Characterization of HIV Drug Resistance Mutations Among Patients Failing First-Line Antiretroviral Therapy From a Tertiary Referral Center in Lusaka, Zambia

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Abstract

In settings of resource constraint, an understanding of HIV drug resistance can guide antiretroviral therapy (ART) at switch to second-line therapy. To determine the prevalence of such HIV drug resistance mutations (HIV DRM), we used an in-house sequencing assay in the *pol* gene (protease and partial reverse transcriptase) in a cohort of patients suspected of failing a first-line regimen, which in Zambia comprises two nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI). Our analysis cohort (n=68) was referred to the University Teaching Hospital in Lusaka from November 2009 to October 2012. Median duration on first-line ART to suspected treatment failure was 3.2 years (IQR 1.7–4.7 years). The majority of patients (95%) harbored HIV-1 subtype C virus. Analysis of reverse transcriptase revealed M184V (88%), K103N/S (32%), and Y181C/I/V (41%) DRMs, with the latter conferring reduced susceptibility to the salvage therapy candidates etravirine and rilpivirine. Three patients (5%) had major protease inhibitor (PI) resistance mutations: all three had the V82A mutation, and one patient (Clade J virus) had a concurrent M46I, Q58E, and L76V DRM. HIV-1 genotyping revealed major and minor DRMs as well as high levels of polymorphisms in subtype C isolates from patients failing first-line antiretroviral therapy. Closer monitoring of DRM mutations at first-line failure can inform clinicians about future options for salvage therapy.

Keywords

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Introduction

The administration of combination antiretroviral therapy (ART) in patients with HIV is effective in suppressing viral replication (Egger et al., 2002), prolonging AIDS-free years of life (Berrey et al., 2001), preventing horizontal transmission (Cohen et al., 2011), and vertical transmission (Connor et al., 1994). Development of HIV-1 drug resistance mutations (HIV DRMs) and polymorphisms to the viral proteins protease and reverse transcriptase, however, can undermine the effectiveness of treatment programs, particularly in resource-limited settings where treatment options beyond a "second line" are limited (Gupta et al., 2012). Consequently, monitoring of clinical isolates for HIV DRMs is critical not only for the management of patients, but also for policy makers forecasting drug needs following initial treatment failure. The World Health Organization (WHO) recommends that resistance testing should be performed for public health drug resistance surveillance in resource-constrained settings (Bennett et al., 2008). In this study, the HIV *pol* region (protease and reverse transcriptase) was sequenced at the time of suspected first-line treatment failure in a cohort of patients referred to the University Teaching Hospital in Lusaka, Zambia. The prevalence of HIV *pol* DRMs and polymorphisms were determined using a locally developed in-house HIV DRM genotyping assay.

Materials and Methods

Patient Characteristics

We conducted a retrospective analysis of HIV-infected patients (>15 years old) suspected of treatment failure during first-line ART who were referred to the University Teaching Hospital's Advanced Treatment Centre. In Zambia, first-line ART regimens comprise two nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs)—among them tenofovir (TDF), zidovudine (ZDV), stavudine (d4T), abacavir (ABC), lamivudine (3TC), and emtricitabine (FTC) during the period of this analysis—along with a non-nucleoside reverse transcriptase inhibitor (NNRTI), either efavirenz (EFV) or nevirapine (NVP). Second line regimens included two NRTIs and a boosted protease inhibitor (Zambian Ministry of Health, 2010). HIV DRM testing was ordered at the discretion of the attending clinician, and the national guidelines specifies that a viral load >1,000 copies/ml after 6 months of therapy is considered virological failure and advises a change to a second line regimen (Zambian Ministry of Health, 2010). We included demographic information and medical history (including current treatment course) to characterize our population, including pharmacy refill-based medication possession ratio (MPR) as a measure of drug adherence (Chi et al., 2009; Kauf et al., 2012; Roth et al., 2012; Vinikoor et al., 2013). Ethics boards at the University of Zambia (Lusaka, Zambia), the University of Alabama at Birmingham (Birmingham, AL, USA), and the University of North Carolina in Chapel Hill (Chapel Hill, NC, USA) approved the use of programmatic data for outcomes analysis.

