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Von Eije, K.J.

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Early versus late viral escape upon anti-HIV-1 shRNA pressure

Karin J. von Eije, Ying Poi Liu, Boaz Aronson and Ben Berkhout

4.1 Abstract

RNA interference (RNAi) is a cellular mechanism in which small interfering RNAs (siRNAs) mediate sequence-specific gene silencing of the targeted mRNA. Previously we have described the effective inhibition of HIV-1 by the stable expression of a single shRNA. But when a mono-shRNA approach is used, viral escape can occur after the generation of a single point mutation in the 19-nucleotide target sequence. In Chapter 2 we describe a large scale escape study in which early escape viruses were sampled. This yielded various escape routes per shRNA. Now we test whether late sampling of viruses yields a different viral escape pattern. We found for Tat-Rev cultures that the escape pattern at late sampling was even more restricted than in the early samples. Interestingly, double mutants are not a preferred escape route and only one double mutant became fixated in one of the 27 cultures. In the early escape study, the viral variations resembled the natural sequence variation of HIV-1 isolates. This became even more apparent in the late sampled escape population. For the shRNA targeting the Tat-Rev overlapping reading frame, we made several molecular clones of escape mutants. These clones were tested for their virus production capacities, also in the presence of the shRNA inhibitor. All these clones were able to produce virus levels similar to wild-type virus and, in contrast to the wild-type virus, they were able to produce virus in the presence of the shRNA inhibitors.
4.2 Introduction

In Chapter 2 we described several early viral escape routes upon the induction of shRNA pressure. In this large scale shRNA escape study, all four tested shRNAs target highly conserved regions in the \textit{gag}, protease (PR), integrase (IN) and tat-rev sequences. Compared with the viral escape routes of an shRNA targeting the non-essential \textit{nef} gene, the shRNAs targeting highly conserved regions induced a restricted escape pattern. Whereas a variety of escape routes were apparent for the shNef inhibitor; deletion of the target region, point mutations in the target region and RNA structural changes were observed \cite{318}, only point mutations within the target region were observed for the shRNAs targeting highly conserved regions. A mixed pool of mutants could be detected in various escape cultures. Mostly a single and sometimes a second mutation occurred in the target region. In Chapter 2 the escape pattern was studied immediately after the initial round of virus replication, which showed a whole scala of escape routes. Possibly at this stage the selection for the most RNAi resistant and fittest viral mutants is not yet optimal. This issue is important because the selection of RNAi-resistant, yet attenuated HIV-1 variants is of clinical relevance, as it may result in a reduced viral load and delayed disease progression.

In this Chapter we set out to compare the early and late sampled escape routes upon shRNA pressure. For this study we selected several interesting viral escape cultures from the escape study described in Chapter 2. We passaged the selected viral supernatants onto fresh shRNA-expressing cells, allowing the most fit and RNAi-resistant viruses to be selected. In the early samples we observed some double mutants and it can be expected that their number will increase over time, since this might increase the level of RNAi resistance. On the other hand, a further loss of viral fitness may occur when double mutants arise. In the previous study we already noted that mutations were not just random point mutations, as further restrictions were apparent for all shRNA inhibitors. This was most notable for the PR shRNA, for which non-silent changes were profoundly counter-selected. Targeting the Tat-Rev overlap sequences provided another interesting restriction pattern: non-silent changes occurred preferentially in Rev, and not in Tat, and the few amino acid changes selected in Tat are conservative in nature. We interpreted these results in terms of viral replication fitness. Many target mutations are able to provide RNAi-resistance, but viral replication can be reduced by mutation of important regulatory sequences and/or essential amino acids.

If the escape pattern becomes more restricted in the late sampled escape viruses, this would be good news for the development of a second generation therapy against this small set of fit and RNAi-resistant escape variants. In such a second generation approach, several shRNAs that target wild-type and the observed escape variant sequences are included in a combination therapy. For PR we observed 7 escape routes in the early sampled cultures, so one could consider the use of a second generation therapy by adding 7 shRNAs that target these mutant sequences \cite{45}. The absence of more non-silent mutations indicates that this PR domain is critical for enzyme
function and we expect that amino acid substitutions generate viruses with reduced fitness. For PR most mutations were already silent, but this was not the case for Tat-Rev and a more restricted escape pattern at late sampling might occur, which would make it an interesting candidate for a second generation approach.

