MAJOR ARTICLE

Human Immunodeficiency Virus-1 Sequence Changes and Drug Resistance Mutation Among Virologic Failures of Lopinavir/Ritonavir Monotherapy: AIDS Clinical Trials Group Protocol A5230

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Background. The mechanism of virologic failure (VF) of lopinavir/ritonavir (LPV/r) monotherapy is not well understood. We assessed sequence changes in human immunodeficiency virus-1 reverse-transcriptase (RT) and protease (PR) regions.

Methods. Human immunodeficiency virus-1 *pol* sequences from 34 participants who failed second-line LPV/r monotherapy were obtained at study entry (SE) and VF. Sequence changes were evaluated using phylogenetic analysis and hamming distance.

Results. Human immunodeficiency virus-1 sequence change was higher over drug resistance mutation (DRM) sites (median genetic distance, 2.2%; Q1 to Q3, 2.1%–2.5%) from SE to VF compared with non-DRM sites (median genetic distance, 1.3%; Q1 to Q3, 1.0%–1.4%; $P < .0001$). Evolution over DRM sites was mainly driven by changes in the RT (median genetic distance, 2.7%; Q1 to Q3, 2.2%–3.2%) compared with PR (median genetic distance, 1.1%; Q1 to Q3, 0.0%–1.1%; $P < .0001$). Most RT DRMs present at SE were lost at VF. At VF, 19 (56%) and 26 (76%) were susceptible to efavirenz/nevirapine and etravirine (ETV)/rilpivirine (RPV), respectively, compared with 1 (3%) and 12 (35%) at SE. Participants who retained nonnucleoside reverse-transcriptase inhibitor (NNRTI) DRMs and those without evolution of LPV/r DRMs had significantly shorter time to VF.

Conclusions. The selection of LPV/r DRMs in participants with longer time to VF suggests better adherence and more selective pressure. Fading NNRTI mutations and an increase in genotypic susceptibility to ETV and RPV could allow for the reuse of NNRTI. Further studies are warranted to understand mechanisms of PR failure.

Keywords. drug resistance mutation; hamming distance; HIV-1 sequence evolution; LPV/r failures; phylogenetic.

The use of boosted protease inhibitor (PI) therapy is increasing in resource-limited settings as second-line therapy; however, this increase also raises the likelihood of viral failures while on a PI. Mechanisms of virologic failure (VF) of boosted PI, the development of resistance, and the options for additional treatment are poorly understood. Studies to date have observed very little or no PI resistance mutations in the protease (PR) region alone. In cross-sectional studies of lopinavir/ritonavir (LPV/r) recipients with viremia in South Africa, <10% had major LPV/r resistance mutations [1–3]. In contrast, studies of subtype C second-line failure in India [4], studies in the private sector drug resistance testing [5], as well as studies among

pediatric patients in South Africa [6] have shown that sequential PI polypharmacy and prolonged VF increase the frequency of major PR mutations in resource-limited settings [7, 8]. This suggests that even with aggressive adherence monitoring and counseling, drug resistance and mutations in the PR region account for less than half of those failing a PI-based regimen. In other studies, alternative genotypic changes in the *gag* and *env* regions have been associated with boosted PR failure in the absence of major PI mutations [9–12].

Studies of the patterns of nucleoside reverse-transcriptase inhibitor (NRTI)-associated and nonnucleoside reverse-transcriptase inhibitor (NNRTI)-associated mutations after transition to PI-based regimens among human immunodeficiency virus (HIV)-1 infected individuals with subsequent VF during a second-line boosted PI-based treatment are limited. Evidence of either gains or losses of NNRTI mutations, particularly Y181C and K103N, have been found among women and infants after single-dose nevirapine (NVP) [13–15].

In this study, we assessed changes in PR and reverse transcriptase (RT) of HIV-1 and their associations with covariates among participants with VF in the AIDS Clinical Trials Group (ACTG) A5230 study receiving LPV/r monotherapy after failure of a first-line regimen. Genotypic and evolutionary analyses were conducted

to identify potential mechanism(s) of VF and drug resistance among recipients of a boosted PI for second-line treatment.