HIV-1 Viral Load Measurement and Genotyping

HIV-1 viral load was measured by the Roche Amplicor HIV-1 RNA Monitor kit (version 1.5; Roche Molecular Diagnostics, Pleasanton, CA). CD4+ lymphocyte counts were performed using a Beckman Coulter flow cytometer (Beckman Coulter, Inc., Fullerton, CA).

For genotype, viral RNA was extracted, amplified and sequenced using a modified in-house assay adapted from prior reports (Wallis et al., 2010). A 1,200 base pair (bp) amplification fragment was generated from patient virus isolated from 500 ml of blood plasma using the QIAamp viral RNA isolation kit (Qiagen Corporation, Venlo, The Netherlands, performed according to the manufacturer's protocol). Complementary DNA (cDNA) was first generated using a forward strand synthesis (FSS) primer: (CWR: 5′-GCATACTTYCCTGTTTTCAG-3′; HXB2 position 3594–3613) using the Superscript III Reverse Transcriptase (Invitrogen Corporation, Life Technologies, Inc., Carlsbad, CA). At the completion of the cDNA synthesis, the remaining RNA was treated with RNaseOUT (Invitrogen Corp., Life Technologies), and the synthesis mixture was purified using a GeneJet DNA (Thermo Scientific Corporation, Waltham, MA) purification kit. A nested PCR reaction was performed on the cDNA eluate using Platinum Taq DNA polymerase (Invitrogen Corporation, Life Technologies) according to previously published amplification methods (Seu et al., 2014): a 1,200 bp sequence was first amplified using forward primer (CWF: 5′-GAAGGACACCAAATGAAAGAYTG-3′; HXB2 nucleotide position 2,044–2,066) and reverse primer (LSR1: 5′-ACTGTTTTACATCATTAGTGTGGG -3′; HXB2 nucleotide position 3,651–3,628) and a second round PCR amplification using a forward primer (LSF1: 5′-TCAGAGCAGACCAGAGCCAACAGCCCCA-3′; HXB2 nt position 2,136–2,163) and reverse primer (Rev7: 5′-ATCCCTGGGTAAATCTGACTTGCCCA-3′; HXB2 position 3,370–3,345). Amplicons were directly analyzed via population-based Sanger Sequencing using BigDye Terminator chemistry and protocols recommended by the manufacturer (Applied Biosystems, Foster City, CA), and sequencing reaction products were analyzed with an ABI 3130XL genetic analyzer (Applied Biosystems). Bi-directional DNA strands were sequenced using overlapping fragments, and individual sequence fragments for each amplicon were assembled, inspected, and edited using Sequencher software version 5.0 (Gene Codes Corporation, Ann Arbor, MI)—capable of resolving down to 20% of minority variations at a nucleotide position. Fasta sequences of the concatenated amplicons were submitted to the Stanford Database (http://hivdb.stanford.edu/index.html) to generate an HIV-1 drug resistance report. The *pol* gene encompassing reverse transcriptase and protease was analyzed. HIV DRM and polymorphisms were analyzed according to both the drug resistance profile on the curated website as well as 2013 IAS–USA guidelines (Johnson et al., 2013).

HIV-1 Protease Phylogenetic Analysis

Protease sequences (amino acids 1–99) were aligned using the ClustalX multiple sequence alignment tool using the Clustal method and phylogenetic trees were generated by using the neighbor-joining method with the PHYLIP software package (version 3.52c; Joseph Felsenstein, University of Washington, Felsenstein, 1985). The SEQBOOT program was carried out to generate 100 data sets that represent randomly re-sampled versions of the input-aligned sequences, to test the reliability of the final tree topology. Evolutionary distances were estimated by the character-based maximum likelihood method DNAML using the transition/transversion ratio of 2, with global rearrangements and randomized input order of sequences. The results from the random datasets were then summarized by constructing a majority rule consensus tree with CONSENSE. The phylogenetic relationships were determined by the NJ-plot program (version 2.3; M. Gouy, University of