4.3 Materials and Methods

Plasmid construction, lentiviral vector production and transduction
See Chapter 2 for materials and methods. Tat-Rev-resistant HIV-1 LAI molecular clones were generated by site directed mutagenesis \[210\]. pLAI was digested with SalI and BamHI and the Tat-Rev fragment was cloned into pBSK to generate pBSK-Tat-Rev. Mutations were introduced into pBSK-Tat-Rev by site-directed mutagenesis, verified by sequence analysis, and the mutant fragment was subsequently cloned back into pLAI and sequence checked.

HIV-1 infection on SupT1-shRNA cells
SupT1-shRNA (5-ml cultures, \(2.5 \times 10^6\) cells) were infected with the HIV-1 escape variants, initially 1 \(\mu l\) of viral supernatant was added, for the cultures that did not show signs of replication within 9 days a new infection with 50 \(\mu l\) of viral supernatant was started. Virus replication was monitored by syncytia formation. When virus replication was observed, cell free virus supernatant was passaged onto fresh SupT1-shRNA cells (10 \(\mu l\)) and virus replication was monitored. Viruses were passaged five times in total, and subsequently sequence analyzed in the target region.

Sequencing proviral target regions
See method description in Chapter 2

Transfection of molecular Tat-Rev clones
Cotransfections were performed in the 48-well format, 100,000 293T cells were seeded in 0.5 ml DMEM with 10% FCS without antibiotics. Transfection was performed the next day when the cells reached a confluence of approximately 70%. DNA lipofectamine mix was prepared according to the manufacturers protocol (Invitrogen) and added to the cells. To measure virus production of the HIV-1 molecular clones LAI-wild-type and LAI-Tat-Rev mutant HIV-1 plasmids (100 ng) were transfected on the cells. The culture supernatant was collected at forty-eight hours post-transfection and supernatant was used to measure CA-p24 by ELISA. Experiment was performed three times and average and standard deviation were calculated. To test for viral inhibition with the Tat-Rev shRNA we cotransfected LAI-wt or LAI Tat-Rev molecular HIV-1 plasmids (100 ng) with 10 or 100 ng JS1-shTat-Rev plasmid in the cells. Supernatant was collected at twenty-four hours post transfection and CA-p24 was measured by ELISA.
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4.4 Results

4.4.1 Experimental design for detection of the late HIV-1 shRNA escape variants

The four potent shRNA inhibitors target *gag*, *protease (PR)*, *integrase (IN)*, and *tat-rev* sequences (Chapter 2, Figure 2.1A). These were studied for early escape, and we now set out to study the viral population after long-term exposure to shRNA pressure. We selected in total 27 escape cultures from the previous escape study. As a selection criterium, only cultures without detectable wild-type virus in the early escape analysis were included. Cultures varied from having only a single mutant or containing a mixed mutant population (Table 4.1, left side). Viruses were passaged onto the SupT1 T cell line that was transduced with the JS1-shRNA lentiviral vectors (Chapter 2, Figure 2.1B). When virus replication became apparent, as determined by syncytia formation, cell-free supernatant was passaged onto fresh shRNA cells. The average time for syncytia formation to occur was between 7-17 days at passage 2 and at passage 4 this was between 3 and 9 days post infection. This reduction in days could be an indication for improved viral fitness in the viral population over time. After five passages the integrated HIV-1 pro-viruses were sequenced as previously described (Chapter 2).

4.4.2 Early versus late escape variants

In Table 4.1 the early and late escape variants are depicted. The virus population in each culture can evolve in different ways: the culture can theoretically stay the same (no change), a mutation that is present in the early mixture can become dominant (mutant selection), if a new mutation became dominant we named this a *de novo* mutation and if a virus acquires an additional mutation that becomes dominant we named this the selection of a double mutant. The viral population showed 9 × no change as the population was already dominant, 10 × mutant selection, 7 × *de novo* mutation and 1 × the selection of a double mutant. Thus the majority of the virus populations still demonstrated ongoing evolution, most likely to generate a more fit and/or RNAi-resistant phenotype. Outside of the target regions no fixated mutations became apparent in the sequenced regions as was also observed for the early escape viruses.

