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OPINION

Noncanonical HIV drug resistance mutations: need to close existing gaps

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An increasing number of people with HIV (PWH) are failing treatment without HIV drug resistance in the drug target region. While sub-optimal adherence is likely the cause of treatment failure in many PWH, resistance emerging at noncanonical (HIV drug resistance mutations occurring outside the drug target site) drug target sites is also plausible. Noncanonical drug resistance mechanisms have been identified for integrase strand transfer inhibitors (INSTIs), protease inhibitors (PIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs) and NRTIs. Overall, they may act by restoring viral fitness caused by mutations in the drug target sites, enhance resistance when occurring with mutations at the drug target sites, independently cause resistance even in the absence of drug resistant mutations (DRMs) at the drug target site, and prime the emergence of resistant variants with DRMs at drug target sites. However, the clinical relevance of non-canonical HIV drug resistance mechanisms beyond in vitro and small in vivo studies is still needed and could include the assessment of such mechanisms in clinical trials and implementation studies. This information would be vital in guiding effective management of PWH with viral nonsuppression despite good adherence as well as informing public health surveillance strategies.

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Introduction

In recent years, the efficacy and safety of antiretroviral therapy (ART) for the treatment of HIV-1 has greatly improved, bringing the 2030 HIV elimination goals within reach. However, the emergence and transmission of HIV drug resistance (HIVDR) remains a threat to achieving the 2030 HIV elimination goals. Rising population levels of HIVDR decrease treatment effectiveness, increase treatment costs, worsen HIV-related morbidity and mortality, and contribute to onward transmission, including vertical transmission in pregnant and breastfeeding women [1]. Thus, there is a need to continue to address the public health risk posed by HIVDR. HIVDR often occurs as a result of sub-optimal treatment adherence, which permits selective drug pressure that selects for drug-resistant variants that can continue accumulating mutations as long as viremia persist.

Conventionally, resistant variants have mutations at or near the antiretroviral drug binding site that alter key interactions between the drug and its target, thus allowing for continuous viral replication despite the presence of the drug [2]. For this reason, our understanding of HIVDR has focused chiefly on mutations at or near drug class target sites [3]. However, HIVDR pathways involving drug resistance mutations (DRMs) outside the drug target sites have also been observed [4–14]. While the proportion of people with HIV (PWH) at risk of having drug resistance due to noncanonical mutations (i.e., resistance mediated by mutations outside of the drug target site) remains unknown, a recent study from South Africa showed that up to 71% of viremic PWH with detectable ritonavir-boosted protease inhibitors (bPIs) drug levels and 88% with detectable integrase strand transfer inhibitors (INSTIs) drug levels who had viral nonsuppression did not have detectable resistance mutations within the PI and IN regions of HIV-1, respectively [15]. While intermittent nonadherence may explain most instances of treatment failure without resistance, mutations outside of the drug target sites could explain some cases. Moreover, noncanonical mutations can also compensate for loss of viral fitness caused by mutations in the drug target sites or enhance resistance when occurring with mutations at the drug target sites [16].

In the current era of highly effective ART, where most PWH fail without DRM at the drug target site, understanding all possible mechanisms for treatment failure, including possible noncanonical mechanisms, is critical and may impact the treatment management of PWH. In this article, we review the known mechanisms of drug resistance located outside of the target sites of the commonly used drug classes (summarized in Table 1), including INSTIs, nonnucleoside and nucleos(t)ide reverse transcriptase inhibitors, and bPIs and propose the need to close the knowledge gaps related to possible clinical relevance, which may inform future population-level surveillance and individual treatment management of PWH.

Resistance to integrase inhibitors mediated by mutations outside the integrase gene

Integrase strand transfer inhibitors (INSTIs) are by far the most commonly used anchor drugs in low- and middle-income countries (LMICs), with dolutegravir (DTG), a second-generation INSTI, being used by more than 90% of PWH [17]. Overall, second generation INSTIs have a high genetic barrier to resistance, with resistance rarely reported among ART-naïve PWH and infrequently detected in ART-experienced INSTI-naïve PWH [18].

