

Recurrent and Novel Evolutionary Pathways Drive in vitro HIV-1 Lenacapavir Resistance with Diverse Phenotypic Consequences

Corresponding Author: Dr Masahiro Yamashita

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Overall, important study that attempts to elucidate HIV-1 viral evolution with regards to development of resistance to a novel class of antiretroviral agents (small molecule capsid inhibitors with lenacapavir as first-in-class) in context of varying drug selection pressures and implications for viral fitness and infectivity. I like the efforts to assess how additional accrued mutations may modify or modulate viral fitness- viz exploring how there may be compensatory mutations as a viral evolutionary strategy to improve/regain fitness. this has important implications for and relevance for a drug that is already approved for HIV treatment and is likely to be deployed widely globally for HIV prevention in the coming months/years.

my

Introduction- the statement- "two individuals who acquired HIV-1 during LEN-based PrEP carried the LEN-resistant N74D variant either through selective transmission of this variant or via de novo evolution." leaves room to suggest that it could have been transmitted resistance. this is inaccurate as studies have shown that N74D is a treatment emergent mutation that is unlikely to be circulating in the community given that lenacapavir has not been used outside the context of small clinical trials thus lending greatest likelihood that it was exposure emergent. see <https://pubmed.ncbi.nlm.nih.gov/32154864/>

Methods-

focus on variant with Q67H and N74D, limits generalizability of tis study as there have been multiple patterns of resistance mutation emergence- singly and accrued in combination in clinical studies of LEN. thus observed evolution patterns described in the paper may have limited applicability. for example in the Capella study, there is heterogeneity in pattern of resistance mutation emergence: foe example, some individuals developed M66I/M first, and others had T107A/S- so there is no one clear pattern of mutation accrual. <https://journals.sagepub.com/doi/full/10.1177/13596535231220754>

- while i understand dose escalation approaches are great to define viral evolution/adaptation in in-vitro models, in the clinical space, maybe except for a patient population such as in Capella where lenacapavir is continued because of limited treatment options even in the face of emergent resistance; the opposite scenario is likely to occur when used for Hlv prevention where infections occur as drug levels wane/ fall below target levels, such that viral mutational "escape" in that context may behave differently- will like authors to address this point.

- not much work on HIV-2 has been conducted in this space (susceptibility to and evolution of resistance to capsid inhibitors)- this has relevance for parts of the world where there are significant background rates of HIV-1/HIV-2 coinfection or HIV-2 mono-infection. experiments on HIV-2 will expand global relevance of this study

Discussion:

- of course in-vitro systems may present different results than in-vivo experience, but how do authors explain detection of novel mutations in their in-vitro systems that have not been elicited in broad ranging preclinical and clinical development studies for lenacapavir or its precursor GS-CA-1 such as E187K that they characterize in so much detail?
rug

Reviewer #2

(Remarks to the Author)

In this study, Orris et al. employed an accelerated evolution approach to study HIV's resistance pathways to lenacapavir (LEN). Their key findings include (1) despite generally accepted sequence conservation in HIV capsid, the virus can develop novel and complex pathways to escape LEN effects, (2) M66I serves as a key bridge in resistance pathways, (3) a variant with combination of M66I+Q67H+E213K became LEN-dependent. Overall, this is a very well-written manuscript. Experimental methods and analyses were robustly performed. Results from the study provide important insights to our understanding of LEN resistance pathways.

Major comments.

1. Clinical relevance of the author's findings should be further clarified. For example, in their culture duration, what was the estimated rounds of replication were they expecting (given the known RT error rate in LAI). How does this compare to a viremic individual not on therapy? Essentially, what is the likelihood that one or more of these mutations would occur in a natural setting? Have any of these mutation and mutation combinations been observed in vivo without LEN pressure?
2. One of the key findings is on drug concentration dependence. Since LEN is long acting, this study may suggest that individuals receiving LEN should be carefully monitored for suboptimal concentration – please include in the discussion. Is there a threshold that the authors would suggest?

Minor comments.

Results.

Line 103-109 and Figure 1. Please clarify in-text: Was this done in the absence or presence of LEN?

Line 127-128. Please clarify that the intolerance is within the context of subtype B LAI.

Line 153-162 and Figure 2. Are you sure Fig 2a is Q67 and Fig 2b is N74? Both wildtypes were shown in the logo plot in 2b.

Line 310. Is E187K naturally occurring in the absence of LEN?

Line 414-416 and Figure 8c. It is unclear to me why the authors started the experiment with a strain containing N74D (in addition to Q67FHWY). Please clarify the rationale.

Discussion.

Observations were based on LAI and subtype B, which should be discussed as a limitation in the discussion section.

Methods.

Line 801-802. Sequencing platform and error estimates not reported. Illumina, PacBio and Nanopore each have different baseline error rates.

Reviewer #3

(Remarks to the Author)

This manuscript represents a comprehensive assessment of the acquisition by HIV-1 of Lenacapavir resistance mutants in vitro. Its nicely done and well controlled. My comments are largely related to interpretation and can mostly be covered by text changes.

1. Would have liked to have seen at least one experiment with a primary isolate of HIV-1. Would the mutations acquired be the same, particularly for a founder clone given LEN is used for prophylaxis.