4.4.3 Late sampling reduces the number of viral escape routes for PR and Tat-Rev

The number of escape routes stays equal over time for the Gag and IN shRNA escape cultures, with 2 and 8 escape routes respectively. But PR yields 8 escape routes in the early analysis and only 4 escape routes in the late cultures. Tat-Rev virus populations that initially shows 14 different mutants, yield a restricted pattern of 6 routes when viruses are sampled at a later stage. The positions that are mutated
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Table 4.1: Early versus late viral escape. A selection of viral supernatants derived from the previous large scale escape study was passaged five times in total on the corresponding shRNA expressing cell line. The cultures that are depicted are (A) Gag, (B) PR, (C) IN and (D) Tat-Rev. On the left the early input virus from the first passage and on the right the late (fifth passage) detected viral sequences are depicted. The amount of TA-clones analyzed for each mutant are depicted at the right side of the mutant sequence.
in the 19-nucleotide sequence remains similar for Gag, PR and IN, but for Tat-Rev initially 9 positions are used, whereas late sampling shows only five positions.

Previously we had reported that the preferred escape route was the G-to-A mutation in the early sampled escape viruses, in concordance with the fact that this mutation is the most prevalent in HIV-1. There is a small reduction in the amount of G-to-A mutations in the late sampled viruses. And for the transversions that are more difficult to generate than transitions, their number slightly increases for the late sampled viruses. The modest increase in transversions could be due to the more disruptive nature of Pu-Pu or Py-Py pairing compared with Pu-Py pairing (see also Chapter 2), resulting in an increased RNAi-resistant phenotype.

4.4.4 Double mutants are not the preferred escape route under RNAi pressure

The RNAi-resistant viral variants require a balance between an RNAi resistant phenotype and the maintenance of viral fitness. In the early escape study we detected multiple double mutants. We hypothesized that these are more resistant and thus would eventually be favored over single mutants. As the generation of double mutants is a rare event compared with single mutants, we expected that late sampling might allow the selection of more double mutants. In the early escape cultures we observed 9 different double mutants, always as a minority population, but none of these double mutants were still present in the late sampled virus populations. In the late passage, four new double mutants are present, but 3 remain a minority population and only one is dominantly present in the culture (IN, culture A). Thus, the selection of double mutants is also at a later sampling stage not the preferred escape route, possibly due to the fact that multiple mutations in these highly conserved regions render the virus less fit. Alternatively, the virus may require more time to select appropriate double mutations that do not affect viral fitness. Another possibility is that the double mutant is not superior in RNAi resistance compared to the single mutants.

4.4.5 Natural variation

As extensively discussed in Chapter 3 viral sequence diversity as induced by RNAi escape does resemble the sequence variation of natural HIV-1 isolates. We reasoned that the selection of fit HIV-1 variants underlies this common evolution result. We wondered if the RNAi-induced sequence variation would resemble the natural variation even more closely for the late sampled viruses. We plotted the number of escape mutants that occurred per position for all 27 cultures used in this study (Figure 4.1). Clones that were detected only once in a culture were excluded from the analysis. For methods see Chapter 3. The early Gag, PR and IN cultures did already resemble the natural sequence variation, and no dramatic changes were detected in later samples. For the Tat-Rev escape viruses, early mutations at position 7, 11 and 13 are not detected in the natural variation and also disappeared in the late samples.
Figure 4.1: Mutations per position for the early and late evolution samples and natural HIV-1 variation. For methods see Chapter 3. (A) Composite of mutations observed within the 19-nucleotide targets in the early sampled viral escape cultures. (B) Composite of mutations observed within the 19-nucleotide targets in the late sampled viral escape cultures. (C) Composite of natural sequence variation within the same four 19-nucleotide targets in HIV-1 strains from the Los Alamos HIV-1 sequence database [131].
4.4.6 The Tat-Rev overlap case

Viral resistance against RNAi is an interplay between the impact on replication efficiency of a mutant and the RNAi resistance gained by certain mutations. The overall pool of escape viruses remained mostly stable for Gag, PR and IN in the early compared with the late sampled viruses, but the Tat-Rev virus pool did undergo some significant changes. Initially a wide variety of mixed populations was observed in the Tat-Rev virus pool, which became more skewed in the late sampled virus population. The Tat-Rev overlap may provide a special situation. First, codon changes silent for Tat and Rev are nearly impossible due to the overlapping reading frames. Only two possibilities exist for such a Tat-Rev-silent escape: A6C and A18C. These Tat-Rev-silent mutants were never detected. Second, the Tat domain encodes the basic stretch of amino acids (48GRKKR52) that encodes an important region, harboring the nuclear localization signal and the TAR-binding domain [81]. Third, this target also contains the rev start codon at position 1 to 3. Thus these many different functions that are encoded in this small nucleotide stretch makes it an interesting target to study in further detail. The Tat-Rev region yielded various minority double mutants in the early sampled virus population, but none of these could be detected in the late sampled viruses. We set out to test if this was due to the loss of viral fitness.

