Forty-eight hours post-transfection, the cells were assayed for siRNA activity.

siRNA-mediated anti-*tat* activity

Anti-*tat* siRNA activity was accessed in transiently transfected 293T cells expressing the luciferase reporter gene from the HIV-1 genomic clone pNL4-3.Luc.R$^-$.E$^-$ [25, 26], which is replication incompetent due to two frameshifts that rendered the clone *env*$^-$ and *vpr*$^-$. In addition, the firefly luciferase gene was inserted into the pNL4-3 *nef* gene. In this plasmid, luciferase expression is driven by the HIV-1 promoter and is therefore *tat*-dependent. To determine the anti-*tat* activity of the cloned siRNAs, 293T cells were plated, $1x10^6$ per well, in 60 mm dishes. After 24 hours, the cells were transfected with 2 µg pNL4-3.Luc.R$^-$.E$^-$ and either 2 µg or 4 µg of pSRNGsi5860, pSRNGsi5892, or pSRNGsiCtrl, a previously cloned control siRNA targeted to the scrambled HIV-1 *tat* sequence located at 6010-6028 (Fig. 1A). These transfection mixtures resulted in a 1:1 or 1:2 HIV-1 to siRNA plasmid ratio allowing dose-dependent siRNA activity to be measured. Transfection efficiency was analysed 48 hours post-transfection by observing GFP expression using an Olympus CKX41 inverted microscope with epifluorescence.

Luciferase activity was analysed using the Steady-Glo Luciferase kit (Promega). Briefly, 48 hours post-transfection, the medium was removed from the transfected cells and replaced with 1.1 ml Glo Lysis buffer. Each plate was rocked briefly to ensure complete coverage of the lysis buffer and then incubated at room temperature for five minutes. The cell lysate was centrifuged to remove cell debris and aliquoted into multiple 1.5 ml tubes. Protein samples were stored at -80°C until analysed. The protein concentration of each sample was determined using a BCA protein assay (Pierce), and luciferase activity was measured in 50 µg total protein.

The assay was conducted in a 96-well format and included three measurements of each sample taken from two independent experiments. Luciferase activity was reported in relative light units (RLU) as measured using a Genios luminometer (Tecan).

Results

The HIV-1 NL4-3 *tat* genomic sequence was analysed for the presence of potential siRNA target sites. Two sites were identified and used to generate siRNA constructs. The sense and antisense strand corresponding to each siRNA was synthesized, annealed, and cloned into the retroviral vector, pSuper.retro.neo+GFP. PCR screening was used to identify siRNA-positive clones that were subsequently sequenced to verify insertion of the specific siRNA construct. The resulting plasmids were designated pSRNGsi5860 and pSRNGsi5892. Successful cloning of si5860 and si5892 placed each under control of the RNA Polymerase III H1 promoter (Fig. 2). In addition, each siRNA vector expresses neomycin phosphotransferase and the green fluorescent protein (GFP) from a single transcript separated by an internal ribosome entry site and under the control of the phosphoglycerokinase promoter (Fig. 2).

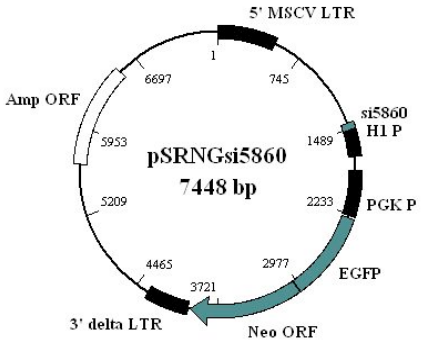

Figure 2. Generation of anti-tat siRNA retroviral vectors. Two siRNAs designed to target the HIV-1 *tat* mRNA at nucleotides 5860-5878 and 5892-5910 were synthesized and cloned into the retroviral vector, pSuper.retro.neo+GFP. The pSRNGsi5860 plasmid map is shown.

To determine the ability of the constructs to inhibit *tat* expression, each siRNA-expression plasmid was co-transfected into 293T cells along with the HIV-1 genomic clone, pNL4-3.Luc.R$^-$.E$^-$. In this plasmid, luciferase expression is under the control of the HIV promoter/enhancer and is therefore *tat*-dependent. For this assay 293T cells were transiently co-transfected with 2 µg of pNL4-3.Luc.R$^-$.E$^-$ and either 2 µg or 4 µg of pSRNGsi5960, pSRNGsi5892, or pSRNGsiCtrl. Therefore the plasmid ratio of HIV-1 to siRNA was either 1:1 or 1:2, allowing a determination to be made regarding any dose dependency of the siRNA constructs. Transfection efficiency, which was evaluated visually by observing GFP expression, suggested that reporter gene expression was higher in the 1:2 ratio transfections (Fig.3 B, D, and F) as compared to the 1:1 ratio transfections (Fig. 3 A, C, and E).

