Development of a therapeutic interfering particle against human immunodeficiency virus

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Author
Franz, Kate McKenzie

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Development of a Therapeutic Interfering Particle against Human Immunodeficiency Virus

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Chemistry

by

Kate McKenzie Franz

Committee in charge:

Leor Weinberger, Chair
Alexander Hoffmann
Scott Rifkin

2011
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Chair

University of California, San Diego

2011
DEDICATION

For L.J.F. and H.J.F.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature Page</td>
<td>iii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>vii</td>
</tr>
<tr>
<td>Abstract of the Thesis</td>
<td>viii</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>4</td>
</tr>
<tr>
<td>Chapter 1: Motivations for the Design of a Therapeutic Interfering Particle</td>
<td>9</td>
</tr>
<tr>
<td>Chapter 2: Design of the Therapeutic Interfering Particle</td>
<td>11</td>
</tr>
<tr>
<td>Chapter 3: HIV-1 Infection Dynamics in the presence of TIP and Quantifying P</td>
<td>14</td>
</tr>
<tr>
<td>Chapter 4: TIP Mobilization</td>
<td>21</td>
</tr>
<tr>
<td>Chapter 5: Discussion</td>
<td>30</td>
</tr>
<tr>
<td>References</td>
<td>33</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1 HIV-1 and TIP gene maps Above is a gene map showing protein-coding regions of HIV-1. Below is a schematic of the HIV-1 regions included in the TIP .................................................................11

Figure 2 HIV-1 infection dynamics in the presence of TIP Populations containing varying levels of TIP integrations were infected with pNL4-3-GFP-IRES-NEF virus. HIV-1 infection progression was monitored over time with flow cytometry .........................................................15

Figure 3 TIP expression (mCherry) in CEM-T4 cells as measured by flow cytometry .................................................................................................16

Figure 4 P measured in cytoplasmic fraction of TIP isoclonal cell lines Three SC cell lines and naïve CEM-T4 cells were infected with HIV-R7/E/GFP .............................................................................18

Figure 5 P calculated from the cytoplasmic and virion fractions of 293T cells 293T cells were transfected with equal weights of pNL4-3 or pNL4-3-GFP-IRES-NEF and a TIP construct ..........................................................20

Figure 6 TIP mobilization by pNL4-3 ..................................................................................................................21

Figure 7 TIP mobilization by pNL4-3-GFP-IRES-NEF .................................................................................18

Figure 8 TIP mobilization by pLAi.2 ..................................................................................................................23

Figure 9 Infection dynamics of pNL4-3-GFP-IRES-NEF virus in the presence of TIP in infected CEM-T4 cells TIP and HIV-1 virus was generated from co-transfected 293T cells ...........................................24

Figure 10 Change in TIP expression (mCherry fluorescence) in CMFDA-stained cells .................................................................26

Figure 11 Change in TIP expression (mCherry fluorescence) in CMFDA-stained cells .................................................................27

Figure 12 Change in TIP expression (mCherry fluorescence) in CMFDA-stained cells .................................................................28

Figure 13 Fraction of unspliced TIP in 293T cytoplasm .................................................................32
LIST OF ABBREVIATIONS

HIV-1: Human Immunodeficiency Virus Type-1

AIDS: Acquired Immunodeficiency Syndrome

ART: Anti-retroviral Therapy

TIP: Therapeutic Interfering Particle

gRNA: genomic RNA

$P$: Fold expression of TIP gRNA over HIV-1 gRNA

VSV-G: Vesicular Stomatitis Virus Glycoprotein

LTR: Long Terminal Repeat

GFP: Green Fluorescent Protein

mCh: mCherry Fluorescent Protein
ABSTRACT OF THE THESIS

Development of a Therapeutic Interfering Particle against Human Immunodeficiency Virus

by

Kate McKenzie Franz

Master of Science in Chemistry
University of California, San Diego, 2011
Leor Weinberger, Chair

Recent theoretical work has proposed a new paradigm of infectious disease intervention through the spread of Therapeutic Interfering Particles (TIPs). Instead of solely focusing on treatment of individuals with a particular disease, TIPs inhibit spread of a pathogen at the population level and decreases prevalence of the pathogen over time. TIPs are degenerate viruses that can only replicate in the presence of wild-type virus, act to inhibit the growth of the wild-type virus, and can be transferred between individuals. They have no protein-coding capability and consist of only the necessary cis-acting elements for replication and mobilization.

