New insights into inhibition of human immunodeficiency virus type 1 replication through mutant tRNA\(^{\text{Lys3}}\)

Chengxiang Wu\(^{1,2}\), Vivek R Nerurkar\(^3\) and Yuanan Lu\(^*\)

Abstract

Background: Host cellular tRNA\(^{\text{Lys3}}\) is exclusively utilized by human immunodeficiency virus type 1 (HIV-1) as a primer for the replication step of reverse transcription (RTIon). Consequently, the priming step of HIV-1 RT constitutes a potential target for anti-HIV-1 intervention. Previous studies indicated that a mutant tRNA\(^{\text{Lys3}}\) with 7-nucleotide substitutions in the 3’ terminus resulted in aberrant HIV-1 RTion from the trans-activation response region (TAR) and inhibition of HIV-1 replication. However, the mutant tRNA\(^{\text{Lys3}}\) also directed HIV-1 RTion from the normal primer-binding site (PBS) with potentially weakened anti-HIV-1 activity. To achieve improved targeting of HIV-1 RTion at sites not including the PBS, a series of mutant tRNA\(^{\text{Lys3}}\) with extended lengths of mutations containing up to 18 bases complementary to their targeting sites were constructed and characterized.

Results: A positive correlation between the length of mutation in the 3’ PBS-binding region of tRNA\(^{\text{Lys3}}\) and the specificity of HIV-1 RTion initiation from the targeting site was demonstrated, as indicated by the potency of HIV-1 inhibition and results of priming assays. Moreover, two mutant tRNA\(^{\text{Lys3}}\)s that targeted the IN-encoding region and Env gene, respectively, both showed a high anti-HIV-1 activity, suggesting that not only the TAR, but also distant sites downstream of the PBS could be effectively targeted by mutant tRNA\(^{\text{Lys3}}\). To increase the expression of mutant tRNA\(^{\text{Lys3}}\), multiple-copy expression cassettes were introduced into target cells with increased anti-HIV-1 potency.

Conclusions: These results highlight the importance of the length of complementarity between the 3’ terminus of the mutant tRNA\(^{\text{Lys3}}\) and its target site, and the feasibility of targeting multiple sites within the HIV-1 genome through mutant tRNA\(^{\text{Lys3}}\). Intervention of the HIV-1 genome conversion through mutant tRNA\(^{\text{Lys3}}\) may constitute an effective approach for development of novel therapeutics against HIV-1 replication and HIV-1-associated diseases.

Keywords: Mutant tRNA\(^{\text{Lys3}}\), Reverse transcription, HIV-1 inhibition

Background

RTion, or the conversion of viral RNA (vRNA) into DNA, is a key step in the life cycle of HIV-1, and it may take place before budding as early as in virus producer cells [1,2]. The reaction is catalyzed by virion-associated reverse transcriptase (RT), and initiated with a cellular primer. Although various primer molecules can be used to initiate RTion in vitro, all retroviruses employ cellular tRNA [3-9]. DNA sequence analysis of the HIV-1 provirus reveals tRNA\(^{\text{Lys3}}\) to be the primer for HIV-1 RTion [10,11].

A prerequisite for the initiation of HIV-1 RTion is formation of a properly folded initiation complex of vRNA and tRNA\(^{\text{Lys3}}\). An 18-nucleotide residue at the 3’ terminus of the tRNA\(^{\text{Lys3}}\) anneals complementarily to the PBS of vRNA, and primes template-dependent DNA synthesis [12]. Upon annealing, the primer is extended and a cDNA is synthesized and termed (−)-strand strong-stop DNA or (−)ssDNA. The (−)ssDNA is released and anneals to the 3’ terminus of the vRNA, and primes further (−)-strand DNA synthesis and generates a full-length (−)-strand DNA that is used as a template for (+)-strand DNA synthesis. Along with (−)-strand DNA synthesis,
RNaseH degrades the RNA template with the exception of two polypurine tracts (PPTs) that resist cleavage: one immediately upstream of the U3 region (3′-PPT) and the other at the center of the vRNA (cPPT). These PPTs are responsible for priming (+)strand DNA synthesis. The 3′-PPT-primed (+)strand DNA synthesis terminates at the first modified base in the tRNALys3 molecule and this product is termed (+)strand strong-stop DNA or (+)ssDNA [13,14], with the tRNA removed by RNaseH. A second strand-transfer takes place through annealing of the (+)ssDNA to the 3′ end of the full-length (−)strand DNA, followed by (+)strand DNA synthesis. Eventually, full-length double-stranded viral DNA is formed and integrated into the host cell genome through the viral integrase protein. For alpha and gamma-retroviruses and lentiviruses, these obligatory steps in genome conversion are chaperoned by a major virion protein of the inner core, the nucleocapsid protein encoded by Gag that serves as a key cofactor of the RT enzyme [15-21].

Different tRNAs are utilized by various retroviruses. Although many different tRNAs exist in an infected cell, each retrovirus is dedicated to its own tRNA [22-24]. For example, although a single point mutation in the HIV-1 PBS that results from the infrequent usage of a low abundant tRNA^Lys5 variant has been observed [25,26], no spontaneous mutations or tRNA switches have been reported, except that primer specificity is less stringent for the murine leukemia virus [27-29]. Previous tRNA-switch study through forced selection of a HIV-1 variant that used a non-self tRNA primer- tRNA^Lys1,2 resulted in severe replication defect, and reversion to the wild-type PBS-Lys3 sequence was the most frequent escape route [30].

Due to specific interactions between HIV-1 and tRNA^Lys3, antiretroviral strategies targeting this unique property have been proposed and tested. tRNA^Lys3 derivatives with mutations in their 3′-terminal sequence, were previously demonstrated to inhibit HIV-1 replication through induction of aberrant RTion products [31-33]. However, the described antiviral effect was minimal due to a limited alteration of the sequence. In this study, a series of mutant tRNA^Lys3's were constructed with extended mutations in the 3′ terminus (up to 18 bp of complementarity to their targeting sites) with or without a combined A58U mutation. These mutants were shown to be encapsidated into progeny HIV-1 virions and reduced their infectivity. When the mutants were transduced into human lymphocyte-derived cells using an improved retroviral vector system [34], the transduced cells showed potent inhibition of HIV-1 replication, with the potency of anti-HIV-1 activity correlating with the complementarity between the mutated 3′ PBS-binding region of the mutant and its targeting site.

### Results

#### Design and cloning of mutant tRNA^Lys3 genes

To strengthen mutant tRNA^Lys3-based anti-HIV-1 activity through extended mutation of the 3′ terminal sequence, and targeting other portions of the viral genome, mutant tRNA^Lys3 genes with various length of mutation targeting either the TAR, IN-encoding region or Env, with up to 18 bases complementary to their target sites, were constructed through a fusion-PCR-based strategy (Figure 1A and B). Among these genes (Figure 1C), an 8-nucleotide mutation in the 3′ end of Mt8TD resulted in a 12-base pair (bp) complementarity to the TAR; a 10-nucleotide mutation in the 3′ end of Mt10TD conferred a 15-bp complementarity to the TAR. Similarly, an 11-nucleotide mutation in Mt11TD resulted in a total of 16-bp complementarity to the TAR, and a 13-nucleotide mutation in Mt13TD produced an 18-bp complementarity to the TAR. Besides these mutations, an extra A58U mutation in the Mt11TD-A58U was included to interfere with the termination of the (+)ssDNA product as previously reported [31,32]. In addition, an extra G44C mutation in Mt11TD-G44C was performed to examine if it was necessary to maintain the native secondary structure. Finally, two mutants, named Int and Env, were constructed with a 7-nucleotide mutation resulting in an 18-bp complementarity to the IN-encoding region and Env gene respectively. It is noteworthy that the CCA ends at the 3′ terminus of the wild-type and mutant tRNA^Lys3's are added post-transcriptionally and are complementary to the binding sites. The number of mutated bases in each mutant and its complementarity to the targeting site are summarized in Table 1.

To facilitate high efficiency and stable expression in human cells, these mutant tRNA^Lys3 genes were cloned into an optimized double-copy retroviral vector (Figure 1D). Due to duplication of the 3′ U3 region during retroviral vector-mediated transduction of target cells, copy number of the mutant tRNA^Lys3 gene in transduced cells is theoretically doubled as previously described [35,36].

