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Emergence and persistence of nevirapine (NVP) resistance in breast milk after single-dose NVP administration

Sarah E. Hudelson[1], **Michelle S. McConnell**[2], **Danstan Bagenda**[3,4], **Estelle Piwowar-Manning**[1], **Teresa L. Parsons**[5], **Monica L. Nolan**[6], **Paul M. Bakaki**[7], **Michael C. Thigpen**[2], **Michael Mubiru**[4], **Mary Glenn Fowler**[1], and **Susan H. Eshleman**[1]

[1]Dept. of Pathology, Johns Hopkins Univ. School of Medicine, Baltimore, MD [2]Epidemiology Branch, Division of HIV/AIDS Prevention, Centers for Disease Control and Prevention, Atlanta, GA [3]Makerere Univ, School of Public Health, Uganda [4]Makerere Univ. - Johns Hopkins Univ. Research Collaboration, Kampala, Uganda [5]Dept. of Clinical Pharmacology, Johns Hopkins Univ. School of Medicine, Baltimore, MD [6]Dept. of Strategic Information, mothers2mothers, Cape Town, South Africa [7]Dept. of Epidemiology and Biostatistics, Case Western Reserve Univ., Cleveland, OH

Abstract

OBJECTIVE—Single-dose nevirapine (sdNVP) can reduce the risk of HIV vertical transmission. We assessed risk factors for NVP resistance in plasma and breast milk from sdNVP-exposed Ugandan women.

METHODS—Samples were analyzed using the Roche AMPLICOR HIV-1 Monitor Test Kit, v1.5, and the ViroSeq HIV-1 Genotyping System. NVP concentrations were determined by liquid chromatography with tandem mass spectroscopy.

RESULTS—HIV genotypes (plasma and breast milk) were obtained for 30 women 4 weeks after sdNVP (HIV subtypes: 15A, 1C, 12D, 2 recombinant). NVP resistance was detected in 12 (40%) of 30 breast milk samples. There was a non-significant trend between detection of NVP resistance in breast milk and plasma (p=0.06). There was no association of HIV resistance in breast milk with median maternal pre-NVP viral load or CD4 cell count, median breast milk viral load at 4 weeks, breast milk sodium >10 mmol/L, HIV subtype, or concentration of NVP in breast milk or plasma.

CONCLUSIONS—NVP resistance was frequently detected in breast milk 4 weeks after sdNVP exposure. In this study, we were unable to identify specific factors associated with breast milk NVP resistance.

Address correspondence and reprint requests to: Susan H. Eshleman, MD/PhD, Professor, Pathology, Director, HIV Genotyping Laboratory, The Johns Hopkins Medical Institutions, Ross Building 646, 720 Rutland Ave., Baltimore, MD 21205, (410) 614-4734 (phone) - (410) 502-9244 (fax) - seshlem@jhmi.edu.

Keywords

nevirapine; HIV-1; breast milk; Uganda; vertical transmission; nevirapine resistance

INTRODUCTION

Many HIV-infected women have detectable nevirapine (NVP) resistance after receiving single-dose nevirapine (sdNVP) for prevention of mother-to-infant HIV transmission (MTCT) [1]. Emergence of NVP resistance in maternal plasma after sdNVP is associated with high maternal pre-NVP plasma viral load, low maternal pre-NVP CD4 cell count, HIV subtype (C>D>A), and pharmacokinetic factors associated with NVP exposure (e.g. decreased oral clearance) [1].

NVP is transferred to breast milk after sdNVP exposure [2–4], and transmission of NVP-resistant HIV to infants by breast-feeding has been documented [5,6]. In Zimbabwean women with subtype C HIV infection, NVP resistance was detected in breast milk from 13 (65%) of 20 women 8 weeks after sdNVP administration [7,8]; resistance test results could not be obtained for 12 other women, most of whom had undetectable breast milk HIV RNA. Factors associated with emergence and persistence of NVP resistance in breast milk have not been described.

In Uganda, most HIV infections are caused by subtypes A and D. The risk of developing NVP resistance in plasma HIV after sdNVP is lower in women infected with subtype A and D HIV, than in women infected with subtype C HIV [9]. We analyzed NVP resistance in breast milk from Ugandan women following sdNVP administration.

