Background: Selection and persistence of non-nucleoside reverse transcriptase inhibitor (NNRTI)-associated mutations during treatment interruptions (TIs) has been attributed to the long plasma half-life of these drugs. However, little is known about the contribution of variable NNRTI plasma levels before a TI. We evaluated the selection of NNRTI-related mutations and the coefficient of variation of NNRTI plasma concentrations during different TI periods.

Methods: The selection of NNRTI-related mutations was examined in 50 HIV type-1 (HIV-1)-infected patients on a virologically suppressive regimen who underwent TI guided by CD4+ T-cell counts and plasma viraemia. Population and clone-based sequencing of the reverse transcriptase coding region was performed using plasma HIV-1 RNA samples during TI and proviral DNA from peripheral blood mononuclear cells before TI. NNRTI plasma concentrations were determined by HPLC.

Results: In 7/50 treated patients, de novo and transient NNRTI-related mutations appeared when treatment was interrupted. Emergence of resistant variants (including K103N, Y181C or G190S) after interruption was associated with a higher coefficient of variation in NNRTI plasma concentrations during the treatment period. Moreover, minority HIV-1 variants containing different resistance patterns (V106I/A, K103R/E or Y188C/D/H) were detected regardless of NNRTI concentrations.

Conclusions: The emergence of NNRTI-associated mutations during TI appears to be associated with the variation of NNRTI plasma concentrations during the preceding treatment period. The selection of minority HIV-1 variants with different patterns of NNRTI resistance in the absence of drug pressure should be considered for the efficacy of future NNRTI-containing antiretroviral regimens.

Introduction

Antiretroviral treatment interruption (TI) strategies frequently result in viral replication in the presence of decreasing plasma drug levels, with the risk of selecting drug resistance mutations [1]. Different studies have reported the selection of de novo non-nucleoside reverse transcriptase inhibitor (NNRTI)-resistant mutations after discontinuation of a virologically suppressive regimen in patients with chronic HIV type-1 (HIV-1) infection [2–5]. Studies focused on the persistence of NNRTI-related mutations during the interruption period have only analysed patients whose antiretroviral therapy has failed, and thus reveal huge individual variability [6,7]. It has also been reported that potential archiving of resistant variants in proviral DNA can lead to further virological failure when treatment is restarted [8]. Repeated discontinuation and resumption of certain antiretroviral drug regimens has been reported to lead to drug resistance in individuals who are chronically infected with HIV-1, particularly in those receiving antiretroviral drugs that are metabolized slowly or have a low genetic barrier to attain resistance [5,9].

In this study, we evaluated the selection of major and minor HIV-1 variants containing NNRTI-associated mutations among virologically suppressed patients who underwent TI guided by CD4+ T-cell count and plasma viral load (pVL). Moreover, we explored the association...
between the emergence of NNRTI-resistant mutations and the variability of NNRTI plasma concentrations.

Methods

Patients were selected from a cohort of 100 Caucasian individuals who underwent TI guided by both CD4+ T-cell count and pVL [3]. From this cohort, we examined all patients (n=50) who received highly active antiretroviral therapy (HAART) regimens containing two nucleoside reverse transcriptase inhibitors and one NNRTI for longer than one year. At enrolment, all patients had CD4+ T-cell counts >500 cells/mm³ and plasma HIV-1 RNA <50 copies/ml. All drugs were withdrawn simultaneously (at baseline) and patients were monitored monthly up to 96 weeks. The criteria for HAART resumption were pVL >100,000 copies/ml or CD4+ T-cell count <350 cells/mm³. Selection of NNRTI-associated resistance mutations was determined in plasma HIV-1 RNA during viral rebound at each TI, in available retrospective plasma samples before the initiation of an NNRTI-containing regimen and in proviral DNA from peripheral blood mononuclear cells (PBMCs) immediately before the interruption. Population-based genotyping of the HIV-1 reverse transcriptase coding region was performed using the TruGene™ HIV-1 Genotyping Kit and the TruGene™ DNA Sequencing System (Siemens Medical Solutions Diagnostics, Barcelona, Spain). Clonal sequence analysis was performed using plasma HIV-1 RNA at viral rebounds during the different interruption cycles and proviral DNA from PBMCs before treatment discontinuation. In those patients who only interrupted treatment once, clonal analysis was performed longitudinally during the interruption period. The cloning procedure was performed using the pGEM®-T Easy Vector System (Promega, Madrid, Spain) according to the manufacturer’s recommendations. Between 20 and 45 purified clones were sequenced and NNRTI-associated mutations were categorized according to the International Aids Society Drug Resistance Mutation Group [10]. Phylogenetic trees constructed using the neighbour-joining method were used to rule out any contamination between samples. NNRTI plasma concentrations were determined using blood samples collected from the 50 eligible patients the day before initiating the first interruption cycle (12 h after the last dose of nevirapine and 8 h after the last dose of efavirenz). Additionally, in patients who re-initiated an NNRTI-containing regimen, NNRTI plasma levels were sequentially assessed during the treatment resumption periods (at three to five time points). Plasma concentrations were assessed by HPLC. Our laboratory follows the KKGT quality assurance programme from the Dutch Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology of the Radboud University Medical Centre (Nijmegen, the Netherlands).