#### Retroviral vector-mediated transduction

A prerequisite for functional analysis of the mutant tRNA^Lys3 is that they are expressed stably at a high level in human cells. This was accomplished through transduction of a human T lymphocyte-derived cell line, CEM-SS, with high-titer retroviral vector stocks. Since the retroviral vector carries an eGFP gene as a reporter [34], transfected 293T packaging cells and transduced CEM-SS cells were examined for eGFP expression (Figure 2A). To generate high-titer vector stocks for enhanced efficiency of gene transduction and expression, vector viruses harvested from transfected 293T cells were concentrated through a one-step ultracentrifugation method and vector titer exceeded 10^8 IU/mL (Figure 2B). Comparative analysis showed that despite differences in titers of retroviral constructs containing
different mutant tRNA<sup>Lys</sup> genes, no clear pattern of influence on vector production was observed, and the differences in titers were possibly due to variations among transfections. When the concentrated vector stocks were used to transduce CEM-SS cells at a multiplicity of infection (MOI) of 100, approximately 90-100% of the cells became GFP positive on day 3 post infection (pi) through a single transduction (Figure 2C). This allowed direct use of the transduced cells, without any selection or cell cloning, for functional analysis of the mutant tRNA<sup>Lys</sup> through HIV-1 challenging. Furthermore, transduction and expression of the mutant tRNA<sup>Lys</sup> were confirmed by PCR and RT-PCR (data not shown).

**Inhibition of HIV-1 replication**

Anti-HIV-1 activities of the mutant tRNA<sup>Lys</sup> were firstly evaluated by examining the relative sensitivity of the transduced CEM-SS cells to HIV-1 infection and the capability

---

**Table 1 Length of mutation and complementarity, and targeting sites, of mutant tRNA<sup>Lys</sup>**

| Name  | Length of mutation | Length of complementarity | Targeting site |
|-------|-------------------|--------------------------|----------------|
| Mt8TD | 8                 | 12                       | TAR            |
| Mt10TD| 10                | 15                       | TAR            |
| Mt11TD| 11                | 16                       | TAR            |
| Mt11TD-G44C | 11            | 16                       | TAR            |
| Mt11TD-AS8U | 11        | 16                       | TAR            |
| Mt13TD| 13                | 18                       | TAR            |
| Env   | 7                 | 18                       | IN-coding region |
| Int   | 7                 | 18                       | Env            |

---

**Figure 1** Construction and retroviral vector-mediated delivery of mutant tRNA<sup>Lys</sup>. (A) Sequence alignment of primer-template for the amplification of mutant tRNA<sup>Lys</sup> through PCR. The Mt11TD is used as an example and mutated bases are shown in bold italic. (B) Schematic illustration of the fusion-PCR used to amplify full-length mutant tRNA<sup>Lys</sup> genes. (C) Maps of the mutant tRNA<sup>Lys</sup> with mutated bases highlighted in darkened background. (D) Retroviral vector-mediated delivery of the mutant tRNA<sup>Lys</sup> genes.
of the cells to inhibit HIV-1 replication. This was done by infecting the mutant tRNA<sub>Lys</sub>-expressing cells with a replication-competent HIV-1 stock, with relative capability of the cells to inhibit HIV-1 replication determined using the median tissue culture infectious dose (TCID<sub>50</sub>) assay. As shown in Figure 3A, the transduced cells expressing various mutants all showed significantly lower TCID<sub>50</sub> titers of the HIV-1 stock compared with the non-transduced cells or cells transduced with the wild-type tRNA<sub>Lys</sub> (p < 0.001). Furthermore, cells expressing mutant tRNA<sub>Lys</sub> with increasing mutation in their 3′ PBS-binding regions generally showed significantly lower TCID<sub>50</sub> titers (p < 0.001). Interestingly, cells transduced with the wild-type tRNA<sub>Lys</sub> did not significantly change their virus production (p > 0.05). Cells transduced with Mt11TD-A58U, Mt13TD, Int, and Env seemed the most refractory to HIV-1 replication, with significantly higher TCID<sub>50</sub> reductions in cells expressing Mt13TD, Int, and Env than that in cells expressing Mt11TD-A58U (p < 0.001). Correspondingly, these four mutants had relatively more potent anti-HIV-1 activities than the others. To further analyze the anti-HIV-1 effects of these mutants, the transduced cells were infected with
HIV-1 at MOI of 0.1, and cell-free supernatants from the infected cell cultures were tested for HIV-1 P24 production every two days for 35 days pi. Figure 3B shows that P24 accumulated rapidly from day 5 pi in control cells transduced with the wild-type tRNALys3 and reached a peak concentration of $1.2 \times 10^7$ pg/mL on day 13 pi. In contrast, depending on the respective mutant expressed in the cells, the replication kinetics of HIV-1 was delayed by 3–10 days with significantly decreased production of P24 by 2–3 logs ($p < 0.001$).

**Multiple copy mutant tRNALys3 delivery**

Using BLAST, twenty examples of a 234-bp sequence of the tRNALys3 gene were found in the human genome database (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=MegaBlast&PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&DBSEARCH=true&QUERY=&SUBJECTS=). Similarly, multiple copies of tRNA\textsuperscript{Lys1} and tRNA\textsuperscript{Lys2} genes were found. Therefore, we hypothesize if mutant tRNA\textsuperscript{Lys3} levels are increased in transduced cells, more mutant would be encapsidated into progeny HIV-1 virions upon infection, and the mutant tRNA-mediated anti-HIV-1 effect will be more effective because of enhanced competition against wild-type tRNA\textsuperscript{Lys3}. To test this hypothesis, multiple copies of the Mt13TD gene (one of the mutants with potent anti-HIV-1 activity, Figures 3A and 3B) were subcloned into the retroviral vector and subsequently packaged and transduced into CEM-SS cells.

Although titers of retroviral vectors were not clearly influenced by insertion of single-copy of the mutant tRNA\textsuperscript{Lys3} genes, the retroviral constructs carrying multiple copies of the Mt13TD gene showed an apparent pattern of significant decrease in titers ($p < 0.001$) (Figure 4A). The vector titer from the triple copies of Mt13TD construct dropped from $3.03 \pm 0.25 \times 10^6$ IU/mL to $2.25 \pm 0.35 \times 10^5$ IU/mL compared with that of the construct carrying single-copy of Mt13TD—a 13-fold decrease, which was also seen where the titer derived from the construct with 12 copies of the Mt13TD gene dropped to $3.72 \pm 0.12 \times 10^3$ IU/mL. To overcome the challenge due to decreased titers from these vector constructs, clones of transduced cells were obtained through a limiting-dilution method and tested for viral inhibition with replication competent HIV-1.

In spite of marked variations among different clones, Figure 4B shows that cells transduced with the construct carrying 3 copies of Mt13TD showed lower TCID\textsubscript{50} titers compared to cells transduced with single-copy of the gene. Consistently, cells transduced with the construct carrying 6 copies of the gene generally showed lower TCID\textsubscript{50} titers than cells transduced with 3 copies. Furthermore, cells transduced with the construct carrying 12 copies showed the lowest TCID\textsubscript{50} titers, with exception of two clones.

Based on the results from the TCID\textsubscript{50} test, two of the clones transduced with the 12-copy construct were further evaluated by challenging with HIV-1 at MOIs of 0.1 and 1.0 respectively. As shown in Figures 4C and 4D, these two clones showed significant reduction of HIV-1 replication than cells transduced with a single copy of the same gene or the wild-type tRNA\textsuperscript{Lys3}, especially the latter ($p < 0.001$). When challenged at MOI 1.0, peak production of P24 from cells transduced with the wild-type tRNA\textsuperscript{Lys3} occurred at day 6 pi with massive cell death, and P24 production decreased sharply following that time point. When these control cells were infected at MOI 0.1, the peak production of P24 occurred on day 12 pi, with the absolute concentration of the peak level 1.26 times higher than that of the cells.
infected at MOI 1.0. In contrast, when challenged at MOI 1.0, the cells transduced with the vector carrying single-copy of Mt13TD accumulated concentration of P24 2 logs lower with occurrence of the peak level delayed to day 9 pi. Following that time point, the P24 level remained relatively stable with a slight decrease. Similarly, when challenged at a lower MOI of 0.1, the P24 accumulated slower with concentrations greater than 2 logs lower compared with that of the cells transduced with the wild-type tRNA\(^{\text{Lys}}\). In addition, the occurrence of peak concentration of P24 was delayed by 3 weeks, and the P24 levels remained relatively stable with only a slight increase following that time point.

In respect of challenging cells transduced by the construct carrying 12 copies of the Mt13TD gene at both MOIs, the data were drastically different. As shown in Figure 4D, in cells infected at MOI of 0.1, P24 accumulation occurred significantly slower compared to that in cells transduced with the wild-type tRNA\(^{\text{Lys}}\) gene, with 3 logs lower concentration of P24 at its peak, and the P24 concentrations remained fairly stable. Moreover,
these cells are much more potently inhibitory to HIV-1 replication compared to cells transduced with the vector containing single-copy of the Mt13TD gene. When challenged at MOI 1.0, the peak level of P24 was more than 2 logs lower compared to that of cells transduced with the wild-type tRNA\textsuperscript{Lys}\textsubscript{3} gene and 4 times lower than that of cells transduced with a single-copy Mt13TD (Figure 4C). Once the peak concentration of P24 occurred, P24 levels dropped to one log lower compared to that of cells transduced with a single-copy of Mt13TD.

