

Analysis of Drug Resistance in Children Receiving Antiretroviral Therapy for Treatment of HIV-1 Infection in Uganda

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Abstract

We analyzed drug resistance in HIV-infected Ugandan children who received antiretroviral therapy in a prospective, observational study (2004–2006); some children had prior single-dose nevirapine (sdNVP) exposure. Children received stavudine (d4T), lamivudine (3TC), and nevirapine (NVP); treatment was continued if they were clinically and immunologically stable. Samples with >1,000 copies/ml HIV RNA were analyzed by using the ViroSeq HIV Genotyping System (ViroSeq). Subtype A and D pretreatment samples also were analyzed with the LigAmp assay (for K103N, Y181C, and G190A). ViroSeq results were obtained for 74 pretreatment samples (35 from sdNVP-exposed children (median age, 19 months) and 39 from sdNVP-unexposed children (median age, 84 months). This included 39 subtype A, 22 subtype D, 1 subtype C, and 12 inter-subtype recombinant samples. One sample had nonnucleoside reverse transcriptase inhibitor (NNRTI) resistance, one had nucleoside reverse transcriptase inhibitor (NRTI) resistance, and three had protease inhibitor (PI) resistance. Y181C was detected by using LigAmp in five pretreatment samples [four (14.8%) of 37 samples from sdNVP-exposed children, one (4.2%) of 24 samples from children without prior sdNVP exposure; $p = 0.35$]. Among children who were not virally suppressed at 48 weeks of treatment, all 12 tested had NNRTI resistance, as well as resistance to 3TC and emtricitabine (FTC); three had resistance to other NRTIs. Seven of those children had a ViroSeq result at 96 weeks of treatment; four of the seven acquired resistance to additional NRTIs by 96 weeks. In Uganda, clinically and immunologically stable children receiving nonsuppressive antiretroviral treatment regimens are at risk for development of drug resistance.

Introduction

ACCESS TO HIGHLY ACTIVE antiretroviral therapy (HAART) for people living with HIV/AIDS in Africa has increased significantly over the last 5 years. However, implementation of antiretroviral treatment programs for children has lagged behind that for adults. More than 2 million children are estimated to be living with HIV infection, most (~90%) in sub-Saharan Africa.

In 2006, almost 800,000 children were considered eligible for HAART; however, only ~5% of those were receiving antiretroviral treatment.¹ Challenges of providing antiretroviral therapy for children in sub-Saharan Africa include the following: limited pediatric drug formulations and treatment options, inadequate numbers of well-trained staff, the

complexity of pediatric antiretroviral treatment, the cost and complexity of laboratory assays needed for diagnosis before 18 months of age, and the need for a caretaker to ensure adherence to the treatment regimen.²

Most cases of pediatric HIV infection result from mother-to-child transmission of HIV (MTCT). In sub-Saharan Africa, the most widely used antiretroviral drug regimen for prevention of MTCT single-dose nevirapine (sdNVP).³ NVP-resistant HIV variants are selected in many infants who are HIV-infected despite sdNVP prophylaxis.⁴ These variants usually fade from detection in infants over time, but are still detectable in some infants more than a year after sdNVP exposure.^{5,6} Prior sdNVP exposure can compromise subsequent antiretroviral therapy in children.⁷ In 2001, results from the PACTG 377 trial demonstrated that continuing treatment

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of HIV-infected children with an antiretroviral regimen after virologic failure is associated with accumulation of additional resistance mutations.⁸ In resource-limited settings, where children receiving HAART often do not have access to viral load monitoring, children may be maintained on non-suppressive regimens, increasing the likelihood that resistance will develop.