Lyon). Representative sequences from selected HIV-1 clades were obtained from Los Alamos National Laboratories (http://www.hiv.lanl.gov/content/index) in order to represent the 68 isolates within the global group M HIV-1 clades. Brackets were used to indicate the clustering of clades (A–J), and branch numbers to represent bootstrap values >75. The GenBank accession numbers of the 68 sequences of the pol region (nucleotide position 2,253–2,550 relative to HXB2) presented in this study are as follows: KM513662–KM513729.

Results

Patient Characteristics

Demographic and clinical characteristics of the patients are described in Table I. 68 HIV-positive patients on PI sparing first-line therapy (46% male, median age 34.6, interquartile range [IQR] 26.9–41.7) who had viral loads above 1,000 copies/ml had HIV DRM testing performed from November 2009 to October 2012. Among these patients, the median viral load was 29,050 copies/ml (IQR 8,652–79,680) and the median $CD4^+$ T cell counts were 144.5 (IQR 44.5–241.7) assessed at the time of the HIV DRM test. Adherence was generally poor, with only 52% shown to have an MPR >95% during the preceding 12 months.

Prevalence of NRTI and NNRTI Drug Resistance Mutations

Genetic analysis of the 5′ half of reverse transcriptase (RT), amino acids 1–265 (HXB2 nucleotide position 2,550–3,345), demonstrated prevalent NRTI and NNRTI DRMs (Johnson et al., 2013). Assessment of the overall prevalence of first line therapy patients with suspected treatment failure for at least one DRM (n = 67, 98%), one NRTI DRM (n = 61, 90%), or NNRTI DRM (n = 67, 98%) is shown (Fig. 1A). Within this cohort, a summary is provided for the prevalence of drug resistance mutations according to first line regimens prescribed: ZDV + 3TC + NNRTIs, D4T + 3 TC + NNRTIs, ABC + 3TC + NVP, and TDF + 3TC/FTC + NNRTIs (Table I). Notably, the prevalence of NNRTI DRM prevalence was high (at or near 100%) in all first line therapy in this population (Fig. 1B).

Prevalence of Drug Resistance Mutations to Salvage Therapy Options

As expected, the most common NRTI DRM was M184V (selected by 3TC and FTC) present in 60 patient isolates (88%), followed by K65R (selected by TDF, d4T, abacavir [ABC], didanosine [DDI]) (n = 21, 31%). A wide range of mutations conferring multi-NRTI resistance were also observed, including M41L (n = 7, 10%), A62V (n = 15, 22%), D67N (n = 10, 15%), K70R (n = 10, 15%), V75I (n = 2, 3%), F77L (n = 1, 1%), Y115F (n = 6, 9%), F116Y (n = 1, 1%), Q151M (n = 1, 1%), L210W (n = 3, 4%), T215Y/F (n = 7, 10%) and (n = 5, 7%), and K219Q/E (n = 4, 6%) and (n = 7, 10%). The mean number of thymidine analog mutations (TAMs) per patient currently prescribed thymidine analogs was 0.9 out of the 46 patients (range 0–4 TAMs per patient) (Fig. 2A). The most prevalent NNRTI mutations were Y181C/I/V (n = 24, 35%), (n = 3, 4%), and (n = 1 (1%), respectively, K103N/S (n = 21 (32%) and n = 1 [1%]), G190A/S/E (n = 20 (29%), n = 1 (1%), and (n = 2, 3%), respectively, V108I (n = 13, 19%), and V106A/M (n = 2, 3%) and (n = 10, 15%) (Fig. 2B). Assessment of NNRTI DRMs to rilpivirine (Fig. 2C) and etravirine (Fig. 2D) revealed the prevalence of several mutations conferring reduced susceptibility to these potential

options for salvage therapy. Of note, there was a high prevalence of Y181C/I/V (n = 28, 41% cumulatively) that confers reduced susceptibility to both rilpivirine (RPV) and etravirine (ETR), and Y188L (n = 5, 7%) which leads to reduced susceptibility to RPV.