2. Should “in vitro” be included in the title to distinguish this work from in vivo studies?

3. All LEN resistance was studied in the T-cell line MT4. My understanding is that this line is selected because its super permissive to HIV replication and particularly to HIV mutants that are defective in other cells, particularly primary cells. I guess this might skew results because maybe some mutations may only be replication competent in MT4. Or at least more replication competent in MT4. In this regard please address the following points in the text.

3a Do we know if HIV-1 replication in MT4 selects change in the absence of inhibitor?

3b What do we know about selection of resistance in vitro vs in vivo for other inhibitors, has this ever been compared. Eg does in vitro replication in the presence of eg integrase inhibitors or RT inhibitors select the same resistance profile. Does this support or undermine the present study.

3c line 572, considering points above I don't think sequence selection is an assay sensitivity issue its due to MT4 providing different selective pressure than in vivo replication. This point should be discussed.

3d I think the authors should go beyond the statement on line 561 that the mutants may have a defect in primary cells, particularly since MT4 are the most permissive T-cell line. Can the authors test 1 or 2 key resistance mutants for replication in some primary cells? Do they get the same level of resistance/replication? Agreed, this would make the paper more compelling.

4 line 562 I don't think that there is an assumption that conserved viral sequences are inherently immutable. Most drug resistance studies undermine this notion.

5 Do the authors expect capacity of resistance mutations to replicate to mirror capacity to transmit? Any thoughts on this particularly in the context of LEN as prophylaxis.

6 Line 627, isn't it clear that inhibition of nuclear entry by LEN is occlusion of the FG binding site required to bind the FGs in the NPC channel? Agreed, could add discussion of Nup153 and disordered nups (reference Dickson 2024 and Fu 2024, Nature) in addition to CPSF6. The observation that L56I does not infect non-dividing cells may (sup fig 10a) may reflect disrupted N57 (the adjacent residue) binding to FG nups.

7 Is the different resistance profile at different LEN concentrations related to the different concentration dependent modes of LEN inhibition. Eg isn't the 2 or 3 phase inhibition curve described in various studies due to low conc LEN inhibition FG cofactor binding/nuclear entry and high conc LEN breaking cores open and inhibiting RT. Are these different profiles indicative of different resistance mutations for each of these. Is this revealed by analysing LEN sensitivity curve shapes?

8 There are several cases of data not shown. I'm not sure that's permissible anymore given the unreliability of and difficulty evaluating unseen data. Please remove reference or show the data.

9 Discussion line 649 should discuss the fact that stabilisation of hexamers can cause destabilisation of the viral cores because the hexamers are flattened. See (Faysal 2024 Elife) which they have already referenced here.

10 Why are there two abstracts?

11 Figure 1 b (right) legend, please state how many HIV-1/SIVcpz sequences were used to make this?

12 Figure 2, legend says 'a' is 67 and 'b' is 74 but the data looks like it is the other way around.

13 Supplementary figure 2, why was only N74 repeated and not Q67? Data requires repeats.

14 Figure 3b and Figure 6a, might be nice to see how infectious each of these variants are in the absence of LEN compared to WT (I think the data has been normalised to this).

15 Figure 4b, I think it would be useful to also show these residues on a capsid structure with LEN bound eg pdb 6VKV to see how close these are to the binding site. This would help to support the statement in line 316.

16 Figure 7 and Figure 9 legend, please state how many replicates are used to generate stats?

17 The statistics described in line 368 comparing the two mutants are not shown in figure 7e, which instead compares each mutant to WT. Perhaps the 'ns' is meant to be between the red and blue bars not grey and red?

18 Line 371, LEN is known to disrupt capsid cores, supporting the data shown, perhaps some of these papers could be referenced here eg Faysal 2024 Elife.

19 Line 670, could add discussion of E75 and E213 binding to RRR motif of Nup153 (Shen PNAS 2023) therefore the trimeric interface may be important for nuclear import as well as stability.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

I have no additional comments. The authors have fully addressed all of my concerns. This is a very well-designed study and a well-written manuscript.

(Remarks on code availability)

The authors have not made the code available yet. They wrote, "Custom scripts used for HIV-1 capsid evolution analyses are available at a private GitHub repository and will be made publicly available upon acceptance of the manuscript. The public repository will be archived and assigned a DOI at that time."

Reviewer #3

(Remarks to the Author)

The authors have addressed the points I made effectively. I have no further comments on the manuscript

(Remarks on code availability)

No code included

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Reviewer #1

Overall, important study that attempts to elucidate HIV-1 viral evolution with regards to development of resistance to a novel class of antiretroviral agents (small molecule capsid inhibitors with lenacapavir as first-in-class) in context of varying drug selection pressures and implications for viral fitness and infectivity. I like the efforts to assess how additional accrued mutations may modify or modulate viral fitness- viz exploring how there may be compensatory mutations as a viral evolutionary strategy to improve/regain fitness. this has important implications for and relevance for a drug that is already approved for HIV treatment and is likely to be deployed widely globally for HIV prevention in the coming months/years.

Response: We thank the reviewer for their careful evaluation of the manuscript and for the helpful comments provided. We appreciate that the reviewer found our study important. We believe that the revisions have improved the clarity and strength of the manuscript.

