Citation: Molecular Therapy–Nucleic Acids (2012) 1, e26; doi:10.1038/mtna.2012.22

www.nature.com/mtna

LETTER TO THE EDITOR

HIV-1 Escape From RNAi Antivirals: Yet Another Houdini Action?

Molecular Therapy–Nucleic Acids (2012) **1,** e26; doi:10.1038/mtna.2012.22; advance online publication 12 June 2012

To the Editor: Since the identification of HIV-1 as the primary cause of AIDS, much effort has been made to develop therapies that potently inhibit virus replication. Unfortunately, HIV-1 uses an error-prone replication machinery that allows the virus to rapidly adapt to new conditions. As a consequence, early single-drug therapies failed because of the rapid appearance of drug-resistant virus variants. The simultaneous use of multiple drugs prevents viral escape because mutations are required in multiple drug targets. This combinatorial antiretroviral therapy significantly improved the prospects of HIV-1 infected patients. However, multidrug-resistant viruses can emerge and drug toxicity is an issue, thus new antiviral therapies are needed.

Gene therapy based on RNA interference (RNAi) seems to be a promising new strategy to fight chronic HIV-1 infection. Arming cells with small interfering RNAs (siRNAs) triggers degradation of the complementary HIV-1 RNA genome. A single siRNA can yield a potent antiviral effect, but the virus can escape by acquiring mutations in the siRNA-target sequence. Combinatorial RNAi approaches prevent viral RNAi escape because it requires acquisition of mutations in all siRNA-target sites. However, in their recent article in Molecular Therapy titled "HIV Develops Indirect Cross-resistance to Combinatorial RNAi Targeting Two Distinct and Spatially Distant Sites"[1], Shah *et al*. suggest that HIV-1 can escape from combinatorial RNAi by the selection of mutations in regions of the viral genome other than the actual RNAi targets, thus seriously challenging the potential of combinatorial RNAi in the fight against HIV-1.

The authors generated T-cell lines that stably produced a single or two siRNAs (Ldr3 and TatB2) that were developed in previous studies.[2,3] In patients undergoing RNAi-based gene therapy, only a fraction of the cells would be modified. To mimic this situation, the authors mixed siRNA-producing cells with unmodified cells. In agreement with previous studies, the chosen siRNAs inhibited HIV-1 replication and the combinatorial approach was most effective. Nevertheless, break-through virus replication was observed within 32 days of follow-up, which resulted in a high virus titer. The percentage of cultures in which the virus escaped from RNAi suppression was ~25% for Ldr3 cells, ~65% for TatB2 cells, and ~10% for Ldr3+TatB2 cells. The authors sequenced parts of the HIV-1 genome to identify sequence changes that are responsible for viral escape. Single point mutations were frequently observed in the siRNA targets, but only in part of the virus population. This inspired the authors to look for alternative escape scenarios by sequencing other parts of the HIV-1 genome. Mutations were detected in all regions, but only the U3 promoter region acquired statistically more mutations in the Ldr3 and Ldr3+TabB2 cells than in unprotected and TatB2 cells.

The replication capacity was determined for 12 candidate escape viruses with U3 mutations but no changes in the siRNA-target sequence. Cells were infected and the viral burst size was calculated as the cumulative viral titer by integrating the 10-day replication curves. Based on differences observed in this burst size, 4 of the 12 virus variants were found to replicate more efficiently than the wild-type virus in the siRNA-expressing cells in which they had been selected, suggesting that these viruses had developed siRNA resistance. Two of the four resistant viruses showed an increased burst size in Ldr3 cells in which they had been selected and the unrelated TatB2 cells, which the authors interpret as cross-resistance. Finally, the authors show that two of the four "RNAi-resistant" virus variants can produce more viral transcripts than wild-type virus upon infection of cells, suggesting that RNAi evasion was due to transcriptional upregulation.

Summarizing, the authors suggest that HIV-1 can develop indirect resistance to RNAi therapy, including a combinatorial attack, by acquiring mutations in the unrelated U3 promoter region. We, however, believe that there are alternative possible explanations for these findings. In our view, the authors dismiss the direct escape route too easily and have not demonstrated that the U3 mutations cause general siRNA escape.

