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# Reply to "Coadaptive Stability of Interfering Particles with HIV-1 When There Is an Evolutionary Conflict"

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We thank Drs. Ke and Lloyd-Smith for their letter (1) highlighting the critical discrepancies between the claims of their recent simulation study (2) and the results derived by evolutionary calculations in our analytic study (3). In contrast to our direct calculations, Ke and Lloyd-Smith argue that therapeutic interfering particles (TIPs)—generated by conditionally replicating vectors—would stably cross dimerize with HIV and indefinitely coevolve with the virus. We wish we could share their optimism, given the need for lifelong anti-HIV therapy. In fact, one of us (L. S. Weinberger) first proposed TIPs over 10 years ago (4) and has spent years trying to engineer TIPs in the lab. Unfortunately, Ke and Lloyd-Smith's optimism is misplaced when it comes to genome dimerization: contrary to their claims, HIV can simply mutate the sequence that initiates efficient dimerization (the dimerization initiation sequence [DIS]) and avoid cross dimerization. Ke and Lloyd-Smith's misplaced optimism stems from an incorrect belief that the fitness cost of mutation precludes a rapidly mutating retrovirus from accumulating fitness-enhancing mutations. This mistake is absolutely critical. If Ke and Lloyd-Smith's argument were correct—that resistance-conferring mutations that come with a cost could not persist or accumulate—there would be no drug resistance against antiretrovirals, and antibiotic resistance would be similarly rare.

How did Ke and Lloyd-Smith miss this basic evolutionary fact? They began with a disbelief in the accumulation of resistance mutations that carry a fitness cost and constructed an argument that the DIS cannot mutate so that TIP and HIV indefinitely cross dimerize. Their simulations (2) then simply postulate a lack of evolution in the DIS by fixing the dimerization efficiencies as equal for HIV-HIV homodimers and HIV-TIP heterodimers. This result is built into their model in disagreement with genomic and patient data. They appear to dismiss the evidence that the DIS diverges in different HIV subtypes. As many readers know, HIV-1 subtype B exhibits a consensus palindromic sequence GCGCGC "kissing loop" DIS, while subtypes A, C, G, and others exhibit the consensus sequence GTGCAC. This divergence in the DIS restricts efficient cross dimerization and recombination between HIV subtypes (5), and DIS divergence would similarly restrict HIV-TIP cross dimerization. Ke and Lloyd-Smith argue that despite the observed DIS divergence in HIV, the DIS will not mutate within subtypes due to the high fitness cost that DIS variants impose. If true, this would greatly simplify TIP design. However, as we detail below, this argument is not consistent with the data.

First, DIS variants do exist within subtypes (e.g., canonical subtype-B viruses appear to have evolved the subtype-C kissing loops, see Los Alamos HIV database accession numbers AB604946 and AB604948). Second, within individual patients, sequenced clones exhibit DIS mutations (6), despite these patients being coinfected with HIV subtypes encoding identical DIS consensus sequences. The evolution of these DIS mutations has even been longitudinally tracked within these patients (6), and our analysis of DIS mutation (3) is consistent with these patient data. These patient data are striking in part because they were obtained using traditional sequencing approaches. Modern ultradeep sequencing of patient DIS sequences is likely to uncover greater DIS variation.

The existence of these DIS variants makes sense, given the speciation of HIV subtypes (i.e., clades): DIS variation has been hypothesized to reduce intersubtype recombination and reflect selection pressure for conservation of the HIV genome within subtypes (5, 6). Since DIS variants exist, the selection advantages of DIS mutation appear to outweigh the costs. For TIP-HIV cross dimerization, the selection pressure for DIS mutation and divergence will be even stronger, since cross dimerization between HIV subtypes generates recombinants that are viable (i.e., they propagate a portion of each subtype's genetic information). Conversely, TIP-HIV cross dimerization generates recombinants that are far less viable (7). In order to further demonstrate why TIP-HIV cross dimerization will be evolutionarily unstable, below we use established evolutionary theory to calculate that the DIS has a strong likelihood of divergence, and we directly quantify how quickly DIS divergence will occur.

Despite the data, Ke and Lloyd-Smith argue that natural selection will weed out HIV DIS mutants, because DIS mutants often bear a lower replicative fitness than the wild type, but mutational costs are only half of the story. In reality, natural selection automatically calculates a cost-benefit analysis: if the selective advantages of a mutation outweigh its fitness costs, the mutation will be selected for. Our analysis in reference 3 rests on this cost-benefit framework, finding cross dimerization to be evolutionary unstable. Ke and Lloyd-Smith appear to have made a critical error in their reading of our study when they assert that our analysis depends upon on an assumption of neutral selection. Neglecting this cost-benefit analysis, Ke and Lloyd-Smith incorrectly assert that our analysis depends upon on an assumption of neutral selection (i.e., that DIS double mutants that reestablish a palindromic sequence occur at no cost to HIV fitness). In fact, we did not limit our analysis to neutral selection, and we explicitly consider the case of significant cost to HIV (see reference 3, p. 2085, column 1, paragraph 2, and Fig. S1 in the supplemental material). Because of the large fitness advantage conferred by evading TIP targeting,

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factoring in the existence of mutational cost to the DIS palindrome does not alter the conclusion that genome stealing is unstable. Double mutations in the DIS cause a relatively small 2- to 3-fold decrease in HIV's burst size (8–10). In contrast, TIP competition for HIV genomes decreases HIV's burst size by the much larger product of $\sim 1 + mP$ where $m$ is the number of integrated interfering-particle proviruses and $P$ is the fold increase in interfering-particle genomic RNA relative to HIV genomic RNA (3). Based on published reports, our analysis indicates that $mP$ will be ~10 to 100 (see reference 3, Fig. S3A for the large parameter range $\eta > 2$ and $P > 5$). Therefore, despite DIS mutations that abrogate cross dimerization having a 2- to 3-fold cost on HIV's burst size, these mutations would boost HIV's burst size by a net factor of 3 to 30.