Introduction- the statement- "two individuals who acquired HIV-1 during LEN-based PrEP carried the LEN-resistant N74D variant either through selective transmission of this variant or via *de novo* evolution." leaves room to suggest that it could have been transmitted resistance. this is inaccurate as studies have shown that N74D is a treatment emergent mutation that is unlikely to be circulating in the community given that lenacapavir has not been used outside the context of small clinical trials thus lending greatest likelihood that it was exposure emergent. see <https://pubmed.ncbi.nlm.nih.gov/32154864/>

Response: We agree. Although we initially considered both possibilities, as the reviewer correctly noted, this observation is most consistent with a *de novo* mutation. Accordingly, we have revised the sentence as follows:

"Given that N74D is rarely observed among LEN-untreated HIV-1-infected individuals, this is most consistent with de novo evolution rather than selective transmission."

Methods-

focus on variant with Q67H and N74D, limits generalizability of tis study as there have been multiple patterns of resistance mutation emergence- singly and accrued in combination in clinical studies of LEN. thus observed evolution patterns described in the paper may have limited applicability. for example in the Capella study, there is heterogeneity in pattern of resistance mutation emergence: foe example, some individuals developed M66I/M first, and others had T107A/S- so there is no one clear pattern of mutation accrual. <https://journals.sagepub.com/doi/full/10.1177/13596535231220754>

Response: We appreciate the reviewer's point regarding the heterogeneity of lenacapavir resistance pathways observed in clinical studies. While M66I has been reported as an early resistance mutation in studies such as CAPELLA, prior in vitro work has shown that M66I alone severely impairs viral infectivity and fails to acquire any secondary mutation under LEN pressure. Our study provides a mechanistic framework for how Q67H/N can facilitate the acquisition and tolerance of M66I, thereby defining plausible evolutionary trajectories that may occur in vivo. This is a point already discussed in the original manuscript (line 624). Notably, several of the mutational combinations examined here have also been observed in the CAPELLA study.

We agree that lenacapavir resistance can emerge through multiple heterogeneous pathways, which is further underscored by our new observation that the CH505 virus carrying Q67H adapted under lenacapavir pressure without acquiring M66I. While our

study does not aim to capture the full spectrum of resistance patterns, it highlights specific, clinically relevant trajectories and points to important areas for future investigation.

- while i understand dose escalation approaches are great to define viral evolution/adaptation in in-vitro models, in the clinical space, maybe except for a patient population such as in Capella where lenacapavir is continued because of limited treatment options even in the face of emergent resistance; the opposite scenario is likely to occur when used for HIV prevention where infections occur as drug levels wane/ fall below target levels, such that viral mutational "escape" in that context may behave differently- will like authors to address this point.

Response: This is a great point, and we agree that drug concentration is likely a key factor shaping viral evolution and adaptation. We have added the following description to the fifth paragraph of the Discussion:

"This difference may reflect the use of a dose-escalation strategy in our adaptation scheme. If drug concentrations instead remain fixed or were changed only modestly in a manner that more closely mirrors pharmacokinetic dynamics in individuals receiving LEN, alternative resistance pathways may be selected. Indeed, the complex evolutionary patterns observed, including both M66I-containing mutational signatures and M66I-independent trajectories, may be driven by heterogeneity in drug exposure."

- not much work on HIV-2 has been conducted in this space (susceptibility to and evolution of resistance to capsid inhibitors)- this has relevance for parts of the world where there are significant background rates of HIV-1/HIV-2 coinfection or HIV-2 monoinfection. experiments on HIV-2 will expand global relevance of this study

Response: We agree that HIV-2 represents an important and understudied context for capsid inhibitor resistance with significant global relevance. The present study focuses on mechanistic and evolutionary analysis of lenacapavir resistance in HIV-1 using clinically observed mutations and established experimental systems. Extending these analyses to HIV-2 would require the development of replication-competent HIV-2 systems and dedicated evolutionary studies, which are beyond the scope of the present work.

Discussion:

- of course in-vitro systems may present different results than in-vivo experience, but how do authors explain detection of novel mutations in their in-vitro systems that have not been elicited in broad ranging preclinical and clinical development studies for lenacapavir or its precursor GS-CA-1 such as E187K that they characterize in so much detail?

Response: In our experiments, E187K emerged under conditions of strong and sustained lenacapavir pressure, including dose-escalation and high drug concentrations. While physiologically active LEN levels can approach ~200 nM, sustained selection at such high exposure is likely to occur only in limited contexts, given the current patterns of LEN deployment and the rarity of functional LEN monotherapy *in vivo*. This may explain why E187K has not been detected in prior preclinical or clinical studies. We have added the following clarification to the eighth paragraph of the Discussion:

"The absence of E187K in prior studies may reflect the rarity of sustained selection under high LEN exposure; although physiologically active LEN concentrations can approach ~200 nM, such levels are unlikely to be commonly or persistently achieved in vivo."

In addition, characterization of E187K and other CA-CTD mutations (such as E213K) was motivated by a mechanistic interest in understanding how the capsid lattice can further adapt under strong selective pressure, providing fundamental insight into the constraints and plasticity of the HIV-1 capsid.

Reviewer #2

In this study, Orris et al. employed an accelerated evolution approach to study HIV's resistance pathways to lenacapavir (LEN). Their key findings include (1) despite generally accepted sequence conservation in HIV capsid, the virus can develop novel and complex pathways to escape LEN effects, (2) M66I serves as a key bridge in resistance pathways, (3) a variant with combination of M66I+Q67H+E213K became LEN-dependent. Overall, this is a very well-written manuscript. Experimental methods and analyses were robustly performed. Results from the study provide important insights to our understanding of LEN resistance pathways.

