Original article

Short-course Combivir after single-dose nevirapine reduces but does not eliminate the emergence of nevirapine resistance in women

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Background: In the Treatment Options Preservation Study (TOPS) trial, 4 or 7 days of Combivir (CBV; zidovudine/lamivudine) with maternal single-dose nevirapine (sdNVP) significantly reduced the emergence of NVP resistance as determined by virus population genotyping. To detect NVP resistance with greater sensitivity, we analysed TOPS samples by allele-specific real-time PCR (ASP).

Methods: In a random subset of women from each arm of the trial, plasma samples from before and 6 weeks after sdNVP were analysed using ASP at codons 103, 181, 184 and 190.

Results: Samples were analysed from 27 women in the sdNVP arm and 24 each in the CBV 4-day (sdNVP/CBV4) and 7-day (sdNVP/CBV7) arms. ASP detected NVP-resistant variants in week 6 samples from 70% of women in the sdNVP arm, 29% in the sdNVP/CBV4 arm and 33% in sdNVP/CBV7 arm (P<0.01 for sdNVP/CBV4 or sdNVP/CBV7 versus sdNVP; P=1.0 for sdNVP/CBV4 versus sdNVP/CBV7). Lamivudine resistance was detected by ASP in only 1 of 51 women who received CBV.

Conclusions: Short-course CBV significantly reduced but did not eliminate the emergence of NVP resistance after sdNVP. NVP-resistant variants were detected in about one-third of women despite CBV treatment, but the duration of persistence and clinical impact of these variants in response to antiretroviral therapy is uncertain.

Introduction

A single dose of nevirapine (sdNVP) administered to HIV-infected women during labour and to their infants within 72 h of birth reduces peripartum mother-to-child transmission (MTCT) of HIV-1 [1–3]. Because of its long half-life and low genetic barrier to resistance, sdNVP produces measurable plasma levels of NVP for as long as 3 weeks that can select NVP-resistant variants. Such variants have been detected by virus population genotyping in 19–75% of mothers and 33–87% of their infants who have received sdNVP [4–8]. Recent studies using more sensitive detection methods show that NVP-resistant variants can persist in plasma as a minor fraction of the virus population not detected by virus population genotyping for up to 2 years after sdNVP [5,7,9]. These low-frequency NVP-resistant variants can reduce the efficacy of subsequent NVP-containing antiretroviral therapy [10,11]. A recent trial (A5208/OCTANE) comparing the efficacy of initial
randomly selected for ASP analysis. For SGS analysis, a random subset of 15 women, 5 from each treatment arm, were selected from the 75 women selected for ASP analysis. The TOPS trial was approved by the South African Medicines Control Council (trial number 1100.1413) and ethics committees of the Universities of the Witwatersrand, Pretoria, KwaZulu-Natal, South Africa, and the Pharma-Ethics Independent Research Ethics Committee. The trial design was registered in the ClinicalTrials.gov register (number NCT00144183) and all participants provided written informed consent for HIV-1 resistance testing. Testing of samples by ASP and SGS was approved by the NIH Office of Human Subjects Protection.

Viral RNA extraction

Viral RNA from plasma samples was extracted as previously described and resuspended in 40 μl of 5 mM Tris-HCl [7,17,18].

Standard genotype analysis

Standard genotype analysis was performed on all specimens at an accredited laboratory in South Africa, employing the TruGen 334 HIV-1 genotyping kit and OpenGene DNA sequencing system (Bayer Diagnostics, Tarrytown, NY, USA), and data were analysed using the Stanford University HIV Drug Resistance Database [21]. The resistance testing laboratory was blinded to treatment allocation.

Allele-specific RT-PCR

ASP testing was performed on pre-intervention and week 6 postpartum samples without knowledge of treatment assignment or results of population genotype. First-round, RT-PCR amplification of a 637-base-pair fragment of pol including reverse transcriptase codons 22–234 was performed using HIV-1 subtype C-specific primers with the PCR product monitored by SYBR green fluorescence as published previously [7,17,18]. All primers used were specifically designed to amplify subtype C virus found in South Africa (primer sequences in Additional file 1 [18]). Viral RNA from each time point was tested in triplicate. No-template controls and HIV RNA standards (3×10⁶ to 300 copies) were included in the same 96-well assay plate. The products of the first amplification were diluted to 10⁶ total copies per reaction and second rounds of PCR were performed in parallel using allele-specific primers to amplify codon-specific mutant or wild-type sequences at codons 103, 181, 184 and 190, and non-specific primers to amplify all templates in the PCR reactions (primer sequences in Additional file 1 [18]). To confirm amplification specificity, PCR products were subjected to thermal denaturation analysis at 70–80°C, with readings taken every 0.1°C for 3 s. Samples that did not yield the correct melting temperature (Tm) for the amplified
Combivir reduces NVP resistance