MATERIALS AND METHODS

Source of samples

Breast milk and plasma samples were obtained from HIV-infected women enrolled in an observational study in Kampala, Uganda (“Pathophysiology of Breast Milk”, 2003–2004). Women received sdNVP according to national treatment guidelines; none of the women received any other antiretroviral drugs, consistent with the standard of care in Uganda at the time of the study. We tested samples from 51 women; this included all available samples from women whose infants were HIV-infected by 6 weeks of age (n=17), and twice as many samples from women whose infants were HIV-uninfected at 6 weeks of age (n=34).

Preparation of breast milk supernatants

Breast milk was stored at room temperature and processed within 30 hours of collection. Breast milk supernatants were prepared as follows: breast milk was centrifuged at 300 g for 15 minutes, the upper lipid layer was discarded, and the supernatant was removed from the cell pellet and transferred to a separate tube. This process was repeated 3–5 times to ensure that removal of lipids was complete. Supernatant samples were stored at −70°C.

HIV viral load and CD4 cell count determination

Plasma HIV viral loads were measured using the Roche AMPLICOR Monitor test kit v1.5 (Branchburg, NJ, lower limit of detection: 400 copies/ml). HIV RNA was extracted from breast milk supernatant using the Boom method [10], and was measured using the Roche AMPLICOR Ultrasensitive Monitor test kit (lower limit of detection: 50 copies/ml) [10]. CD4 cell counts were measured using a FACS Calibur flow cytometer.

HIV genotyping and subtyping

HIV genotyping was performed using the ViroSeq™ HIV-1 Genotyping System v2.7 (Celera, Alameda, CA); a nested PCR procedure was used to amplify breast milk samples with <500 copies/ml HIV RNA [11,12]. HIV subtypes were determined by phylogenetic analysis of *pol* region, as described [13].

Analysis of NVP

Plasma and breast milk NVP concentrations were determined using a liquid chromatography tandem mass spectroscopy assay. The lower limit of quantification was 10 ng/ml.

Statistical methods

Statistical analyses consisted of computation of medians and proportions. Owing to the small sample size, statistical significance for comparison of proportions and medians was tested using Fisher's Exact test and the Wilcoxon test, respectively. All statistical tests were done using the R Software [14], against a two-sided 0.05 alpha significance level. For statistical analysis, plasma viral loads <400 copies/ml were assigned a value of 200 copies/ml, plasma viral loads >750,000 were assigned a value of 750,000 copies/ml, and breast milk viral loads <50 copies/ml were assigned a value of 25 copies/ml.

Ethical considerations

Written informed consent was obtained from all women for participation in the Pathophysiology of Breast Feeding study. The study was approved by the National AIDS Research Committee and the Uganda National Center for Science and Technology in Uganda, by the Western Institutional Review Board for Johns Hopkins University, and by the U.S. Centers for Disease Control and Prevention in Atlanta, GA.

GenBank accession numbers

The GenBank accession numbers for 30 breast milk and 30 plasma HIV sequences are: GU059279-GU059338.

RESULTS

We analyzed breast milk samples collected from 51 Ugandan women 4 weeks after sdNVP administration. Genotyping results were obtained for all 10 breast milk samples with viral loads > 500 copies/ml using standard genotyping procedures (median: 1,208, range 503–8,509 copies/ml), and for 21 (51.2%) of 41 samples with viral loads <500 copies/ml using a nested PCR procedure (median: 25, range 25–200 copies/ml). Overall results were obtained for 31 (60.8%) of the 51 breast milk samples. HIV genotyping results were also obtained for plasma from these 31 women. Results from one woman were excluded because of a possible sample mix-up. The final data set used for analysis included results from 30 paired maternal plasma and breast milk samples (HIV subtypes: 15A, 1C, 12D, 2 intersubtype recombinant).

Twelve (40%) of 30 breast milk samples had at least one NVP resistance mutation; three of those samples had more than one mutation (Table 1). The most common mutations detected were K103N and Y181C. NVP resistance in breast milk was not associated with HIV transmission; NVP resistance was identified in breast milk from 4/11 (36.4%) transmitters vs. 8/19 (42.1%) non-transmitters (p=1.0). Among breast milk samples with NVP resistance, the median number of mutations detected was higher for the 11 women who transmitted HIV to their infants (2.0) than for the 19 women whose infants were HIV-uninfected (1.0); however, this difference was not statistically significant (p=0.18).

Among the 30 women, five had NVP resistance mutations detected in plasma only, four had NVP resistance mutations detected in breast milk only, eight had NVP resistance mutations detected in both samples, and 13 did not have NVP resistance mutations detected in either sample. Five of the eight women who had NVP resistance mutations detected in both plasma and breast milk had different mutations detected in the two samples (Table 1).