Response: The reviewer provides an overall positive assessment of the manuscript (noting that it is well-written, robustly performed, and provides important insights). We appreciate these comments and have revised the manuscript accordingly, addressing each point in detail below.

Major comments.

1. Clinical relevance of the author's findings should be further clarified. For example, in their culture duration, what was the estimated rounds of replication were they expecting (given the known RT error rate in LAI). How does this compare to a viremic individual not on therapy? Essentially, what is the likelihood that one or more of these mutations would occur in a natural setting? Have any of these mutation and mutation combinations been observed *in vivo* without LEN pressure?

Response: We thank the reviewer for raising the question of clinical relevance and the likelihood that the identified mutations could arise in a natural setting. Rather than introducing a separate discussion section, we addressed this point within the existing Discussion paragraph focused on evolutionary trajectories involving M66I, as this context best reflects our interpretation of the data.

Given the high mutational rate of HIV-1 (approximately one genome in three acquiring at least one mutation per replication cycle) and the extraordinarily large number of virions produced daily in untreated infection, the mutational supply *in vivo* is orders of magnitude greater than that typically achieved *in vitro*, even in large-scale culture systems. Consequently, single-nucleotide substitutions such as Q67H or M66I are expected to arise stochastically *in vivo*. However, in the absence of LEN pressure, such variants are unlikely to be observed, which would be constrained by competition with WT virus and associated fitness costs.

Although double mutants could, in principle, be generated at very low frequency given the large mutational supply *in vivo*, their persistence and evolutionary impact in drug-free conditions would be expected to be rare, particularly for combinations involving M66I, which is strongly fitness-impaired on its own. Accordingly, rather than invoking selection of rare pre-existing double mutants, our data support a stepwise evolutionary model in which compensatory substitutions such as Q67H or A105T enable the emergence and maintenance of highly resistant M66I-containing variants specifically under LEN pressure. We have clarified this interpretation in the 6th paragraph of the Discussion section.

“Notably, substitutions such as M66I are not frequently observed in the absence of LEN pressure. However, given the high mutational supply generated during untreated HIV-1 replication, single-nucleotide substitutions are expected to arise stochastically in vivo but are unlikely to be detected unless they reach sufficient frequency, which would be constrained by fitness and competition with WT virus. While double mutants could in principle be generated at very low frequency, their persistence in the absence of selection would be expected to be rare due to associated fitness costs.”

2. One of the key findings is on drug concentration dependence. Since LEN is long acting, this study may suggest that individuals receiving LEN should be carefully monitored for suboptimal concentration – please include in the discussion. Is there a threshold that the authors would suggest?

Response: We appreciate the reviewer’s comment regarding drug concentration dependence in the context of long-acting LEN. Our study does not address pharmacokinetic thresholds, and the LEN-dependent variants described here emerged only under high LEN concentrations exceeding those used clinically. As such, we do not believe our data support proposing concentration-based monitoring strategies or defining a specific threshold. Instead, and consistent with the emphasis already made in the Discussion, we consider virologic and genetic monitoring in the setting of breakthrough infection to be the most informative and practical approach for identifying and interpreting such rare phenotypes. For this reason, we did not expand the main text further beyond the existing discussion.

Minor comments.

Results.

Line 103-109 and Figure 1. Please clarify in-text: Was this done in the absence or presence of LEN?

Response: We thank the reviewer for pointing out this ambiguity. The experiment shown Figure 1 was performed in the absence of LEN. We have clarified this by adding “in the absence of LEN” to the main text (Results section, second paragraph) and to the legend of Figure 1.

Line 127-128. Please clarify that the intolerance is within the context of subtype B LAI.

Response: We agree with the reviewer. We have explicitly mentioned that this observation was made with the LAI backbone. has now been modified as follows:

“Sequence analysis of variants selected five days post-expansion revealed highly variable degrees of mutational tolerance in these four positions, with an unexpected diversity of viable variants found in three of the four mutant libraries using an LAI backbone.”

Additionally, this point is briefly revisited in the Discussion of the revised manuscript in light of our new results using the subtype C CH505 capsid; further discussion is provided below.

Line 153-162 and Figure 2. Are you sure Fig 2a is Q67 and Fig 2b is N74? Both wildtypes were shown in the logo plot in 2b.

Response: We apologize for this oversight and thank the reviewer for drawing our attention to it. The figures have been corrected such that Figure 2a shows N74 and Figure 2b shows Q67.

Line 310. Is E187K naturally occurring in the absence of LEN?

Responses: E187K appears to be extremely rare in the absence of lenacapavir (LEN) selection. We surveyed approximately 8,000 HIV-1 p24 (capsid) sequences from the Los Alamos HIV Sequence Database and identified E187K in 5 out of 8,003 sequences (<0.1%). Given this very low frequency, it is difficult to determine whether these represent bona fide naturally occurring variants or reflect sequencing errors or annotation artifacts. Overall, these data indicate that E187K is not a common naturally occurring polymorphism in untreated HIV-1 populations.

Line 414-416 and Figure 8c. It is unclear to me why the authors started the experiment with a strain containing N74D (in addition to Q67FHWY). Please clarify the rationale.

Response: We apologize for the lack of clarity. The experiment was not initiated with an N74D-containing virus. Selection was started from the M66I+Q67H background, and N74D arose *de novo* under lenacapavir pressure and became nearly fixed at 200 nM. Upon further escalation of drug concentration to 400 nM, an additional mutation, A77V, was acquired on the M66I+Q67H+N74D background, resulting in the quadruple mutant shown in Figure 8c. We have revised the text to explicitly clarify the sequential emergence of these mutations.

