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LETTER TO THE EDITOR

Response to "HIV Escape From RNAi Antivirals: Yet Another Houdini Action?"

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To the Editor: In his 1909 book *Handcuff Secrets*, Harry Houdini lifted the shroud of secrecy surrounding his performances and revealed that his mystifying capacity to escape shackles and chains was based in painstaking effort and compromise. Shaking off straightjackets for example required dislocating his shoulders, performance after performance, year after year. In the current issue of Molecular Therapy *Nucleic Acids*, Berkhout and Das argue that our recent findings of indirect HIV escape from RNA interference (RNAi)[1] have been misinterpreted as a "Houdini action" (citation). However, the "escape act" that we observed is not an illusion but a painful additional example of HIV's recurring ability to evade antiviral therapies.

In our recent work,[1] we propagated HIV *in vitro* in the presence or absence of antiviral RNAi for 32 days. Extensive sequencing of virus that escaped RNAi, as well as control virus propagated in parallel cultures for the same period of time in the absence of RNAi, enabled distinction between mutations due to RNAi-mediated selective pressure versus those arising from drift or general selection for replication. Subsequent analysis of over 400 individual sequences revealed a statistically significant increase in nucleotide diversity in the U3 region of the long terminal repeat (LTR) for virus exposed to RNAi. Armed with this in-depth statistical analysis, we then introduced individual, mutant U3 regions back into the original parent HIV strain and clonally tested the roles of these specific LTR mutations on viral replication in the presence or absence of antiviral RNAi. Four such variants showed significantly enhanced replication compared to the wild-type virus in RNAi-protected cells. The RNAi-resistant variants also exhibited enhanced transcriptional activity, and their replicative advantage in RNAi-protected cells was reduced by addition of a RNAi-enhancing small molecule. In concert with our prior investigation,[2] these findings support the hypothesis that HIV can evolve as a stronger promoter to overwhelm the RNAi pathway with a large number of transcripts. Unfortunately, this indirect escape also resulted in cross-resistance to combinatorial RNAi targeting two distinct and spatially distant sites in the HIV genome, thus complicating antiviral RNAi design.

In the general spirit of open scientific exchange, we appreciate Berkhout and Das' discussion, and we take this opportunity to discuss a number of questions they raise. One claim is that the mutants found to be indirectly resistant to RNAi are not truly resistant. As shown in **Supplementary Figure S3**, however, the four mutant viruses replicate to maximum titers of 10^4–10^5 IU/ml over 10 days in RNAi-protected cells, whereas the wild-type parental strain maximally replicates to only 10^2–10^3 IU/ml over the same period. As one example, mutant LK-7 replicates to a maximum titer of 8×10^4, 10^5, and 2×10^3 IU/ml in the presence of Ldr3, TatB2, or a combination of both short hairpin RNAs (shRNAs), respectively, whereas the wild-type strain replicates to a maximum of 10^3, 4×10^3 or 10^2 IU/ml under identical conditions. In their prior work, Berkhout and Das have stated that a number of their variants exhibiting a tenfold to 100-fold increase in replicative titer compared to parental virus within 10 days of culture in RNAi-expressing cells are resistant to the RNAi.[3] Following the same standard, we conclude that our variants are likewise RNAi resistant. In addition, the mutants in RNAi-protected cells do not replicate as rapidly as wild-type virus in unprotected cells, which is to be expected since the mutants still contain the RNAi targets, and which is consistent with our model of indirect resistance. They key point is that by any metric (e.g., titers at any time point over the 10-day time course, overall burst size, replication rate) the U3 mutants replicate to a much greater extent than the wild-type strain on RNAi-protected cells. Furthermore, over time, and with additional opportunity for evolutionary adaptation by transcriptional fine-tuning, one could anticipate that these differences would become even more pronounced.

As another concern, Berkhout and Das state that mutants that exhibit resistance to only one small interfering RNA (siRNA) are in fact demonstrating specific resistance to the TatB2 siRNA; however, this is also a misinterpretation of our model. Mutants TC-19 and SCK-1, which exhibit resistance to only TatB2, are not necessarily specifically resistant to TatB2. It is likely that they do not exhibit resistance to Ldr3 or the combination because the transcriptional activity of these mutants is not sufficiently high to overwhelm the RNAi pathway primed with Ldr3, which was much more effective than TatB2 at suppressing HIV in our long-term study (see **Figure 2a**[1]). Mutant LK-7, the mutant with the highest transcriptional activity, exhibited resistance to TatB2, Ldr3, and the combination. This result is consistent with our model of indirect cross-resistance, and suggests that a certain transcriptional threshold must be exceeded to generate cross-resistance to highly effective siRNAs and combinations.

Berkhout and Das also imply that the eight variants with mutations in the U3 region that did not demonstrate indirect resistance are inconsistent with our model, because they contain mutations in U3 that are "similar" to those in the RNAi-resistant mutants. However, we would not expect all mutations in the U3 region to result in resistance because some

are known to be neutral. We cited important work published by Wang and colleagues who found that some specific mutations we observed (e.g., from the HIV nuclear factor (NF)-κB consensus sequence GGGACTTTCC to GAGACTTTCC) significantly reduced affinity for the p50 homodimer,[4] which could in turn enhance HIV transcription by decreasing binding of the transcriptionally repressive p50 homodimer to the NF-κB sites.[5] This same binding affinity study[4] elegantly demonstrated that not all mutations are "created equal," and other mutations in the NF-κB consensus sequence (for example, GGGGCTTTCC) do not alter p50 homodimer binding. Thus, some U3 mutations may have a significant impact on promoter activity and RNAi resistance, and others may not.