Protease Inhibitor Drug Resistance Mutations

Despite a lack of prior exposure to protease inhibitors, three patients (5%) had one or more major PI DRM (full length protease 1–99 amino acids). V82A was detected in all three patients, with concurrent M46I, Q58E, and L76V in one of the three patients harboring the clade J HIV-1 virus. (Supplementary Fig. 1A) Genotypic assessment of prevalence of PI-specific DRM are also shown: lopinavir/r, indinavir/r, and darunavir/r (Supplementary Fig. 1B–D).

Phylogenetic Analysis

The majority of isolates (n = 65, 95%) were identified as HIV-1 subtype C during phylogenetic analysis of *pol* amino acid positions 1–99 of protease (HXB2 nucleotide [nt] position 2253–2550). Sequences representative of HIV-1 clades were obtained from Los Alamos National Laboratories (http://www.hiv.lanl.gov/content/index) and represent global group M HIV-1 subtypes. Brackets indicate the clustering of clades, and branch numbers represent bootstrap values >75. Non-clade C protease sequences were identified in three individuals and were HIV-1 subtypes A, B, and J (Fig. 3). Phylogenetic review showed two main branches of clade C virus among generated HIV sequences from patients. One main group (n = 24, 35%) clustered around sequences from viruses isolated in Zambia (ZM) and Malawi (MW); another group (n = 19, 28%) clustered around sequences from isolates from South Africa (ZA) Somalia (SO), Tanzania (TZ), and Botswana (BZ). Lastly, another group (n = 22, 32%) clustered around Clade C sequences from Israel (IL) and Georgia (GE). This phylogenetic analysis indicates the broad sequence diversity of the HIV protease gene from a sample population in Lusaka, Zambia.

Discussion

In this retrospective study, we sequenced the HIV-1 *pol* gene in ART patients suspected of first-line failure using an in-house genotyping assay in Zambia, thus providing critical HIV drug resistance mutation (HIV DRM) information. We observed a high prevalence of the Y181C/I/V (41%) and L100I (2%), an important consideration as these NNRTI DRMs confers reduced susceptibility to the third line salvage candidate etravirine. We performed a genetic analysis of HIV protease sequences that revealed the level of genetic diversity of HIV clades in Zambia, with sequences deposited to Genbank (accession IDs: KM513662–KM513729). Lastly, we performed all virological sequencing assays for HIV DRM analysis at the clinical diagnostic laboratory in the same urban township as the tertiary care referral center in Lusaka, Zambia.

Our study confirms preliminary reports that subtype C is the predominant clade in Zambia (Tang et al., 2002). Phylogenetic analysis of HIV-1 protease showed three main branches of clade C virus among generated HIV sequences from patients, from southern and eastern Sub-Saharan Africa as well as beyond. Some clades, such as clades C and D, are linked with

faster disease progression, and differing subtypes can have differences in their phenotypic drug susceptibilities (Harrigan et al., 2001; Pant Pai et al., 2012). The overall goal of the phylogenetic analysis was to capture the variability of circulating native HIV-1 genetic strains from this cohort of patients in Lusaka, Zambia. We see that despite the incomplete assessment, we already see a wide range of genetic variability of Clade C viruses that share sequence concordance with other regional Clade C viruses (Fig. 3). Our sequencing analysis reveals many naturally occurring polymorphisms in HIV-1 subtype C isolates from PI drug-naive individuals. Because of this, careful attention needs to be paid to these patients when switching to second-line therapy, such as ritonavir-boosted lopinavir-based ART. Such polymorphisms could lead to rapid treatment failure and development of drug-resistant HIV-1 mutants in individuals initiating lopinavir/ritonavir-based second-line regimens (Wainberg and Brenner 2012). The breadth of sequence diversity underscores the need for further HIV-1 genotyping studies, particularly in light of different drug susceptibilities to HIV-1 nucleotide polymorphisms at these loci.