“Both substitutions reside within the drug-binding site and were maintained at higher drug concentrations. A notable de novo mutation that emerged during selection of the M66I+Q67H background was the well-known LEN resistance mutation N74D, which became nearly fixed at 200 nM; continued selection at 400 nM subsequently led to acquisition of A77V, resulting in the M66I+Q67H+N74D+A77V genotype.”

Discussion.

Observations were based on LAI and subtype B, which should be discussed as a limitation in the discussion section.

Response: We agree with the reviewer. The initial experiments were performed using LAI, a subtype B virus, and we now explicitly discuss this as a limitation in the Discussion. In addition, prompted by this comment and a related comment from another reviewer, we performed dose-escalation adaptation experiments using a virus carrying the CH505 capsid (subtype C), starting from the Q67H background. Notably, the adaptation pattern observed with CH505 CA differed from that observed with LAI CA; in contrast to the emergence of M66I in the LAI background, CH505 CA selected for a distinct set of mutations, which are shown in Figure S14. Together, the limitation along with these new findings and their implications for subtype- and background-dependent adaptation are now discussed in the revised Discussion as follows:

“Finally, our mutagenesis work solely relied on the LAI CA backbone. Hence, the mutational tolerance we observed may not be universal across other strains or subtypes. Recent work, however, has shown that the pattern of early-emerging LEN resistance mutations is highly similar across different subtypes. In contrast, our in vitro adaptation using a virus harboring the capsid with a Q67H substitution from a T/F strain of HIV-1

(CH505) displayed a distinct evolutionary trajectory from that observed in our LAI-based adaptation, which recurrently selected for the strong M66I substitution. Thus, while the mechanistic basis for these differential evolutionary pathways remains to be defined, these results highlight the importance of capsid sequence context in LEN resistance evolution."

Methods.

Line 801-802. Sequencing platform and error estimates not reported. Illumina, PacBio and Nanopore each have different baseline error rates.

Thank you for pointing out this oversight in our manuscript. In the revised manuscript, information on the sequencing platforms has been added as follows.

"The resulting libraries were submitted to GENEWIZ for Illumina sequencing or AmpSeq using the Element AVITI sequencing platform, which yields estimated per-base error probabilities of <0.1% per nucleotide."

"For CA sequence analysis of adapted variants, PCR-amplified products covering the full coding sequence of HIV-1 CA were submitted for Oxford Nanopore sequencing at Plasmidsaurus, which is characterized by raw single-read per-base error probabilities of approximately 1–5% per nucleotide."

Reviewer #3

This manuscript represents a comprehensive assessment of the acquisition by HIV-1 of Lenacapavir resistance mutants in vitro. Its nicely done and well controlled. My comments are largely related to interpretation and can mostly be covered by text changes.

Response: The reviewer's comments were helpful in improving the manuscript. It should be also noted that the positive evaluation of the work as "nicely done and well controlled" is appreciated. All points raised have been addressed in the responses below.

1. Would have liked to have seen at least one experiment with a primary isolate of HIV-1. Would the mutations acquired be the same, particularly for a founder clone given LEN is used for prophylaxis.

Response: We agree with the reviewer that evaluating resistance evolution in a primary isolate or transmitted/founder (TF) context is important, particularly given the use of lenacapavir (LEN) in prophylaxis settings. After submission of our manuscript, Naik *et al.* published a study examining resistance mutations across a diverse collection of HIV-1 strains and found that resistance profiles are largely conserved across HIV-1 subtypes (PMID: 40965168).

Thus, this question has now been addressed independently; however, we also became interested in exploring evolutionary pathways beyond the initial amino acid substitution. Accordingly, we performed an additional dose-escalation adaptation experiment using a newly generated LAI-based chimeric virus carrying the capsid (CA) sequence from the clade C TF virus CH505 which we then added Q67H substitution to.

This virus was successfully adapted to higher LEN concentrations, demonstrating that LEN resistance evolution is not restricted to the LAI CA background. During adaptation, we identified the emergence of a K70R substitution. Notably, the Q67H+K70R combination has been observed in the CAPELLA clinical study, further supporting the

recurrent evolutionary patterns emphasized in this manuscript. With continued adaptation, an additional substitution (A174T) emerged, indicating engagement of both recurrent and novel evolutionary pathways.

Importantly, this adaptation trajectory differed from the LAI-based pathway characterized by acquisition of M66I, suggesting that capsid genetic background influences accessible LEN resistance routes. While defining the precise determinants underlying these background-dependent differences is beyond the scope of the current study, the emergence of clinically observed substitutions (Q67H+K70R) in a transmitted/founder-derived capsid context directly addresses concerns raised by Reviewer 2 regarding the clinical relevance of laboratory strain-based adaptation. Together, these data strengthen the clinical relevance of our findings and support the conclusion that both recurrent and context-dependent evolutionary pathways can contribute to LEN resistance.

This has been added to the last paragraph of the Results section and discussed in the paragraph before the final paragraph of the Discussion section. Given that these changes span multiple paragraphs, we refer the reviewer to the indicated sections of the revised manuscript.

2. Should “in vitro” be included in the title to distinguish this work from in vivo studies?

Response: We agree with the reviewer. To clearly distinguish this work from in vivo studies, we have revised the manuscript title to include “in vitro” as follows:

“Recurrent and Novel Evolutionary Pathways Drive *in vitro* HIV-1 Lenacapavir Resistance with Diverse Phenotypic Consequences.”