Table 1. Frequency of nevirapine mutations 103N, 181C and 190A measured by allele-specific real-time PCR in week-6 plasma samples

<table>
<thead>
<tr>
<th>Treatment arm</th>
<th>Patients, n</th>
<th>Patients with NNRTI-resistance mutations, %</th>
<th>103N (AAC or AAT; 0.1%)</th>
<th>181C (TGT; 0.3%)</th>
<th>190A (GCA; 0.1%)</th>
<th>Any mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median, %</td>
<td>Range, [n]</td>
<td>Median, %</td>
<td>Range, [n]</td>
<td>Median, %</td>
</tr>
<tr>
<td>sdNVP</td>
<td>27</td>
<td>11</td>
<td>1.3–82</td>
<td>7</td>
<td>1–58</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(18)</td>
<td>(14)</td>
<td></td>
<td>(8)</td>
</tr>
<tr>
<td>sdNVP+CBV 4</td>
<td>24</td>
<td>2</td>
<td>0.3–13</td>
<td>1.6</td>
<td>0.8–49</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(5)</td>
<td>(4)</td>
<td></td>
<td>(4)</td>
</tr>
<tr>
<td>sdNVP+CBV 7</td>
<td>24</td>
<td>0.7</td>
<td>0.4–10</td>
<td>10.5</td>
<td>1.3–16</td>
<td>0.6</td>
</tr>
<tr>
<td>CBV7 days</td>
<td></td>
<td></td>
<td>(6)</td>
<td>(3)</td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>Combined</td>
<td>75</td>
<td>3.8</td>
<td>0.3–82</td>
<td>7.0</td>
<td>0.8–58</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(29)</td>
<td>(20)</td>
<td></td>
<td>(14)</td>
</tr>
</tbody>
</table>

*The frequencies of 103N AAC and AAT mutations were combined. †Limit of detection of mutant frequency. ‡Number of patients with mutation detected by ASP.

Results

Plasma samples (pre-intervention and 6 weeks postpartum) from 75 randomly selected women were analysed by ASP; 27 in the sdNVP alone arm, and 24 each in the 4-day (sdNVP/CBV4) and 7-day (sdNVP/CBV7) CBV arms. SGS analysis was performed on plasma samples from 15 women, 5 in each arm.

Standard genotype analysis

Population genotype analysis had been performed for the primary end point of the TOPS trial on plasma samples obtained 6 weeks postpartum from the 75 women selected for ASP analysis. Population genotype detected NVP resistance in 18 (24%) of 75 women: 14 of 27 (52%) in the sdNVP alone arm, 4 of 24 (17%) in the sdNVP/CBV4 and 4 of 24 (17%) in the sdNVP/CBV7 arm.

NVP resistance in sdNVP alone arm

All pre-therapy samples were below the limit of detection for mutants at each codon tested (0.1% for codons 103 and 190 and 0.3% for codons 181 and 184). sdNVP alone resulted in the emergence of NVP resistance in the majority of patients 6 weeks postpartum with 19 of 27 (70%) and 3 of 5 (60%) having NVP resistance mutations as analysed by ASP and SGS, respectively (Table 1 and Figures 1A and 2A). The K103N mutation was detected most commonly (18 of 27 patients) with a median population frequency of 11% and a population frequency >50% in 6 of 18 women (Table 1 and Figure 1A). Although the G190A mutation was detected in fewer patients (8 of 27) and its population frequency was never >50%, the median frequency of 11% was the same as for K103N. Y181C was detected in 14 patients and the median population frequency was 7%. Of 19 women with NVP resistance,
Figure 1. Nevirapine resistance mutations detected by allele-specific PCR

Population frequency of nevirapine (NVP) resistance mutations detected by allele-specific real-time PCR (ASP) in samples obtained 6 weeks after single-dose NVP (sdNVP) at codons 103, 181 and 190 in women receiving (A) sdNVP alone, (B) sdNVP plus Combivir for 4 days or (C) sdNVP plus Combivir for 7 days. Vertical bars show data from individual women.