Table 4.2: Tat-Rev molecular clones. The generated molecular clones with escape mutations in the 19-nucleotide targets. The Tat-Rev target is shown from 5′ to 3′, positions 1 to 19. The type of mutation is indicated and the amino acid changes for Tat and Rev are shown in the middle and right columns respectively.

| mutant     | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | Tat | Rev |
|------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| G11A       |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | S  |
| G8A+G11A   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | E  |
| A13G+C15A  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | G  |
| A6C        |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

In Table 4.2 the generated molecular LAI HIV-1 clones are depicted. We selected one single mutant (G11A) and two double mutants (G8A+G11A and A13G+C15A). These molecular clones were selected from the early escape mutants, that were absent in the late sampled viruses, therefore we expect these variants to not be optimally viral fit. We also included the Tat-Rev-silent A6C mutant that was not observed in the evolution experiment.

We tested if the generated Tat-Rev mutants could produce virus. For this we cotransfected the pLAI wild type and mutant plasmids in 293T cells and measured CA-p24 as a measure of virus production at 48 hours post transfection. We set the virus production of the LAI wild type at 1 (Figure 4.2A). The variant that were observed in the evolution experiment, although all disappeared at the later stage, were able to produce virus at a level that is similar to that of the wilt-type virus. Also the man-made Tat-Rev-silent A6C was able to produce virus. We next set out to test if the mutant viruses exhibited different levels of RNAi resistance. To
Figure 4.2: Tat-Rev single versus double mutants. (A) Viral production of the Tat-Rev molecular clones. Viral CA-p24 production was measured for the molecular clones and corrected with the control. (B) Virus production in the presence of Tat-Rev shRNA.
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test this, we cotransfected the molecular clone plasmids with two concentrations of shRNA (Figure 4.2B). The wild-type clone did not produce virus, in contrast to all mutant molecular clones that show an RNAi-resistant phenotype, that is normal production of virus at both shRNA concentrations. Thus, also the double mutants are fit and display an RNAi resistant phenotype. Virus replication studies and especially competition assays are required to detect subtle differences in fitness.

4.5 Conclusion

Here we studied the difference between early and late sampled RNAi-resistant HIV-1 variants. When viral escape mutants are sampled after five passages the viral escape pattern becomes more restricted for the Tat-Rev cultures. For the PR and IN cultures it becomes modestly more restricted. Most likely only the optimally RNAi-resistant and most fit viruses remain present after five passages. For Tat-Rev the viral escape pattern moves even more to the sequence variation observed in natural HIV-1 isolates. Interestingly, we had expected that the number of double mutants would increase over time but this did not happen. Only one culture gets a dominant double mutant, sometimes de novo mutations are made or mixed cultures get a selection of one or two mutants. In the early cultures more escape variants are present for Tat-Rev. Certain mutations become fixed over time and furthermore the number of positions used for viral escape in the target decreases. The easy to generate G-to-A mutations becomes less prominent and the number of more difficult transversions increases slightly. As reported in Chapter 2 no fixed mutations outside of the target sequence could be observed.

A small selection of Tat-Rev molecular HIV-1 clones was tested for viral fitness and shRNA resistance. All tested molecular clones were able to produce normal amounts of virus in the absence and presence of an shRNA. To get a better understanding about the viral fitness constraints and shRNA resistance more research is warranted. In subsequent studies we will test more molecular clones for the tat-rev target region. In addition, replication studies and competition assays will be performed to detect small differences in fitness with and without shRNA inhibitors. Because of the multitude of viral escape routes observed, we conclude that a second generation approach requires a significant number of shRNAs, and targeting multiple regions instead of a single target region might be a better therapeutic RNAi approach for HIV-1 [287].

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