In this study, we analyzed antiretroviral drug resistance in children who received HAART in a prospective, observational study, "A study of the effectiveness of generic highly active antiretroviral therapy (HAART) for the treatment of HIV infected Ugandan children."^{9,10}

Methods

Source of samples

Samples were obtained from HIV-infected children who received HAART in the Makerere University–Johns Hopkins University (MU-JHU) Research Clinic, in Kampala, Uganda. Ninety-two children were enrolled in the study from 2004 to 2006; 44 of the children had been exposed to sdNVP for prevention of MTCT. None of the children had received HAART before study enrollment, and all of the children met the 2003 World Health Organization (WHO) criteria for antiretroviral therapy in resource-limited settings. The children received a combination of stavudine (d4T), lamivudine (3TC), and nevirapine (NVP). Children weighing <9 kg were initiated on syrup formulations of d4T, 3TC, and NVP; children weighing >9 kg received a fixed-dose formulation of the same three drugs (Triomune tablets, CIPLA, India).

Children were followed up for ≥ 48 weeks of treatment. Adherence counseling was provided to caregivers throughout the study; adherence was monitored by using pill counts and reports from caregivers. Clinical data were collected, and CD4 cell count and HIV viral load were measured before HAART initiation, and at 12, 24, 36, 48, 72, and 96 weeks of therapy. Plasma samples were stored for HIV-resistance testing. Therapy was considered to have failed if there was inadequate weight gain, increasing WHO clinical staging, or a >30% decline in CD4 count/percent >12 weeks after the start of therapy. Detection of HIV RNA did not lead to a change of therapy if the child was clinically stable and the CD4 cell count was not declining. In this substudy, children were categorized as virally suppressed if their viral load was <1,000 copies/ml throughout the study period, and they were categorized as nonsuppressed if they had >1,000 copies/ml at any study visit. This cut-off value was selected because the volume of plasma available for genotyping was low (0.25 ml per sample); with only 0.25 ml plasma for testing, a viral load of $\geq 1,000$ copies/ml is needed for amplification in the genotyping assay. Three children were changed to a different antiretroviral regimen because of adverse reactions, all within 2 weeks of starting therapy. Two of the three children had baseline samples and were included in the analysis of baseline resistance; however, these two children were not included in the analysis of resistance at 48 or 96 weeks and were excluded from the analysis of viral suppression. The majority of children had a significant decline in HIV RNA after HAART initiation, and the response to antiretroviral therapy was similar among children with and without prior sdNVP exposure.⁹

Laboratory methods

Methods used for analysis of HIV RNA, HIV DNA (for HIV diagnosis in infants), and CD4 cell counts were described in a previous report.¹¹ The ViroSeq HIV Genotyping System v2.7 (Celera Diagnostics, Alameda, CA) was used for HIV genotyping, by using 0.25 ml plasma samples with at least 1,000 copies/ml HIV RNA. Bidirectional sequence data were obtained for HIV reverse transcriptase (amino acids 41–335) for 88 (93.6%) of 94 samples with resistance test results (74 pretreatment samples and 20 follow-up samples). HIV subtypes were determined by phylogenetic analysis of *pol* region sequences.¹² Phylogenetic analysis confirmed that the sequences from each child were grouped together, with no evidence of sample mix-up or cross-contamination (not shown). For subtype A and D HIV variants, NVP resistance mutations (K103N=AAC, 0.5% assay cutoff; Y181C=TGT, 1% assay cutoff; G190A=GCA, 0.5% assay cutoff) were detected and quantified by using the LigAmp assay, as described.¹³

Statistical methods

Statistical analysis consisted of computation of medians and proportions. Owing to the small sample size, statistical significance for comparison of proportions and medians was tested by using Fisher's Exact test and the Wilcoxon test, respectively. All statistical tests were done by using the R Software,¹⁴ against a two-sided 0.05 alpha significance level.

Informed consent

Guidelines of the U.S. Department of Health and Human Services and the authors' institutions were followed in the conduct of this research. Informed consent was obtained from the mothers of children enrolled in the observational study; the study was approved by Makerere University Faculty of Medicine Research and Ethics Committee in Uganda.

GenBank submission

HIV sequences obtained in this study were submitted to GenBank [Accession numbers: HM037809–HM037882 (baseline), HM037883–HM037894 (48 weeks), and HM037895–HM037902 (96 weeks)].