INSTIs act by blocking the integration of viral DNA into the host genome. Resistance against INSTIs is mainly mediated by mutations at or near the active site that induce conformational changes, reducing the binding affinity of INSTIs and/or increasing the energy barrier for high-affinity binding [19]. However, these structural changes also impact the function of the integrase enzyme, which is partially restored by compensatory mutations. Besides INSTI resistance mediated by DRMs at the drug target site, four alternative pathways involving mutations outside the integrase gene have been proposed, which include, mutations in the *nef* gene at the 3-prime polypurine tract (3'-PPT) – a primer vital for the reverse transcription process; mutations in the long-terminal repeat region; mutations in the nucleocapsid region; and mutations in the envelope gene [4–10].

The 3'-PPT acts as a hybridization site for the RNA primer for the plus-strand DNA synthesis during the reverse transcription process. The RNA primers define the ends of the linear viral DNA used for the integration step, and it has been proposed that mutations in the 3'-PPT tract may modify the viral DNA and affect the integration step [20]. Resistance mediated by mutations in the 3'-PPT was first cited by Malet *et al.* in an *in vitro* study where they showed that these mutations result in high-level phenotypic DTG resistance [20]. Their findings were also observed *in vivo* by Wijting *et al.* in a person living with HIV failing DTG monotherapy without resistance in the integrase gene but with a set of mutations in the highly conserved 3'-PPT region. However, in studies involving site-directed mutants and patient-derived recombinant viral constructs, Wei and Sluis-Cremer [21] and Smith *et al.* [22] did not find evidence for reduced susceptibility to INSTIs for mutants with mutations in the 3'PPT region. Although these data

Table 1. Summary of noncanonical HIV drug resistance mechanism mediated by mutations outside of the drug target gene for the commonly used drugs in low-and middle-income settings.

Drug class	Noncanonical DRM site	Resistance mechanism	Projected impact	Research gaps	Potential clinical monitoring
Integrase strand transfer inhibitors	3'PPT region of the *nef* gene[12,13,16,17]	Unknown but integration-independent replication mechanism has been hypothesized [16,17] ∗	*Low* as it's expected to be rare [18]	-Determining the clinical relevance of the observed resistance mechanism in patients failing treatment -Determining the minimal set of clinically significant DRMs -Determining the pheno-to-geno correlation of the minimal set of clinically relevant DRMs -Determining the proportion of failure attributable to the noncanonical DRMs -Determining the frequency of occurrence overall and across subtypes -Determining transmissibility during infection -Determining the impact on the efficacy of pre and postexposure prophylaxis -Development of methods suitable to assess resistance occurring from the nonconventional mechanisms	-Use of whole genome sequencing -Use of phenotypic tests
	env gene[21,22]	Enhancement of cell-to-cell replication mediated by mutations leading to high-level INSTI resistance [21,22]	High as the mutations lead up to up to 4000-fold increased resistance to INSTIs [21,22]		
	Nucleocapsid region[20]	Epistatic resistance affecting NC, RT and IN functions leading to DTG resistance [20]	Moderate as the mutations lead to a 3–5-fold increased resistance [20]		
	5' terminal bases of the long terminal repeat[19]	Decreases the binding of INSTIs to integrase gene [19]	High as the mutations lead up to up to 30-fold increased resistance to INSTIs [19]		
Protease inhibitors	*Gag gene*[30,31,34].	Increased affinity of the protease enzyme to the natural substrate relative to the PIs [30,31,34]	High and also affect high genetic-barrier 2nd generation PI Darunavir with up to 13-fold increased resistance [30,31,34]		-Inclusion of *gag* and *env* region that has the minimum set of mutations known to impact PI efficacy in HIVDR tests -Use of whole genome sequencing -Use of phenotypic tests
	env gene[32,36]	Mutations in the *env* gene impact the ability of PIs to inhibit viral entry [32]	High as mutations lead to up to 10-fold increased resistance to the PIs [32]		
		Enhancement of cell-to-cell replication mediated by mutations in the *env* gene [36]	Moderate as the mutations lead to 5.7-fold increased resistance to the PIs, including DRV-r [36]		
Reverse transcriptase inhibitors	Connection sub-domain of RT and RNase H domain[38–40].	Reduces RNaseH cleavage, thereby allowing more time for dissociation of NNRTIs, permitting re-initiation of DNA synthesis[38–40]	*Low* as INSTI and tenofovir will replace NNRTIs and zidovudine, respectively, in LMICs according to the current global guidance		-Inclusion of the connection domain/RNAse H site region that has the minimum set of mutations known to impact the efficacy of reverse transcriptase inhibitors in HIVDR tests -Use of whole genome sequencing -Phenotypic tests
		Enhances selective excision of zidovudine [38–40].			
	env gene	Enhancement of cell-to-cell replication mediated by mutations in the *env* gene			

