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Impact of Nevirapine (NVP) Plasma Concentration on Selection of Resistant Virus in Mothers Who Received Single-Dose NVP To Prevent Perinatal Human Immunodeficiency Virus Type 1 Transmission and Persistence of Resistant Virus in Their Infected Children▽

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Nonnucleoside reverse transcriptase inhibitor resistance following the use of single-dose nevirapine (sdNVP) for the prevention of mother-to-child transmission (PMTCT) remains a concern. In the ANRS-1201/1202 Ditrame study, conducted in Abidjan, Côte d'Ivoire, a short-course regimen of zidovudine was associated with sdNVP for PMTCT. In this study, we estimate the frequency of NVP resistance and its relationship with NVP concentration in mothers. Genotypic resistance analysis was performed on mothers' plasma samples at week 4 postpartum (PP) and on human immunodeficiency virus (HIV) DNA in peripheral blood mononuclear cells (PBMC) when an NVP resistance mutation was detected. The same tests were performed for the infected children at week 4, month 3, and month 12. Mothers' NVP plasma concentrations were measured at 48 h PP. Twenty-one (33%) of the 63 women selected had NVP-resistant (NVP-R) virus at week 4 PP. The median plasma NVP concentration was 598 ng/ml for the mothers without NVP-R virus compared to 851 ng/ml for the mothers harboring NVP-R virus ($P = 0.014$). NVP-R mutations were detected in the HIV DNA of 15/20 women. Plasma NVP-R mutations were detectable in 6 of 26 infected children at week 4. All 6 children had detectable NVP-R mutations in HIV DNA of PBMC. Blood samples taken at month 3 (1 child) and month 12 (1 child) revealed the persistence of NVP-R mutations in plasma and cells. Emergence of NVP-R virus in mothers is strongly correlated with a high level of plasma NVP concentration, owing to a prolonged postpartum period of viral replication under NVP selective pressure. The follow-up of the cohort demonstrates the prolonged archive of resistant virus.

Nevirapine (NVP) administered in a single dose (sdNVP) to mother and to baby, has become one of several antiretroviral regimens used for the prevention of mother-to-child transmission (PMTCT) of human immunodeficiency virus (HIV) in developing countries (21, 31). Several program reports of the use of sdNVP have documented transmission rates similar to those reported in clinical trials: between 8.7% and 22% (22). Concern remains about the selection of nonnucleoside reverse transcriptase inhibitor (NNRTI) resistance mutations following the use of sdNVP for PMTCT. Only one point mutation in the viral codon confers resistance, and the long half-life of NVP, which may be present up to 20 days following a single dose in mothers, contributes to this viral selection (5, 23). The emergence of NVP-resistant virus has now been widely reported, with detection by standard genotyping techniques ranging from 15 to 75% in mothers (6, 9, 10, 20). The risk of resistance is affected by the time of sampling, CD4 count, viral load, and viral subtype (13). The addition of sdNVP to zidovudine (ZDV) or ZDV-lamivudine short-course regimens may further reduce transmission to below 5% (7). This latter regimen has become the World Health Organization's recommended regimen for those women who do not require ongoing highly active antiretroviral therapy (HAART) (31). In Abidjan, Côte d'Ivoire, we have evaluated the combination of a short course (sc) of ZDV and sdNVP with a transmission rate of 6.5% (7). We are now reporting on the occurrence of resistant virus in plasma samples with this drug regimen, on the persistence of the resistant virus in the peripheral blood mononuclear cells (PBMCs), and on the link between plasma NVP concentration and selection of NVP resistance mutations.