We used a rational design approach to synthesize a TIP against Human Immunodeficiency Virus Type-1 (HIV-1). Employing a joint computational-experimental methodology, we quantified the inhibition of
HIV-1 replication by TIP in cell culture and the relative fold-increase of TIP genomic RNA expression compared to HIV-1 genomic RNA expression. Our results show that the presence of TIPs in a cell culture decreases HIV-1 spread by 67% by day 8 post-infection. Increased mobilization of TIP virions and cell-to-cell transfer correlated with greater control of HIV-1 replication within a cell culture. Although we did detect differences in packaging efficiency between TIP genomic RNA and HIV-1 genomic RNA, we proposed this could be compensated for through engineering even higher production values of TIP genomic RNA in the cell than our models originally predicted.
Introduction

Currently, there are an estimated 33 million people living with Human Immunodeficiency Virus Type-1 (HIV-1) and Autoimmune Deficiency Syndrome (AIDS) globally, a number that has nearly quadrupled since the 1990’s. The majority of these people live in resource-poor settings, in some countries where the weight of disease burden lies, HIV-1 prevalence is close to 30%.

As no successful vaccine strategies have yet been developed, current treatment for HIV-1 infection is primarily comprised of anti-retroviral therapy (ART). These drugs act to inhibit specific steps in the HIV-1 replication cycle. For example, protease inhibitors block HIV-1 protease to impede maturation of newly budded viral particles rendering new particles non-infectious. ART is very effective and has been shown to reduce HIV-1 viral loads by 4- to 5-fold compared to untreated titer in patients. Since HIV-1 transmission rates are correlated to viral load, a population with high ART treatment rates should decrease HIV-1 prevalence and incidence. However, due to the rapid rate of evolution, HIV-1 can quickly and successfully evolve to replicate even in the presence of drug and most HIV-1 positive patients experience treatment failure. This means that in order to stay ahead of HIV-1 evolution, new drugs must constantly be developed. Even with treatment, ART is not a cure. HIV-1 is maintained as latent provirus in cellular reservoirs, even with
effective ART, eradication of HIV-1 infection is not possible within a HIV-1 infected person’s lifespan (Finzi et al., 1997).

Not only are there biological and scientific barriers to use of ART, there are also economic and logistical barriers to accessing ART in the developing world, where the majority of HIV-1 infections exist. ART is very expensive and as a result is used almost exclusively in the developed world.

As mentioned previously, no effective vaccine has been developed. Limited knowledge of the immune system correlates of protection has created difficulty in designing an effective vaccine strategy (Barouch, 2008). Further barriers include determining how to fund and roll-out a vaccine in a resource-poor setting.

Our lab has proposed a third paradigm of population-level disease control that is capable of overcoming biological, economic, and logistical barriers to HIV-1 treatment, namely Therapeutic Interfering Particles, or TIPs. TIPs expand upon the idea of transmissible immunization observed in the case some live-attenuated vaccines, like Oral Polio Vaccine. However, there are some key differences. Like live-attenuated vaccine, TIPs can transmit between individuals but transmission can only occur from an individual already infected with wild-type pathogen.

Recent theoretical work from our lab modeled the effect of ART, optimistic theoretical vaccines and TIPs on population level prevalence and incidence of HIV-1 in a resource-poor setting (Metzger et al., 2011). The
model utilized data that was collected from antenatal clinics in Malawi. Findings from the model suggested that a TIP capable of decreasing HIV-1 viral load between 0.5-1.5 logs could decrease HIV prevalence from 29% to 2-8% within 50 years. For comparison, a vaccine that was 50% effective, administered to 95% of the population was predicted to decrease prevalence to around 20%. The impressive control of HIV by TIPs predicted by our model has motivated our research efforts in the lab to design a TIP
Materials and Methods

Plasmids The TIP-coding construct SC was created using sequence data for pNL4-3 as described in the text. The sequence was sent to GenScript (Piscataway, NJ) for synthesis. The construct was then cloned into the CL-G (Huang and Baltimore, 1970; Miyoshi et al., 1998) backbone between the Pmel and MfeI sites. Ld2mCh was generated by replacing the GFP sequence of CL-G with the sequence for mCherry, a gift from Roger Tsien, using standard cloning techniques. pNL4-3 (Adachi et al., 1986) and pNL4-3-GFP-IRES-NEF contain complete infectious HIV-1 provirus and were a gift from Douglas Richmond. To generate pNL4-3-GFP-IRES-NEF, GFP and the encephalomyocarditis virus (ECMV) IRES were inserted upstream of nef. pLAI.2 (Peden et al., 1991) is a complete infectious HIV-1 provirus and was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pLAI.2 from Dr. Keith Peden, courtesy of the MRC AIDS Directed Program.