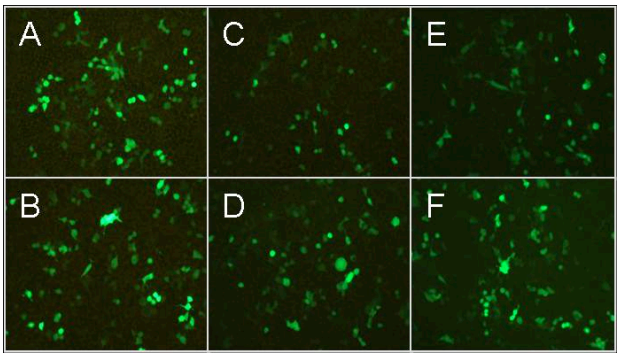

Figure 3. Analysis of transfection efficiency. 293T cells were transiently transfected with 2 µg pNL4-3.Luc.R$^-$E$^-$ and either 2 µg or 4 µg of each siRNA-expression plasmid. (A, B). 2 µg and 4 µg pSRNGsiCtrl. (C, D). 2 µg and 4 µg pSRNGsi5860. (E, F). 2 µg and 4 µg pSRNGsi5892. Forty-eight hours post-transfection, cells were observed for GFP expression as an indication of transfection efficiency. This analysis indicated efficient transfection and suggested increased GFP expression in cells transfected at the 1:2 plasmid ratio.

To measure luciferase activity, transfected cells were lysed to obtain total cytoplasmic protein. Six 50 µg total protein samples, three samples taken from each of two independent experiments, were analysed to determine the relative luciferase activity associated with each HIV-1/siRNA plasmid combination. The results of these tests indicated that si5892 was active against *tat* in a dose dependent manner (Fig. 4, pSRNGsi5892). To this end, 293T cells transfected at a 1:2 HIV-1/5892siRNA plasmid ratio showed a significantly lower luciferase activity than identical cells transfected at a 1:1 HIV-1/5892 siRNA ratio ($p = 0.002$). In comparison, 293T cells transfected at a 1:2 HIV-1/5860siRNA plasmid ratio did not indicate significantly different luciferase activity as compared to the cells transfected at a 1:1 HIV-1/si5860 plasmid ratio. However, luciferase activity at the 1:2 plasmid ratio trended lower than that of the 1:1 plasmid ratio (Fig. 4, pSRNGsi5860). Additionally, the control siRNA (pSRNGsiCtrl) showed no dose-dependent activity.

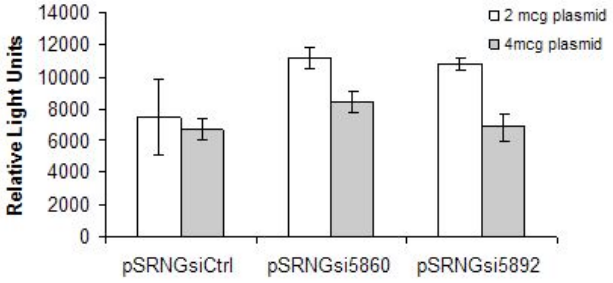

Figure 4. Anti-*tat* siRNA luciferase assay. 293T cells were transiently transfected with 2 µg of the HIV-1 genomic clone pNL4-3.Luc.R$^-$.E$^-$ and either 2 µg or 4 µg of the siRNA-expression plasmids, pSRNGsiCtrl, pSRNGsi5860, or pSRNGsi5892. Luciferase activity was measured 48 hours post-transfection in transfected cell lysates. This analysis indicated a significant dose dependent decrease in luciferase activity as a function of pSRNGsi5892 plasmid levels ($p = 0.002$). No significant differences in luciferase activity were observed with pSRNGsiCtrl or pSRNGsi5860.

Each measurement represents the mean and standard error from six samples taken from two independent experiments.

Conclusions

The primary goal of this project was to design and clone two siRNA constructs targeted to HIV-1 *tat*. Subsequently, an analysis of the HIV-1 *tat* genomic sequence revealed two potential siRNA target sites located at nucleotides 5860-5878 and 5892-5910, respectively (Fig. 1). To determine which of these sequences provides a more attractive target, each of the 19-nucleotide sequences was integrated into a siRNA model based on the Oligoengine design for cloning into their retroviral vector, pSuper.retro.neo+GFP. The resulting DNA oligonucleotides were synthesized and cloned to create the siRNA constructs, pSRNGsi5860 and pSRNGsi5892 (Fig 2.)

The relative anti-*tat* activity of these siRNA constructs was tested in a transient assay in which each was co-transfected along with the HIV-1 genomic clone, pNL4-3.Luc.R$^-$.E$^-$. In this plasmid, luciferase expression is under control of the HIV-1 promoter and is therefore *tat*-dependent. Co-transfections with pNL4-3.Luc.R-.E- and the siRNA plasmids at either a 1:1 or a 1:2 plasmid ratio indicated that one si5892, acted in a dose dependent manner to reduce luciferase activity (Fig. 4). In comparison, si5860, showed no significant ability to reduce luciferase activity in a dose-dependent manner.

Co-transfection of pNL4-3.Luc.R$^-$.E$^-$ with anti-*tat* reagents, such as the siRNA constructs described here, provides a convenient and relatively simple assay to determine the effectiveness of such reagents without the need for more elaborate methods. However, because this is an indirect measure of anti-*tat* activity, follow-up analyses must be made to verify these results. There are a number of factors that may impact the relative activity of the siRNAs in this system. Primarily, the transient nature of the experiment makes it difficult to control plasmid uptake by the transfected cells. It is likely that some cells have altered HIV-1/siRNA plasmid ratios. However, the variation among the samples was relatively small, indicating that this assay is a reasonable system for initial screening of anti-HIV siRNAs. In addition to determining if either siRNA construct act directly on *tat*, it will also be important to test the ability of the pSuper vectors to generate retroviral particles to deliver the siRNA constructs. These assays will allow a more realistic check of siRNA activity and potential efficacy.

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