Sequencing of RT revealed genotypes with multiple TAMs from patients with exposure to ZDV and d4T, reflecting the influence of these historical agents for first-line treatment in Zambia. The most prevalent NNRTI mutations were Y181C/I/V (41%), G190A/S/E (34%), K103N/S (32%), and Y188L (7%). These results are of particular relevance as Y181C/I/V and Y188L (and to a lesser extent, G190A/S/E) renders second-generation NNRTI drugs (e.g., ETR, RPV) ineffectual for potential salvage therapies (Johnson et al., 2013). Our study also included an assessment of minor DRMs as well as documented HIV-1 polymorphisms. Minor DRMs emerge later than major mutations and in isolation do not have a substantial effect on phenotype. Some may improve replication of viruses containing major mutations (Johnson et al., 2013). Furthermore, polymorphisms not conferring drug resistance were also seen, especially at RT codon positions 67, 69, 70, 74, 75, and 215, and protease positions 63 and 89, which may indicate viruses on the pathway toward additional mutations that could ultimately result in HIVDR if selected under drug pressure (Wainberg and Brenner, 2012).

A prior study of antiretroviral drug-näve HIV-1-infected Zambian adults (n=28) demonstrated a high frequency of pre-existing minor mutations in the protease gene— including I93L (92%), L89M (79%), and M36I (79%)—which corroborates results from our current study (Handema et al., 2003). Another Zambian study compared therapy-näve patients before (n = 30) and after (n = 66) the availability of free-of-charge ART, showing no statistically significant difference in major PI DRMs (V82I: 10% vs. 3%, $P = 0.20$), NNRTIs (K103N: 0% vs. 1%, $P = 1.0$), or NRTIs (M184V: 0% vs. 1%, $P = 1.0$). However, authors observed an increase in the number of minor, borderline, or partial resistance mutations in the later cohort (Gonzalez et al., 2010). These findings are in line with the data presented in our study. Preexisting minor mutations as well as naturally occurring HIV polymorphisms may have an impact on drug susceptibility; as such the monitoring of HIV genotypes in patients is warranted to guide the switching of ART regimens.

The major limitation of our study is its limited external validity. This population, referred to the University Teaching Hospital's Advance Treatment Centre, is a subset of those receiving care in Lusaka. Furthermore, viral load and HIV drug resistance testing were requested at the discretion of the clinical staff based on medical history; however, we did not collect

information about criteria used for each individual. Because of this fact, the cohort represented in this report may represent a group of patients who have severe complications in HIV treatment, which in turn may explain the high prevalence of those with 1 NRTI or 1 NNRTI DRM. This finding is consistent with a recent study in Thailand, South Africa, India, Malawi, and Tanzania showing that, of 148 patients with genotypic resistance testing after first-line antiretroviral therapy (ART) failure, 93% (n = 138) and 96% (n = 142) had at least one NRTI and NNRTI DRM, respectively. (Wallis et al., 2014) Additionally, only 16% in our cohort reported use of the TDF-based regimens that have been incorporated into first-line treatment for most Sub-Saharan African countries (World Health Organization, 2010). In fact, Zambia was one of the first in the region to roll out TDF-based ART (Chi et al., 2010). Given the time that elapsed between the implementation of TDF (2007) and our analysis cohort (2009–2012), the relatively low prevalence of its use in our study could suggest better patient outcomes with TDF-based ART. Large-scale studies of patient outcomes, ones that include virologic outcomes and HIV resistance testing, are needed to confirm this finding. Finally, we recognize that the small sample size may lead to imprecision to our estimates. A follow-up study of HIV DRM within a larger and better-defined group of first-line ART failing patients may necessary to confirm our findings here.