Generation of lentiviral vectors. Lentiviral vectors were generated by FuGENE HD transfection (Roche, Indianapolis, IN) of a 3-plasmid system in 293T cells. 30µL of FuGENE HD was mixed with 300 µL of DMEM. 5.6µg of pΔ8.9, 2.8µg of pVSV-G, and 2.2µg of SC were added to the mix and incubated for 20 minutes at room temperature. Transfection complexes were added dropwise to a 10 cm dish of 80% confluent 293T cells. 293T
supernatant containing lentiviral vectors were harvested and clarified through a 0.45\(\mu\)m syringe-filter 32 hours post transfection.

**Cell lines.** All SC cell lines were created by infecting low-passage, naïve CEM-T4 cells with SC-encoding lentiviral vectors at an MOI of 0.1.

Inducible, isogenic TIP cell lines were isolated by single-cell sorting on a FACS. First, live cells were gated using forward- and side-scatter measurements. Next, the live population was gated via the fluorescent reporter protein expression histogram. Single cells not expressing reporter protein were sorted into one cell per well in a 96-well plate. All sorted isogenic cell lines were expanded and 5x10⁵ cells were infected with pNL4-3-EGFP-IRES-NEF virus. Cell lines that expressed mCherry protein after infection were identified as TIP-encoding cell lines.

CEM-T4 cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. J.P. Jacobs. Cells were maintained in RPMI-1640 (Mediatech, Manassas, VA) supplemented with 9% fetal calf serum (Mediatech, Manassas, VA) and 1% penicillin and streptomycin (Mediatech, Manassas, VA). Cells were grown at cell densities of 2x10⁵-2x10⁶ cells/mL.

293T cells were maintained in DMEM (Mediatech, Manassas, VA) supplemented with 9% fetal calf serum and 1% penicillin and streptomycin. Cells were grown on 10cm dishes and maintained between 20-80% confluency.
All cells were incubated at 37°C, 5% CO₂.

**Virus preparation.** pNL4-3-GFP-IRES-NEF virus was generated by transfecting 1x10⁶ CEM-T4 cells with 1µg of proviral DNA according to the Lipofectamine 2000 (Invitrogen, Carlsbad, CA) procedure. Cells were expanded to 30mL culture and virus-containing supernatants harvested by centrifugation of the cell culture at 400xg for 10 minutes and clarified through a 0.45 µm filter.

**HIV-1 infection and mobilization assays.** All pNL4-3-EGFP-IRES-NEF virus infections were done at an MOI of 0.01. 5x10⁵ cells were suspended in 0.5mL of virus-containing RPMI in a 12-well dish. Cells were incubated with the virus at 37°C, 5% CO₂ for 18hr and then washed with PBS and resuspended in 2mL of RPMI supplemented with 9% FCS, penicillin, and streptomycin.

For the TIP mobilization assays, 1µg of an HIV molecular clone and 1µg of a TIP construct were co-transfected into 10⁶ 293T cells using the FuGENE HD reagent protocol. Virus containing supernatants were harvested and clarified through 0.45µm syringe-filter 48 hours post transfection. 5x10⁵ naïve CEM-T4 cells were incubated at 37°C, 5% CO₂ in 0.5mL of virus-containing 293T supernatant for 20 hrs and were then washed with PBS and resuspended in 1mL of RPMI supplemented with 9% FCS, 1% penicillin and streptomycin. Flow cytometry time points were taken 1, 4, and 8, 9, 11 days post-infection.
Flow cytometry. All flow cytometry measurements were taken on a Partec CyFlow® ML I CyFlow® space instrument. Cells were live-gated via forward scatter and side scatter. Data was collected for at least 10,000 cells in the live gate per sample taken. All data analysis was performed using FlowJo (Treestar, Ashland, OR).