**Potential adverse impact by mutant tRNA\textsuperscript{Lys}\textsubscript{3}**

To determine whether transduction and expression of mutant tRNA\textsuperscript{Lys}\textsubscript{3} resulted in any adverse impact on target cells, transduced cells were examined for their growth kinetics and morphology where no obvious alteration was found at different passage numbers (data not shown). Subsequent MTT assays also found no statistically significant difference between non-transduced cells and those transduced with mutant tRNA\textsuperscript{Lys}\textsubscript{3}s (P > 0.05) (Figure 5A). To rule out the possibility that mutant tRNA\textsuperscript{Lys}\textsubscript{3} may interfere with the translation machinery in transduced cells, expressions of two reporter genes, luciferase [37] and hTNFR-Fc [38], were examined. Figure 5B and C indicate that expression of these reporter genes were not significantly influenced by the transduction and expression of mutant tRNA\textsuperscript{Lys}\textsubscript{3} (P > 0.05).

**Encapsulation and priming assay**

Analysis was performed at the molecular level to test the hypothesis that the improved anti-HIV-1 activities of the mutant tRNA\textsuperscript{Lys}\textsubscript{3}s were conferred through improved priming or directing RTion of HIV-1 to their targeting sites rather than the normal PBS. Retroviral plasmid containing the mutant tRNA\textsuperscript{Lys}\textsubscript{3} gene was respectively co-transfected with a defective HIV-1-based vector system, with RT-PCR using RNAs extracted from the HIV-1 virions harvested from the co-transfections employed to examine encapsidation of the mutant tRNA\textsuperscript{Lys}\textsubscript{3}s. As shown in Figure 6A, when the RT-PCR products were separated through 2.0% agarose gel electrophoresis, DNA bands of 76 bp corresponding to the size of the mutants were detected. When the HIV-1-based packaging plasmid was omitted from the co-transfections, the mutants were not detected under the same experimental conditions. This indicated that the mutant tRNA\textsuperscript{Lys}\textsubscript{3} genes were expressed from the...
retroviral constructs and encapsidated into the progeny HIV-1 particles.

In addition, their relative anti-HIV-1 activities were evaluated through a one-replication-cycle assay with replication-defective HIV-1-based vectors harvested from the co-transfections. This was accomplished through titration of the vectors in CEM-SS cells. Figure 6B shows that titers of the HIV-1-based vectors were significantly reduced when they were prepared through co-transfection with the mutant tRNALys3 (p < 0.001), with 15-20-fold reductions when the vector system was co-transfected with Mt11TD-A58U, Mt13TD, Env, and Int respectively. Moreover, a significant difference in the level of reduction was demonstrated, with a pattern consistent with the previous TCID50 assay (Figure 3A).

To characterize the efficiency and specificity of mutant tRNALys3-primed HIV-1 RTion, the mutant tRNALys3-containing HIV-1 virions were used to infect CEM-SS cells, with DNAs extracted from the infected cells and subjected to PCR amplifications. Two primers, the forward primer specific to a site in the U3 region of HIV-1 3' LTR of the RTion product following the first strand transfer event and the reverse primer specific to the mutated PBS-binding region of respective mutant tRNALys3, were used for the amplifications (more detail in Methods). For HIV-1 virions containing mutant targeting the TAR, PCR product amplified from RTion products primed from the TAR would be 226 bp in size. In case that the mutant tRNALys3 primes RTion from the normal PBS as previously reported [33], another PCR product with the expected size of 395 bp will be generated. Moreover, amplification efficiencies of the products would be approximately the same since the same primers were used and the PCR products are similar in size. Consequently, if both products were amplified, concentrations of the end products would reflect the relative starting amount of the templates. Figure 7A shows that Mt8TD made a fair amount of non-specific priming from the PBS, as indicated by the relative intensity of the 395-bp band to that of the 226-bp band. When the length of mutations was extended in other mutants, such as Mt10TD, non-specific
priming was still detected but the relative intensity of the 395-bp band decreased notably. This tendency continued with Mt11TD. In case of Mt13TD that contained 18 bp complementarity to the TAR (Figure 1C and Table 1), the product that would reflect non-specific priming of RTion from the PBS was nearly non-detectable. Similarly, priming of HIV-1 RTion by two of the mutants that target the IN-encoding region or Env gene was examined and characterized. Two PCR reactions were employed for each mutant (details specified in Methods). After separation of the PCR products, robust DNA bands amplified from the RTion products primed from their targeting sites were detected. In contrast, the PCR product that would reflect the RTion product primed non-specifically from the PBS was not detectable (Figure 7B).

Quantification of transduction and expression of mutant tRNA^{Lys3}

Due to significant variations in the anti-HIV-1 activities of different cell clones transduced with the multiple-copy Mt13TD constructs (Figure 4B), it was speculated that these variations might be caused by difference in the expression of Mt13TD among different clones. Real-time PCR and RT-PCR were employed to quantify the copy number of the Mt13TD gene and the relative expression level of Mt13TD versus that of the wild-type tRNA^{Lys3}. Table 2 shows copy numbers of the Mt13TD gene in cells transduced with the multiple-copy constructs were largely determined by the vector constructs used, with exception for one cell clone transduced with vectors carrying either 3 or 9 copies of Mt13TD respectively, and two cell clones transduced with the construct carrying 12 copies of genes. However, levels of Mt13TD varied notably among cells transduced with the same vector construct. Among cell clones transduced with different multiple-copy constructs, relative expression of Mt13TD varied even more dramatically (Table 3). In particular, expression level of Mt13TD was less than that of the wild-type tRNA^{Lys3} in all cell clones regardless of the copy number of Mt13TD that was introduced into the cells. However, cell clones transduced with more copy numbers of Mt13TD generally tended to have
higher expression levels, and this is consistent to their anti-HIV-1 activities as evaluated in previous tests (Figure 4B).

## Discussion

### Rationale for designing the mutant tRNA^{Lys3}

tRNAs are an essential part of the protein translation mechanism in cells and are recognized by many intracellular proteins including the 5′ and 3′ tRNA processing enzymes [39] and tRNA aminoacyl transferases [40,41]. Retroviruses selectively encapsidate tRNAs as primers, resulting in an increased concentration of certain tRNAs inside the virions compared with the cytoplasm of the infected cells [42-45]. For the selective incorporation of tRNA^{Lys3} into HIV-1, both the vRNA and interactions between the tRNA^{Lys3} and vRNA are dispensable since viral particles lacking an RNA genome are still able to incorporate the wild-type set of tRNA^{Lys3} [45]. However, selective packaging of tRNA^{Lys3} is affected in virions lacking a functional RT domain [45-47], and the centrally located thumb subdomain of RT is indispensable [48].

In HIV-1 virions, all tRNA^{Lys} isoacceptors are enriched. The ratio of tRNA^{Lys3} versus tRNA^{Lys1,2} are the same in cells and virions, with approximately 8 and 12 molecules per particle respectively [49]. The tRNA^{Lys} molecules are encapsidated during particle assembly via interactions between the Gag-Pol precursor and a protein complex composed of the cellular lysyl-tRNA synthetase (LysRS) and the Gag protein [45,48,50-52]. Changing the intracellular levels of LysRS, by either overexpression or siRNA-mediated silencing, results in a concomitantly altered level of tRNA^{Lys} in virus particles. This suggests that LysRS may be the limiting factor for tRNA^{Lys} packaging [53-55]. The presence of other tRNA synthetases in HIV-1 virions has also been analyzed [51,56], with only LysRS detected among eight synthetases screened. Approximately 20–25 LysRS and 20 tRNA^{Lys3} molecules are present per virus particle, indicating an approximately equimolar stoichiometry [51]. These previous studies provided the supporting foundation for this study of anti-HIV-1 replication by designing and constitutive expression of the mutant tRNA^{Lys3}.

In designing the mutant tRNA^{Lys3}, we extended the length of mutations in the 3′ terminal PBS-binding region to enhance their binding specificity and efficiency of directing the RT onset of HIV-1 to new targeting sites. In addition, corresponding mutations to maintain the natural secondary structure of tRNA^{Lys3} were made (Figure 1C) with the natural promoter and terminator sequences needed for transcription and post-transcriptional processing maintained. Integrity of the anti-codon domain that is important for interactions between the tRNA^{Lys3} and LysRS was also kept intact. To facilitate efficient transcription and processing of the mutant tRNA^{Lys3} genes, the 5′ and 3′ flanking sequences that were derived from three of the most efficiently expressed cellular tRNA^{Lys3} loci [57] were included. Consequently, except for the mutations mentioned, the remaining parts of the tRNA^{Lys3} were not altered (Figure 1C). Because both vRNA and interactions between tRNA and vRNA are dispensable in the tRNA^{Lys3} encapsidation process [45], encapsidation of the mutant tRNA^{Lys3} is unlikely to be influenced, as confirmed by RT-PCR tests (Figure 5A).