Results

Analysis of pretreatment samples

Samples collected before HAART initiation (pretreatment samples) were available for 83 (90.2%) of the 92 children. HIV genotyping results were obtained by using the ViroSeq system for 74 (89.2%) of the 83 samples (HIV subtypes: 39 subtype A, 22 subtype D, one subtype C, and 12 inter-subtype recombinant). The 74 children with results included 35 children with a history of sdNVP exposure (median age, 19 months; range, 7–75 months) and 39 children without prior sdNVP exposure (median age, 84 months; range, 24–148 months). No significant differences were found in demographic or laboratory variables for children with *versus* without pretreatment ViroSeq test results (Table 1).

Antiretroviral drug resistance mutations were detected in pretreatment samples from two (2.7%) of the 74 children (Table 2); both children were 1 year old and had a history of prior sdNVP exposure. One child had resistance to nucleoside

TABLE 1. CHARACTERISTICS OF STUDY SUBJECTS WITH VERSUS WITHOUT A PRETREATMENT VIROSEQ TEST RESULT

	Total	No ViroSeq result	ViroSeq result obtained	p Value
No. of subjects	92	18 ^a	74	
Median age at baseline (mo)	51.5	47.0	51.5	0.66 ^b
Male gender (%)	53.9	58.9	52.8	0.78 ^c
Median CD4 cell count (cells/mm ³)	506.0	592.5	492.0	0.35 ^b
Median log ₁₀ viral load (copies/ml)	5.6	5.3	5.6	0.55 ^b
Prior sdNVP exposure (%)	47.8	50.0	47.3	1.00 ^c

^aNine children did not have a pretreatment sample; samples from nine children failed genotyping.
^bWilcoxon rank-sum test.
^cFisher's Exact test.

reverse transcriptase inhibitors (NRTIs), with possible resistance to zidovudine (ZDV) and stavudine (d4T), reflecting the presence of the T215A mutation; that child was virologically suppressed throughout the study period (followed up to week 48). The other child had resistance to nonnucleoside reverse transcriptase inhibitors (NNRTIs), with resistance to NVP and delavirdine (DLV), and possible resistance to efavirenz (EFV), reflecting the presence of the Y181C mutation; that child had >1,000 copies/ml of HIV RNA at the 12-, 24-, 36-, and 48-week study visits (followed up to week 48). HIV from all 74 children had polymorphisms in HIV protease that are associated with reduced susceptibility to protease inhibitors (PIs, e.g., M36I, K20R). However, only three samples had genotypes with predicted PI resistance. In all three cases, the

samples had possible resistance to tripranavir (TPV); all three samples had I13V, M36I, K45R, and H69K; one sample also had L10I, and two samples also had K20R.

For 51 of the 61 children with subtype A or D HIV infection, sufficient DNA remained after genotyping to permit analysis with a sensitive point-mutation assay, LigAmp. This assay was used to detect and quantify NVP resistance mutations K103N, Y181C, and G190A. None of the 51 samples had K103N or G190A detected. However, five (9.8%) of 51 samples had Y181C detected at low levels (Table 2). The proportion of children who had Y181C detected before HAART initiation was higher among children with prior sdNVP exposure [four (14.8%) of 27] than among children without sdNVP exposure [one (4.2%) of 24], but this difference was not statistically significant ($p = 0.35$). The four sdNVP-exposed children who had Y181C detected initiated HAART at 6, 12, 16, and 16 months of age; the one child without prior sdNVP exposure who had Y181C detected initiated HAART at 5 years, 4 months of age. Three (20%) of 15 children who were not virally suppressed on HAART had Y181C detected before treatment, compared with only two (5.6%) of 36 children who were virally suppressed on HAART ($p = 0.14$).