We examined persistence of NVP resistance in the 12 women who had NVP resistance detected in breast milk at 4 weeks post-partum (Table 1). Eleven of those women had a 10-week sample available for testing; one sample failed to amplify, leaving 10 follow-up samples with genotyping results. NVP resistance mutations were still detected in four of the 10 women by 10 weeks post-partum. The six women who did not have NVP resistance in breast milk at 10 weeks had only one NVP resistance mutation detected at 4 weeks. In contrast, among the four women who still had NVP resistance mutations detected at 10 weeks, three had multiple NVP resistance mutations detected at the earlier time point. Among the four women who had NVP resistance detected in breast milk at 10 weeks, the number of mutations detected at 10 weeks was lower than the number of mutations detected at 4 weeks; in three of the four cases, only K103N was detected in the 10-week sample.

We analyzed the association of breast milk resistance at 4 weeks with clinical and laboratory factors (Table 2). NVP resistance was detected in plasma more frequently among women who had NVP resistance detected in breast milk, but the association was not statistically significant (p=0.06, Table 2). We found no significant association of breast milk resistance with median maternal pre-NVP viral load, maternal pre-NVP CD4 cell count, median breast milk viral load at 4 weeks, median pre-NVP white blood cell count, or breast milk sodium >10 mmol/Lat 1 or 4 weeks; none of the 30 women had clinical mastitis. Detection of NVP resistance in breast milk was not associated with HIV subtype.

We also measured NVP concentrations in plasma and breast milk. At 1 week post-partum, NVP was detected (>10 ng/ml) in all 29 evaluable plasma samples and in 28 (96.6%) of 29 evaluable breast milk samples. At 2 weeks post-partum, NVP was detected at >10 ng/ml in 21 (72.4%) of 29 plasma samples and in 16 (69.6%) of 23 breast milk samples. At 2 weeks, detection of NVP in breast milk at >10 ng/ml was associated with detection of NVP in plasma at >10 ng/ml (p<0.001). The median NVP concentrations in plasma and breast milk were similar (at 1 week, for plasma: 168 ng/ml (range 16–744), for breast milk: 117 ng/ml (range <10–576), p=0.16; at 2 weeks, for plasma: 16 ng/ml (range, <10–83), for breast milk: 15 ng/ml (range <10–64), p=0.58). The concentration of NVP was <10 ng/ml in plasma and breast milk from all 30 women by 4 weeks post-partum. There was no association of NVP resistance in breast milk at 4 weeks with either detection of NVP >10 ng/ml in breast milk or plasma, or the level of NVP in either breast milk or plasma, at either study visit (1 or 2 weeks, Table 2).

DISCUSSION

We detected NVP resistance in 40% breast milk samples collected 4 weeks after sdNVP exposure; most samples had subtype A or D HIV. Results were not obtained for 20 additional samples with <500 copies /ml of HIV RNA. We did not identify any clinical or laboratory factors associated with detection of NVP resistance in breast milk at 4 weeks post-partum, possibly due to small sample size.

In a previous study, NVP resistance was detected in breast milk from 13 (65%) of 20 Zimbabwean women with subtype C HIV at 8 weeks post-partum [7]. It is difficult to compare those results to results from our study [7], because the two studies analyzed resistance at different times (4 weeks vs. 8 weeks post-partum), used different methods for

HIV genotyping, and included samples with different HIV subtypes (predominantly A and D vs. all subtype C).

Administration of sdNVP alone to women in labor continues to be part of regimens for prevention of MTCT in resource-limited settings. Regimens that combine sdNVP with other antiretroviral drugs (e.g. antenatal zidovudine or lamivudine) are also recommended by the World Health Organization for prevention of MTCT. Studies are needed to test whether women receiving those regimens have a reduced risk for acquiring NVP resistance than women who receive sdNVP alone. Recent studies also show that use of extended NVP infant prophylaxis reduces post-natal HIV transmission through breastfeeding [15,16]. Further studies are needed to evaluate whether emergence or persistence of NVP-resistant HIV in breast milk increases the risk of post-natal transmission in infants receiving NVP-based regimens for prevention of post-natal HIV transmission, or is associated with transmission of NVP-resistant strains to infants during breastfeeding.