### Improved inhibition of HIV-1 replication

We report that mutant tRNA^{Lys3} with extended mutations in the PBS-binding region were effectively expressed and

---

### Table 2: Quantification of copy number of Mt13TD in cells transduced with the multiple-copy vector constructs

|   | TD-1 | TD-3 | TD-6 | TD-9 | TD-12 |
|---|------|------|------|------|-------|
| 1 | 2.14±0.17 | 6.08±0.17 | 12.14±0.23 | 18.05±0.26 | 24.11±0.19 |
| 2 | 5.98±0.26 | 12.06±0.28 | 18.03±0.27 | 24.1±0.27 |       |
| 3 | 4.07±0.25 | 12.02±0.13 | 18.04±0.16 | 23.95±0.23 |       |
| 4 | 6.07±0.08 | 12.05±0.24 | 18.07±0.16 | 24.01±0.13 |       |
| 5 | 6.04±0.11 | 12.19±0.17 | 17.99±0.12 | 23.98±0.17 |       |
| 6 | 6.07±0.1 | 11.99±0.26 | 13.08±0.23 | 19.8±0.17 |       |
| 7 | 6.02±0.13 | 11.84±0.47 | 18.06±0.3 | 10.05±0.09 |       |
| 8 | 6.01±0.1 | 11.97±0.1 | 18.0±0.12 | 23.98±0.17 |       |
| 9 | 5.98±0.18 | 12.06±0.25 | 18.03±0.18 | 24.05±0.08 |       |
| 10 | 6.06±0.11 | 12.05±0.11 | 18.1±0.05 | 24.04±0.15 |       |
| 11 | 6.09±0.03 |       |       |       |       |
| 12 | 5.95±0.08 |       |       |       |       |

Note: TD-1,3,6,9,12, retroviral vector carrying 1,3,6,9 and 12 copies of Mt13TD gene respectively; 1–12, cell clones derived from transduced cells with the multiple-copy retroviral constructs.

### Table 3: Relative expression of Mt13TD versus wild-type tRNA^{Lys3} in cells transduced with multiple-copy vector constructs

|   | TD-1 | TD-3 | TD-6 | TD-9 | TD-12 |
|---|------|------|------|------|-------|
| 1 | 6.91±0.65 | 7.48±0.79 | 20.74±0.83 | 22.7±0.41 | 27.01±0.13 |
| 2 | 18.32±0.63 | 17.4±1.18 | 23.11±0.34 | 57.87±0.41 |       |
| 3 | 3.98±0.26 | 15.48±0.43 | 45.42±0.33 | 82.35±0.78 |       |
| 4 | 20.16±0.81 | 22.66±0.32 | 42.48±0.38 | 48.68±0.45 |       |
| 5 | 20.71±0.81 | 26.15±0.35 | 42.72±0.18 | 77.26±0.87 |       |
| 6 | 19.38±1.13 | 48.74±0.35 | 6.8±0.39 | 0±0 |       |
| 7 | 18.33±1.22 | 43.43±0.62 | 65.78±0.76 | 41.1±0.24 |       |
| 8 | 22.79±0.41 | 41.1±0.4 | 40.03±0.45 | 54.37±0.65 |       |
| 9 | 24.89±0.23 | 38.54±1.26 | 39.76±0.64 | 55.74±0.55 |       |
| 10 | 23.19±0.35 | 23.14±0.18 | 28.3±1.03 | 35.18±0.33 |       |
| 11 | 19.14±0.83 |       |       |       |       |
| 12 | 22.9±0.15 |       |       |       |       |

Note: the expression level of wild-type tRNA^{Lys3} is arbitrarily set as 100%, and the relative percentages of expression of Mt13TD are shown.
encapsidated into progeny HIV-1 virions and they redirected the RTion of HIV-1 to targeting sites with improved specificity and efficiency, which concomitantly heightened the inhibition of HIV-1 replication. Furthermore, besides the TAR, sites downstream of the PBS such as the IN-encoding region and Env gene could be effectively targeted. Moreover, enhanced anti-HIV-1 activity was observed when these mutations were combined with a previously reported A58U mutation [32]. This indicates the mutations in the 3’ PBS-binding region conferred anti-HIV-1 activities in a different mechanism from that of the A58U mutation, which could give rise to an additive anti-HIV-1 effect.

**Transduction and expression of mutant tRNA^{lys}_{3}**

For efficient delivery and expression of the mutant tRNA^{lys}_{3} in human cells, a retroviral vector system was optimized [34] and employed. The retroviral vector was used because it does not process inherent capability of inhibiting HIV-1 replication as previously demonstrated [32,33]. This offers an advantage for the analysis of anti-HIV-1 activities compared to a HIV-1-based vector that has been previously shown to process potent inherent inhibition of HIV-1 replication [58]. Furthermore, titers of the retroviral vectors with a single-copy of the mutant tRNA^{lys}_{3} were not significantly affected, indicating the mutants did not interfere with infectivity of the retroviral vector. Although titers of vector preparations were significantly reduced when multiple copies of M13TD were introduced, such reduction might not be directly related the mutant tRNA^{lys}_{3}. Rather, a more plausible explanation is the insertion of a large DNA fragment in the LTR that might hinder vRNA processing and transduction of target cells as previously reported with lentiviral vectors [59]. Rather than being used as a delivery tool, an HIV-1-based vector system was employed for the one-replication-cycle infection assay and provided supplemental evidence to those obtained through HIV-1 challenging tests. When the mutant tRNA^{lys}_{3} constructs were co-transfected with the HIV-1-based vector system, vector titers were significantly reduced by more than one log for four of the mutants tested (Figure 6B). These results indicates a marked hindrance of the RTion process within the HIV-1 virions, which is consistent with potencies of the anti-HIV-1 activities of the mutants as determined by TCID50 assay and HIV-1 challenge tests (Figures 3A and 3B).

**Transduction of multiple copies of mutant tRNA^{lys}_{3}**

Naturally, all tRNA^{lys}_{3} isoacceptors are enriched in the HIV-1 virions with the same ratio of tRNA^{lys}_{3} versus tRNA^{lys}_{1,2} between cells and virions [49]. In addition, there are multiple copies of these genes in the human genome. To improve the encapsidation of mutant tRNA^{lys}_{3} through increasing its concentration in transduced cells, we hypothesized that delivery of multiple copies of the gene may further boost its anti-HIV-1 effects. As anticipated, transduction of multiple copies of the M13TD gene into CEM-SS cells resulted in decreased HIV-1 replication (Figures 4B and 4C). Furthermore, we examined the copy numbers of M13TD in these clones and the expression level of M13TD as compared to that of its wild-type counterpart. We demonstrated the potency of inhibition of HIV-1 replication in the cell clones was more closely determined by the relative level of expression of M13TD, rather than by the copy number of this gene that was introduced into the cells (Figure 4B and C and Tables 2 and 3). As for the variations in expression of the gene, it could be attributed to the position effect of the integration site. For the four clones with less copies of M13TD as expected, we hypothesize that some irregular event such as recombination was the cause, since retroviral vectors are unstable with repeat sequences [60]. Nevertheless, none of these is directly associated with the mutant tRNA^{lys}_{3} but are more likely to be associated with the delivery system. Future optimizations on the vector system or change of the delivery vector may possibly resolve these issues.

**Implications of targeting multiple sites**

Due to high mutation rates of HIV-1, drug resistance mutations constitute a major concern that confronts current antiretroviral strategies. When mutant tRNA^{lys}_{3} targeting a single site of the HIV-1 genome is expressed, potential viral resistance is possible through mutations in the PBS to acquire complementarity to the mutant tRNA^{lys}_{3} or in the site being targeted to reduce its complementarity to the mutant tRNA^{lys}_{3}. Therefore, the mutant tRNA^{lys}_{3}-mediated anti-HIV-1 strategy would be more effective if multiple sites within the HIV-1 genome could be targeted. To test this concept, two tRNA^{lys}_{3} mutants targeting the IN-encoding region and Env gene, respectively, were tested. These targeting sites were selected because only a 7-base substitution in the 3’ PBS-binding region of the tRNA^{lys}_{3} allowed an 18-bp complementarity to the sites respectively (Figure 1A). RTion priming tests indicated that these mutants were as effective as others targeting the TAR (Figure 7B) with high specificity and efficiency, showing no detectable priming activity from the PBS. Both TCID50 assay and HIV-1 challenging tests indicated these mutants could lead to inhibition of HIV-1 infection to similar potencies as M13TD (Figure 3A and 3B). These findings clearly suggest that the mutant tRNA^{lys}_{3}-mediated inhibition of HIV-1 replication is not limited to targeting sites upstream the PBS and other portions of the vRNA could be effectively
targeted. Moreover, this makes it feasible for simultaneous delivery of multiple mutants that target various portions of the vRNA, which would provide a strong genetic barrier for spontaneous evolution of resistant HIV-1 genome.