Several studies have shown that HIV-1 and other viruses can escape from RNAi pressure by a single mutation in the siRNA-target sequence.[4–9] The data presented in **Table 1** of Shah et al. show that such mutations are present in two (out of three) Ldr3 and three (out of three) TatB2 cultures. Mutations were also observed in eight (out of nine) combinatorial siRNA cultures, either in one or both siRNA targets. The authors dismiss the simple scenario that these target mutations cause viral escape because they were not present in the complete virus population. However, this may relate to the use of mixed cell cultures. The escape viruses will never outcompete the wild-type virus that can replicate unhindered in the unmodified cells (**Figure 1**). This effect will be magnified when the RNAi-resistant viruses exhibit a replication deficit. Such fitness losses are likely when well-conserved viral sequences are targeted, as is the case for the chosen siRNAs.[10] Remarkably, the "no-fixation" argument that was used to dismiss direct target site mutations, was not used to scrutinize the indirect U3 region mutations. Supplementary Table S2 of Shah et al. shows that very few U3 mutations became fixed in the many cultures tested. Of these, the most

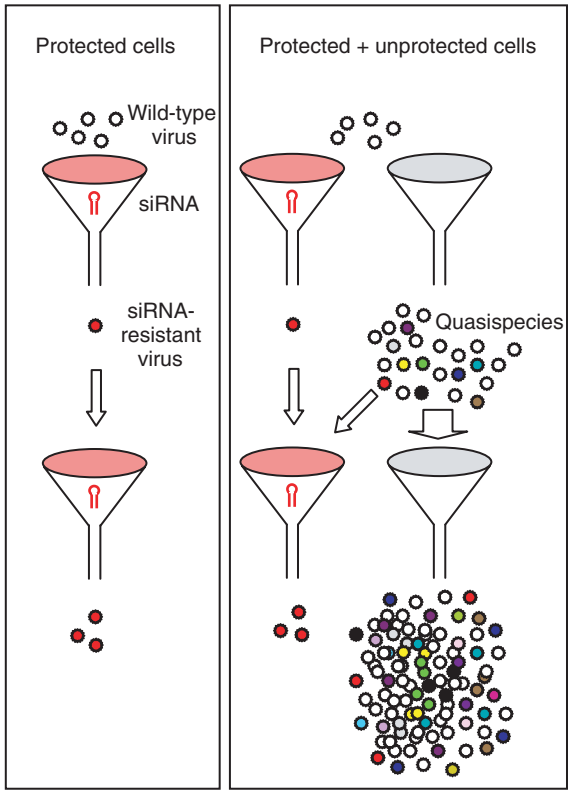

Figure 1 HIV-1 escape from siRNA therapy in different culture systems. Virus evolution is usually studied in pure cultures of siRNA-expressing cells that are protected against HIV-1 infection (left panel). Variants will be generated at a low rate because the block in virus replication is not absolute. Only the siRNA-resistant variant (red circle) will be able to replicate and spread on these cells (passage through the red funnel). Shah *et al.* used mixed cell cultures of protected and unprotected cells to study HIV-1 evolution under siRNA pressure (right panel). The bulk of virus replication will occur in the unprotected cells (passage through gray funnel), leading to the rapid generation of a viral quasispecies by spontaneously acquired mutations. This quasispecies may also contain a siRNA-resistant variant that is able to replicate in the protected cells. The latter mutant could also be generated in the protected cells, but at a reduced rate due to siRNA-mediated suppression of virus replication. The outcome of these two evolution scenarios is strikingly different: the siRNA-resistant HIV-1 variants dominate the culture in the left panel, but remain as minority variants in the right panel. siRNA, small interfering RNA.

frequent change (T-1G, observed in three cultures) has been detected repeatedly in HIV-1 evolution studies in the absence of any RNAi pressure (see e.g., ref. [11,12]).