Analysis of samples from children on HAART

Fifteen (20.3%) of the 74 of the children who had a pretreatment ViroSeq result had detectable HIV RNA at week 48; 14 of those children had a 48-week sample, and results were obtained for 12 (85.7%) of the 14 samples (Table 3). All 12 samples had resistance to the NRTIs, 3TC and emtricitabine (FTC), reflecting the presence of the M184V mutation. Three samples had resistance to other NRTIs. One sample had resistance to tenofovir (TDF) with possible resistance to abacavir (ABC) and didanosine (ddI), reflecting the presence of the K65R mutation. One sample had possible resistance to ddI, possibly related to the D67G and T69N mutations. One sample had

TABLE 2. DETECTION OF NRTI AND NNRTI RESISTANCE BEFORE HAART INITIATION

Virally suppressed ^a	sdNVP exposed	No. tested	ViroSeq		LigAmp (subtype A and D only)	
			No. results	No. (%) with resistance	No. tested	No. (%) with resistance
Yes	No	29	25	0	16	1 ^d
	Yes	27	24	1 ^b	20	1 ^e
	Total	56	49	1 (2.0%)	36	2 (5.6%)
No	No	16	14	0	8	0
	Yes	11	11	1 ^c	7	3 ^{e,f,g}
	Total	27	25	1 (4.0%)	15	3 (20.0%)
Overall		83	74	2 (2.7%)	51	5 (9.8%)

Pretreatment samples were analyzed with the ViroSeq HIV Genotyping System for detection of mutations in HIV protease and reverse transcriptase. Fifty-one of the 61 samples with subtype A or D HIV were also tested with the LigAmp assay for detection of selected NVP-resistance mutations (K103N, Y181C, and G190A); 10 subtype A or D samples had insufficient DNA remaining after genotyping for analysis with the LigAmp assay. The table shows the number of samples tested with each assay (no. tested), the number of samples with results with ViroSeq (no. with results), and the number of samples with resistance mutations detected by using each assay (no. with resistance). For children who had one or more resistance mutations detected in the pretreatment sample, the age of the child, HIV subtype, mutation(s) detected, and level of mutation detected (for LigAmp) are detailed in the footnotes.

^aChildren were characterized as virally suppressed if their viral load was <1,000 copies/ml throughout the study period.

^bOne-year-old child with subtype D HIV; T215A detected by ViroSeq.

^cOne-year-old child with subtype A HIV; Y181C detected by ViroSeq and LigAmp (at 1.4%).

^dFive-year-old child with subtype D HIV; Y181C detected by LigAmp (at 2.6%).

^eThis child was 1.4 years old with subtype D HIV; Y181C detected by LigAmp (at 5.2%).

^fThis child was 1.4 years old with A/F recombinant HIV; Y181C detected by LigAmp (at 2.3%).

^gSix-month-old child with subtype D HIV; Y181C detected by LigAmp (at 3.9%).

TABLE 3. DETECTION OF NRTI AND NNRTI RESISTANCE AFTER 48 OR 96 WEEKS ON HAART IN CHILDREN WHO WERE NOT VIRALLY SUPPRESSED

Study subject	sdNVP	Visit	NRTI mutations	NRTI resistance	NNRTI mutations	NNRTI resistance
1		48	M184V+E44D+ T69N+V118I	3TC, FTC	K103N+V108I	DLV, NVP, EFV
2	Yes	48	M184V+T69N+K70R	3TC, FTC	Y181C	DLV, NVP, (EFV)
3	Yes	48	M184V	3TC, FTC	Y181C	DLV, NVP, (EFV)
4	Yes	48	M184V+T69N+V75I	3TC, FTC	V108I+Y181C	DLV, NVP, (EFV)
5	Yes	48	M184V	3TC, FTC	Y181C	DLV, NVP, (EFV)
6		48	M184V+K65R	3TC, FTC, <i>TDF, (ABC), (ddI)</i>	Y181C	DLV, NVP, (EFV)
		96	M184V+A62V	3TC, FTC	Y181C	DLV, NVP, (EFV)
7		48	M184V+V75I	3TC, FTC	G190A	NVP, (EFV)
		96	M184V+V75I+ T215F/I/S	3TC, FTC, (ZDV), (d4T), (ABC)	G190A	NVP, (EFV)
8		48	M184V	3TC, FTC	K103N+G190A	DLV, NVP, EFV
		96	M184V	3TC, FTC	K103S +G190A	DLV, NVP, EFV
9		48	M184V	3TC, FTC	A98G+Y181C+G190A	DLV, NVP, EFV
		96	M184V+D67N+ K70R+K219Q	3TC, FTC, (AZT), (d4T)	A98G+G190A	NVP, (EFV)
10		48	M184V+V75I	3TC, FTC	G190A	NVP, (EFV)
		96	M184V	3TC, FTC	G190A	NVP, (EFV)
11		48	M184V+D67G+T69N	3TC, FTC, (ddI)	Y181C	DLV, NVP, (EFV)
		96	M184V+M41L+D67G+ T69N+V118I+T215F	3TC, FTC, ddI, d4T, ZDV, (ABC)	Y181C	DLV, NVP, (EFV)
12	Yes	48	M184V+T215S/Y	3TC, FTC, (ZDV), (d4T), (ABC)	Y181C	DLV, NVP, (EFV)
		96	M184V+M41L+L210W+ T215Y	3TC, FTC, ZDV, d4T, ABC, (ddI), (TDF)	Y181C	DLV, NVP, (EFV)