METHODS

Participant Samples

The ACTG 5230 is a single arm, open-label, multicenter, pilot study to evaluate the safety and efficacy of LPV/r monotherapy in PI-naive individuals failing an initial NNRTI-containing regimen in Thailand, South Africa, India, Malawi, and Tanzania. CD4 cell counts and HIV-1 ribonucleic acid levels (viral load [VL]) were available as part of the study at screening. Plasma samples from ACTG A5230 participants at the time of screening and VF were tested for HIV-1 drug resistance testing.

Population Genotype Analysis

Population-based genotyping was performed using the Celera Diagnostics ViroSeq (Abbott Molecular, Abbott Park, Illinois) drug resistance assay, per manufacturer's instructions. A 1.7-kb amplicon was generated by RT-initiated polymerase chain reaction encompassing the entire PR and partial RT. Sequencing was performed with an ABI Prism 3100-*Avant* Genetic Analyzer (Applied Biosystems). Human immunodeficiency virus-1 drug resistance and subtype were determined from PR and RT sequences.

Data Analysis

Thirty-four participants had study entry (SE) and VF sequences available for analysis. Within the HIV-1 *pol* sequence, we interrogated 987 nucleotide positions (329 amino acids: PR codon 1–99 and RT codon 1–230). There were 46 DRM sites including 31 RT and 15 major PR mutation sites based on the International AIDS Society-USA 2014 update of the DRM in HIV-1 [16]. For each participant, paired HIV-1 sequences (at time of SE and VF) were used to characterize the HIV-1 sequence evolution using 2 different approaches [17, 18]. (1) Hamming distance [18] measured the percentage mismatch in nucleotides between HIV-1 sequences obtained at screening and the time of VF. For matched and mismatched nucleotides, the distance was assigned a value of 0 and 1, respectively. This Hamming distance is normalized by the sequence length but does not take into account the time span between the 2 isolates. (2) Phylogenetic analysis was used to calculate nucleotide substitution rates for each participant based on the Tamura-Nei (TN93) model. Pairwise TN93 distances were computed and normalized by follow-up time using PolEvolution scripts in the HyPhy package [19]. The TN93 model corrects for biases in unequal base composition and differences in transition/transversion rates seen in nucleotide sequence evolution of HIV-1.

Rank-sum tests were used to compare genetic distances and time to VF between groups. Spearman coefficients (r) were used for the correlations between genetic distances and continuous covariates (age, SE VL, SE CD4, VL at VF, and time to VF). Fisher exact tests were used for associations between changes

(binary) in mutations from SE to VF and categorical covariates (sex, race/ethnicity, and HIV-1 subtype).

RESULTS

Thirty-four participants had *pol* sequence data available at SE and VF (median VL) at SE = 4.6 $\log_{10}$ copies/mL (Q1 to Q3, 3.9–5.0). The median duration from SE to VF was 48 weeks (Q1 to Q3, 31–80). At SE, 91% and 97% of participants had at least 1 NRTI or NNRTI mutation, respectively, and 1 participant had 1 major PI mutation. The most common mutation(s) at SE for NRTI was M184V/I (79%), and the most common mutations for NNRTI were Y181C (53%) and K103N (41%) (Table 1). At VF, the majority of RT mutations presented at SE were lost: only 26% (7 of 27) of the participants retained the M184V/I and 22% (4 of 18) retained the Y181C. However, K103N was retained among 79% (11 of 14) of participants with this mutation at SE. Among the minor LPV/r-associated mutations present in >10% at SE (L63P, L10I/F/V, and K20R), 71% (15 of 21) remained at VF. Additional participants' characteristics and corresponding HIV-1 resistance mutations are provided in the Supplementary Tables 1 and 2.