Most importantly, the replication assays used do not convincingly demonstrate an RNAi-resistance effect of the U3 mutations. The viral burst size is a rather unusual and dangerous measure for virus replication as improved virus replication can cause increased cell death that translates into a lower burst size. Only four of the 12 U3-mutated viruses showed an increased burst size when tested on siRNA-expressing cells. Only two of these four variants showed cross-resistance, which would mean that the U3 mutations in the other two variants provide resistance against a specific siRNA. The other eight variants did not show any RNAi resistance, whereas these viruses carried similar mutations in the U3 region. In fact, the replication curves in the **Supplementary Figure S3** of Shah et al. show that the four "RNAi-resistant" virus variants are similarly inhibited by the siRNAs as the wild-type virus, which demonstrates that they are not RNAi resistant.

Is there perhaps any evidence for a transcriptional impact of the U3 mutations on the HIV-1 long terminal repeat (LTR) promoter? To answer this question, Shah et al. infected cells with the virus variants and quantified the intracellular viral transcripts after 3 days. This is however not a proper assay to measure transcription, because the read out is dependent on several processes, including RNA genome packaging (determining the RNA content of the virus stock), virus infection, reverse transcription of the RNA genome, integration of the newly made viral DNA, transcription, RNA processing, and translation. Thus, the difference measured for U3-mutated viruses does not simply reflect different transcriptional activity of the U3 promoter, but can be due to a difference in another process. Again, this phenotype was not seen for all tested U3 mutants, but only for two of the four selected variants. Only one of these two showed cross-resistance, whereas the proposed mechanism would cause general RNAi resistance.

In toto, the role of the siRNA-target site mutations in RNAi escape was dismissed too easily and the RNAi-resistance effect of the newly identified U3 changes is questionable. In addition, the proposed escape by upregulation of viral transcription was not properly tested. In numerous HIV-1 evolution studies, we observed that every escape culture had acquired a mutation in the target site.[8] This was subsequently verified by recloning and retesting, demonstrating that a single target site mutation is required and sufficient for resistance, although some cultures acquire a second mutation over time to reach high-level resistance. One notable exception was described that represents a more indirect escape route. We infrequently observed the selection of a point mutation just outside the actual target sequence, which causes a rearrangement of the local RNA structure that restricts access of the RNA machinery.[13,14]

The Schaffer laboratory previously reported a similar indirect compensatory mechanism by which HIV-1 evades antiviral RNAi action.[15] Specifically, evidence was presented that HIV-1 selects up-mutations in the viral promoter to overcome RNAi pressure imposed by siRNAs against the TAR motif. Also then, the idea was that tuning of viral gene expression by promoter upregulation represents a general mechanism by which viruses escape from RNAi therapy. However, we have argued that the observed promoter changes do reflect a general improvement of the attenuated HIV-1 strain that was used in these evolution experiments and not a general RNAi escape route.[16] In fact, exactly the same HIV-1 promoter modification—duplication of the three Sp1 binding sites to form six sites—was reported previously for this attenuated virus by spontaneous virus evolution in extended cultures in the absence of any RNAi pressure.[17] The absence of direct viral escape routes by mutation of the target sequence may relate to the mediocre potency of the anti-TAR siRNAs used in the Leonard study.[15] These siRNAs gave only 20% knock-down in transient transfection assays with a reporter construct, which may relate to the stable hairpin structure of the viral TAR target.[18]

In the absence of more convincing evidence against the efficacy of a combinatorial RNAi attack, it seems imperative to further develop this promising antiviral track. We are still

impressed by the finding that a combinatorial RNAi approach with four siRNAs is able to permanently block virus evolution.[3] This concept is currently being tested in humanized mice as preclinical test system for a future ex vivo gene therapy.[19] We realize that there may be many hurdles along this route, but viral escape by indirect routes does not seem to be a major issue.