3. All LEN resistance was studied in the T-cell line MT4. My understanding is that this line is selected because its super permissive to HIV replication and particularly to HIV mutants that are defective in other cells, particularly primary cells. I guess this might skew results because maybe some mutations may only be replication competent in MT4. Or at least more replication competent in MT4. In this regard please address the following points in the text.

3a Do we know if HIV-1 replication in MT4 selects change in the absence of inhibitor?
What was the long-term evolution study?

Response: Yes, long-term passaging of HIV-1 in MT-4 cells in the absence of antiviral drugs can select for adaptive changes related to growth in this cellular environment, as shown in prior experimental evolution studies (e.g., Bertels *et al.*: PMID: 31251344). However, in our study, the mutations analyzed were selected under lenacapavir pressure, and most of the resistance-associated substitutions we tested either increased viral fitness or conferred resistance rather than reflecting general adaptation to MT-4 cells. Accordingly, we do not believe that MT-4-specific adaptation is a major driver of the resistance pathways described here.

3b What do we know about selection of resistance in vitro vs in vivo for other inhibitors, has this ever been compared. Eg does in vitro replication in the presence of eg integrase inhibitors or RT inhibitors select the same resistance profile. Does this support or undermine the present study.

Response: Both outcomes have been reported. For multiple antiretroviral classes, including reverse transcriptase and integrase inhibitors, most resistance mutations

identified through *in vitro* selection are also observed *in vivo*, and many have been validated clinically. However, certain resistance mutations that arise *in vivo* require multi-nucleotide changes and are therefore less readily accessed by standard *in vitro* selection approaches.

This distinction was a key motivation for our experimental design. As noted in the 1st paragraph of the Results section, we used site-saturation mutagenesis to systematically probe less-accessible, multi-nucleotide drug-resistance mutations that frequently emerge in clinical settings but may be underrepresented in conventional *in vitro* selection experiments. Thus, rather than undermining our conclusions, prior comparisons between *in vitro* and *in vivo* resistance evolution support the rationale and relevance of the present study.

3c line 572, considering points above I don't think sequence selection is an assay sensitivity issue its due to MT4 providing different selective pressure than *in vivo* replication. This point should be discussed.

Response: We agree that differences in cellular environment can contribute to differences in selective pressure between *in vitro* and *in vivo* settings. Additionally, we realized that N74S emerged in one of the Q67N dose escalation experiments (Supplementary Fig. 6). To clarify this point, while incorporating the reviewer's comment and our discovery, we have revised the sentence as follows:

"Our inability to detect N74S, which appeared alongside Q67H in one LEN-treated individual, may be due to a potential auxiliary role in supporting DRMs at position 67, differences in drug pressure and/or cellular environment between in vitro and in vivo settings."

3d I think the authors should go beyond the statement on line 561 that the mutants may have a defect in primary cells, particularly since MT4 are the most permissive T-cell line. Can the authors test 1 or 2 key resistance mutants for replication in some primary cells? Do they get the same level of resistance/replication? Agreed, this would make the paper more compelling.

Response: We agree that directly assessing replication competence in primary CD4⁺ T cells is important given the high permissiveness of MT-4 cells. In response to this comment, we examined the infectivity and spreading capacity of representative lenacapavir-resistant variants in primary CD4⁺ T cells. This is now shown in Fig. S5 and mentioned in text as follows:

"We next assessed the viral infectivity and replicative capacity of Q67H and Q67N in primary CD4⁺ T cells. Single-round infectivity was comparable between Q67H and Q67N, while Q67H showed a modest trend toward higher replicative capacity relative to Q67N that did not reach statistical significance. As controls, M66I and N74D exhibited markedly impaired infectivity and replication in primary CD4⁺ T cells (Supplementary Fig. 5)."

As expected, the highly resistant M66I mutant exhibited severely impaired infectivity, whereas Q67H and Q67N retained infectivity and spreading kinetics comparable to wild-type virus. In contrast, the N74D variant showed impaired replication in primary CD4⁺ T cells, consistent with our prior observations and confirmed here. Together, these results indicate that, for most representative variants, replication competence in primary CD4⁺ T cells parallels that observed in MT-4 cells.

This is now described in the revised manuscript as follows:

“We next assessed the viral infectivity and replicative capacity of Q67H and Q67N in primary CD4⁺ T cells. Single-round infectivity was comparable between Q67H and Q67N, while Q67H showed a modest trend toward higher replicative capacity relative to Q67N that did not reach statistical significance. As controls, M66I and N74D exhibited markedly impaired infectivity and replication in primary CD4⁺ T cells (Supplementary Fig. 5).”

The sentence in the Discussion section has been changed as follows:

“It is also possible that some viable variants identified in this study have a defect in replicating in primary CD4⁺ T cells, although limited analyses of HIV-1 mutants in primary CD4⁺ T cells show infectivity and replicative properties similar to those in MT-4 cells.”

We note that these experiments were designed to address replication and infectivity, which was the primary concern raised here. A systematic evaluation of drug resistance levels in primary cells would require additional assay optimization and we believe this point falls outside the focus of the current work.

4 line 562 I don't think that there is an assumption that conserved viral sequences are inherently immutable. Most drug resistance studies undermine this notion.