Samples collected from children at 48- and 96-week study visits (48 and 96 weeks on antiretroviral therapy) were analyzed with the ViroSeq system. Five children had a history of single-dose nevirapine exposure (sdNVP, Yes). The table shows the mutations associated with resistance to nucleoside reverse transcriptase inhibitors (NRTIs) and nonnucleoside reverse transcriptase inhibitors (NNRTIs). Mutations shown in italics were detected only at 48 weeks; mutations shown in bold were detected only at 96 weeks. The ViroSeq interpretations of NRTI and NNRTI drug susceptibility are also shown for each sample. Parentheses indicate a prediction of possible resistance; italics indicate that resistance or possible resistance was detected at 48 weeks only; bold indicates that resistance or possible resistance was detected at 96 weeks only. Antiretroviral drugs are abbreviated as follows: 3TC, lamivudine; FTC, emtricitabine; ZDV, zidovudine; ddI, didanosine; d4T, stavudine; ABC, abacavir; TDF, tenofovir; DLV, delavirdine; EFV, efavirenz; NVP, nevirapine.

possible resistance to ZDV, d4T, and ABC, reflecting the presence of mutations at codon 215 (T251S/Y).

All 12 samples also had resistance to NNRTIs, including resistance to NVP ($n=12$), DLV ($n=10$), and (EFV, $n=2$; Table 3). The most common NNRTI mutation detected was Y181C, which was detected in eight (66.7%) of the 12 samples; one sample also had V108I. The eight samples with Y181C included the 48-week sample from the child who had Y181C detected by ViroSeq and LigAmp before treatment, and the 48-week sample from one of the four children who had Y181C detected by LigAmp only before treatment. The other five samples were from children who did not have Y181C detected before HAART initiation. Different patterns of NNRTI mutations were detected at 48 weeks in children with *versus* without prior sdNVP exposure (Table 3). All five samples from children with prior sdNVP exposure had Y181C; one sample also had V108I. In contrast, samples from seven children without prior sdNVP exposure had up to three NVP resistance mutations detected (mean, 1.6 mutations), four with G190A, three with Y181C, two with K103N, one with V108I, and one with A98G (Table 3). All 12 of the 48-week samples had at least one mutation associated with reduced PI susceptibility; however, only one had a genotype predictive of

PI resistance (possible resistance to TPV, reflecting the presence of L10V, in addition to M36I, K45R, and H69K); this child had the same protease genotype before treatment. Two other children with possible TPV resistance at baseline did not have a 48-week sample for analysis.

Seven of the 12 children had >1,000 c/ml HIV RNA at the 96-week visit, and had a 96-week sample available for analysis (one child died after the 48-week visit, and four children were followed up for 48 weeks only). In four of the seven samples, additional mutations were detected at 96 weeks, with new or increased resistance to NRTIs (Table 3). One child acquired new possible resistance to ZDV, d4T, and ABC; one acquired new resistance to ddI, d4T, and ZDV with new possible resistance to ABC; one acquired new possible resistance to ZDV and d4T; and one acquired new resistance to ZDV, d4T, and ABC, with new possible resistance to ddI and TDF. No major differences in NNRTI resistance were found between the 48- and 96-week visits, with one exception: in one child, the 48-week sample had evidence of resistance to all three NNRTIs (A98G, Y181C, and G190A were detected), but the 96-week sample only had evidence for resistance to NVP and possible resistance to EFV (A98G and G190A were detected without Y181C).