In conclusion, this study documents the HIV-1 drug resistance profile and genetic diversity in patients within the public health system in Zambia. Our major finding was the high frequency of NNRTI DRMs that may compromise the effectiveness of third-line salvage candidates such as etravirine and rilpivirine. Closer monitoring of DRM mutations at first-line failure can better inform clinicians and healthy policy advisors about what future ART regimens should comprise at a regional level. Ongoing surveillance for population antiretroviral drug resistance—using lower cost testing assays that can be performed in local settings—is urgently needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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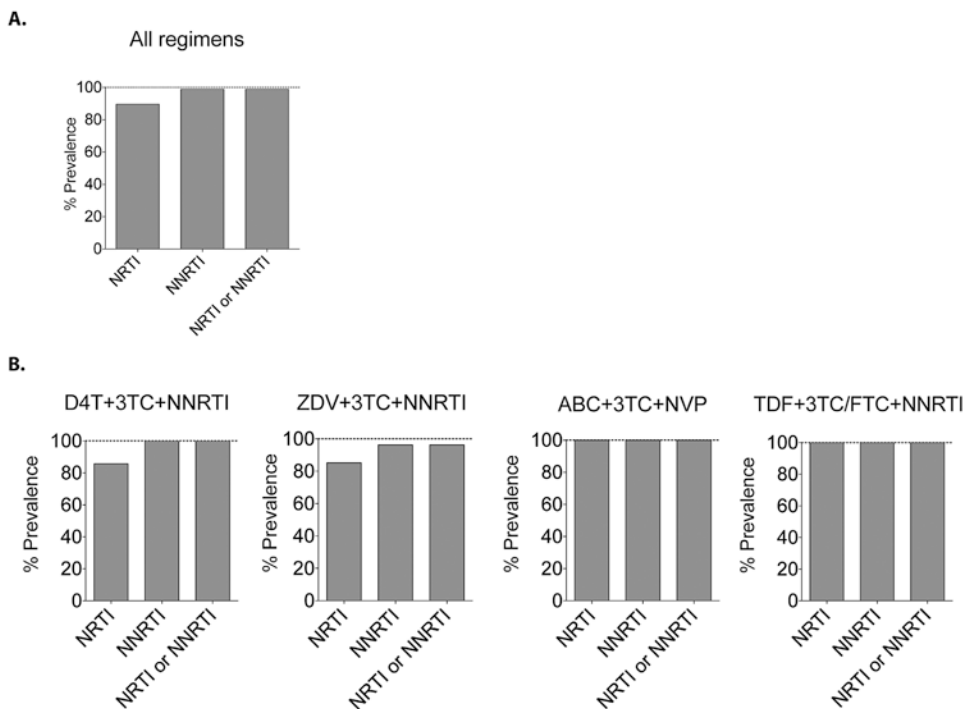Zambian Ministry of Health. Adult and adolescent antiretroviral therapy protocols. Lusaka: Printech Press; 2010.