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MATERIALS AND METHODS

Study population. The ANRS 1201/1202 Ditrame plus study was an open-label intervention cohort conducted in Abidjan, Côte d'Ivoire. HIV type 1 (HIV-1)-infected consenting women started oral ZDV 300 mg twice daily at ≥36 weeks

TABLE 1. Baseline characteristics of women (transmitting and nontransmitting mothers included or not in the resistance study) (ANRS DITRAME PLUS 1201/1202, Abidjan, Côte d'Ivoire)

Parameter	Result for women:			P^a	P^b
	Included in the resistance study		Not included in the resistance study (335 nontransmitting mothers)		
	21 transmitting mothers	42 nontransmitting mothers			
Median HIV-1 RNA VL (log_{10} copies/ml) (IQR)[c]	4.87 (4.57–5.19)	4.59 (4.04–4.9)	4.40 (3.68–4.74)	0.007	0.34
HIV RNA VL (log_{10} copies/ml) (n [%])					
3.53–4	0 (0.0)	10 (28.8)		0.014	
4–5	13 (61.9)	25 (59.5)			
5 and above	8 (38.1)	7 (16.7)			

[a] Transmitting mothers versus nontransmitting mothers included in the resistance study.
[b] Nontransmitting mothers included in the resistance study versus nontransmitting mothers not included.
[c] VL, viral load in plasma; IQR, interquartile range.

of gestation complemented by an oral dose of 600 mg ZDV and 200 mg NVP at the beginning of labor. Neonates received ZDV syrup (2 mg/kg of body weight/6 h) for 7 days and a single dose of NVP syrup (2 mg/kg) on day 2. Pediatric HIV infection was diagnosed by plasma HIV RNA viral load at 4 weeks and then confirmed at 6 weeks. A postpartum component to reduce postnatal HIV transmission was also proposed to every women enrolled in the peripartum program. Three hundred sixty-one women were included in the transmission analysis, and the 6-week transmission probability was 6.5% (95% confidence interval [CI], 3.9 to 9.1%). Detailed methods and results have been previously reported (7, 26).

NVP resistance analysis study subjects. All transmitting women with an available plasma sample ($n = 21$) were included in this substudy and compared with a sample of nontransmitting mothers ($n = 42$) (2 nontransmitting mothers per 1 transmitting woman). Among the 335 nontransmitting women, to be representative of the HIV viral load, we selected 14 women with a viral load of $\geq$3.53 log_{10} copies/ml and $\leq$4.2 log_{10} copies/ml, 14 women with a viral load of $\geq$4.21 log_{10} copies/ml and $\leq$4.68 log_{10} copies/ml, and 14 women with a viral load of $\geq$4.69 log_{10} copies/ml.

Women were excluded from this selection when the viral load, $<$3.53 log_{10} copies/ml at baseline, provided insufficient plasma HIV-1 RNA for genotyping ($n = 82$). Plasma samples were tested at 4 weeks postpartum. Additionally, for women who had NVP resistance (NVP-R) mutations at week 4, plasma samples were tested at delivery and cell samples were tested at week 4. For three women, plasma and cells samples were available at month 12.

HIV-1 was genotyped from all infected children ($n = 26$) diagnosed at 4 to 6 weeks of age. Additionally, for children who had NVP-R mutations at 4 weeks, cell samples were also tested at week 4. For 2 children, plasma and cell samples were available at month 3, and for one child, samples were available at month 12 as well.

HIV-1 viral load measurements. Plasma HIV-1 RNA levels were quantified on site using the ANRS-approved test based on a real-time reverse transcriptase PCR (RT-PCR) assay. The threshold of the technique is at 2.30 log_{10} copies/ml as previously described (27). The CeDReS lab in Abidjan participated in the ANRS quality assurance program throughout the study period.

HIV-1 genotypic resistance tests. The HIV-1 RT and protease genes were amplified from plasma HIV RNA and PBMC HIV DNA and then sequenced as previously described (3). Bidirectional sequences were obtained in the region of interest for all samples analyzed in this report. For quality control, NVP-R mutations present as amino acid mixtures were identified only if the corresponding nucleotide mixture was present in the sequences of both DNA strands. RT and protease-associated resistance mutations were identified according to the 2005 International AIDS Society definition list (www.iasusa.org) (18). HIV drug resistance was defined according to the 2005 ANRS HIV-1 genotypic resistance interpretation algorithm (www.hivfrenchresistance.org).

