Baseline PI susceptibility by HIV-1 Gag-protease phenotyping and subsequent virological suppression with PI-based second-line ART in Nigeria

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Objectives: Previous work showed that gag-protease-derived phenotypic susceptibility to PIs differed between HIV-1 subtype CRF02_AG/subtype G-infected patients who went on to successfully suppress viral replication versus those who experienced virological failure of lopinavir/ritonavir monotherapy as first-line treatment in a clinical trial. We analysed the relationship between PI susceptibility and outcome of second-line ART in Nigeria, where subtypes CRF02_AG/G dominate the epidemic.

Methods: Individuals who experienced second-line failure with ritonavir-boosted PI-based ART were matched (by subtype, sex, age, viral load, duration of treatment and baseline CD4 count) to those who achieved virological response (‘successes’). Successes were defined by viral load <400 copies of HIV-1 RNA/mL by week 48. Full-length Gag-protease was amplified from patient samples for in vitro phenotypic susceptibility testing, with PI susceptibility expressed as IC50 fold change (FC) relative to a subtype B reference strain.

Results: The median (IQR) lopinavir IC50 FC was 4.04 (2.49–7.89) for virological failures and 4.13 (3.14–8.17) for virological successes (P = 0.94). One patient had an FC >10 for lopinavir at baseline and experienced subsequent virological failure with ritonavir-boosted lopinavir as the PI. There was no statistically significant difference in single-round replication efficiency between the two groups (P = 0.93). There was a moderate correlation between single-round replication efficiency and FC for lopinavir (correlation coefficient 0.32).

Conclusions: We found no impact of baseline HIV-1 Gag-protease-derived phenotypic susceptibility on outcomes of PI-based second-line ART in Nigeria.

Introduction

Prevalence of virological failure for first-line antiretroviral therapy can be as high as 30%,1 with high-level resistance to NNRTI, tenofovir and cytosine analogues common in resource-limited settings and compounded by prior undisclosed ART.2,3 Second-line ART recommended by WHO comprises a ritonavir-boosted PI and two NRTIs, commonly lopinavir or atazanavir.4 PIs are the second- and last-line therapy for the majority of HIV-infected patients worldwide as access to third-line therapy is still limited.5 Virological failure with PIs as second-line therapy occurs in around 20% of individuals.5–8 In contrast to first-line therapy, which with >80% develop drug resistance mutations, only around 10%–20% develop major resistance mutations to PIs by week 48.6,7,9,10 and this proportion increases over time.5

It is known that proteins such as Gag and Env can affect susceptibility to PIs even in the absence of known major resistance mutations in the protease gene.11–15 There are limited data on changes in gag following treatment failure with PIs in the non-B subtypes that dominate low- and middle-income countries.15–20 It appears that in around 15% of patients failing boosted PI (bPI) without major protease mutations, a decrease in phenotypic susceptibility to the drug appears to occur when gag-protease is phenotyped.21–23 Therefore it is conceivable that underlying phenotypic susceptibility resulting from variation in genes such as gag and env might impact clinical responses to PI.

We previously showed that gag-protease-derived phenotypic susceptibility differed between CRF02_AG and subtype G-infected patients who went on to successfully suppress viral replication
versus those who experienced virological failure (VF) of lopinavir/ritonavir monotherapy as first-line treatment in a clinical trial. In order to determine the relevance of this finding for real-world settings in the context of tenofovir disoproxil fumarate + lamivudine or zidovudine + lamivudine with ritonavir-boosted PI (lopinavir or atazanavir) we analysed the relationship between PI susceptibility and the outcome of PI-based second-line ART in Nigeria, where subtypes CRF02 AG and G dominate the epidemic.

**Patients and methods**

**Study participants**

This study involved retrospectively testing samples from patients attending for HIV care at University of Abuja Teaching Hospital (UATH) who experienced second-line failure (HIV-1 RNA >1000 copies/mL after >6 months on treatment) on a lopinavir/ritonavir- or atazanavir/ritonavir-containing regimen, without any major PI mutations, who were selected as ‘cases’. They were matched to ‘controls’, who had achieved virological suppression lasting up to 12 months (HIV-1 RNA <400 copies/mL) with a similar age, sex, baseline CD4 count and duration of treatment. Baseline (pre-PI) plasma samples from these matched pairs were retrospectively retrieved.