Fig. 1.
Sequences were uploaded onto the Stanford Drug Database program (http://sierra2.stanford.edu/sierra/servlet/JSierra), and both Drug Resistance Mutations (DRM) and polymorphisms were analyzed according to both the drug resistance profile on the curated website as well as the 2013 IAS guidelines. (**A**) Assessment of the overall prevalence of first line therapy patients with suspected treatment failure. (**B**) Prevalence of drug resistance mutations according to first line regimens prescribed.
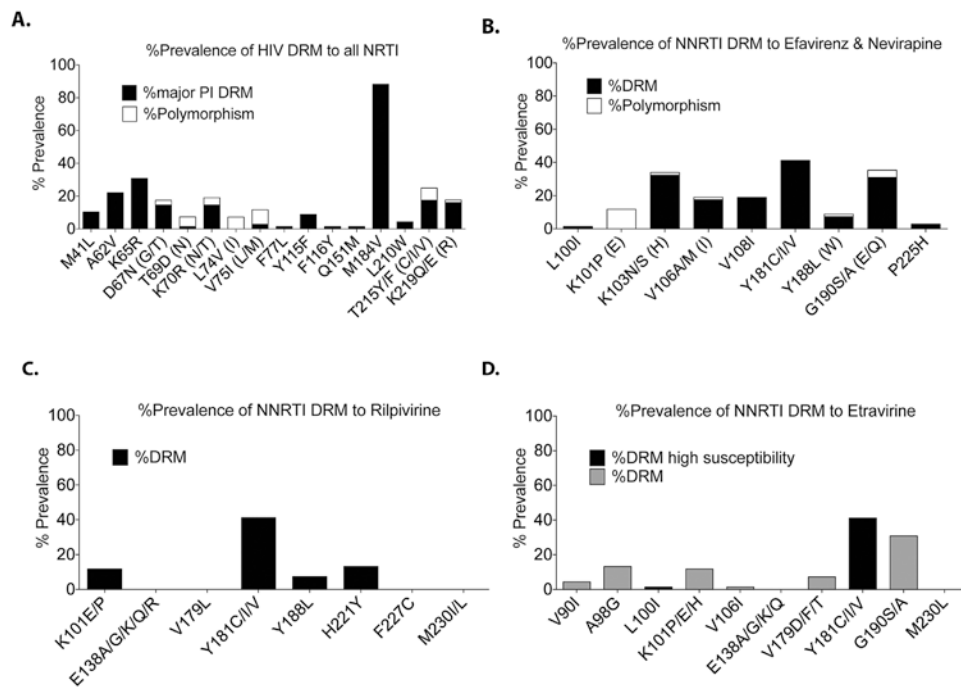
Fig. 2.
(**A**) All NRTI DRMs. (**B**) NNRTI-Efavirenz and Nevaripine DRMs. (**C**) NNRTI-Rilpivirine
DRMs. (**D**) NNRTIEtravirine DRMs. Alphanumerical symbols refer to the amino acid
position of the drug resistance mutation. The first letter represents the wild type residue,
followed by the amino acid position, followed by the major/minor drug resistance mutation.
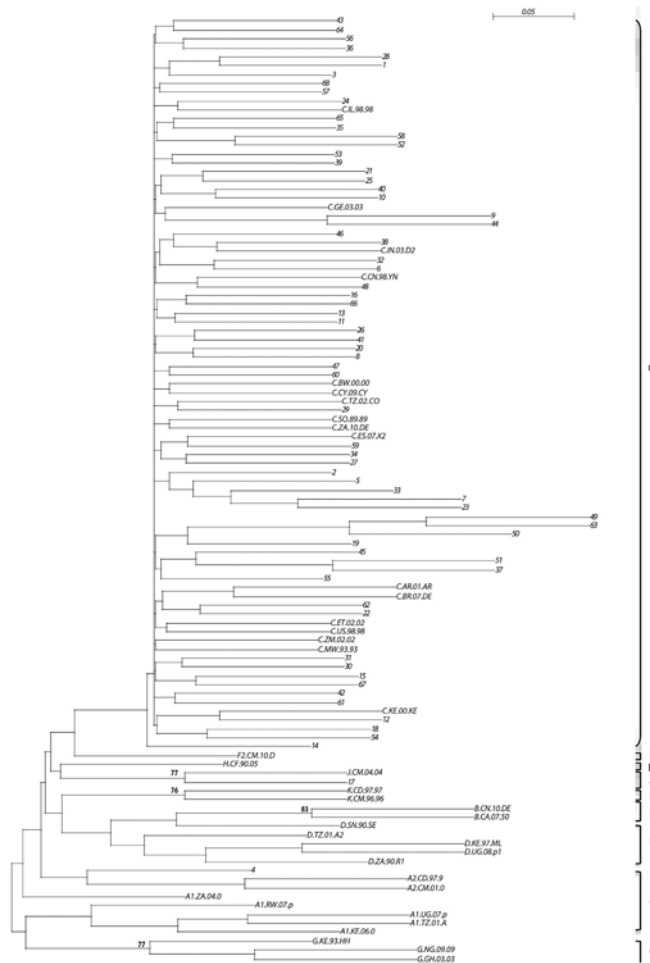In parentheses are polymorphisms at this genetic locus.