Ben Berkhout[1] and Atze T Das[1]

[1]Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center (AMC), University of Amsterdam, Amsterdam, The Netherlands. Correspondence: Ben Berkhout, Laboratory of Experimental Virology, Department of Medical Microbiology, Academic Medical Center (AMC), Meibergdreef 15, K3-110, 1105 AZ Amsterdam, The Netherlands. E-mail: b.berkhout@amc.uva.nl

1. Shah, PS, Pham, NP and Schaffer, DV (2012). HIV develops indirect cross-resistance to combinatorial RNAi targeting two distinct and spatially distant sites. *Mol Ther* **20**: 840–848.
2. Lee, SK, Dykxhoorn, DM, Kumar, P, Ranjbar, S, Song, E, Maliszewski, LE *et al.* (2005). Lentiviral delivery of short hairpin RNAs protects CD4 T cells from multiple clades and primary isolates of HIV. *Blood* **106**: 818–826.
3. ter Brake, O, Konstantinova, P, Ceylan, M and Berkhout, B (2006). Silencing of HIV-1 with RNA interference: a multiple shRNA approach. *Mol Ther* **14**: 883–892.
4. Das, AT, Brummelkamp, TR, Westerhout, EM, Vink, M, Madiredjo, M, Bernards, R *et al.* (2004). Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. *J Virol* **78**: 2601–2605.
5. Boden, D, Pusch, O, Lee, F, Tucker, L and Ramratnam, B (2003). Human immunodeficiency virus type 1 escape from RNA interference. *J Virol* **77**: 11531–11535.
6. Holz, CL, Albina, E, Minet, C, Lancelot, R, Kwiatek, O, Libeau, G *et al.* (2012). RNA interference against animal viruses: how morbilliviruses generate extended diversity to escape small interfering RNA control. *J Virol* **86**: 786–795.
7. Nevot, M, Martrus, G, Clotet, B and Martínez, MA (2011). RNA interference as a tool for exploring HIV-1 robustness. *J Mol Biol* **413**: 84–96.
8. von Eije, KJ, ter Brake, O and Berkhout, B (2008). Human immunodeficiency virus type 1 escape is restricted when conserved genome sequences are targeted by RNA interference. *J Virol* **82**: 2895–2903.
9. Gitlin, L, Stone, JK and Andino, R (2005). Poliovirus escape from RNA interference: short interfering RNA-target recognition and implications for therapeutic approaches. *J Virol* **79**: 1027–1035.
10. ter Brake, O, von Eije, KJ and Berkhout, B (2008). Probing the sequence space available for HIV-1 evolution. *AIDS* **22**: 1875–1877.
11. Berkhout, B and Klaver, B (1995). Revertants and pseudo-revertants of human immunodeficiency virus type 1 viruses mutated in the long terminal repeat promoter region. *J Gen Virol* **76** (Pt 4): 845–853.
12. Das, AT, Harwig, A, Vrolijk, MM and Berkhout, B (2007). The TAR hairpin of human immunodeficiency virus type 1 can be deleted when not required for Tat-mediated activation of transcription. *J Virol* **81**: 7742–7748.
13. Westerhout, EM, Ooms, M, Vink, M, Das, AT and Berkhout, B (2005). HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. *Nucleic Acids Res* **33**: 796–804.
14. Westerhout, EM and Berkhout, B (2007). A systematic analysis of the effect of target RNA structure on RNA interference. *Nucleic Acids Res* **35**: 4322–4330.
15. Leonard, JN, Shah, PS, Burnett, JC and Schaffer, DV (2008). HIV evades RNA interference directed at TAR by an indirect compensatory mechanism. *Cell Host Microbe* **4**: 484–494.
16. Berkhout, B (2009). A new Houdini act: multiple routes for HIV-1 escape from RNAi-mediated inhibition. *Future Microbiol* **4**: 151–154.
17. Berkhout, B, Verhoef, K, van Wamel, JL and Back, NK (1999). Genetic instability of live, attenuated human immunodeficiency virus type 1 vaccine strains. *J Virol* **73**: 1138–1145.
18. Low, JT, Knoepfel, SA, Watts, JM, ter Brake, O, Berkhout, B and Weeks, KM (2012). SHAPE-directed discovery of potent shRNA inhibitors of HIV-1. *Mol Ther* **20**: 820–828.
19. ter Brake, O, Legrand, N, von Eije, KJ, Centlivre, M, Spits, H, Weijer, K *et al.* (2009). Evaluation of safety and efficacy of RNAi against HIV-1 in the human immune system (Rag-2(-/-)gammac(-/-)) mouse model. *Gene Ther* **16**: 148–153.

Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)