Determination and interpretation of NVP plasma concentrations. The time interval between the 200-mg sdNVP intake and sampling between day 1 and day 5 after delivery was recorded. NVP plasma concentrations ($C_{observed}$) were determined by a specific and validated high-performance liquid chromatography assay coupled with UV detection after liquid-liquid extraction with an inter and intraday variability of 10% (30). The NVP assay was validated over concentration ranges of 50 to 10,000 ng/ml. To compare the measured concentrations according to the time interval between dosing and sampling, a pharmacokinetic analysis was performed using a noncompartmental analysis according to a previously described method (23). The maternal NVP plasma concentrations were pooled to calculate a median elimination half-life ($t_{1/2}$) in the population using WinNonlin 1.1 software (Pharsight Corporation, Cary, NC). Considering the terminal elimination phase, the initial NVP plasma concentrations were extrapolated and normalized to 48 h ($C_{48\ h}$). The $C_{48\ h}$ was calculated for each patient with the following equation: $C_{48\ h} = C_{observed} \times \exp(-\beta \times [48\ h - \text{interval}])$, with the coefficient $\beta = \ln 2/t_{1/2}$, assumed to be identical in all patients.

Phylogenetic analysis. Phylogenetic analyses were performed by estimating the relationships among *pol* sequences and reference sequences of HIV-1 genetic subtypes and circulating recombinant forms obtained from the Los Alamos Database (http://hiv-web.lanl.gov). Nucleotide sequences were aligned with the CLUSTAL W program version 1.7 (28). Phylogenetic reconstruction was performed using a Kimura two-parameter model and the neighbor-joining method with 100 bootstrapped data sets (14).

Statistics. Group comparisons used Student's t test or nonparametric Mann-Whitney test for quantitative variables and chi-square test or Fisher's exact test for qualitative variables. All factors potentially associated with NVP-R mutations were studied in univariate and then in multivariate logistic regression analyses. Statistical analyses were processed with STATA software, version 8.0 (STATA, College Station, TX).

RESULTS

Characteristics of women at baseline. Overall, 63 women with available samples were included in this study. For the transmitting mothers, appropriate samples were available only from 21 of the 26 transmitting women. For the nontransmitting mothers, 42 were selected among the 335 nontransmitting women (Table 1). The median HIV RNA plasma viral load of the nontransmitting mothers included in the resistance analysis was not statistically different from those not included in the resistance study ($P = 0.34$). The median viral load of the transmitting mothers was significantly higher than among the nontransmitting mothers (4.87 versus 4.59 log_{10} copies/ml, $P = 0.007$).

NVP-R mutations in women at baseline, at week 4, and at 12 months postpartum. Four weeks after delivery, plasma NVP-R mutations were detected in 21 of 63 women (33.3%; 95% CI, 21.4 to 45.3%). No ZDV-associated mutation was observed. The most commonly selected mutation was K103N, and NVP resistance profiles are further described in Table 2. The frequency of NVP-R virus among women whose infants were or were not detected as infected at 4 weeks was not significantly different (7/21, 33.3% in transmitting mothers versus 14/42, 33.3% in nontransmitting mothers). Delivery samples were available for all women (except one) who had NVP-R mutations detected at week 4; all 20 samples lacked detectable NVP-R mutations. For 20 of 21 women who had plasma NVP-R mutations, frozen blood cells taken at 4 weeks post-

TABLE 2. Characteristics of mothers who had NVP-resistant virus at week 4 postdelivery (ANRS DITRAME PLUS 1201/1202, Abidjan, Côte d'Ivoire)