Fig. 3.
The protease sequences obtained were aligned using the ClustalX multiple sequence alignment tool using the Clustal method. Phylogenetic trees were generated by using the neighbor-joining method with the PHYLIP software package (version 3.52c; Joseph Felsenstein, University of Washington). Sequences representative of HIV-1 clades were obtained from Los Alamos National Laboratories (www.LANL.gov) andrepresent global group M HIV-1 subtypes. Brackets indicate the clustering of clades, and branch numbers represent bootstrap values >75. Subtype nomenclature are denoted as the following: A.BC.xx, where "A" represents the HIV clade, "BC" represents the two letter country abbreviation, and "xx" represents the first two numbers of the sequence accession numbers on LANL. Country codes are represented as Ghana (GH), Nigeria (NG), Kenya (KE), Tanzania (TZ), Uganda (UG), Rwanda (RW), South Africa (ZA), Cameroon (CM), Democratic Republic of Congo (CD), Senegal (SN), Botswana (BW), Zambia (ZM), Malawi (MW), Ethiopia (ET), Somalia (SO), Central African Republic (CF), China (CN), Canada (CA), United States of America (US), Brazil (BR), Argentina (AR), Spain (ES), Cyprus (CY), Georgia (GE), India (IN), and Israel (IL). Patients within the study cohort are denoted as numerical values from 1 to 68.

Table I

Clinical and Biological Characteristics of 68 Zambian Patients Screened for HIVDR at First Line Failure

Characteristic	N	Value
Age (years [IQR])	52	34.6 (26.9–41.7)
Male (No. [%])	68	31 (46%)
WHO stage (No. [%])	66	
I		9 (13%)
II		19 (28%)
III		31 (46%)
IV		7 (10%)
BMI (kg/m^2) median [IQR]	48	21.8 (19.3–24.6)
CD4 count (cells/mm^3) (Value [IQR])	60	144.5 (44.5–241.7)
Hemoglobin (g/dl) value [IQR]	60	10.9 (10.0–12.8)
Time on antiretroviral treatment (years [IQR])	68	3.2 (1.7–4.7 years)
Most recent antiretroviral therapy	68	
ZDV + 3TC + EFV		1 (1.5%)
ZDV + 3TC + NVP		26 (38%)
D4T + 3TC + EFV		3 (4%)
D4T + 3TC + NVP		18 (27%)
ABC + 3TC + NVP		1 (1.5%)
TDF + 3TC/FTC+ EFV		8 (12%)
TDF + 3TC/FTC + NVP		11 (16%)
Medication Possession Ratio >0.95 (value, %)		
Previous 3 months	67	46 (69%)
Previous 6 months	64	38 (59%)
Previous 12 months	62	32 (52%)
Viral Load at time of HIVDR testing-copies/ml (Interquartile Range)	68	29,050 (8,652–79,680)

AIDS, Acquired immunodeficiency syndrome; HAART, Highly Active Antiretroviral therapy; HIV, Human immunodeficiency virus; NNRTI, Non-Nucleoside/Nucleotide reverse transcriptase inhibitor; NRTI, Nucleoside/Nucleotide reverse transcriptase inhibitor; PI, Protease inhibitor. Antiretroviral therapy (ART) regimens taken during this course of follow-up are depicted on the graph as either combination (commas) or fixed dose (slash) drugs. 3-letter abbreviations for ARTs are as follows: Nucleoside reverse transcriptase inhibitors (NRTIs) [abacavir- **ABC**; emtricitabine- **FTC**; lamivudine- **3TC**; stavudine- **D4T**; tenofovir- **TDF**; zidovudine -**ZDV**], non-nucleoside reverse transcriptase inhibitors (NNRTIs) [efavirenz- **EFV**, nevirapine- **NVP**].