Proposed anti-HIV-1 mechanisms through mutant tRNA\textsuperscript{Lys3}

Upon HIV-1 infection of cells that express mutant tRNA\textsuperscript{Lys3}, both wild-type and mutant tRNA\textsuperscript{Lys3} are encapsidated into the progeny virions and are capable of initiating RTions from the PBS and the targeting sites, resulting in aberrant RTion products. Furthermore, due to the RNaseH-mediated degradation of vRNA, a gap between the site being targeted and the PBS is generated. Consequently, integrity of the viral genome is disrupted. This would lead to abortion of the genome conversion and non-productive infection of the cell (Figure 8). Consequently, replication cycle of the virus is bleached.

However, due to competition between the wild-type and mutant tRNA\textsuperscript{Lys3}'s and presence of high concentrations of the wild-type tRNA\textsuperscript{Lys3} in the cell, some progeny virions may carry the natural set of tRNA\textsuperscript{Lys3} without mutant tRNA\textsuperscript{Lys3} where infectivity of this type of progeny virus is not affected. In addition, priming of RTion by the wild-type tRNA\textsuperscript{Lys3} and complete synthesis of the (−)ssDNA before disruption of the viral genome through RTion primed by the mutant tRNA\textsuperscript{Lys3} also may take place in some virions due to encapsidation of insufficient amount of mutant tRNA\textsuperscript{Lys3}. This will lead to successful conversion of the viral genome and productive infection of the cell. These possibilities may explain why CEM-SS cells transduced with mutant tRNA\textsuperscript{Lys3} showed significantly reduced support to HIV-1 replication but did not completely eliminate the viral infection. However, impact of these possibilities can be minimized through increasing expression/concentration of the mutant tRNA\textsuperscript{Lys3} in target cells by transduction of multiple copies of the gene, or simultaneous transduction of multiple mutants that target various portions on the HIV-1 genome.

Conclusions

We demonstrated that the potency of anti-HIV-1 activity of the mutant tRNA\textsuperscript{Lys3} correlated with the length of complementarity between the mutated PBS-binding region and the targeting site, and we showed that increasing the concentration of mutant tRNA\textsuperscript{Lys3} in cells through transduction of multiple copies of the gene further augmented its anti-HIV-1 potency. We also targeted effectively various sites widely distributed in the HIV-1 genome, which would provide an effective means of fighting the evolution of resistance HIV-1 mutants. Because of the specific associations between HIV-1 and tRNA\textsuperscript{Lys3}, off-target side effects that are associated with other anti-HIV-1 approaches can be avoided, which may offer significant advantages over conventional anti-HIV-1 methods such as antisense RNA or RNA interference. Inhibition of HIV-1 replication through mutant tRNA\textsuperscript{Lys3} may represent a novel and effective gene therapy approach against HIV-1-associated diseases.

Methods

Mutant tRNA construction and cloning

Mutant tRNA\textsuperscript{Lys3} with various lengths of mutations in their PBS-binding region that targeted new sites in HIV-1 genome were generated through PCR-based mutagenesis as previously described [33] with minor modifications. Briefly, a primer including the coding sequence of tRNA\textsuperscript{Lys3} and the desired mutations, as specified in Table 4, was synthesized (integrated DNA techniques, IDT) and used as a template for PCR amplification with primers FatRNA and RbtRNA. The PCR product was named fragment A. A second PCR used primers FbtRNA and RbtRNA and human genomic DNA extracted from 293T cells as template, with the PCR product named fragment B. A third fusion-PCR used primers FatRNA and RbtRNA, with fragments A and B as templates, and gave rise to a 234-bp fragment. Using primers FatRNA and RbtRNA, the wild-type tRNA\textsuperscript{Lys3} gene was amplified from human genomic DNA, and a mutant tRNA\textsuperscript{Lys3} gene containing the A58U mutation was amplified from plasmid pPPT-PGK-A58U [31] (a kind gift from Dr. Pannelles Vicente, University of Utah). The amplified genes were subsequently cloned into the StuB I restriction site of the plasmid pSV-N2A-GFP [34].

To construct the vector with three copies of Mti13TD, the plasmid with one copy was first digested with Bgl II and blunted with DNA polymerase I Klenow fragment to insert the second copy, and the resultant plasmid was digested with Sac II and blunted to insert the third copy. To construct the vector with six copies, the plasmid with three copies was digested with Mlu I and blunted and inserted with a three-copy fragment cut from the same plasmid with Nhe I and blunted with Klenow fragment. To construct the vector with nine copies, the plasmid with six copies was digested with Mlu I and blunted, and inserted with the three-copy fragment. To construct the vector with twelve copies, the plasmid with nine copies was digested with Mlu I and blunted and inserted with the three-copy fragment. To prevent self-ligation, linearized plasmid DNAs were treated with calf intestinal alkaline phosphatase (New England Biolabs, NEB) as previously described [61] before ligations.

Delivery of mutant tRNA\textsuperscript{Lys3} into target cells

Retroviral vector were packaged and used to transduce CEM-SS cells as previously described [34,62]. Detection
of mutant tRNA_{Lys} in transduced cells was performed by PCR with primers specific to the mutated regions of the mutants and genomic DNA extracted from the cells as template. Primers (F) 5′-TAGACCATAGCTCAG-3′ and (R) 5′-TGGTTAGACCAGATC-3′ were used for detection of the mutants targeting the TAR; primers (F) 5′-TTTATTATAGCTCAGTC-3′ and (R) 5′-TGGGTTATTACAGGG-3′ were used for detection of the mutant targeting the Env gene; and primers (F) 5′-GGTGGGGTAGCTCAG-3′ and (R) 5′-TGGGGGTGGAGGTGG-3′ were used for detection of the mutant targeting the IN-encoding region. To examine expression of the mutant tRNA_{Lys}, RT-PCR was used with the same primers. Briefly, total RNA was extracted from transduced CEM-SS cells using the acid guanidinium isothiocyanate/phenol-chloroform method [63]. First strand synthesis was done using MoMuLV RT (NEB) following the vendor’s manual with 100 ng antisense primer specific to the gene to be detected. One microliter from the reaction was used as template for PCR amplification, with the PCR product separated with 2% agarose gel and visualized through ethidium bromide staining.

**Cells and viruses**

293T cells and CEM-SS cells were routinely maintained and split as previously described [62]. Replication competent HIV-1 virus was generated through transient transfection of 293T cells with plasmid pHIV-thy (from Dr. Planelles) and used for HIV-1 challenging tests.
Table 4 Primers used for PCR-based amplification of mutant tRNA_{Lys3} derivatives

| Primer name | Primer sequence (5' to 3') |
|-------------|-----------------------------|
| FAtRNA      | CAAGCTTATAAGAAAAAGCTCTCTGGAAG |
| RatRNA      | CGGCAATGGGTTTTCTTCTCTGACG   |
| FbRNA       | GTCTTGGTTTTGTGACACT        |
| RibRNA      | GTTCGAAATAAAGGAGGTGGCACGAAACGAC |
| Mt8TD       | GCTCTGGTGAAGAGAGACAGCTAGTCCGTAGTCGAGCTAGCATGAGGTTTATATCTGAGGGTGGTCAAGGCCCTCTGTTAAGGATGCTTTGGC |
| Mt10TD      | GCTCTGGTGAAGAGAGACAGCTAGTCCGTAGTCGAGCTAGCATGAGGTTTATATCTGAGGGTGGTCAAGGCCCTCTGTTAAGGATGCTTTGGC |
| Mt11TD      | GCTCTGGTGAAGAGAGACAGCTAGTCCGTAGTCGAGCTAGCATGAGGTTTATATCTGAGGGTGGTCAAGGCCCTCTGTTAAGGATGCTTTGGC |
| Mt11TD-ASBU | GCTCTGGTGAAGAGAGACAGCTAGTCCGTAGTCGAGCTAGCATGAGGTTTATATCTGAGGGTGGTCAAGGCCCTCTGTTAAGGATGCTTTGGC |
| Mt13TD      | GCTCTGGTGAAGAGAGACAGCTAGTCCGTAGTCGAGCTAGCATGAGGTTTATATCTGAGGGTGGTCAAGGCCCTCTGTTAAGGATGCTTTGGC |
| Int         | GCTCTGGTGAAGAGAGACAGCTAGTCCGTAGTCGAGCTAGCATGAGGTTTATATCTGAGGGTGGTCAAGGCCCTCTGTTAAGGATGCTTTGGC |
| Env         | GCTCTGGTGAAGAGAGACAGCTAGTCCGTAGTCGAGCTAGCATGAGGTTTATATCTGAGGGTGGTCAAGGCCCTCTGTTAAGGATGCTTTGGC |

Note: mutated bases are shown in bold italic.