Population frequency of nevirapine (NVP) resistance mutations detected by allele-specific real-time PCR (ASP) in samples obtained 6 weeks after single-dose NVP (sdNVP) at codons 103, 181 and 190 in women receiving (A) sdNVP alone, (B) sdNVP plus Combivir for 4 days or (C) sdNVP plus Combivir for 7 days. Vertical bars show data from individual women.
Combivir reduces NVP resistance

13 (68%) had multiple mutations detected, 7 of whom had all three NVP resistance mutations (103N, 181C and 190A).

SGS revealed that viral variants with mutations at codons 103, 106, 181, 188 and 190 comprised a substantial fraction of the circulating virus population in 3 of 5 women 6 weeks after sdNVP, although generally not exceeding 30% (Figure 2A). The overall percentage of genomes with any drug resistance mutation was 45%. Mutations at codons 106 (A/M) and 188 (C), which were not analysed by ASP, were detected in approximately 10% of genomes from 2 of 5 patients who received sdNVP alone. Mutation at codon 108 (I) was detected at low frequency (2%) in 1 of 5 patients. None of the mutations listed above (103, 106, 108, 181, 184, 188 and 190) was linked on the same genome in any of the patients. The two patients (P4 and P11; Figure 2A) without NVP-resistant genomes detected by SGS also did not have mutations detected by ASP.

NVP and NRTI resistance in the sdNVP/CBV4 arm

In the TOPS study, the addition of CBV for 4 days after sdNVP significantly reduced the proportion of patients with NVP resistance detected using standard population genotyping by about fivefold from 59% with sdNVP alone to 12% with sdNVP/CBV4 [14]. ASP analysis confirmed that CBV for 4 days significantly reduced the proportion of women with NVP resistance detected, from 70% in the sdNVP arm to 29%, or about 2.5-fold ($P=0.005$). In addition, the median population frequency of NVP-resistant mutants decreased from 11% to 2% (Table 1). Of the 7 women with NVP resistance, 5 had multiple NVP-resistance mutations detected by ASP (Figure 1B). Only 2 of 5 patients analysed by SGS had NVP-resistance mutations (Figure 2B), and the remaining 3 patients were negative for mutations when analysed by both SGS and ASP. The total percentage of genomes that have NVP-resistance mutations detected by SGS was 13.5% compared with 45.2% in the sdNVP-alone arm. Lamivudine-resistant variants were detected by ASP in only 1 of 24 patients with a frequency of 2% M184I. This patient did not have NNRTI resistance. The M184V mutation was not detected by ASP in any patient. SGS did not detect M184I/V or other nucleoside reverse transcriptase inhibitor (NRTI)-resistance mutations including thymidine analogue mutations (TAMs) in postpartum samples.
Figure 2. Nevirapine resistance mutations detected by single-genome sequencing

Proportion of genomes showing the specified nevirapine (NVP) and lamivudine resistance mutations detected by SGS in samples obtained 6 weeks after single-dose NVP (sdNVP) in women receiving (A) sdNVP alone, (B) sdNVP plus Combivir for 4 days or (C) sdNVP plus Combivir for 7 days. Vertical bars show data from individual women. None indicates genomes with no resistance mutations.
NVP and NRTI resistance in the sdNVP/CBV7 arm

CBV for 7 days also significantly reduced the proportion of patients with NVP resistance detected by ASP from 70% with sdNVP alone to 33% (Table 1) with sdNVP/CBV7 (P=0.012), but the extent of reduction in resistance was less than that observed by population genotype (59.2% for sdNVP alone versus 7.3% for sdNVP/CBV7 [14]). There was no significant difference in the proportion of women with NVP-resistant variants detected by ASP between the 4- and 7-day CBV arms (Table 1; P=1.0). However, only 2 of 8 patients with NVP resistance detected by ASP in the sdNVP/CBV7 arm had multiple NVP-resistance mutations detected compared with 5 of 7 in the sdNVP/CB4 arm (Figure 1C), although this difference was not significant (P=0.13). NVP-resistance mutations were not detected by SGS in any of the 5 women analysed in the sdNVP/CBV7 arm, but one had low frequency NVP-resistant variants detected by ASP at codon 103 (0.4%). Lamivudine-resistance mutations were not detected by ASP and neither lamivudine- nor other NRTI-resistance mutations were detected by SGS (Figure 2C).