RNA isolation and quantitative PCR For cytoplasmic RNA isolations, 10⁶ CEM-T4 cells were washed in PBS and then resuspended in 175µL of buffer RLN and 1000U/mL of RNasin (Promega, Madison, WI). Cells were incubated on ice for 5 minutes and then centrifuged at 500xg for 5 minutes to pellet the nuclei. The supernatant containing the cytoplasmic RNA was pipetted into 600µL of buffer RLT. One volume of ethanol was added to the homogenized mixture and RNA isolation was completed using a RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was suspended in RNase-free water and stored at -70C until cDNA synthesis.

cDNA synthesis was performed using the Quantitect cDNA Synthesis kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. 1µg of RNA was reverse transcribed using random hexamer primers to eliminate any positional bias of transcribed sequence. cDNA was diluted 1:20 or 1:100 in molecular grade water and stored at -20C until use in quantitative PCR.

Quantitative PCR reactions were created using Brilliant SYBR Green 2x Master Mix (Agilent Technologies, Santa Clara, CA), 200nM primer concentrations (SC primer set was assayed at 250nM), 10ng cDNA, and
molecular grade water. Genomic HIV-1 RNA was detected using the following primers: 5’-GAATTTGCCAGGAAGATGGA-3’ and 5’-GCAGCCAATCTGAGTCAACA-3’. Genomic SC RNA was detected with the following primers: 5’-TCCAGTTTGAAAGGACCAG-3’ and 5’-TTGAAGCGCATGAACTCCTT-3’. Genomic Ld2mch RNA was detected using the following primer sets: 5’-ACCCGACAGGCCCGAAGGAA-3’ and 5’-CCTCCTCGCCCTTGCTCACC-3’. Detecting all TIP RNA species was accomplished by amplifying a short sequence from mCherry. The following primers were used: 5’-AGATCAAGCAGAGGCTGA-3’ and 5’-ATGGTGTAGTCCTCGTTGTG-3’. Protein phosphatase 1A (PP1A) was used as a housekeeping gene in the qPCR assays. The following primers were used: 5’-CCCACCGTGTTCTTCGAC-3’ and 5’-CCAGTGTAGTCAGAGCAGA-3’. All primers were tested at an annealing temperature of 55C.
Chapter 1: Motivations for the Design of a Therapeutic Interfering Particle

Intracellular dynamics of Human Immunodeficiency Virus Type-1 (HIV-1) genomic RNA transcription and packaging of a singly infected cell can be represented by the following equations (Metzger et al., 2011):

\[
\frac{dG_W}{dt} = \theta - k_{pkg} \cdot G_W^2
\]
\[
\frac{dG_W}{dt} = k_{pkg} \cdot G_W^2
\]

HIV-1 genomic RNA (\(G_W\)) is transcribed and exported to the cytoplasm at a rate \(\theta\) and gRNA dimerization and packaging occurs at a rate, \(k_{pkg}\). This model can be expanded to consider the case of a cell that becomes dually infected with HIV-1(\(G^A_W\)) and TIP (\(G^A_T\)):

\[
\frac{dG^A_W}{dt} = \theta - k_{pkg} \cdot G^A_W^2 - k_{pkg} \cdot G^A_W \cdot G^A_T
\]
\[
\frac{dG^A_T}{dt} = P \cdot \theta - k_{pkg} \cdot G^A_T^2 - k_{pkg} \cdot G^A_W \cdot G^A_T
\]
\[
\frac{dG^A_{WW}}{dt} = k_{pkg} \cdot G^A_W^2
\]
\[
\frac{dG^A_{TW}}{dt} = 2k_{pkg} \cdot G^A_W \cdot G^A_T
\]
\[
\frac{dG^A_{TT}}{dt} = k_{pkg} \cdot G_T^2
\]
This model assumes the formation of homozygous HIV-1 virions $(G^\Delta_{WW})$, homozygous TIP virions $(G^\Delta_{TT})$, and heterozygous virions $(G^\Delta_{TW})$ occurs in a binomial distribution, as recently reported (Chen et al., 2009). This implies that there is no difference between $k_{pkg}$ for HIV-1 genomes and $k_{pkg}$ for TIP genomes. The TIP genomic production value, $P$, is defined as relative fold expression of TIP genomic RNA compared to HIV genomic RNA. Assuming that packaging reagents are in excess and that genomic RNA is the limiting reagent for RNA packaging as reported (Chen et al., 2009); one can see through inspection of the model that as $P$ increases, the proportion of heterozygous virions produced increases. This effectively "wastes" HIV-1 genomes, as heterozygous virions are non-infectious, and decreases the number of homozygous HIV-1 virions produced (An et al., 1999). In addition, the number homozygous TIP virions increase with $P$.