Berkhout and Das' statement that our transcriptional activity assay is not valid is also based on misinterpretation of the methods. We measured transcriptional elongation using a quantitative PCR method first developed by the esteemed Eric Verdin, Warner Greene, and colleagues.[5–7] The method is versatile and has been adopted by other groups,[8,9] because it allows one to gather quantitative information on both initiated and fully elongated transcripts from actual integrated virus (compared to transient transfection of micrograms of a reporter plasmid, which can exhibit artifacts). For this assay, we infected cells with mutant or wild-type stocks that were quantified by measuring infectious titers, which we find to be more biologically relevant than p24 concentrations. By infecting with the same number of infectious units per cell (i.e., equal multiplicity of infection, or MOI), we removed any variation that could otherwise be introduced into the steps of genome packaging, reverse transcription, and integration. Furthermore, we quantified viral message levels at steady-state, 3 days postinfection, which are in turn directly related to transcription of the nearly identical viral mRNA sequences.

Berkhout and Das also claim that transcriptional enhancement was only seen for 50% of mutants, and thus is not consistent with our model. Although we did not see an enhancement of transcription for all resistant variants, we do not claim that this mechanism is mutually exclusive of all other mechanisms of resistance, and others may be at play. Again, we simply wish to draw attention to alternative mechanisms of resistance. Moreover, Berkhout and Das do not offer a satisfying explanation for why enhancement of the RNAi pathway using enoxacin results in synergistic inhibition of RNAi-resistant mutants if indirect resistance is not a factor.

As another concern, Berkhout and Das believe that the Sp1 duplications we previously observed "reflect a general improvement of the attenuated HIV-1 strain used in these experiments rather than a general RNAi escape mechanism." This interpretation is inconsistent with the data and controls we conducted at the time of publication.[2] First, at the same time as enabling transcriptional upregulation and overwhelming RNAi in the protected cells, the Sp1 duplications consistently reduce viral replication in unprotected cells. That is, HIV "dislocates its shoulder" and reduces fitness in unprotected cells in order to overcome RNAi in the protected cells. This finding, along with the fact that we never observed such Sp1 duplications in HIV that was extensively propagated in parallel cultures without the RNAi, indicates that our observed mutations did not help, but in fact harmed, the wild-type HIV strain under the baseline culture conditions used. Moreover, we carefully cloned the Sp1 mutations into a full-length strain and rigorously quantified replication of the wild-type and mutant strains in the presence and absence of antiviral RNAi to demonstrate that the Sp1 duplications are not merely a reflection of general improvement of an attenuated strain, but *bona fide* indirect resistance to RNAi.[2]

Berkhout and Das also claim that in our original publication direct escape routes were not observed due to mediocre RNAi knockdown in which we targeted the TAR hairpin with RNAi.[2] They cite a knockdown of 20%, which was first characterized by a reporter assay knockdown of green fluorescent protein (GFP) (in which the target construct also overexpressed Tat, making potent knockdown highly challenging and thus underestimating the true antiviral potency of the anti-TAR siRNA). More importantly, inhibition of infectious HIV titer was nearly complete.[2] Furthermore, given the highly conserved and functionally important nature of the structured TAR hairpin,[2] where mutations are strongly disfavored, we believe that we observed indirect escape precisely because this targeted region was not amenable to direct escape. At any rate, our more recent work[1] utilized potent, previously published shRNAs,[10] and we again observed indirect mechanisms of HIV escape.

There was also discussion about the "dangerous" burst size metric, which we simply defined as the sum of all virus produced over the 10 days of culture, i.e., the integral under the replication time course curve. This metric is generally useful for comparing the replication of many mutants under many conditions. For rapidly replicating virus, it is possible that bursts sizes can be reduced relative to wild-type virus in the absence of RNAi because of excessive cell death early in the time course. We addressed this nuance at the time of publication and referred to complete replication time courses (**Supplementary Figure S3**) to clarify the subtlety. Regardless, as mentioned above, titers for RNAi-resistant mutants were higher than those of the wild-type virus in the RNAi-protected cells throughout the 10-day time course, indicating that the burst size metric accurately captures virus dynamics.

We share with Berkhout and Das a firm belief that RNAi must continue to play a central role in the search for new classes of antiviral therapies, but, as with highly active antiretroviral therapy, combinatorial therapies are very effective but may well not be escape-proof. We also emphasize that the infectious disease literature is brimming with examples of surprising drug evasion mechanisms. As the field further advances into the genomic age, researchers are encouraged to sequence entire genomes, beyond the intended target, and investigate all paths and mechanisms for viral escape. We must choose to contend with painful realities, and set aside distracting illusions.

Priya S Shah[1,4] and David V Schaffer[1–3]

[1]Department of Chemical and Biomolecular Engineering, University of California, Berkeley, California, USA; [2]Helen Wills Neuroscience Institute, University of California, Berkeley, California, USA; [3]Department of Bioengineering, University of California, Berkeley, California, USA; [4]Current address: Department of Cellular and Molecular Pharmacology, University of California, San Francisco, California, USA.
Correspondence: David V Schaffer University of California, Berkeley, California 94720, USA. E-mail: schaffer@berkeley.edu

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