Four children were virally suppressed through week 48, but had >1,000 c/ml HIV RNA at 96 weeks; three of those children also had >1,000 c/ml HIV RNA at week 72. All four children had 96-week samples available for testing; however, three of those samples failed to amplify in the ViroSeq assay. In the remaining child, the 96-week sample had evidence of 3TC and FTC resistance (with M184V detected), as well as evidence of NVP resistance with possible EFV resistance (with G190A detected). Before HAART initiation, that child had no evidence of NRTI or NNRTI resistance.

Discussion

Several other studies have evaluated antiretroviral drug resistance in HIV-infected African children on HAART.^{15–18} Those studies include children on a variety of antiretroviral regimens (e.g., two NRTIs with either one PI or one NNRTI). Because of the differences in design among the studies, it is difficult to compare the risk of resistance in the different cohorts. In general, NRTI and NNRTI resistance were commonly detected among children with suboptimal viral suppression.

By using the ViroSeq system, we detected NVP resistance before treatment in only one of 74 children, 35 of whom had a history of prior sdNVP exposure. That child did not achieve viral suppression on the NVP-containing treatment regimen. With the LigAmp assay, we detected Y181C in five children, one of whom did not have a history of prior sdNVP exposure; we do not know if detection of Y181C in that child reflects an inaccurate NVP exposure history, or natural occurrence of Y181C as a minority variant in the viral population. Our validation studies of the LigAmp assay included comparison of LigAmp results with other methods for minority variants analysis, including phenotypic selection in a yeast system,¹⁹ allele-specific PCR (ASPCR),²⁰ and characterization of cloned variants. We have not detected false-positive results with LigAmp. In contrast to LigAmp, false-positive results are not uncommon with ASPCR. False-positive results in ASPCR usually reflect mismatches at binding sites in the control (nonmutant) reaction; this leads to an underestimation of wild-type HIV, with a consequent overestimation of mutant HIV. A major difference between LigAmp and ASPCR is that in LigAmp, mutant DNA is directly quantified in a fixed amount of amplified DNA. Mutant data are not normalized to wild-type data, as in ASPCR. In LigAmp, mismatches at oligonucleotide-binding sites can lead to underestimation of mutant DNA, but do not lead to false-positive results.²⁰ For these reasons, although we cannot rule out the possibility of a false-positive result, we believe that this is unlikely to be the explanation for mutation detection in these children. A trend was noted toward detection of Y181C before treatment in children who did not achieve viral suppression on HAART, but the association was not statistically significant.

In our cohort, all 12 of the children who had a viral load >1,000 copies/ml at 48 weeks and had a genotyping result had both NNRTI and NRTI resistance. Continuing treatment for an additional 48 weeks (to week 96) led to enhanced resistance to NRTIs, reflecting selection of HIV with thymidine analogue mutations. With this combination of NNRTI and NRTI resistance, children remain susceptible to only the protease inhibitors in the currently recommended second-line regimen for children (ABC+ddI+lopinavir/ritonavir). In this cohort, im-

munologic and virologic treatment responses were similar in sdNVP-exposed *versus* sdNVP-unexposed children.¹⁰ In this study, children who were not virally suppressed developed NNRTI resistance, regardless of prior sdNVP exposure.

These findings demonstrate that resistance emerges frequently in children receiving HAART who are not virally suppressed, even if they are clinically and immunologically stable. Use of viral load monitoring should be considered if feasible to reduce the emergence of multidrug resistance in children undergoing antiretroviral treatment. This is particularly important in resource-limited settings where the pediatric formulations of antiretroviral drugs are limited.

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Author Disclosure Statement

None of the authors has a commercial or other association that might pose a conflict of interest, with the following exception: Dr. Susan Eshleman is a co-inventor of the LigAmp assay, and Johns Hopkins University has filed a patent application with the U.S. Patent and Trademark Office. The inventors may receive royalty payments if the patent is awarded and licensed.

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