Our lab recently proposed a multi-scale model, referred to as the "Metzger model," which links HIV-1 infection dynamics at the population, individual, and intracellular level. This allows one to make predictions about the population-level prevalence of HIV-1 given particular intracellular parameters. Specifically, the Metzger model predicts that in a resource-poor setting with an HIV-1 prevalence of 29%, a TIP intervention with $5 < P < 25$ would decrease HIV-1 prevalence to between 2-8% after 50 years, much lower than what is predicted after a vaccine and ART intervention.
Chapter 2: Design of the Therapeutic Interfering Particle

Therapeutic Interfering Particles, or TIPs, are conditionally replicating viruses that lack protein-coding sequences. For this reason, TIPs can only mobilize when HIV-1 super-infects a TIP-infected cell. In order to efficiently mobilize TIPs must contain all the cis-acting elements necessary for cellular transcription, reverse transcription, integration, dimerization, and packaging. A literature search informed the rational design of our TIP, which is named SC.

**Figure 1 HIV-1 and TIP gene maps** Above is a gene map showing protein-coding regions of HIV-1. Below is a schematic of the HIV-1 regions included in the TIP. The only protein-coding ability the TIP has is for a fluorescent reporter protein that enables visualization of TIP expression and mobilization within a cell culture.

All sequences and structural elements included in the SC were from pNL4-3, a recombinant molecular clone of HIV-1 and the primary strain of...
virus used in the subsequent experiments. The SC plasmid contains the 5' and 3' long terminal repeat (LTR) and all sequence between the end of the 5'LTR and start codon for gag (Guan et al., 2000; Luban and Goff, 1994; McBride and Panganiban, 1997; McBride et al., 1997), this includes the packaging signal (ψ) which has a dimer initiation signal (DIS) to ensure the SC can dimerize with wild-type pNL4-3 genomic RNA and form heterozygous virions (Moore et al., 2007). The first few hundred base pairs of the gag coding sequence were included in the SC plasmid, as mutations to sequences flanking the gag start codon cause packaging defects (Nikolaitchik et al., 2006). The central polypyrimidine tract (cPPT) which primes positive-strand DNA synthesis during reverse transcription and is required for optimal HIV-1 replication kinetics (Charneau et al., 1992) was also included in the TIP design. Nuclear export is blocked for unspliced RNAs, however, HIV-1 can circumvent this requirement by utilizing the Rev-Responsive Element (RRE), an RNA structural element located in the middle of the env-coding sequence of HIV. HIV-1 protein Rev binds the RRE and acts as an adapter for the CRM1 nuclear export pathway to export unspliced RNA to the cytoplasm (Fischer et al., 1995; Neville et al., 1997). We included the RRE minimal sequence in addition to a few hundred base pairs of flanking sequence found in pNL4-3. Sequences which contained splice acceptors and donors were not included in the SC design, with exception for the major splice donor (MSD) which is located in the packaging signal of
HIV-1 and splice acceptor 4a (Schwartz et al., 1990; Watts et al., 2009), which was placed in front of a fluorescent reporter protein, mCherry, coding sequence. This is the only coding sequence in the TIP to enable TIP mobilization and expression to be monitored via flow cytometry and will be removed in subsequent TIP designs.
Chapter 3: HIV-1 Infection Dynamics in the presence of TIP and Quantifying P