Comparison of population genotype and ASP for NVP resistance

As noted earlier, among the 75 women studied, 18 (24%) had NVP resistance detected by population genotyping. Of these 18 women, 15 (83%) had NVP resistance detected by ASP and 3 had mutations detected at codons not examined by ASP (106, 108 and 188). Among the women who were negative for NVP resistance by standard genotyping, the proportions with NVP resistance detected by ASP were 31% (5 of 16), 15% (3 of 20), and 24% (5 of 21) in the sdNVP, sdNVP/CB4 and sdNVP/CB7 arms, respectively. Overall, the proportions of women with NVP resistance detected by either population genotyping or ASP were 74% (20 of 27), 37.5% (9 of 24) and 33% (8 of 24), in the sdNVP, sdNVP/CB4 and sdNVP/CB7 arms, respectively.

Discussion

Prevention of MTCT of HIV-1 continues to be a major challenge in resource-poor settings where ART for pregnant women is not routinely available [1]. In some settings, sdNVP alone is still used to prevent HIV-1 transmission to infants [3], even though NVP-resistant variants are selected in the majority of women and these resistant variants can decrease the effectiveness of subsequent ART with NVP-containing regimens [10,11]. Thus, reducing the emergence of NVP resistance after sdNVP is an important goal. In the TOPS trial, initiation of short-course CBV at the same time as sdNVP significantly reduced the proportion of women with new NVP resistance mutations detected by population genotype analysis 6 weeks post-therapy [14]. Specifically, 59.2%, 11.7% and 7.3% of women in the sdNVP, sdNVP/CBV4 and sdNVP/CBV7 arms, respectively, had NVP resistance detected. In the present study, we used more sensitive methods, ASP and SGS, to identify NVP resistance that could have been missed by population genotyping. Testing for NVP resistance using more sensitive methods is important because the presence of low-frequency NNRTI-resistant variants, below the detection limit of population genotype methods, is strongly associated with the failure of NNRTI-containing regimens [11,13,22-26].

Our results corroborate the main finding of the TOPS trial [14] and provide additional insights into the efficacy of short-course CBV in preventing NVP resistance. ASP analysis revealed a significant, albeit smaller, reduction in the frequency of NVP resistance with 4 or 7 days of CBV. To illustrate, by population genotyping, only 11.7% and 7.3% of women in the 4- and 7-day CBV arms had NVP resistance detected. By contrast, 29% and 33% of women in the 4- and 7-day CBV arms, respectively, had NVP resistance detected by ASP and 37.5% and 33%, respectively, with the two methods combined. More frequent detection of mutants by ASP is not surprising given its higher sensitivity than that of standard genotyping, but the emergence of NVP-resistant mutants in about one-third of women in both the CBV 4- and 7-day arms was not anticipated.

Although the SGS analyses were restricted to 15 patients, the results are also consistent with the findings of the TOPS trial, with both 4 and 7 days of CBV showing suppression of NVP resistance compared with sdNVP alone, and 7 days of CBV showing greater suppression of NVP resistance than 4 days of CBV (Figure 2). In addition, SGS and ASP results were consistent with each other (Figures 1 and 2). For the codons analysed by ASP, all mutations detected by SGS were also detected by ASP at approximately similar frequencies. There was only one instance (in patient P66) in which a mutation was detected by ASP but not by SGS. In this instance, the mutant frequency was <1% and well below the sensitivity of 45 single genome sequences, which is 90% sensitivity for mutants at 5% frequency. Conversely, there were 4 women who had drug-resistant mutants detected by SGS at sites that were not included in the ASP assay (106, 108 and 188), although each of these women had NVP-resistance mutations detected by ASP at codons 103, 181 or 190. Only 9 of the 22 mutations detected by SGS were detected by standard sequencing (data not shown), which is consistent with prior reports showing improved sensitivity of SGS compared with population genotyping [16,19]. Additionally, patients P8 and P28 had multiple mutations detected by SGS
and ASP but population genotype failed to detect any resistance mutations.

ASP analysis revealed no significant difference between the 4- and 7-day CBV arms in the proportion of women with NVP-resistant variants (P=1.0). However, 5 of 7 patients receiving sdNVP/CBV4 had multiple NVP mutations detected by ASP compared with only 2 of 8 patients receiving sdNVP/CBV7, but this difference was not significant (P=0.13).