Primary virus preparation was used to infect CEM-SS cells, and virus-containing supernatant was collected on day 9 when maximal amount of syncytia were observed, aliquoted in 1.0 mL and stored at −80°C until used.

Cytotoxicity tests

MTT assay [64] was performed as previously described [38] with minor modifications. Briefly, cells non-transduced or transduced with mutant tRNA_{Lys3} were inoculated in triplicates at 1.0 × 10^5 cells/well in 96-well plate in 100 μl RPMI-1640 medium with 2% FBS including three wells without cell as blanks, and cultured at 37 C with 5% CO2. Each well was treated with 10 μl MTT (5 mg/mL) for 4 h at 37 C on day 3 post inoculation, followed by addition of 100 μl DMSO. Plate was gently swirled and left with cover in the dark for 4 hours at room temperature. To measure the absorbance, plate was read at 570 nm using a microplate reader (Beckman Coulter AD340). The optical densities (OD) were compared and used for evaluating cell growth and viability.

A lentiviral vector expressing the firefly luciferase gene and eGFP was constructed through cutting the cDNA of luciferase from pNL-CMV-Luc (from Dr. Planelles) with Xho I and Mlu I, and ligated into the plasmid pHR-hTNFR-Fc-eGFP [38] that was cut by Xho I and Asc I. Resultant plasmid, named pHR-luc-eGFP, and pHR-hTNFR-Fc-eGFP were respectively packaged as previously described [65], and used to infect 1.0 × 10^5 CEM-SS at MOI 10.0. On day 7 following infection, supernatant of the cultured cells were collected and examined for sTNFR-Fc expression as previously described [38]. Test for luciferase activity was done with the dual luciferase assay kit (Promega). Following the vendor’s manual, cell lysates were prepared from 1.0 × 10^6 cells using 200 μl 1× PLB through the passive lysis method, and 20 μl from each lysate were sampled for the test. Luminescence was measured in a Turner luminometer-96 (Turner Designs, Sunnyvale, CA). The readings, counts per second (CPS), were used to evaluate luciferase activities.

TCID_{50} assay

TCID_{50} assay was performed as previously described [33] with minor modifications. Briefly, CEM-SS cells at the exponential growth stage were seeded into 96-well plates at 5 × 10^3 cells/well in 100 μL RPMI1640 medium with 10% heat-inactivated fetal bovine serum (FBS). The HIV-1 virus stock was serially diluted 10-fold with RPMI1640 medium without serum, and 100 μL/well of each virus dilution was inoculated into 4 wells with cells along with control wells receiving the same amount of virus-free medium. The infected cells were examined daily for syncytia formation and TCID_{50} readings were determined on day 15 pi.

HIV-1 challenge and P24 assay

HIV-1 challenge and P24 assay was done as previously described [58] with minor changes. Briefly, 4 × 10^5 cells transduced with mutant tRNA_{Lys3} in the exponential growth phase were pelleted with a bench top centrifuge at 3000 rpm for 3 minutes, washed once with 1.0 mL RPMI1640 medium without serum, pelleted, and then
resuspended in 1.0 mL of diluted HIV-1 virus at the desired MOI. After adsorption at 37°C for 90 minutes, cells were pelleted and washed for three times with 1.0 mL RPMI1640. After the third washing and pelleting, supernatant was discarded. Cells were resuspended in 6.0 mL RPMI1640 medium containing 10% heat-inactivated FBS, and incubated at 37°C in a T-25 flask. Every 2 or 3 days following the infection, 0.5 mL of cell-free supernatant was collected from the flasks and used for P24 assay through an antigen capture enzyme-linked immunosorbent assay (ELISA) (Coulter Immunology, Hialeah, FL).

**Mutant tRNA<sup>lys</sup> encapsidation assay**

To confirm encapsidation of mutant tRNA<sup>lys</sup>, retroviral vector plasmid containing the mutants were respectively co-transfected with a three-plasmid HIV-1-based vector system [62]. For negative controls, transfections omitting the packaging plasmid were performed. Supernatant conditioned by transfected cells were collected and titrated on CEM-SS cells. In addition, 35 μL supernatant was concentrated into 0.1 mL through an ultracentrifugation method [34]. Viral RNA was extracted using the QIAamp viral RNA mini kit (Qiagen) with detection of mutant tRNA<sup>lys</sup> from the RNA extractions performed through RT-PCR using the same primers as used for the detection of the mutants form transduced CEM-SS cells.

**Priming assay**

To characterize the specificity and efficiency of RTion primed by mutant tRNA<sup>lys</sup>, a competitive PCR-based method was employed. Briefly, HIV-1-based vectors with mutant tRNA<sup>lys</sup> were prepared and concentrated as previously described, with 0.1 ml of concentrated vector used to infect 1.0 × 10<sup>6</sup> CEM-SS cells. For negative controls, infections were done with the same amount of vector plasmid were performed. Supernatant collected from the flasks and used for DNA extraction as previously described. RTion is initiated from the PBS as previously reported ([65]). Briefly, following activation of the iTaq™ polymerase for 10 min at 95°C, 40 cycles (15 s at 95°C and 30 s at 72°C) and primer annealing at 60°C, the PCR product is 226 bp in size. The primer sets were specific to the wild-type tRNA<sup>lys</sup> and (R) 5′-TAGACCATAGCT-CAGTCGGGTAGACATCAG-3′ (F) 5′-TAGTTAGACCATAGCT-GCCCGGATTTGTAAGGATCG-3′, and primers specific for Mt13TD were (F) 5′-TGGTTAGACCATAGCT-GCCCGGATTTGTAAGGATCG-3′ and (R) 5′-TGGTTAGACCATAGCT-GCCCGGATTTGTAAGGATCG-3′. To examine the relative expression of Mt13TD versus wild-type tRNA<sup>lys</sup>, total cellular RNAs were extracted from the cells, with 2.0 μg RNAs used for RTion as previously mentioned, using the wild-type tRNA<sup>lys</sup>- and Mt13TD-specific reverse primers. Subsequently, copy numbers of the wild-type tRNA<sup>lys</sup> and Mt13TD were respectively determined through real-time PCR.

### Table 5 Primers used for priming assay

| Primer name | Primer sequence (5′ to 3′) |
|-------------|----------------------------|
| F-tRNA | GGAGGTTTGTACAGCGCTAGCAT |
| F-Env | GCAGTAAATGTAGCATGTAATGCAAC |
| F-Int | TAAAGATTAAGAAAAATATTAGGACAGGTAAGAG |
| R-WT-tRNA | GTCCCTGTCGGGCGCA |
| R-Mt8TD | GTCCCTCGTGTCAACCA |
| R-Mt10TD | GTGCCGTCTGTCAACCA |
| R-Mt11TD | GTGACATGTGCTCAACCA |
| R-Mt13TD | TCAGATCTGCTCAACCA |
| R-env | GTTCCTTACACCC |
| R-int | GTCCCTGTAATACCC |