In order to determine the inhibition of HIV-1 replication in the presence of TIP, I first created TIP isoclonal cell lines. To do so, I generated lentiviral vectors coding for the SC construct and transduced naïve CEM-T4 cells at a multiplicity of infection of 0.1. In order to monitor accurately if an HIV-1 infected cell was co-expressing TIP, I used fluorescence-activated cell sorting (FACS) to isolate isoclonal cell lines that were selected for to induce SC expression (mCherry) only in the presence of HIV-1 (GFP). Figure 2 shows the pNL4-3-GFP-IRES-NEF, an HIV-1 proviral clone which has GFP-IRES-NEF cloned into the nef reading frame, virus infection progression (MOI 0.01) in a population of naïve CEM-T4 cells, an isoclonal population of TIP-transduced cells, a population that consists of 67% TIP infected cells and 33% naïve CEM-T4 cells, and a population that consists of 33% TIP infected cells and 67% naïve CEM-T4 cells. These two mixed naïve CEM-T4/TIP populations were created in order to monitor subsequent TIP mobilization events.
Figure 2 HIV-1 infection dynamics in the presence of TIP. Populations containing varying levels of TIP integrations were infected with pNL4-3-GFP-IRES-NEF virus. HIV-1 infection progression was monitored over time with flow cytometry.

Suppression of pNL4-3-GFP-IRES-NEF replication was observed for all populations containing TIP integrations. By day 8, the naïve CEM-T4 population was 30.6% infected with pNL4-3-GFP-IRES-NEF, whereas the 100% TIP-transduced population was only 10.8% infected. Higher percentage of TIP-transduced cells within an infected population correlated with lower pNL4-3-GFP-IRES-NEF spread.

However, in the mixed naïve CEM-T4/TIP-transduced populations, the mCherry signal, a marker for TIP expression, never increased beyond the starting concentrations of TIP (Figure 3). Transfer of supernatants from day 12 infected cultures to naïve CEM-T4s did not result in a transfer of mCherry
signal and qPCR assays did not detect TIP virion RNA in the supernatant of the infected cultures (data not shown). This data implied that the TIP was not mobilizing and that the suppression of pNL4-3-GFP-IRES-NEF was due to an unknown TIP intracellular interference.

**Figure 3** TIP expression (mCherry) in CEM-T4 cells as measured by flow cytometry

The intracellular model of TIP/HIV-1 dynamics proposed by the Metzger model assumes that the level of TIP mobilization and HIV-1 inhibition is directly dependent on the fold expression of TIP gRNA over HIV-1 gRNA, represented by the parameter $P$. Although it is not possible to relate the HIV-1 inhibition in cell culture to a particular $P$ because the system is not at steady state, we can test whether the SC $P$ is high enough in
TIP/HIV-1 dually infected cells to expect mobilization.

To quantify $P$ for the different TIP-transduced cell lines, SC isoclonal cell lines were infected with HIV-R7/E'/GFP virus pseudo-typed with VSV-G. HIV-R7/E'/GFP is a HXB2 derivative that codes for GFP in the *nef* reading frame and has a frameshift in the *env*-coding region, making the virus replication defective. SC cell lines were infected at an MOI of 0.1 to minimize double integrations of HIV-R7/E'/GFP. Thirty-five hours post infection, the cytoplasmic RNA fraction was isolated, used as a template for cDNA synthesis and quantified using real-time PCR. $P$ was calculated using the $2^{-\Delta \text{Ct}}$ method (Livak and Schmittgen, 2001) and is interpreted as the fold expression of TIP gRNA over HIV-R7/E'/GFP gRNA.
Figure 4. *P* measured in cytoplasmic fraction of TIP isoclonal cell lines. Three SC cell lines and naïve CEM-T4 cells were infected with HIV-R7/E/GFP.

Of the three cell lines analyzed, only one expressed the genomic RNA at a higher level than HIV-1 (SC7, *P*=1.2). The relatively low value for *P* may have been due to a design problem with the SC, loss of gRNA to RNA splicing in the nucleus, or integration-site expression bias. In addition, since the SC construct was not detected at all in the supernatants of infected cultures with *P*=1, there may also be a packaging defect in the SC.

To test whether the TIP would be able to mobilize given a higher *P* and in order to quantify any packaging defect, SC was co-transfected with two different HIV-1 molecular clones into 293T cells. By transfecting different ratios of HIV-1 and SC proviral DNA, *P* can be artificially increased in the
cytoplasm of the 293T cells. A second SC construct was made which contains more of the *gag* coding region, named SC1216 and was also co-transfected with HIV-1 molecular clones. Lastly, since the sequence and structural requirements for a TIP are similar to that of a lentiviral transfer vector lacking an internal promoter, the lentiviral plasmid Ld2mch, which expresses a destabilized form of mCherry protein from the HIV-1 LTR was tested.