Identification no.	Group[a]	$CD4^+$ cell count at baseline/mm^3	HIV-1 plasma RNA at baseline (log_{10} copies/ml)	Resistance mutation at delivery[b]	NVP-R mutation(s) at 4 wk postpartum detected in:	
					Plasma	PBMC
1	T	85	5.3	NA	103N	67D/N, 103N
2	T	356	4.21	No mutation	103N	103N/K
3	T	107	4.81	No mutation	103N/K	103N/K
4	T	71	4.79	No mutation	103N/K, 106V/A	103N/K
5	T	328	5.16	179I	103N/K, 179V/I	103N/K, 179I
6	T	623	5.01	No mutation	103N/K, 106V/A	106V/A
7	T	306	5.55	No mutation	103N/K, 106V/A	103N/K, 106V/A
8	NT	307	4.99	No mutation	103N/K, 106A/V, 188Y/C, 190G/A	No mutation
9	NT	333	4.61	No mutation	103N/K, 181Y/C	181Y/C
10	NT	245	4.93	No mutation	103N/K	No mutation
11	NT	238	4.65	No mutation	103N/K, 106A/V, 188Y/C, 190G/A	106A
12	NT	336	5	No mutation	103N/K	No mutation
13	NT	150	5.23	No mutation	103N/K, 106A/V	103N/K
14	NT	111	5.4	No mutation	103N/K	103N/K
15	NT	244	4.95	No mutation	103N/K	No mutation
16	NT	968	3.86	No mutation	103N/K	103N
17	NT	329	4.66	No mutation	106V/A	106V/A
18	NT	118	5.54	No mutation	103N/K	NA
19	NT	270	4.61	No mutation	103N/K	No mutation
20	NT	614	3.71	No mutation	103N/K, 181Y/C	181Y/C
21	NT	224	4.2	No mutation	103N/K	103N/K

[a] T, transmitting mothers; NT, nontransmitting mothers.
[b] NA, not available.

partum could be tested. DNA NVP-R mutations were detected in the PBMC DNA from 15 of 20 women (75%). For three women (one transmitter, two nontransmitters) who had NVP-R mutations on plasma samples taken at week 4, plasma and cell samples were tested at month 12. All three lacked detectable NVP-R mutations in plasma and in HIV DNA PBMCs.

NVP-R mutations in HIV-1-infected infants. Plasma samples collected at day 2 and at 4 weeks after birth were available for all 26 infected children. NVP-R mutations were detected in 6 of the 26 (23%; 95% CI, 8.9 to 43.6%) children at 4 weeks (Table 3). Among these six infected children, four were diagnosed at day 2 (in utero infection), while two were negative at day 2 and then HIV-1 positive at week 4 after birth (intrapartum infection). The six children also had frozen PBMC samples available at 4 weeks, and all of them had NVP-R mutations archived in the HIV DNA from PBMCs. The most commonly detected NVP-R mutation was K103N.

Comparison of NVP resistance pattern observed in HIV-1-infected infants and their mothers. Different patterns of NVP-R mutations were detected at week 4 in women and in infants (Table 3). Two infants had NVP-R mutations at 4 weeks of age, while their mothers had no detectable mutations at that time; both of these 2 children had in utero HIV-1 infection. In two instances where both mother and infant had NVP-R mutations at week 4, the pattern of NVP-R mutations was different (one in utero-infected child and one intrapartum-infected child). In two other cases, the pattern of NVP-R mutations was similar, one child was infected in utero and one children was infected intrapartum.

Persistence of NVP-R mutations in children. We found a persistence of NVP-R viruses in the plasma and in the PBMC for the child with a sample available at 3 months of age and for the child with a sample available at 12 months of age. Child A was infected intrapartum, and the pattern of mutations

TABLE 3. Characteristics of neonates who had NVP-resistant virus at week 4 and at month 3 after birth and characteristics of their mothers at 4 weeks postpartum (ANRS DITRAME PLUS 1201/1202, Abidjan, Côte d'Ivoire)

Identification	Timing of infection	Mother's plasma NVP-R mutation(s) at 4 wk postpartum	Neonate's NVP-R mutation(s) at:			
			4 wk of life detected in:		3 mo of life detected in:	
			Plasma	PBMC	Plasma	PBMC
A	Intrapartum	103N	103N	103N	103N	103N
B	In utero	103N	103N/K, 190A/G	103N/K, 106V/A, 190A/G	103N/K, 190A/G	103N/K, 190A/G
C	Intrapartum	103N/K	106A	106A	NA[a]	NA
D	In utero	103N/K, 106V/A	103N/K, 106V/A	103N/K, 106V/A	NA	NA
E	In utero	No mutation	190A/G	190A/G	NA	NA
F	In utero	No mutation	103N/K	103N/K	NA	NA

[a] NA, not available.