For real-time PCR analysis, 1.0 μl of genomic DNAs or cDNA were amplified in triplicates in 25 μl reaction volumes with 0.2 μM concentrations of each primer using the IQ SYBR GREEN Super mix (Bio-Rad, Hercules, CA). The principle of the real-time PCR has been described elsewhere [65]. Briefly, following activation of the iTaq™ DNA polymerase for 10 min at 95°C, 40 cycles (15 s at 95°C and 30 s at 72°C) and primer annealing at 60°C, the PCR product is 226 bp in size. The primer sets were specific to the wild-type tRNA<sup>lys</sup> and (R) 5′-TAGACCATAGCT-CAGTCGGGTAGACATCAG-3′ (F) 5′-TAGTTAGACCATAGCT-GCCCGGATTTGTAAGGATCG-3′, and primers specific for Mt13TD were (F) 5′-TGGTTAGACCATAGCT-GCCCGGATTTGTAAGGATCG-3′ and (R) 5′-TGGTTAGACCATAGCT-GCCCGGATTTGTAAGGATCG-3′. To examine the relative expression of Mt13TD versus wild-type tRNA<sup>lys</sup>, total cellular RNAs were extracted from the cells, with 2.0 μg RNAs used for RTion as previously mentioned, using the wild-type tRNA<sup>lys</sup>- and Mt13TD-specific reverse primers. Subsequently, copy numbers of the wild-type tRNA<sup>lys</sup> and Mt13TD were respectively determined through real-time PCR.
1 min at 72°C) were performed with the iQ5 real-time PCR detection system (Bio-Rad). The positive controls consisted of the retroviral plasmid containing the gene to be tested. As negative control, samples consisting of distilled water were also subjected to the DNA/RNA extraction procedure and the resulting extracts were amplified. Standard graphs of the CT values obtained from serial dilutions (10 to 10^6 copies) of the retroviral plasmids were constructed, and the CT values from unknown samples were plotted on the standard curves. Subsequently, copy number of the gene was calculated. Copy numbers of the plasmids and the number of human cells that 20 ng genomic DNAs were extracted from were calculated with the formula as following: number of copies/cells = (amount * 6.022×10^{23})/ (length * 1×10^9 * 650) [http://cels.uri.edu/gsc/cndna.html], with amount referring to the amount of DNA present in ng and length referring to the length of plasmid or amount of DNA in a single human cell in bp. The length of mutant tRNALys<sub>3</sub>-containing retroviral plasmid was 9638 bp and length of DNA from a single human cell is about 6.6×10^9 bp [http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/G/GenomeSizes.html]. Relative expression of Mt13TD versus wild-type tRNALys<sub>3</sub> was calculated through dividing the copy number of Mt13TD by the copy number of wild-type tRNALys<sub>3</sub>. Statistical analysis

Origin 6.0 professional software (OriginLab Corporation) was used for two-population t-tests or one-way ANOVA analysis. P < 0.05 was considered statistically significant.

* indicates 0.01 < P < 0.05; ** indicates 0.001 < P < 0.01; *** indicates P < 0.001.

Abbreviations

HIV-1: Human immunodeficiency virus type 1; RTon: Reverse transcription; TAR: Trans-activation response region; PBS: Primer binding site; vRNA: Viral RNA; RT: Reverse transcriptase; R: Repeat region; PPT: Polypurine tracts; LTR: Long terminal repeat; TCID<sub>50</sub>: Median tissue culture infective dose; MOI: Multiplicity of infection; bp: Base pair; pi: Post infection; LysRS: Lysyl-tRNA synthetase; FBS: Fetal bovine serum; ELISA: Enzyme-linked immunosorbent assay; CPS: Count per second; OD: Optical density.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

CW participated in designing the study, carried out the experiments, collected and interpreted the data, and wrote the manuscript. YL participated in coordination of the study and revised the manuscript. YL conceived and designed the study, participated in data analysis and coordination, and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Authors would like to thank Dr. Vicente Planelles for the parental plasmids used in this study, and his critical reading of the manuscript. This study was supported by U.S. Public Health Service grants S11NS043499 from the National Institute of Neurological Disorders and Stroke, RO1MH079717 from the National Institute of Mental Health, and G12RR003061 from the former National Center for Research Resources, National Institutes of Health.

Author details

1 Department of Public Health Sciences, John A. Burns School of Medicine, University of Hawaii at Manoa, 1960 East–west Road, Biomed. Bldg. D105, Honolulu, Hawaii 96822, USA. 2 Department of Microbiology, College of Natural Sciences, University of Hawaii at Manoa, 2538 McCarthy Mall, Snyder 207, Honolulu, HI 96822, USA. 3 Departments of Tropical Medicine, Medical Microbiology and Pharmacology, Asia-Pacific Institute of Tropical Medicine and Infectious Diseases, John A. Burns School of Medicine, University of Hawaii at Manoa, 651 Ilalo Street, BSB 325AA, Honolulu, HI 96813, USA.

Received: 22 April 2013 Accepted: 25 September 2013

Published: 24 October 2013

References

1. Zhang H, Dornadula G, Pomerantz RJ: Endogenous reverse transcription of human immunodeficiency virus type 1 in physiological microenvironments: an important stage for viral infection of nondividing cells. J Virol 1996, 70(3):2809–2834.
2. Mougel M, Houzet L, Darlix JL: When is it time for reverse transcription to start and go? Retrovirology 2009, 6:24.
3. Harada F, Peters GG, Dahlberg JE: The primer tRNA for moloney murine leukemia virus DNA synthesis. Nucleotide sequence and aminocylation of tRNA<sub>51U</sub>. J Biol Chem 1979, 254:10979–10985.
4. Harada F, Sawyer RC, Dahlberg JE: A primer ribonucleic acid for initiation of in vitro Rous sarcoma virus deoxyribonucleic acid synthesis. J Biol Chem 1975, 250:3467–3470.
5. Litvak S, Araya A: Primer transfer RNA in retroviruses. Trends Biochem Sci 1982, 7:361–364.
6. Leis J, Aiyar A, Cobrinik D: Regulation of initiation of reverse transcription of retroviruses. In Reverse transcriptase. Edited by Skalka AM, Goff SP. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1993:93–48.
7. Marquet R, Isel C, Ehresmann C, Ehresmann B: tRNAs as primer of reverse transcriptase activities. Biochimie 1995, 77:113–124.
8. Mak J, Kleiman L: Primer tRNAs for reverse transcription. J Virol 1997, 71:8087–8095.
9. Telefsnitsky A, Goff SP: Retroviral transcriptase and the generation of retroviral DNA. In Retroviruses. Edited by Coffin JM, Hughes SH, Varmus HE. Plainview, NY: Cold Spring Harbor Laboratory Press; 1997:121–162.
10. Wain-Hobson S, Sonigo P, Danos O, Cole S, Alizon M: Nucleotide sequence of the AIDS virus, LAV. Cell 1985, 40:9–17.
11. Guyader M, Emerman M, Sonigo P, Clavel F, Montagnier L, Alizon M: Genome organization and transactivation of the human immunodeficiency virus type 2. Nature 1987, 326:662–669.
12. Goff SP: Retroviral reverse transcriptase: synthesis, structure, and function. J Acquir Immune Defic Syndr 1999, 3:817–831.
13. Ben Artzi H, Shemesh J, Zeelon E, Amit B, Kleiman L, Gorecki M, Panet A: Molecular analysis of the second template switch during reverse transcription of the HIV RNA template. Biochemistry 1996, 35:10549–10557.
14.Auxilien S, Keight G, Le Grice SFJ, Darlix J-L: Role of post-transcriptional modifications of primer tRNALys<sub>3</sub> in the fidelity and efficacy of plus strand DNA transfer during HIV-1 reverse transcription. J Biol Chem 1999, 274:4412–4420.
15. Allain B, Lapadat-Tapolsky M, Berloz C, Darlix J-L: Transactivation of the minus-strand DNA transfer by nucleocapsid protein during reverse transcription of the retroviral genome. EMBO J 1994, 13(4):973–981.
16. Tsuchihashi Z, Brown PO: DNA strand exchange and selective DNA annealing promoted by the human immunodeficiency virus type 1 nucleocapsid protein. J Virol 1994, 68(9):5863–5870.
17. Guo J, Henderson LE, Bess J, Kane B, Levin JG: Human immunodeficiency virus type 1 nucleocapsid protein promotes efficient strand transfer and specific viral DNA synthesis by inhibiting TAR-dependent self priming from minus-strand strong-stop DNA. J Virol 1997, 71(7):1518–1528.
18. Guo J, Wu T, Anderson J, Kane BF, Johnson DG, Gorelick RJ, Henderson LE, Levin JG: Zinc finger structures in the human immunodeficiency virus type 1 nucleocapsid protein facilitate efficient minus- and plus-strand transfer. J Virol 2000, 74(19):8980–8988.
Yu Q, Dark J. The zinc finger of nucleosidase protein of Friend murine leukemia virus is critical for proviral DNA synthesis in vivo. J Virol 1996, 70(3):5791–5798.

Gonsky J, Bacharach E, Goff SP. Identification of residues of the moloney murine leukemia virus nucleosidase critical for viral DNA synthesis in vivo. J Virol 2001, 75(8):5269–5276.

Dark J, Lapadat-Traczyk M, de Rocquigny H, Roques BP. First glimpses at structure-function relationships of the nucleosidase protein of retroviruses. J Mol Biol 1995, 254(4):523–537.

Li X, Mak J, Arts EI, Gu Z, Kleinman L, Wainberg MA, Pamia MA. Effects of alterations of primer-binding site sequences on human immunodeficiency virus type 1 replication. J Virol 1994, 68:6198–6206.

Das AT, Klaver B, Berkhourt B. Reduced replication of human immunodeficiency virus type 1 mutants that use reverse transcription primers other than the natural tRNA3(Lys). J Virol 1995, 69:3000–3007.

Wakefield J, Wolf AG, Morrow CD. Human immunodeficiency virus type 1 can use different tRNAs as primers for reverse transcription but selectively maintains a primer binding site complementary to tRNA(Lys). J Virol 1995, 69:6021–6029.