**Amplification of full-length gag-protease genes**

HIV-1 RNA was manually extracted from archived plasma samples using the QIAamp viral RNA extraction kit. Previously described techniques, full-length gag-protease was amplified and cloned into a subtype B-based (p8.9NSX) vector. Clonal sequencing of up to 10 plasmids (where possible) was performed by standard Sanger sequencing. The variant that most closely represented the consensus (obtained via next-generation sequencing as previously described) was taken forward for phenotypic testing. Sequences were manually analysed using DNA dynamo software (http://www.bluetractorsoftware.co.uk) and MEGA v7.0 software. Protease sequences were analysed for PI resistance mutations using the Stanford Resistance Database (https://hivdb.stanford.edu).

**PI susceptibility and infectivity assays**

PI susceptibility and viral infectivity were determined using a previously described single assay. Briefly, 293T cells were co-transfected with a Gag-Pol protein expression vector (p8.9NSX+) containing cloned patient-derived gag-protease sequences, pMDG expressing vesicular stomatitis virus envelope glycoprotein (VSV-G), and pCSFLW (expressing the firefly luciferase reporter gene with HIV-1 packaging signal).

PI drug susceptibility testing was carried out as previously described. Transfected cells were seeded with serial dilutions of lopinavir and harvested pseudovirions were used to infect fresh 293T cells. To determine strain infectivity, transfected cells were seeded in the absence of drug. Infectivity was monitored by measuring luciferase activity 48 h after infection. Results derived from at least two independent experiments (each in duplicate) were analysed. The IC50 was calculated using GraphPad Prism 5 software. Susceptibility was expressed as a fold change in IC50 compared with the subtype B reference strain (p8.9NSX+). Replicative capacity of these viruses was assessed by comparing the luciferase activity of recombinant virus with that of the WT subtype B control virus in the absence of drug. Equal amounts of input plasmid DNA were used, and it has previously been shown that percentage infectivity correlates well with infectivity/ng p24 in this system. The PI drugs used in this study were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

**Ethics**

Informed consent was obtained from all subjects and ethics approval for virological testing was obtained from the National Research Ethics Committee of Nigeria (NHREC/01/01/2007).

**Statistical analysis**

Differences in PI susceptibility were compared with the Wilcoxon rank-sum test (GraphPad Software Inc., La Jolla, CA, USA), which is robust to data that are not normally distributed.

**Results**

Six matched pairs of patients were included. Table 1 contains clinical and laboratory data on cases, who experienced virological failure (duration), and controls, who suppressed viral replication for 48 weeks. Of note, all pairs but one had a CD4 count <200 cells/mm3. All but one pair was treated with lopinavir-based ART (atazanavir was used in one pair). Table 2 shows NRTI and NNRTI resistance mutations detected prior to second-line initiation. All patients had lamivudine resistance [M184V/I in reverse transcriptase (RT)] and 7/12 (58.3%) had at least moderate resistance to tenofovir (3 with K65R, 3 with K70E and 1 with three thymidine analogue mutations including M41L, L210W and T215Y). All 12 individuals had high-level NNRTI resistance. Two pairs were infected with subtype G viruses and four pairs with CRF02 AG viruses (Table 2). No major mutations in protease were observed in the patients. We analysed sequences for mutations in Gag in cases and controls associated with PI susceptibility or exposure (Table 3).

The median (IQR) lopinavir fold change (FC) was 4.04 (2.49–7.89) for virological failures and 4.13 (3.14–8.17) for virological successes (P = 0.94), as described in Figure 1(a). The median (IQR) atazanavir FC was 2.43 (1.35–9.66) for virological failures and 4.39 (1.60–7.73) for successes (P = 0.47). The median (IQR) darunavir FC was 1.234 (0.84–2.05) for virological failures and 1.529 (1.14–2.319) for successes (P = 0.47).