Transfection efficiency was assessed using flow cytometry. All populations were 40% co-infected with TIP and HIV-1, 40% infected with only TIP, and 20% expressing neither TIP nor HIV-1 reporter proteins. Cytoplasmic RNA was isolated from the 293T cells 48 hours post-transfection and P was calculated as previously described in the thesis. In addition, viral RNA (vRNA) was isolated from clarified 293T supernatant.
**Figure 5 P calculated from the cytoplasmic and virion fractions of 293T cells** 293T cells were transfected with equal weights of pNL4-3 or pNL4-3-GFP-IRES-NEF and a TIP construct. Average ratio of levels of TIP gRNA to HIV-1 gRNA was calculated using a qPCR assay.

The transfection procedure produced $P$ values between 2.8 and 6.1. If the packaging efficiency between HIV-1 and TIP genomes were equal, the $P$ measured in the cytoplasm should equal the $P$ measured in the virion fraction. Comparing the cytoplasmic and virion fractions for all co-transfections, it is clear that there is not equal packaging efficiency between pNL4-3 or pNL4-3-GFP-IRES-NEF and any of the TIP constructs. The highest packaging efficiency observed was with Ld2mCh and was, at best, 25% the packaging efficiency of HIV.
Chapter 4: TIP Mobilization

Since TIP RNA was detectable in the supernatants of the 293T cells, it was important to determine if TIP packaged into infectious virion particles and could be mobilized by HIV-1 in cell culture. 293T supernatants from TIP/HIV-1 co-transfected cells were clarified and used to infect naïve CEM-T4 cells and TIP expression was monitored via flow cytometry. A third HIV-1 molecular clone, pLAI.2 was also tested for its ability to mobilize TIP constructs.

![Graph showing TIP mobilization by pNL4-3](image)

**Figure 6** TIP mobilization by pNL4-3
Figure 7 TIP mobilization by pNL4-3-GFP-IRES-NEF
Figure 8 TIP mobilization by pLAI.2

The highest vector mobilization occurred when the TIPs were co-transfected with pNL4-3. All TIPs were mobilized to the highest level (as measured by fluorescent reporter expression in the CEM-T4s) when co-transfected with pNL4-3. This is interesting to note, since all the TIPs sequences are based off the pNL4-3 genome. pLAI.2 mobilized the vectors to the second highest level, with the vector V1GFP reaching the highest level, followed by Ld2mch. SC and SC1216 had near identical mobilization in all HIV-1 contexts. pNL4-3-GFP-IRES-NEF mobilized the TIPs the least well, despite having sequence identity to the TIP constructs. One possible explanation for this observation could be that the TIP mobilize to a lesser extent because the replication kinetics of pNL4-3-GFP-IRES-NEF are slower.
compared to pNL4-3 (due to a decrease in NEF translation). No mobilization was observed for TIP constructs transfected without HIV-1.

Replication dynamics were measured for pNL4-3-GFP-IRES-NEF virus produced from 293T co-transfection.

![pNL4-3-GFP-IRES-NEF Infection Dynamics](image)

**Figure 9** Infection dynamics of pNL4-3-GFP-IRES-NEF virus in the presence of TIP in infected CEM-T4 cells

TIP and HIV-1 virus was generated from TIP/HIV-1 co-transfected 293T cells.

pNL4-3-GFP-IRES-NEF spread was suppressed most effectively by the presence of Ld2mch in culture, although Ld2mch did not have the highest cytoplasmic $P$ in the 293T cells, its ability to suppress HIV-1 is likely due to its higher packaging efficiency compared to SC and SC1216.