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REFERENCES

1. McConnell MS, Stringer JSA, Kourtis AP, Weidle PJ, Eshleman SH. Use of single-dose nevirapine for the prevention of mother-to-child transmission of HIV-1: Does development of resistance matter? Amer J Obstetrics Gynecology. 2007; 197:S56–S63.
2. Kunz A, Frank M, Mugenyi K, Kabasinguzi R, Weidenhammer A, Kurowski M, et al. Persistence of nevirapine in breast milk and plasma of mothers and their children after single-dose administration. J Antimicrob Chemother. 2009; 63:170–177. [PubMed: 18974161]
3. Muro E, Droste JA, Hofstede HT, Bosch M, Dolmans W, Burger DM. Nevirapine plasma concentrations are still detectable after more than 2 weeks in the majority of women receiving single-dose nevirapine: implications for intervention studies. J Acquir Immune Defic Syndr. 2005; 39:419–421. [PubMed: 16010163]
4. Musoke P, Guay LA, Bagenda D, Mirochnick M, Nakabiito C, Fleming T, et al. A phase I/II study of the safety and pharmacokinetics of nevirapine in HIV-1-infected pregnant Ugandan women and their neonates (HIVNET 006). AIDS. 1999; 13:479–486. [PubMed: 10197376]
5. Eshleman SH, Mracna M, Guay LA, Deseyve M, Cunningham C, Mirochnick M, et al. Selection and fading of resistance mutations in women and infants receiving nevirapine to prevent HIV-1 vertical transmission (HIVNET 012). AIDS. 2001; 15:1951–1957. [PubMed: 11600822]
6. Church JD, Omer SB, Guay LA, Huang W, Lidstrom J, Musoke P, et al. Analysis of nevirapine (NVP) resistance in Ugandan infants who were HIV infected despite receiving single-Dose (SD) NVP versus SD NVP plus daily NVP up to 6 weeks of age to prevent HIV vertical transmission. J Infect Dis. 2008; 198:1075–1082. [PubMed: 18684096]
7. Lee EJ, Kantor R, Zijenah L, Sheldon W, Emel L, Mateta P, et al. Breast-milk shedding of drug resistant subtype C HIV-1 among women receiving single dose nevirapine. J Infect Dis. 2005; 192:1260–1264. [PubMed: 16136470]

8. Kassaye S, Lee E, Kantor R, Johnston E, Winters M, Zijenah L, et al. Drug resistance in plasma and breast milk after single-dose nevirapine in subtype C HIV type 1: population and clonal sequence analysis. AIDS Res Hum Retroviruses. 2007; 23:1055–1061. [PubMed: 17725424]

9. Eshleman SH, Hoover DR, Chen S, Hudelson SE, Guay LA, Mwatha A, et al. Nevirapine (NVP) resistance in women with HIV-1 subtype C, compared with subtypes A and D, after administration of single-dose NVP. J Infect Dis. 2005; 192:30–36. [PubMed: 15942891]

10. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J. Rapid and simple method for purification of nucleic acids. J Clin Microbiol. 1990; 28:495–503. [PubMed: 1691208]

11. Elbeik T, Hoo BS, Campodonico ME, Dileanis J, Fay FF, Bortolozzi RL, et al. In vivo emergence of drug-resistant mutations at less than 50 HIV-1 RNA copies/mL that are maintained at viral rebound in longitudinal plasma samples from human immunodeficiency virus type-1-infected patients on highly active antiretroviral therapy. J Hum Virol. 2001; 4:317–328. [PubMed: 12082398]

12. Mackie N, Dustan S, McClure MO, Weber JN, Clarke JR. Detection of HIV-1 antiretroviral resistance from patients with persistently low but detectable viraemia. J Virol Methods. 2004; 119:73–78. [PubMed: 15158587]

13. Eshleman SH, Guay LA, Mwatha A, Brown ER, Cunningham SP, Musoke P, et al. Characterization of nevirapine resistance mutations in women with subtype A vs. D HIV-1 6–8 weeks after single dose nevirapine (HIVNET 012). J Acquir Immune Defic Syndr. 2004; 35:126–130. [PubMed: 14722443]

14. R-Development-Core-Team. A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. 2007. ISBN 3-900051-07-0: http://www.R-project.org

15. Kumwenda NI, Hoover DR, Mofenson LM, Thigpen MC, Kafulafula G, Li Q, et al. Extended antiretroviral prophylaxis to reduce breast-milk HIV-1 transmission. N Engl J Med. 2008; 359:119–129. [PubMed: 18525035]

16. Six Week Extended-Dose Nevirapine (SWEN) Study Team. Extended-dose nevirapine to six weeks of age for infants to prevent HIV transmission via breastfeeding in Ethiopia, India, and Uganda: an analysis of three randomised controlled trials. Lancet. 2008; 372:300–313. [PubMed: 18657709]

Table 1

NVP resistance detected in plasma and breast milk samples (Uganda, 2003–2004)*.