INSTI, integrase strand transfer inhibitors; NNRTIs, nonnucleoside reverse transcriptase inhibitors; PIs, protease inhibitors. ∗Observed only in specific cell-lines expressing HTLV Tax and not been observed *in-vivo*. ∗∗Use of whole genome sequencing for clinical monitoring is predicated upon identifying clinically relevant DRMs. ∗∗Use of phenotypic tests for clinical monitoring particularly for *env* is also predicated upon availability of multiround assays targeting larger segments of the virus, as well as tests that can be used outside research settings.

are conflicting, recent *in vitro* studies by Dekker *et al.* and Richetta *et al.* [7] show that viral mutants with mutations in the 3'PPT can escape DTG inhibition by integration-independent replication evidenced by the absence of integrated viral DNA but with the presence of unintegrated viral DNA. However, although unintegrated DNA has been shown to support the expression of viral proteins, its role in the formation of infectious virion particles is still debatable, having been demonstrated in cell lines that express HTLV tax [6]. It is worth noting that the selection of mutations in the 3'-PPT region during INSTI failure is likely a rare event, as observed from reviews of sequences of PWH failing INSTI-based ART [23].

The second proposed noncanonical mechanism for INSTI failure involves terminal mutations in the 5' region of LTR. The four terminal bases of LTR play a vital role in the integration of the viral DNA into the host genome and binding of INSTIs. As such, mutations in the four terminal bases have been shown to impact the binding affinity of INSTIs by 2–12-fold when occurring alone but >50-fold when present with canonical INSTI DRMs though only in *in vitro* studies [8].

The third proposed noncanonical mechanism involves mutations in the HIV-1 gag nucleocapsid region, resulting in 3–5-fold resistance [9]. Although the precise mechanism is unknown, Atsuko *et al.* proposed that changes in the nucleocapsid region affects the viral DNA formation leading to DNA with abnormal LTR end which affects DTG binding and its exclusion from the active sites of nucleocapsid and the nucleocapsid–integrase complex [9].

The final proposed noncanonical mechanism involves mutations in the envelope *(env)* gene [5,10]. HIV within the host can spread directly through cell-to-cell infection or through cell-free route [24]. Compared to cell-free infection, cell–cell replication is associated with reduced ART susceptibility [25] but the use of combination ART can effectively inhibit both mechanisms of HIV replication [25]. However, studies show that the selection of mutations in *env* may lead to escape of viral inhibition to most currently available antiretrovirals except for some entry inhibitors by enhancing replication through the cell-to-cell infection route even in the absence of mutations in the drug target site [5,10]. Duyne *et al.* and Hikichi *et al.* showed that mutations in the *env* gene can lead to very high-level resistance of INSTIs by up to 4000-fold [5,10]. Hikichi *et al.* also showed that the reason for INSTI resistance via the cell-to-cell route was due to the greater multiplicity-of-infection (MOI), i.e. infection of a cell by multiple viruses primarily via the cell-to-cell route [5]. As such, the INSTIs cannot effectively stop every integration activity as they are overwhelmed by the high MOI.