(K103N) was similar to the pattern described for the mother's sample. For this child, the mutation K103N persisted in the plasma and in the blood cells until 12 months of age. For child B, infected in utero, mutations K103N and G190A were detected in the plasma samples at week 4 and in the plasma and blood cells at month 3. An additional mutation V106A was detected in the blood cells at week 4. The mother's plasma sample had a detectable 103N mutation.

Phylogenetic analysis. Phylogenetic analysis of the 63 HIV-1 strains revealed 79% CRF02, 13% subtype A, and 8% subtype CRF06. NVP-R mutations were more common in women with subtype CRF02 than in women infected with subtype A.

Pharmacokinetic analysis. A single determination was available for each of the 61 women, for 8 women at day 1, for 13 women at day 2, for 25 women at day 3, for 9 women at day 4, and for 6 women at day 5. Using a median calculated NVP plasma half-life of 69.9 h, the overall median plasma NVP concentration at day 2 postpartum was 684 ng/ml (range, 417 to 954). An important interpatient variability was found. The median NVP concentration was significantly higher for the 20 women who developed NVP-R mutations (851 ng/ml [range, 633 to 1,063 ng/ml]) than the 598 ng/ml (range, 315 to 885 ng/ml) for the 41 women without selection of NVP-R mutations ($P = 0.014$).

Predictive factors associated with NVP-R mutations. In univariate analysis, two factors were associated with the identification of NVP-R mutations: the maternal plasma viral load and the plasmatic NVP concentration. Indeed, the risk for presenting NVP-R mutations was three times higher for each 1 log increase of plasma viral load (odds ratio [OR], 3.1; 95% CI, 1.00–13.28; $P = 0.02$) and for each 100-ng/ml increase of plasmatic NVP concentration (OR, 1.24; 95% CI, 1.05–1.50; $P = 0.01$). The total lymphocyte count was not associated with NVP-R mutations (81% of those with NVP-R mutations had a CD4 count of $<350/mm^3$ versus 57% without NVP-R mutation, $P = 0.06$). In the final multivariate model, these two factors remained associated with the selection of NVP-R mutations: adjusted OR of 3.4 (95% CI, 1.0–12.1; $P = 0.05$) for the plasma viral load at inclusion and adjusted OR of 1.3 (95% CI, 1.1–1.6; $P = 0.006$) for the plasmatic NVP concentration at day 2.

DISCUSSION

The prophylactic regimen of the Ditrame Plus ANRS 1201/02 study conducted a 6-week probability of transmission evaluated at 6.4% (7). In this substudy, we report an overall frequency of NVP resistance mutations of 33.3% in the mothers' plasma samples at 4 weeks postdelivery. In a previous study conducted in Uganda, the frequency of NVP resistance mutations among women receiving sdNVP was 19% (13). In the NVAZ studies, NVP resistance had developed in 69% of mothers at 6 to 8 weeks postpartum (11). Comparable observations have been reported in Thailand when scZDV preceded sdNVP, with frequencies of resistant virus of 32% (19, 20). NVP-resistant virus can also be detected following an sdNVP intake among pregnant women upon combination AIDS-associated retrovirus therapy with incomplete viral suppression. In the PACTG 316 trial, all women received at least scZDV, and 76% received combination therapy in addition to sdNVP. NVP-R mutations were observed in 15% of mothers at week 6 postpartum (6). High baseline plasma viral load and low baseline $CD4^+$ cell count were reported to be associated with the development of NVP resistance (13).

In addition, we present pharmacological results that described a large range of NVP concentrations 2 days after delivery, suggesting a wide interpatient variability for the concentration of NVP in women at delivery, probably related to physiological modifications occurring during pregnancy and labor influencing drug bioavailability, metabolic pathways, and clearance. We also described a significant association between a high level of NVP concentration at 48 h and selection of NVP resistance mutations. Our results demonstrate that, considering the long half-life of nevirapine very close to the previously published median $t_{1/2}$ of 61.3 h (23, 24), a higher concentration was more likely to induce a prolonged viral replication under suboptimal drug selective pressure, therefore increasing the probability of emergence of resistant strains. A limitation of our study is the absence of the maternal plasma viral load at day 2, and we cannot correlate the selection of nevirapine-resistant virus with the level of the viral load.