Evolutionary Change in Protease and Reverse Transcriptase From Study Entry to Virologic Failure

Using Hamming distance to quantify changes in consensus nucleic acid sequence from SE to VF, median percentage mismatch

Table 1. Frequencies of NRTI-, NNRTI-, and LPV/r-Associated Resistance Mutations That Occurred in >10% at SE and Their Changes at VF[a]

Drug Class-Associated Resistance Mutations	SE	VF		
	Frequencies (%)	No. Lost	No. Gained	No. Retained
NRTI				
M184V/I	27 (79)	20	1	7
T215Y	5 (15)	3	0	2
D67N	5 (15)	3	0	2
K65R	5 (15)	5	0	0
T69d	4 (12)	4	0	0
Total	46	35	1	11
NNRTI				
Y181C	18 (53)	14	0	4
K103N	14 (41)	3	0	11
H221Y	9 (26)	7	0	2
G190A/S	8 (24)	8	1	0
K101E	5 (15)	5	0	0
V108I	4 (12)	3	0	1
Total	58	40	1	18
LPV/r				
L63P	10 (29)	2	1	8
L10I/F/V	7 (21)	3	0	4
K20R	4 (12)	1	1	3
Total	21	6	2	15

Abbreviations: DRM, drug resistance mutations; LPV/r, lopinavir/ritonavir; NNRTI, nonnucleoside reverse-transcriptase inhibitor; NRTI, nucleoside reverse-transcriptase inhibitor; SE, study entry; VF, viral failure.

[a] The percentage of DRM at SE is among 34 participants.

Table 2. Sequence Change (%Mismatch) Based on Hamming Distance From SE to VF

Type of HIV-1 Sequence Change	DRM Sites (PR and RT) Genetic Distance (Median, 95% CI)		Non-DRM Sites (PR and RT) Genetic Distance (Median, 95% CI)	
Sequence change from SE to VF	2.2% (2.1%–2.5%)		1.3% (1.0%–1.4%)	
	$P < .0001^{a}$			
	DRM PR	DRM RT	Non-DRM PR	Non-DRM RT
Sequence change from SE to VF	1.1% (0.0%–1.1%)	2.7% (2.2%–3.2%)	1.3% (1.2%–1.6%)	1.2% (1.0%–1.4%)
	$P < .0001^{a}$		$P = .27^{a}$	

Abbreviations: CI, confidence interval; DRM, drug resistance mutations; PR, protease; RT, reverse transcriptase; SE, study entry; VF, viral failure.

[a] Rank tests for difference in genetic distances between groups.

from SE to VF across *pol* sequences was 1.5% (95% confidence interval [CI], 1.2%–1.6%). Focusing on DRM vs non-DRM sites (PR and RT), the HIV-1 sequence change was greater at DRM sites (median percentage mismatch, 2.2%; 95% CI, 2.1%–2.5%) from SE to VF compared with non-DRM sites (median percentage mismatch, 1.3%; 95% CI, 1.0%–1.4%; $P < .0001$). Changes in DRM sites were mainly driven by changes in the RT gene (median percentage mismatch, 2.7%; 95% CI, 2.2%–3.2%) compared with the PR gene (median percentage mismatch, 1.1%; 95% CI, 0.0%–1.1%; $P < .0001$). However, changes in RT and PR genes were similar in non-DRM sites (median percentage mismatch, 1.2% [95% CI, 1.0%–1.4%] vs 1.3% [95% CI, 1.2%–1.6%], respectively; $P = .27$) (Table 2).

Phylogenetic analysis had similar findings with the calculated nucleotide substitution rates (Table 3). From SE to VF, nucleotide substitution rate across *pol* sequence was 1.4×10^{-2} substitutions per site per year (95% CI, 1.3×10^{-2} to 1.6×10^{-2}). For DRM and non-DRM sites, nucleotide substitution rates were 2.9×10^{-2} (95% CI, 2.4×10^{-2} to 3.4×10^{-2}) and 1.2×10^{-2} (95% CI, 1.1×10^{-2} to 1.3×10^{-2}), respectively. In particular, RT DRM sites had higher nucleotide substitution rates (3.8×10^{-2}; 95% CI, 3.1×10^{-2} to 4.5×10^{-2}) compared with PR DRM sites (1.1×10^{-2}; 95% CI, 0.6×10^{-2} to 1.7×10^{-2}; $P < .001$). Substitution rates were similar between PR and RT over non-DRM sites (1.4×10^{-2} [95% CI, 1.2×10^{-2} to 1.7×10^{-2}] vs 1.1×10^{-2} [95% CI, 1.0×10^{-2} to 1.3×10^{-2}]; $P = .49$). The relative rates of nonsynonymous and synonymous substitutions ratio (d_N/d_S) overall for PR and RT regions were above one for all SE-failure samples, indicating that genes are evolving under positive selection and that at least some of the mutations must be advantageous.