Response: We agree that “inherently immutable” overstates the prevailing view. We have therefore revised the sentence to adopt more precise language, emphasizing evolutionary constraint rather than immutability. The revised text now reads:

“Nonetheless, our data challenge the view that highly conserved viral sequences are strictly constrained and demonstrate that even evolutionarily conserved regions can accommodate resistance-associated variation under strong selective pressure.”

5 Do the authors expect capacity of resistance mutations to replicate to mirror capacity to transmit? Any thoughts on this particularly in the context of LEN as prophylaxis.

Response: This is an important question. Our answer is not necessarily. Replicative capacity and transmission fitness are related but distinct properties, and resistance mutations that replicate efficiently in vitro may not transmit with equal efficiency in vivo. Transmission is influenced by additional selective pressures present at the mucosal barrier and during early infection, including innate immune responses.

In this context, we note that certain lenacapavir-resistant variants (e.g., N74D) show increased sensitivity to type I interferon, whereas interferon resistance is a characteristic frequently associated with transmitted founder viruses. This raises the possibility that some resistance mutations may carry a transmission cost even if they retain substantial replicative capacity. While our study was not designed to directly test transmission fitness, this distinction is particularly relevant for considering LEN in prophylaxis settings and represents an important area for future investigation.

6 Line 627, isn't it clear that inhibition of nuclear entry by LEN is occlusion of the FG binding site required to bind the FGs in the NPC channel? Agreed, could add discussion of Nup153 and disordered nups (reference Dickson 2024 and Fu 2024, Nature) in addition to CPSF6. The observation that L56I does not infect non-dividing cells (sup fig 10a) may reflect disrupted N57 (the adjacent residue) binding to FG nups.

Response: We agree that interactions between the capsid lattice and FG-containing nucleoporins, including Nup153, play an important role in HIV-1 nuclear entry and that LEN binding could influence these interactions. However, prior work (PMID: 40231850) has shown that M66I, which impairs infectivity in non-dividing cells, does retain Nup153 binding, indicating that additional capsid-dependent properties likely contribute to this phenotype. Given this complexity, we have chosen not to further elaborate on specific FG-nucleoporin binding models

7 Is the different resistance profile at different LEN concentrations related to the different concentration dependent modes of LEN inhibition. Eg isn't the 2 or 3 phase inhibition curve described in various studies due to low conc LEN inhibition FG cofactor binding/nuclear entry and high conc LEN breaking cores open and inhibiting RT. Are these different profiles indicative of different resistance mutations for each of these. Is this revealed by analysing LEN sensitivity curve shapes?

Response: We agree that, for some capsid-targeting compounds (e.g., PF74), dose-response curve shapes can provide mechanistic insight, and we have previously shown concentration-dependent effects using this approach. However, in our hands, lenacapavir exhibits extremely potent antiviral activity, and we did not observe distinct or multi-phase dose-response behaviors that could be readily interpreted as reflecting separable modes of inhibition. In addition, once highly resistant variants such as M66I emerge, the level of resistance is so pronounced that it becomes difficult to meaningfully resolve concentration-dependent effects based on dose-response curve shape alone. Accordingly, while the reviewer's hypothesis is plausible, our data do not reveal clear evidence for distinct resistance profiles linked to different LEN concentration-dependent modes of action.

8 There are several cases of data not shown. I'm not sure that's permissible anymore given the unreliability of and difficulty evaluating unseen data. Please remove reference or show the data.

Response: We agree with the reviewer and have removed all instances of "data not shown" from the manuscript. Two such cases were present in the original submission, and both have now been addressed.

First, we removed the clause referencing unpublished data and revised the text to link directly to the preceding observations. The revised sentence now reads:

"Various E213K-containing molecular clones were constructed but found to be non-infectious. As these E213K variants emerged upon replication in high LEN concentrations, we hypothesized that variants carrying E213K may depend on LEN for infection."

Second, the result showing that the N183K mutation alone does not confer LEN resistance has now been included in **Supplementary Fig. 9** of the revised manuscript.

"Interestingly, N183K, which emerged after high-dose selection and resides close to E187K, also did not affect LEN sensitivity (Supplementary Fig. 9)."

9 Discussion line 649 should discuss the fact that stabilisation of hexamers can cause destabilisation of the viral cores because the hexamers are flattened. See (Faysal 2024 Elife) which they have already referenced here.

Response: We agree that this is an important conceptual point. We now discuss that hexamer stabilization can be accompanied by hexamer flattening, which introduces lattice-level strain and can destabilize the assembled viral core, as described previously. This clarification helps resolve the apparent paradox between increased core yield and LEN's core-disrupting activity, while remaining consistent with prior observations linking increased core stabilization to enhanced LEN resistance. The revised part is as follows:

“One possible explanation is that stabilization of individual CA hexamers can be accompanied by hexamer flattening, which introduces lattice-level strain and destabilizes the assembled core. Consistent with this view, previous work showed that increased core stabilization, either through mutations or IP6, is associated with enhanced LEN resistance.”

10 Why are there two abstracts?

Response: Apologies for this oversight, and we thank the reviewer for catching it. The second abstract was an outdated version and has been removed from the revised manuscript.