Resistance mutations to NVP are also a concern for infants infected despite AIDS-associated retrovirus prophylaxis. We report a rate of NVP-R mutations of 23% for the infected infants. In two recent clinical trials conducted in Malawi, in infants 6 to 8 weeks of age infected with subtype C virus, NVP resistance mutations were detected in 64% of cases (12). In this latter study, the frequency of the development of NVP resistance was moderate (27%) and very similar to our results (23%) when infants had received sdNVP plus ZDV and maternal sdNVP was avoided than when infants had received sdNVP alone and mothers had received sdNVP (87%) ($P < 0.001$) (12). This latter estimate is substantially higher than the percentage of 46% reported for Ugandan infants who were infected with NVP-resistant HIV-1 in HIVNET 012 (mostly subtype A and D).

Considering the impact of HIV-1 subtype, most of the viral strains of this study are mainly CRF02, as it was previously reported in Ivory Coast (29). In the HIVNET 012 trial, the probability of having NVP resistance was significantly higher in women infected with HIV-1 subtype D than in women with subtype A (36% versus 19%, $P = 0.0035$) (9, 13). Eshleman et al. have recently reported a significantly higher probability of having NVP resistance 6 to 8 weeks after the administration of sdNVP for women infected with subtype C than for women with subtypes A or D (11). In our study, NVP-R mutations were more common in women infected with CRF02 than in women infected with subtype A. The high probability of having nevirapine resistance observed in women with CRF02 strains is of concern, as CRF02 is the most common subtype found in women living in West Africa (29).

Recent studies have used more sensitive resistance assays to detect specific NVP-R mutations in women after administration of sdNVP. Preliminary reports on the use of those assays show that NVP-R mutations can be detected in many women who did not have NVP resistance mutations when population-based sequencing assays were used (15, 17, 25). In addition, in our study, all six children and 75% of the women who developed NVP resistance mutations in plasma had detectable mutations in their PBMCs as well, and the underlying question is

clearly the impact on the response to subsequent HAART initiation. Jourdain et al. found in Thailand that among HIV-infected women who initiated an NVP-based HAART regimen after delivery, exposure to short-course ZDV and sdNVP was associated with a significant decrease in the rate of virological suppression at 6 months (19). Moreover, in our study, resistance mutations persisted in a drug-free environment in circulating and in intracellular HIV strains for the 2 children with HIV RNA and HIV DNA detected in samples taken at month 3 and month 12. This result suggests that HIV-1-resistant strains selected early in infected children massively fuel the cellular reservoir and persist for lengthy periods of time, as was previously described for adults at the time of primary infection (1, 2, 16). In contrast, for the 3 mothers with samples available at month 12, resistant HIV-1 strains selected by PMTCT prophylaxis would not be detectable over time, being overwhelmed by fitter wild-type strains, as seen during chronic disease in patients mainly infected with wild-type virus and who discontinue a failing regimen (8). The persistence of minor variants or proviruses with NVP-R mutations in mother cells could potentially limit the use of NVP or other NNRTIs for subsequent treatment of HIV-1 infection or prophylaxis for PMTCT.

The long-term relevance of the selection of NNRTI resistance after PMTCT regimens is not fully understood, but avoidance of the selection of resistant virus remains an important objective. Recent research advances in PMTCT of HIV-1 have shown ways to reduce the risk of transmission to below 5%, and a low rate of 1.1% of NVP-R virus could be obtained using ZDV plus lamivudine at 3 days postdelivery (4). The challenge for developing countries would be now to implement on a wide scale this kind of PMTCT strategy and to provide unrestricted access to HAART for women who require ongoing treatment, as recently recommended by the WHO (31).

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