Genotypic Resistance

The pattern of drug resistance, estimated by genotypic resistance from SE to VF, among 34 participants, demonstrated that 24 (71%) participants experienced loss of RT mutations (24 [71%] lost NRTI and 23 [68%] lost NNRTI mutations) from SE to VF. The 23 participants who lost NNRTI resistance mutations had greater changes in RT compared with the 11 who retained SE NNRTI mutations (median percentage mismatch, 1.7% [95% CI, 1.3%–1.8%] vs 0.6% [95% CI, 0.3%–1.2%]; $P < .01$). Due to loss of NNRTI mutations, at VF, 19 (56%) and 26 (76%) participants were susceptible to efavirenz (EFV)/NVP and etravirine (ETV)/rilpivirine (RPV), respectively, compared with only 1 (3%) and 12 (35%) participants at SE.

Twenty-one (62%) participants who experienced gain/loss of LPV/r mutations had modestly greater, but not significantly different, HIV-1 sequence changes over RT and PR genes compared with 13 participants who experienced no change in LPV/r mutations (median percentage mismatch, 1.5% [95% CI, 1.2%–2.0%] vs 1.2% [95% CI, 0.5%–1.7%] for RT and 1.3% [95% CI, 1.0%–2.0%] vs 1.0% [95% CI, 0.2%–1.7%] for PR gene). Sequence change over DRM sites and SE CD4 count were significantly correlated ($r = -0.42$, $P = .01$). No other significant correlations between genetic distances and SE VL, VL at VF, and time to VF were detected (over DRM and non-DRM sites, overall and within RT and PR genes).

Time to Virologic Failure and Genotypic Changes

The time from SE to VF was significantly shorter among the 11 participants who retained NNRTI mutations at VF compared with 23 participants who lost NNRTI mutations (median, 22 weeks [Q1 to Q3, 20–48] vs 48 weeks [Q1 to Q3, 22–80],

Table 3. Nucleotide Substitution Rate Based on Phylogenetic Analysis From SE to VF

HIV-1 Sites	Nucleotide Substitution Rate (Substitutions/Site/Year) (95% CI)		
	Overall	PR	RT
DRM sites	2.9×10^{-2} (2.4×10^{-2} to 3.4×10^{-2})	1.1×10^{-2} (0.6×10^{-2}–1.7×10^{-2})	3.8×10^{-2} (3.1×10^{-2} to 4.5×10^{-2})
Non-DRM sites	1.2×10^{-2} (1.1×10^{-2} to 1.3×10^{-2})	1.4×10^{-2} (1.2×10^{-2} to 1.7×10^{-2})	1.1×10^{-2} (1.0×10^{-2} to 1.3×10^{-2})

Abbreviations: CI, confidence interval; DRM, drug resistance mutations; PR, protease; RT, reverse transcriptase; SE, study entry; VF, viral failure.

respectively; $P = .04$). Eleven participants who retained NNRTI mutations had similar SE characteristics with 23 participants who lost NNRTI mutations: ie, age (median age, 40 [Q1 to Q3, 28–47] vs 41 [Q1 to Q3, 34–47]), female sex (55% [6] vs 61% [14]), black race/ethnicity (82% [9] vs 83% [19]), and HIV-1 subtype C virus (82% [9] vs 61% [14]).

Time to VF was also significantly shorter among the 13 (38%) who did not experience changes in LPV/r mutations compared with the 21 participants who experienced any change (median time, 22 weeks [Q1 to Q3, 21–40] vs 48 weeks [Q1 to Q3, 32–80], respectively; $P = .04$). Thirteen participants without change in LPV/r mutations vs 21 with change in LPV/r mutations were somewhat younger (median age, 36 [Q1 to Q3, 31–43] vs 41 [Q1 to Q3, 36–47]) and more likely female (69% [n = 9]) vs 52% [n = 11]), of black race/ethnicity (100% [n = 13] vs 71% [n = 15]), and with HIV-1 subtype C virus (85% [n = 11] vs 57% [n = 12]), although differences between these groups were not statistically significant. Additional factors such as SE VL, SE CD4 count, and change between SE and VF VL were not significantly different between the 2 groups.