Overall, the gain for HIV mutating its DIS far outweighs the cost of mutation. HIV and TIP genomes will thus diverge in their respective DISs. Reciprocally, there is no obvious selection pressure for TIPs to "chase" HIV in the DIS sequence space, since genome stealing merely wastes a portion of TIP genomes. In other words, TIPs do not benefit from genome stealing. In addition to the selection pressure driving the HIV to diverge from TIP DIS, TIPs have an added selection pressure to diverge from HIV in their own DIS. Therefore, despite the fitness costs, DIS divergence is overwhelmingly likely.

Given that selection pressure favors DIS divergence, how quickly is this divergence likely to occur? We now demonstrate that double mutations in the DIS either preexist in an HIV-infected individual or rapidly arise from single mutations (irrespective of which scenario produces these divergent double mutants, they will rapidly transmit through the population if selection pressure exists). The time to emergence of double mutants can be calculated from established mutation-selection theory which states that single mutations in the DIS exist in $N_{single} = \mu N/s$ copies (11)—where the mutation rate per site $\mu = 3 \times 10^{-5}$ (12), the mutation cost $s = \log(2 \text{ to } 3)$ (8–10) which is ~1, and the effective population size $N$ is between $10^5$ (13) and the census size of $10^8$ (14). Accordingly, a double mutant rescuing HIV fitness will be generated in the average time $\sim 1/(\mu N_{single}) = 1/(\mu^2 N)$, which is ~10 to $10^4$ replication cycles (days) (11). Consequently, given the average length of an infection, ~2,000 days, HIV mutants with divergent DISs will replace wild-type HIV with a probability of 0.2 to 1.0 in an average individual! A mutant only need arise in one individual and will then rapidly spread through the TIP-HIV-infected population. This prediction is consistent with data showing the accumulation of multiple DIS variants within a patient (6) and the presence of DIS double mutants in sequenced patient samples (Los Alamos database accession numbers AB604946 and AB604948).

Thus, both theory and sequence data show that the genome-stealing mechanism—which incidentally underlies Ke and Lloyd-Smith's simulation study (2)—is not an evolutionarily stable strategy for designing interfering-particle therapies or conditionally replicating vectors. In contrast, our analysis (3) shows that the capsid-stealing mechanism maintains coevolutionary stability and is as a robust strategy for designing potentially lifelong anti-HIV therapies.

The second point of Ke and Lloyd-Smith's letter highlights their prediction of "three-phase" evolutionary dynamics. In fact, this prediction is built into their model using striking assumptions about the organization of HIV and TIP genomes and the resultant genotype-to-phenotype correspondence. Key among their assumptions is the belief that fitness costs and benefits occur in distinct genomic regions. Ke and Lloyd-Smith then develop a pattern-matching model consisting of HIV and TIP genomes containing a total of 12 bp divided into as-of-yet undiscovered segments designated "P," "D," and "A" regions. Curiously, they assume that only mutations in hypothetical "A" segments confer fitness costs and only to HIV. TIP mutates unfettered in their model (which is surprising, since they raised the neutral selection argument). At no point do they appear to consider the nonneutral selection case. They next assume that the ratio of the TIP-to-HIV expression rate can decrease due to mutations in the "P-segment" of the HIV genome until the P-segments in TIP and HIV have perfect sequence identity, at which point TIP expression becomes completely suppressed (i.e., the expression asymmetry between TIP and HIV, denoted $P$, is 0). However, this scenario does not match the actual genetic constraints faced by HIV and TIPs. TIPs are essentially HIV genomes with large deletions that enable more-efficient expression of RNA, relative to HIV RNA. Because of these deletions, TIPs are replication incompetent in the absence of HIV. Thus, if enhanced expression of the TIP over HIV is generated by mutations/deletions in splice sites, the identical mutation in HIV will not produce $P = 0$ (or even $P = 1$). Instead, this mutation will severely diminish the replication of HIV. Without full-length HIV to act as a helper virus for TIPs, TIPs will be unable to piggyback; neither HIV nor TIPs will be generated. Thus, reciprocal mutations in the HIV provirus to match mutations/deletions in the TIP cannot cancel the enhanced expression asymmetry of the TIP ($P > 1$). Taken together, Ke and Lloyd-Smith's assumptions generate a model that is substantially at odds with the current understanding of the biology of HIV replication.

In summary, coevolutionary stability between interfering particles and HIV will not be maintained by genome cross-dimerization on either flat or realistic fitness landscapes.

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