One patient had an FC >10 for lopinavir at baseline and experienced subsequent virological failure on boosted lopinavir as the PI. We also measured the single-round replication efficiency of patient-derived gag-protease-containing pseudoviruses prior to initiation of second-line boosted PI treatment from patients who either did (success) or did not (failure) suppress viral replication after 48 weeks (Figure 1b). Mean replication efficiency relative to a subtype B reference strain was 117.7% for the successes and 105.8% for failures. There was no statistically significant difference in replication efficiency between the two groups (P = 0.93 by Mann–Whitney U-test).

Finally, we analysed the relationship between single-round replication efficiency and FC to lopinavir in all viruses tested. There was a moderate correlation between these parameters (correlation coefficient 0.32, Figure 2). When a single outlier was excluded from analysis (FC 10.7 with replication efficiency 50.0%), the correlation coefficient increased to 0.78.

**Discussion**

Given the contribution of the highly polymorphic Gag protein and resulting epistatic interactions to PI susceptibility, we hypothesized that patients would respond differently to these drugs, particularly...
in the context of extensive NRTI resistance. We previously reported an association between susceptibility to PI and outcome of first-line ritonavir-boosted lopinavir monotherapy in a clinical trial. Here we performed a similar study in patients about to start second-line combination ART, including ritonavir-boosted lopinavir or atazanavir as well as two NRTIs. We found the difference in phenotypic drug susceptibility (assessed by FC relative to a subtype B reference) was not statistically different between the virological failures (cases) and virological successes (controls) for any of the PIs tested: lopinavir, atazanavir or darunavir.

This negative result could be due to the influence of adherence, in that second-line therapy is used in patients for whom first-line therapy has failed, usually as the result of incomplete adherence.

Therefore, the patient group was enriched for poor adherers, which could have overcome the effects of small differences in susceptibility.

Interestingly, we previously showed that 2/2 patients with FC \( \leq 10 \) prior to PI monotherapy went on to virological failure.\(^{27}\) In this study the only patient with FC \( \leq 10 \) for lopinavir failed treatment with this drug. Further work needs to be undertaken to explore whether a threshold FC of 10 in our assay is relevant in larger datasets.

We also showed here that replication efficiency over a single round was correlated with lopinavir susceptibility prior to initiation of the bPI. We have previously reported similar findings in replication-competent subtype C viruses that contained patient-derived gag and partial protease genes.\(^{13}\) These data suggest that

<table>
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<th>Sample pair</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Baseline(^a) CD4 count (cells/mm(^3))</th>
<th>Second-line PI used</th>
<th>Baseline(^a) viral load (copies of HIV-1 RNA/mL)</th>
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LPV, lopinavir; ATV, atazanavir.
\(^a\)Baseline refers to pre-initiation of second-line therapy.

<table>
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<tr>
<th>NRTI mutations</th>
<th>NNRTI mutations</th>
<th>Baseline(^a) VL (copies of HIV-1 RNA/mL)</th>
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</table>

AZT, zidovudine; 3TC, lamivudine; FTC, emtricitabine; TDF, tenofovir disoproxil fumarate.
\(^a\)Baseline refers to pre-initiation of second-line therapy.

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increased replicative capacity and resistance to PI might involve an overlapping mechanism.

**Limitations**

Limitations of our study include the relatively small sample size, the inclusion of more than one subtype and the possibility of viral recombination through our PCR and cloning strategy. In addition, the process of mapping next-generation sequencing reads to a consensus reference sequence to generate a patient consensus can introduce biases against variation, which may affect the identification of novel drug resistance mutations. Finally, our assay system did not incorporate the native gp160 envelope.

Despite introduction of second-generation integrase inhibitors such as dolutegravir as first-line therapy in areas where pre-treatment resistance is >10%,28,29 bPI will still be used as second-line therapy for those who fail dolutegravir-based first-line...
Table 3. Variation in Gag cleavage and non-cleavage sites and protease regimens. Therefore, research into determinants of responses to PI in non-B subtypes is as important as ever.

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Transparency declarations
None to declare.

References
Gag-protease phenotypic drug susceptibility to PIs