Lastly, the TIP and HIV-1 clones were assayed for ability to spread between CEM-T4s. To accomplish this, naïve CEM-T4s were infected by
293T supernatants as was done before. After the CEM-T4s were infected and washed, an equal number of CEM-T4 cells labeled with CMFDA-Cell Tracker Dye (Invitrogen, Carlsbad, CA) were added to the culture. If the CMFDA-labeled CEM-T4s become infected with TIP, the TIP would have to have been mobilized from a dually infected CEM-T4 cell. TIP expression was measured one day after CMFDA addition to establish a baseline TIP expression and then measured four days post-addition of CMFDA-labeled cells. In addition, to test if mobilization was dependent on HIV-1 replication, nelfinavir, a potent HIV-1 protease inhibitor, was added to replicate wells of each infection. Nelfinavir was added at a concentration of 5 µM, which was the EC_{90} concentration as determined experimentally with pNL4-3-GFP-IRES-NEF virus in CEM-T4 cells. In LD_{50} assays performed, no cell death occurred until nelfinavir concentrations reached 7.5 µM.
Figure 10 Change in TIP expression (mCherry fluorescence) in CMFDA-stained cells
Figure 11 Change in TIP expression (mCherry fluorescence) in CMFDA-stained cells
In the mobilization assays, Ld2mch was the only TIP mobilized to the CMFDA-labeled CEM-T4s and only by pNL4-3 and pNL4-3-GFP-IRES-NEF virus in the absence of HIV-1 protease inhibitor. This suggests that Ld2mch mobilization is HIV-1-dependent. No mobilization of the SC construct was observed, and no mobilization of the either construct by pLAI.2 virus was observed. This mobilization data also helps to further explain the increased suppression of pNL4-3-GFP-IRES-NEF virus replication by Ld2mch compared to SC.

Although mCherry signal increased for all TIP/HIV-1 co-infections, the
lack of mobilization to the CMFDA-labeled cells suggests that the TIP signal was only increasing due to silent TIP integrations becoming induced by HIV-1 superinfection. Again, these data suggest that mobilization is most efficient when the TIP sequence is closely related to the infecting strain of HIV-1 and when replication kinetics of the virus are faster.

It is probable the SC construct did not mobilize due to inefficient packaging by HIV-1, since the SC can mobilize under high P and high HIV-1 MOI settings, as seen in the 293T data. This is an important distinction to make because inefficient packaging can be compensated by engineering a higher P value which could still produce the necessary proportions homozygous HIV-1, TIP and heterozygous virions.
Chapter 5: Discussion

Our lab has recently proposed a theoretical framework for an infectious disease intervention strategy in which conditionally replicating viruses act to inhibit wild-type pathogen spread and are concurrently mobilized by the wildtype pathogen. Though other groups have previously proposed conditionally replicating viral therapies, none have explored how intracellular dynamics affect mobilization (An et al., 1999; Klimatcheva et al., 2001; Levine et al., 2006). Other studies have shown decreased spread of HIV-1 in the presence of these therapies, but have found no evidence supporting how inhibition occurs when a HIV-1 down-regulating element is not encoded by the therapy virus.

The data presented within this thesis showed that the presence of TIP in a cell culture decreased HIV-1 spread by 67% by day 8 post-infection and 50% day 11 (Figure 1). Although these values are not as impressive as the suppression one can obtain through the use of ART, the TIP has an evolutionary advantage. While HIV-1 can evolve resistance to ART, since the TIP is reverse transcribed by the same reverse transcriptase enzyme as HIV-1, it will have similar mutation rates and thus a similar capacity a HIV-1 to evolve. TIP virions were only detected and mobilized from cell-to-cell when $P$ values were artificially increased in the cytoplasm. The observation that TIPs are best packaged in a high MOI environment, like in the
cytoplasm of the transfected 293T cells, is in agreement with early observations of defective interfering particles (replication defective viruses, much like a TIP) in cell culture (Huang and Baltimore, 1970; Perrault and Holland, 1972).

Clearly, the next step in designing an effective TIP is to engineer higher $P$ values in the cytoplasm. This would enable mobilization of the TIP outside of the artificial transfected 293T environment. One promising approach is to completely ablate splicing within the TIP. Currently, the TIP is spliced at at least one position to express mCherry. A qPCR assay to detecting genomic RNA and total of TIP RNA species showed that for the SC, only between 3-5% of TIP RNA is genomic RNA and thus packagable (Figure 13). Ablating splicing in the SC construct could theoretically increase $P$ 20-fold. Previous work by other groups have shown that mutating the major splice donor in the packaging site ablates the majority of spliced RNA species (Bohne et al., 2005), increases viral titer of lentiviral vectors (Koldej and Anson, 2009), and in SIV did not affect dimerization or replication kinetics (Whitney and Wainberg, 2006).
Figure 13 Fraction of unspliced TIP in 293T cytoplasm
References


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