While the overall contribution of the cell-to-cell HIV replication mechanism relative to the cell-free route is unclear, some studies show that the cell-to-cell transmission route has a higher replication efficiency [24,26] and prediction models demonstrate that up to 80% of infections could use this route due to high MOI [27]; however, this assertion remains unproven.

Resistance to protease inhibitors mediated by mutations outside the protease gene

Ritonavir-boosted protease inhibitors are recommended as second-line treatment following DTG failure in most LMICs [28] due to their potency and high genetic barrier to resistance. Nonetheless, 8–37% of PWH switched to bPI-based second-line ART experience viral failure [29] with majority failing without major PI mutations [29]. Three possible explanations have been suggested for treatment failure in the absence of PI mutations: patient self-directed treatment withdrawal with no resistance mutations selected in the absence of drugs; a short mutation selection window attributed to a rapid fall in inhibitory concentration of PIs during nonadherence; and, mediation of resistance by mutations outside of the HIV-1 *pol* region, namely in the *gag* and *env* genes [11,12,30–32].

PIs are substrate or transition state analogues that actively compete with precursor polyproteins, inhibiting the proteolytic activity of the viral protease enzyme. Protease enzyme cleaves the gag and gag-pol polyproteins, resulting in the maturation of noninfectious virion particles to fully infectious viruses. Resistance to PIs is mediated by mutations at or near the catalytic binding site of the drug. Their occurrence lowers the enzyme's affinity to the inhibitors more than for the natural substrate through conformational changes at the substrate binding region. However, these structural changes come with a fitness cost and loss of replication capacity that can be compensated by secondary mutations surrounding the active site [2].

In addition to DRMs in the protease gene, studies have shown that mutations in the *gag* substrate region can compensate for fitness loss imposed by the protease gene's primary mutations and use resistance independent of mutations in the protease gene [11,30,32]. Like mutations in the protease region, these mutations are thought to cause conformational changes that increase the enzyme's affinity toward the natural substrate instead of the PI drug. The most commonly described mutations are those that occur at the *gag* proteolytic cleavage sites [11,30,32]. Mutations in the noncleavage site – particularly in the matrix region – have also been shown to reduce PI susceptibility, but the exact mechanism for this is still under study [31,32]. Overall, mutations in both cleavage and noncleavage sites mediate PI resistance by improving protease binding affinity to the gag substrate, restoring viral capacity, and increasing fitness of the mutant viruses.

A less studied mechanism for PI resistance outside the protease gene involves mutations in the *env* region [12]. Rabi *et al.* showed that up to 50% of the inhibitory

potential of PIs occurs at viral entry; therefore, mutations in the *env* gene may affect PIs' overall activity [12]. Using pseudoviruses, Rabi *et al.* showed that *env* mutations in the gp41 cytoplasmic tail lead to PI resistance, but this comes at a fitness cost to the virus [12].

While the clinical significance of mutations outside of the PI binding region have not been fully ascertained, studies have shown a higher frequency of their occurrence among PWH failing treatment compared to the naïve population with phenotypic confirmation of their potential significance [11,30,32].

A third mechanism, akin to that hypothesized for INSTIs, involves mutations that promote cell-to-cell transmission, leading to resistance to all ART drugs, including PIs [33].

Noncanonical drug resistance mutations affecting reverse transcriptase inhibitors

NRTIs are modified nucleos(t)ide analogs that compete with natural analogs for the reverse transcriptase enzyme. Their incorporation terminates the synthesis of the viral DNA. NNRTIs are noncompetitive inhibitors for the RT enzyme binding to an allosteric site of the enzyme leading to conformational changes that impair polymerase activity. Various mechanisms are involved in resistance for NRTI and NNRTI, involving mutations occurring at or near the drug binding site [34].