DISCUSSION

Continuous metrics of HIV sequence changes demonstrated differences in HIV-1 evolution at DRM and non-DRM sites in participants with VF during second-line LPV/r monotherapy after first-line failure of a NNRTI-containing regimen. Restricting the analysis to non-DRM sites, changes between PR and RT genes were similar. However, under antiretroviral therapy (ART) drug pressure with only LPV/r monotherapy, there was greater HIV-1 evolution from SE to VF in DRM sites compared with non-DRM sites. The changes in DRM sites were largely driven by changes in RT gene compared with PR gene, specifically due to loss of mutations in RT gene region in the absence of NNRTI and NRTI drug pressure. A prominent exception was the persistence of the K103N mutation, which was still present at VF in the majority of those with K103N at SE, despite the absence of NNRTI drug pressure. This contrasts with the changes in other significant RT mutations; the majority of Y181C and M184V mutations were no longer detected (faded) in the absence of drug pressure.

Genotypic assessment of drug susceptibility among first-line ART failures receiving monotherapy with LPV/r provides specific evidence of selective pressure on the PR gene at drug resistance-associated sites in 62% of those with VF. The selection of minor LPV/r resistance mutations among 21 LPV/r monotherapy recipients provides evidence of selective drug pressure. However, the mutations identified contribute only minimally to estimated LPV/r resistance [20]. The absence of PR mutations on LPV/r monotherapy was associated with a shorter time to VF, suggesting less selective pressure, likely due to decreased adherence or drug exposure. Although differences in measured adherence were not observed, the absence of genotypic evidence of PI selective pressure among persons experiencing VF may identify those who will benefit from pharmacokinetic analysis and reinforced adherence counseling.

Re-emergence of wild-type alleles through evolution and selection was more prominent in the RT at codons associated with drug resistance mutations. In contrast to PR, RT gene changes from SE to VF showed that most mutations associated with drug resistance were lost from the consensus sequence, changing the predicted genotypic drug susceptibility. Among individuals with VF of an EFV- and/or NVP-based regimen, genotypic drug resistance to RPV was frequent, whereas resistance to ETV was rare [21]. Moreover, genotypic algorithms may overestimate resistance to ETV and RPV in subtype C virus [22]. Fading of NNRTI mutations during LPV/r treatment may increase the effectiveness of second-generation NNRTI drugs, RPV and ETV in third-line. This suggests the need for clinical studies in the selective reuse of NNRTIs, which have been shown to be effective among treatment-experienced HIV-1 participants with documented evidence of NNRTI resistance [23–25].

The retention of RT drug resistance mutations, albeit in a minority after virologic suppression with LPV/r, is more difficult to explain. Reappearance of NNRTI mutation at K103N after a median of 48 weeks of viral suppression provides evidence for the archiving of this mutation in replication-competent proviral deoxyribonucleic acid [26]. K103N is a commonly transmitted NNRTI drug resistance mutation [27, 28], and it may persist for years despite drug discontinuation [29]. This is in comparison to the marked decrease in M184V, the most common mutation after first-line failure. The evidence for fitness costs of M184V [30] and interaction with tenofovir resistance [31] emphasizes the importance of its continuation in salvage regimens despite genotypic resistance. It is noteworthy to mention that only CD4 at SE was associated with minimal sequence change from SE to VF, which suggests that immune surveillance may mitigate selection of DRM.

CONCLUSIONS

In summary, this study provides evidence of sequence evolution, which was largely driven by the re-emergence of wild-type, susceptible alleles at RT DRM sites between the first-line failure on NNRTI-based regimen and the second-line failure on LPV/r monotherapy. The fading of RT mutations could allow selective reuse of NNRTI regimens, but clinical studies are needed. Evolution at the PR region was limited compared with the RT region, but participants with evolution at LPV/r-associated mutations had longer time to VF on LPV/r monotherapy, possibly due to better adherence and more selective drug pressure. Analysis of adherence, pharmacodynamics, and changes in sequence of *gag* and *env* are warranted to understand mechanisms of PR failure.

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