11 Figure 1 b (right) legend, please state how many HIV-1/SIVcpz sequences were used to make this?

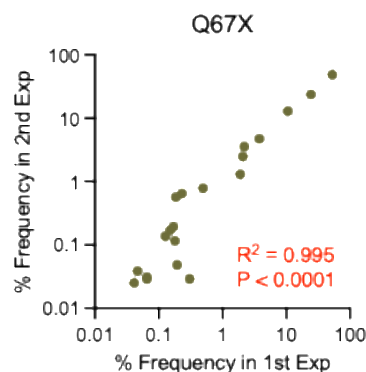
Response: We thank the reviewer for this suggestion. The number of HIV-1/SIVcpz sequences used to generate Figure 1b (12,261) has now been added to the figure legend.

12 Figure 2, legend says ‘a’ is 67 and ‘b’ is 74 but the data looks like it is the other way around.

Response: We thank the reviewer for catching this error. The labeling in the Figure 2 legend has been corrected.

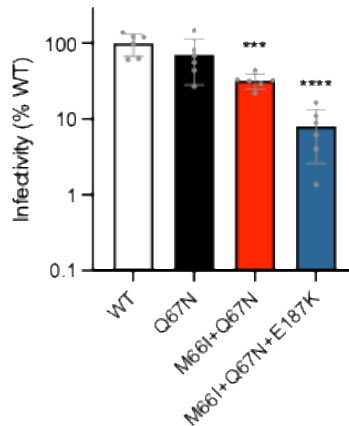
13 Supplementary figure 2, why was only N74 repeated and not Q67? Data requires repeats.

Response: In response to this comment, we performed a replicate experiment for the Q67 library. These data have now been added to Supplementary Figure 2. As shown, there is a strong correlation between the first and second experiments, demonstrating reproducibility.



14 Figure 3b and Figure 6a, might be nice to see how infectious each of these variants are in the absence of LEN compared to WT (I think the data has been normalised to this).

Response: We thank the reviewer for this suggestion. In response, we have added infectivity measurements relative to wild-type virus in the absence of LEN for the cloned variants shown in Figure 6c (see below), demonstrating that acquisition of LEN resistance is accompanied by a reduction in viral fitness.



In contrast, performing an equivalent analysis for Figure 3b is not straightforward, as these samples represent adapted viral populations propagated in the presence of LEN and contain heterogeneous viral material with variable residual drug exposure. Normalizing infectivity in this context would therefore be difficult to interpret. Importantly, the data shown in Figure 6a–c, using molecularly cloned variants, clearly demonstrate a progressive loss of viral infectivity as higher levels of LEN resistance are acquired.

15 Figure 4b, I think it would be useful to also show these residues on a capsid structure with LEN bound eg pdb 6VKV to see how close these are to the binding site. This would help to support the statement in line 316.

Response: We thank the reviewer for this helpful suggestion. In response, we have generated a new supplementary figure (now **Figure S8**) in which the relevant residues are mapped onto a capsid structure with LEN bound (PDB 6VKV), illustrating their spatial relationship to the LEN-binding site and supporting the statement in line 316.

16 Figure 7 and Figure 9 legend, please state how many replicates are used to generate stats?

Response: The following information has been added to the relevant figure legends.

Figure 7c. Left panel: N = 6. Right panel: WT (N = 12), M66I+Q67N (N = 9), and M66I+Q67N+E187K (N = 12)

Figure 7e. Left panel: N = 7. Right panel: WT (N = 6), M66I+Q67N (N = 4), and M66I+Q67N+E187K (N = 6)

Figure 7g. N = 3

Figure 9b. WT (N = 8), Q67N (N = 6), M66I+Q67N (N = 4), and M66I+Q67N+E187K (N = 4).

Figure 9d. N = 4

In addition, upon re-examining the dataset, we noted that the assignment of *P* value (*) for comparing M66I+Q67N+E187K to WT in the right panel of Figure 7C was incorrect, which has now been assigned to **. The revised analysis has been incorporated into the manuscript.

17 The statistics described in line 368 comparing the two mutants are not shown in figure 7e, which instead compares each mutant to WT. Perhaps the 'ns' is meant to be between the red and blue bars not grey and red?

Response: The “ns” annotation does refer to the comparison between WT and M66I/Q67N. Although a visual trend is apparent, this comparison did not reach statistical significance.

18 Line 371, LEN is known to disrupt capsid cores, supporting the data shown, perhaps some of these papers could be referenced here eg Faysal 2024 Elife.

Response: We agree. Accordingly, the sentence has been modified as follows:
“As expected from the known core-disrupting activity of lenacapavir^{27,28}, LEN treatment resulted in a steady decrease in pelletable cores of the WT virus up to 20 nM, at which the curve plateaued (Fig. 7g).”

19 Line 670, could add discussion of E75 and E213 binding to RRR motif of Nup153 (Shen PNAS 2023) therefore the trimeric interface may be important for nuclear import as well as stability.

Response: We agree and have added a brief discussion citing prior work linking residues at the trimeric interface to Nup153 binding and nuclear import (see below). As nuclear entry was not directly examined in this study, we have confined our interpretation in this section to effects on capsid stability.

“Although not addressed here, prior work has shown that residues at the trimeric interface, including E75 and E213, can interact with the RRR motif of Nup153, suggesting that this region may also contribute to nuclear import.”

Finally, we made minor editorial revisions throughout the manuscript to correct grammatical errors. This included small refinements in terminology, primarily related to the use of the term “mutation” (e.g., replacing “mutations in the capsid protein” with “capsid mutations” for consistency and precision). As these changes are distributed throughout the manuscript and do not affect the substance of the work, they are not listed individually but are highlighted in blue