Das AT, Klaer B, Berkhourt B. Sequence variation of the HIV primer-binding site suggests the use of an alternative tRNA3(Lys) molecule in reverse transcription. J Gen Virol 1997, 78:837–840.

Abink TB, Breevers NJ, Berkhourt B. Forced selection of a human immunodeficiency virus type 1 variant that uses a non-self tRNA primer for reverse transcription: involvement of viral RNA sequences and the reverse transcriptase enzyme. J Virol 2004, 78(19):10762–10774.

Renda MJ, Bradel-Tretheway B, Planelles V, Bambara RA, Dewhurst S. Inhibition of HIV type 1 replication using lentiviral-mediated delivery of mutant tRNA(Lys)3(ASU)5. AIDS Res Hum Retroviruses 2004, 20:324–334.

Renda MJ, Rosenblatt JD, Klimatcheva E, Demeter LM, Bambara RA, Planelles V. Mutation of the methylated tRNA(Lys)(3) residue A58 disrupts reverse transcription and inhibits replication of human immunodeficiency virus type 1. J Virol 2001, 75:9671–9678.

Lu Y, Planelles V, Xu X, Palanippan C, Day B, Chalita-Ed P, Amado R, Stephens D, Kohn DB, Bakker A, Fay P, Bambara RA, Rosenblatt JD. Inhibition of HIV-1 replication using a mutated tRNA3(Lys) primer. J Biol Chem 1997, 272:14523–14531.

Wu C, Lu Y. High-titre retroviral vector system for efficient gene delivery into human and mouse cells of haematopoietic and lymphoid lineages. J Gen Virol 2010, 91:1909–1918.

Hantzena P, Sullenger BA, Unger G, Gilboa E. Improved gene expression upon transfer of the adenosine deaminase minigene outside the transcriptional unit of a retroviral vector. Proc Natl Acad Sci USA 1989, 86(10):3519–3523.

Armentano D, Yu SF, Kantoff PW, von Ruden T, Andersson WF, Gilboa E. Effect of internal viral sequences on the utility of retroviral vectors. J Virol 1989, 61:1647–1650.

Baldwin TD. Firefly luciferase: the structure is known, but the mystery remains. Structure 1996, 4(3):223–228.

Cao S, Wu C, Yang Y, Sniderman LF, Maggirwar SB, Dewhurst S. Lu Y. Lentiviral vector-mediated stable expression of stNFRE-Fc in human macrophage and neuronal cells as a potential therapy for NeuroAIDS. J Neuroimmunol 2011, 231:131–138.

Altmann S. Transfer RNA processing enzymes. Cell 1981, 23(1):3–4.

Sampson JR, Dillenzo AB, Behlen LS, Uhlenbeck OC. Role of the tertiary nucleotides in the interaction of yeast phenylalanine tRNA with its cognate synthetase. Biochemistry 1990, 29:2523–2532.

Behlen LS, Sampson JR, Dillenzo AB, Uhlenbeck OC. Lead-catalyzed cleavage of yeast tRNAPhe mutants. Biochemistry 1990, 29:2515–2523.

Waters LC, Mullin BC. Transfer RNA in RNA tumor viruses. Prog Nucleic Acid Res Mol Biol 1977, 20:131–160.

Jiang M, Mak J, Wainberg MA, Pamia MA, Cohen E, Kleinman L. Variable tRNA content in HIV-1188. Biochem Biophys Res Commun 1992, 185:1005–1015.

Jiang M, Mak J, Ladhja A, Cohen E, Klein M, Rovinski B, Kleinman L. Identification of tRNAs incorporated into wild-type and mutant human immunodeficiency virus type 1. J Virol 1993, 67:3246–3253.

Mak J, Jiang M, Wainberg MA, Harmanskold ML, Roksh D, Kleinman L. Role of Pr160gag-pol in mediating the selective incorporation of tRNA(Lys) into human immunodeficiency virus type 1 particles. J Virol 1994, 68:2006–2027.

Peters GG, Hu J. Reverse transcriptase as the major determinant for selective packaging of tRNA’s into avian sarcoma virus particles. J Virol 1980, 36:692–700.

Levin JG, Seidman JG. Effect of polymerase mutations on packaging of primer tRNA during murine leukemia virus assembly. J Virol 1981, 43:403–408.

Khochid A, Javanbakht H, Wise S, Halwani R, Pamia MA, Wainberg MA, Kleinman L. Sequences within Pr160gag-pol affecting the selective packaging of primer tRNA into HIV-1. J Mol Biol 2001, 299:17–26.

Huang Y, Mak J, Cao Q, Li Z, Wainberg MA, Kleinman L. Incorporation of excess wild-type and mutant tRNA3(Lys) into human immunodeficiency virus type 1. J Virol 1994, 68:7676–7683.

Jiang M, Mak J, Huang Y, Kleinman L. Reverse transcriptase is an important factor for the primer tRNA selection in HIV-1. Leukemia 1994, 8:149–151.

Gates S, Javanbakht H, Kim S, Shibata K, Craven R, Rein A, Ewalt K, Schimmel P, Musier-Forsyth K, Kleinman L. Retrovirus-specific packaging of aminocyclic tRNA synthetases with cognate primer tRNAs. J Virol 2002, 76:13111–13115.

Javanbakht H, Halwani R, Cen S, Saadatmand J, Musier-Forsyth K, Gottlinger H, Kleinman L. The interaction between HIV-1 Gag and human lysyl-tRNA synthetase during viral assembly. J Biol Chem 2003, 278:27644–27651.

Gates S, Cen S, Javanbakht H, Kim S, Shibata K, Cen S, Javanbakht H, Kleiman L. Effect of altering the tRNA concentration in human immunodeficiency virus type 1 upon its annealing to viral RNA, Gag-Pol incorporation, and viral infectivity. J Virol 2002, 76:9006–9102.

Guo F, Cen S, Niu J, Javanbakht H, Kleiman L. Specific inhibition of the synthesis of human lysi-tRNA synthetase results in decreases in tRNA (Lys) incorporation, tRNA(Lys)(3)Lys annealing to viral RNA, and viral infectivity in human immunodeficiency virus type 1. J Virol 2003, 77:9817–9822.

Cen S, Javanbakht H, Niu M, Kleiman L. Ability of wild-type and mutant lysyl-tRNA synthetase to facilitate tRNA(Lys) incorporation into human immunodeficiency virus type 1. J Virol 2004, 78:1595–1601.

Halwani R, Cen S, Javanbakht H, Niu M, Kleiman L. Effect of altering the tRNA concentration in human immunodeficiency virus type 1 assembly. J Virol 2004, 78:7533–7546.

Roy KL, Cooke H, Buckland R. Nucleotide sequence of a segment of human DNA containing the three tRNA genes. Nucleic Acids Res 1982, 10:313–322.

Zeng L, Planelles V, Sui Z, Gartner S, Maggirwar SB, Dewhurst S, Ye L, Nenurkar VR, Yagayhara R, Lu Y. HIV-1-based defective lentiviral vectors efficiently transduce human monocyes-derived macrophages and suppress replication of wild-type HIV-1. J Gene Med 2006, 8(1):18–28.

Uritani F, Arumugam P, Higashimoto T, Perumerti A, Mitts K, Xia P, Malik P. Mechanism of reduction in titers from lentivirus vectors carrying large inserts in the 3LTR. Mol Ther 2009, 17(9):1527–1536.

ter Brake G, Tweel A, Heo S, Li Y, Centelles M, van Eij K, Berkhourt B. Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition. Mol Ther 2008, 16:5517–5564.

Wu C, Nenurkar VR, Yanagihara R, Lu Y. Effective modifications for improved homologous recombination and high-efficiency generation of recombinant adenovirus-based vectors. J Virol Methods 2008, 153(2):120–129.

Wu C, Lu Y. Inclusion of high molecular weight dextran in calcium phosphate-mediated transfection significantly improves gene transfer efficiency. Cell Mol Biol (Nisy-in-Grand) 2007, 53:67–74.

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987, 162(1):156–159.
64. Jeffrey ME, Armstrong LS, Martinez AO: A rapid and simple MTT-based spectrophotometric assay for determining drug sensitivity in monolayer cultures. *Methods Cell Sci* 1988, 11:15–17.

65. Heid CA, Stevens J, Livak KJ, Williams PM: Real time quantitative PCR. *Genome Res* 1996, 6:986–994.

doi:10.1186/1742-4690-10-112
Cite this article as: Wu et al: New insights into inhibition of human immunodeficiency virus type 1 replication through mutant tRNA<sup>Lys</sup><sub>3</sub>. *Retrovirology* 2013 10:112.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at www.biomedcentral.com/submit