ID	Subtype	Plasma 4 weeks	Breast milk 4 weeks	Breast milk 10 weeks
64	D	K103N	WT	
117	A	V106A	WT	
52	D	G190A	WT	
5	D	K103N+G190A	WT	
79	D	Y181C+Y188C	WT	
42	A	WT	G190A	WT
17	D	WT	Y188C	WT
8	A	WT	Y181C	Failed
69	A	WT	K103N	WT
39	D	Y181C	Y181C	WT
3	R	Y181C+Y188C	Y188C	K103N
23	D	K103N+Y181C	Y181C	WT
77	R	K103N+Y181C	K103N	WT
30	D	K103N+Y181C+Y188C	K103N+Y181C+Y188C	K103N+G190A
13	D	K103N+V106A+Y188C	K103N	No sample
24	C	K103N+Y181C+Y188C+G190A	K103N+Y181C+Y188C+G190A	K103N
22	A	K103N+V106A+Y181C+G190A	K103N+V106A+G190A	K103N

* Thirteen women had no NVP resistance mutations detected in plasma or breast milk. Results are shown for the 17 women who had at least one NVP resistance mutation detected in either sample. R indicates inter-subtype recombinant; wild type (WT) indicates no resistance mutations detected.

Table 2

Factors associated with detection of NVP resistance in breast milk 4 weeks after sdNVP administration (Uganda, 2003–2004).

	N	BM Resistance	No BM Resistance	P value
Number of women	30	12	18	
Maternal plasma NVP resistance at 4 weeks	30	8/12 (66.7%)	5/18 (27.8%)	**0.06** [c]
Median maternal pre-NVP viral load	30	1002	1035	0.33 [b]
Median maternal pre-NVP CD4 cell count	30	0.371	0.341	0.75 [b]
Median breast milk viral load at 4 weeks	30	352	200	0.37 [b]
Median pre-NVP breast milk WBC	30	6.90	5.85	0.18 [b]
Breast milk sodium ≥ 10 mmol/L at 1 week	22	7/11 (64%)	3/11 (27%)	0.20 [c]
Breast milk sodium ≥ 10 mmol/L at 4 weeks	19	5/10 (50%)	2/9 (22%)	0.35 [c]
HIV subtype[a]	30			0.10 [c]
A		4/12 (33.3%)	11/18 (61.1%)	
C		1/12 (8.3%)	0/18 (0%)	
D		5/12 (41.7%)	7/18 (38.9%)	
Intersubtype recombinant		2/12 (16.7%)	0/18 (0%)	
Non-A		8/12 (66.7%)	7/18 (38.9%)	0.26 [c]
Non-D		7/12 (58.3%)	11/18 (61.1%)	1.00 [c]
Pharmacokinetic analysis [d]				
Detection of NVP in plasma at 1 week	30	12/12 (100%)	18/18 (100%)	1.00 [c]
Detection of NVP in plasma at 2 weeks	29	8/11 (72.7%)	13/18 (72.2%)	1.00 [c]
Median NVP level in plasma at 1 week (range)	30	133 (16–379)	168 (20–744)	0.37 [b]
Median NVP level in plasma at 2 weeks (range)	29	16 (<10–46)	16 (<10–83)	0.98 [b]
Detection of NVP in breast milk at 1 week	29	11/12 (91.7%)	17/17 (100%)	0.41 [c]
Detection of NVP in breast milk at 2 weeks	23	8/10 (80%)	8/13 (61.5%)	0.40 [c]
Median NVP level in breast milk at 1 week (range)	29	82 (<10–226)	132 (31–576)	0.14 [b]
Median NVP level in breast milk at 2 weeks (range)	23	15 (<10–33)	14 (<10–64)	0.70 [b]

[a] P value for all subtypes.

[b] Wilcoxon test

[c] Fisher's exact test

[d] NVP levels below 10 ng/ml were below the limit of quantification of the assay.