Our findings are also generally consistent with the important report by Farr et al. [27] showing suppression of NVP resistance by CBV for 7 days in an observational study conducted in Malawi. In that study, NVP resistance at 6 weeks postpartum, as detected by population genotype combined with ASP, was reduced from 74% with sdNVP alone to 10% with sdNVP+CBV for 7 days. Our study extends this finding to a randomized clinical trial and compares CBV treatment for 4 days with that for 7 days. Of note, the frequency of NVP resistance detected in both the 4- and 7-day CBV arms (37.5% and 33%, respectively) in our study was considerably higher than the 10% frequency reported by Farr et al. [27] after 7 days of CBV (10%). Possible explanations for the discordant results are differences in the patient population studied, the study design (observational versus randomized), medication adherence and the methods used to detect low-frequency variants. Regarding the latter possibility, the mutant detection limit for ASP used in our study was 0.1–0.3% compared with that of 0.5–1.0% reported by Farr et al. [27].

We found that only 1 of 51 women receiving sdNVP/CBV4 had a lamivudine-resistance mutation (M184I). The absence of this mutation in the baseline sample from the same patient implies that it was selected by CBV. The absence of M184V or other NRTI mutations in women from either the 4- or 7-day CBV arms might be due to the shorter half-life of the active forms of lamivudine and zidovudine than nevirapine, or, alternatively, that NRTI-resistant variants decline more rapidly than NVP-resistant mutants after clearance of drug. By contrast, NVP has a long half-life and the K103N mutation seems to have little effect on HIV-1 replicative fitness, resulting in persistence of variants after the drug is cleared.

Our findings raise important additional questions. For example, the long-term effects of the NVP-resistant variants we detected on subsequent treatment outcome are incompletely defined. Two initial studies suggested that the effects of NVP resistance were limited to those women who initiated ART within 6 months after receiving sdNVP [10,28]. By contrast, a more recent study by Coovadia et al. [11] suggested that women who developed K103N mutations after sdNVP had a significantly higher chance of virological failure after initiating ART 18–36 months after sdNVP. Similarly, a study using ASP in 26 women who received sdNVP found minor NNRTI variants in 86% of the women who failed subsequent NVP-containing therapy compared with 32% of women who continued to respond [29]. Finally, A5208/OCTANE Trial 1 was designed to compare protease-inhibitor-based therapy using lopinavir/ritonavir (LPV/r) with NVP-based therapy in women with prior sdNVP. The trial showed LPV/r+ tenofovir and emtricitabine (TDF/FTC) to be superior to NVP+TDF/FTC (P=0.001) for initial ART of women with prior sdNVP>6 months earlier [12]. Population genotype analysis of stored pre-therapy plasma samples revealed NVP resistance mutations in 13% of women in the NVP arm and 73% of these women reached a primary end point of virological failure or death, but this association explained less than half of the primary end points in the NVP arm. Among the 201 women without NVP resistance as determined by standard genotype, 70 (35%) had NVP-resistant variants detected by ASP. Among these 70 women, a primary study end point occurred in 12 of 38 (32%) women in the NVP arm versus 3 of 32 (9%) in the LPV/r arm (HR 3.84 [95% CI 1.06, 13.9]; P=0.040). NVP-resistant variants at frequencies >1% were significantly associated with the primary study end point in the NVP arm (P=0.003), but not in the LPV/r arm [13]. These findings suggest that the persistence of NVP-resistant variants at frequencies as low as 1% of the virus population can negatively influence subsequent treatment response.

This level of NVP-resistant mutant (>1%) was found in 21% of women in the current study 6 weeks after receiving CBV for 4 or 7 days. A crucial issue to be resolved is how long such low-frequency mutants persist after sdNVP with 4 or 7 days of CBV and whether they persist at levels that would influence subsequent treatment response.

In conclusion, by applying sensitive methods to detect resistance, we have shown that short-course CBV therapy significantly reduces but does not eliminate the selection of NVP-resistant variants. The persistence and ultimate clinical impact of low-frequency NVP-resistant variants after sdNVP with short-course CBV or other antiretroviral regimens remains to be determined. Additional strategies are needed to maximize prevention of NVP resistance and optimize subsequent response to antiretroviral therapy in women who have received sdNVP.

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Disclosure statement

MH, DBH and PR are employed by Boehringer Ingelheim, the sponsor of the 1100.1413 study, and DM was employed by Boehringer Ingelheim at the time of the study. JAM and GEG have received research funding, travel grants and speaker’s honoraria from Boehringer Ingelheim and GlaxoSmithKline. JWM is a consultant for Merck, Gilead Sciences and RFS Pharma and owns share options in RFS Pharma. All other authors declare no competing interests.

Additional file


References