However, mutations outside the polymerase region, in the RT connection sub-domain and the ribonuclease (RNase) H domain have also been shown to reduce susceptibility to the first-generation NNRTI and zidovudine (ZDV) [13,14,35]. These mutations lead only to a modest reduction in susceptibility of the RTIs but may significantly enhance resistance when co-occurring with mutations in the RT region. While the exact mechanism of resistance mediated by these (combined) mutations is unclear, it is thought that they reduce RNaseH cleavage, allowing more time for dissociation of the NNRTI, permitting re-initiation of DNA synthesis [13,14,35]. Concerning NRTIs, these mutations are thought to enhance ZDV excision [13,14,35]. However, their presence also increases cross-resistance to lamivudine and tenofovir [36].

In addition to mutations in the connection domain, as observed by Hikichi *et al.*, mutations in the *env* gene can lead to escape of viral inhibition by RTIs through enhancement of the cell-to-cell infection route [33].

Research gaps around the clinical and public health significance of noncanonical HIV drug resistance mutations

Despite existing evidence on the possible importance of drug resistance mediated by mutations outside the drug target genes, the clinical relevance of noncanonical mutations has not been conclusively established and various research gaps remain. Most of the current evidence is based on data from *in vitro* studies or small *in vivo* observational studies. The remaining gaps include identifying the clinical relevance of noncanonical mutations and their potential contribution to treatment failure, if they are transmissible, and if they impact the efficacy of antiretrovirals used for preexposure prophylaxis. There is also a need to identify correlates of noncanonical mutations, including the impact of HIV-1 subtypes, population affected for example ART-naïve vs. experienced among others and the context of their occurrence for example in PWH on sub-optimal therapy or those who are nonadherent. Moreover, there will be a need to determine the management of PWH with clinically relevant noncanonical mutations.

Potential studies could focus on highly adherent populations who fail to resuppress despite evidence of good adherence as measured by objective measurements like therapeutic drug level tests and particularly in PWH being treated with long-acting agents with persistent viremia who do not have mutations in the drug target genes. Such studies could consider the use of whole genome sequencing (WGS) as well as phenotypic tests where capacity is available (Table 1). Next generation sequencing technologies have become more widely accessible in LMICs with drop in prices and the capacity building that was done to support COVID-19 surveillance during the pandemic [37,38]. Routine genotypic assessment of noncanonical DRMs would nonetheless require incorporating the genotypic-to-phenotypic correlation data and/or clinical relevance information into HIVDR interpretation algorithms.

Inclusion of noncanonical resistance mechanisms in clinical trials and implementation studies

Despite increasing attention on the potential impact of noncanonical DRMs, they are not commonly assessed as part of clinical trials or studies assessing for possible causes of treatment failure. Traditionally, US-FDA recommendations recognize the need for clinical trials to also assess resistance mechanisms, including those mediated outside the drug targets [39]. With the increased potency of new antiretrovirals and the fact that most PWH fail treatment without resistance in the drug target genes, it may be prudent for clinical and implementation studies to consider embedding studies assessing for the alternative causes of failure including noncanonical drug resistance mechanisms.

Clinical monitoring and population surveillance of noncanonical mutations

Due to the current knowledge gaps around the clinical relevance and possible public health impact of noncanonical DRMs the need to include these regions in genotyping for surveillance and routine care remains unsettled [13,35].

Understanding the frequency of noncanonical mutation occurrence, defining a minimal set of clinically relevant noncanonical DRMs, if they are shown to be clinically relevant, as well as limiting their possible impact on transmission (if found to be transmissible) is essential in order to inform routine clinical and HIVDR surveillance practices.

In conclusion, the high proportion of PWH failing treatment without resistance in the drug target gene coupled with increasing evidence showing a potential for noncanonical mechanisms of resistance in explaining treatment failure suggest the need to fast-track the ascertainment of clinical relevance of these mechanisms, including assessment of such mechanisms in clinical trials and implementation studies to provide evidence to guide effective management of PWH with viral nonsuppression despite good adherence and inform public health surveillance strategies.

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Conflicts of interest

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