For each treatment period, the percent of time that plasma drug measures were below a suggested therapeutic threshold for efavirenz (trough concentration [Ctrough] >1.1 mg/l) or nevirapine (Ctrough >3.0 mg/l) [11–13] was estimated as (number of weeks/total length of the period) ×100.

 Plasma levels not available at those time points without TIs were estimated using the linear interpolation method. Because the selected thresholds were not fully validated, we repeated the analysis varying them by 25%. Furthermore, the variability of plasma drug concentrations was assessed using the coefficient of variation (CV) and interquartile range (IQR). CV was computed as (std/mean)×100 on the basis of the observed plasma drug concentrations. Only treatment periods >10 weeks were considered for the analysis. Differences in the distribution of the estimated percentages and CV between treatment periods with and without consecutive emergence of major NNRTI-related mutations were assessed using the Mann–Whitney U test.

Results

Population-based sequencing of plasma HIV-1 rebounds revealed that, in 7/50 patients (14%) who received an NNRTI-containing regimen (Table 1, patients A, B, D, E, G, H and I), the drugs selected NNRTI-associated mutations during the interruption. A further two patients presented NNRTI-associated mutations that were only detected at the clonal level (Table 1, patients C and F). The remaining 41 patients, who showed no major NNRTI-resistant variants during the study period, did not restart treatment or restarted treatment with an NNRTI-free regimen and were therefore excluded from this analysis. The most frequent mutation selected in plasma RNA during treatment discontinuations was K103N alone or combined with Y181C (4/7 patients). At the clonal level, additional substitutions (Y188C/D/H [8/9 patients], K103E/R [3/9 patients] and V106I/A [3/9 patients]) were found at residues associated with resistance to NNRTI. NNRTI-related mutations were not detected in retrospective plasma samples or in proviral DNA in 8/9 patients, suggesting de novo selection of these mutations during the TIs. Only one patient (patient I) presented the mutation Y181C in plasma HIV-1 RNA prior to initiating HAART. The presence of this mutation, which re-emerged during TI, could not be explained by selective pressure of the treatment regimen because the patient was taking monotherapy with zidovudine before starting HAART. Moreover, this patient showed alternative NNRTI-related variants in proviral DNA, with 4% of the clones harbouring the K103Q substitution.
NNRTI plasma variability and genotypic resistance

(Table 1). With regard to the presence of other drug resistance mutations, only patient B showed NNRTI-related mutations (T215F and K219Q) in plasma HIV-1 RNA and in proviral DNA before initiating HAART and during the interruption cycles.

We initially tried to correlate efavirenz and nevirapine plasma concentrations at baseline (before TI) with the appearance of NNRTI mutations. We observed that the mean ±SD plasma concentration of patients whose treatment did not select drug resistance mutations during subsequent TIs was 2.8 ±1.4 mg/l for efavirenz (n=11) and 5.4 ±2.3 mg/l for nevirapine (n=30). Thus, NNRTI plasma concentrations consistently above the therapeutic cutoff could contribute to limiting the selection of major HIV-1-resistant mutants when combined with other active drugs in the antiretroviral regimen. When we analysed the pharmacological levels of the six patients whose treatment selected NNRTI-resistant mutants (Figure 1), we found a high variability of results at baseline that did not allow us to establish a statistical association (data not shown). Therefore, of the patients who underwent two or more treatment resumption cycles (patients A to F), we also considered NNRTI plasma concentration levels at several time points during the treatment periods to examine the association between the CV and the selection of HIV-1 mutants. The proportion of weeks with suboptimal NNRTI drug concentrations and the total variability during each treatment period in patients with more than one interruption cycle are shown in Table 2. The percentage of time below the theoretical drug level cutoff in those periods followed by the emergence of major mutations was similar to periods in which mutations did not appear (P=0.524). However, the CV in patients whose treatment selected major mutations (median CV 57.2% IQR 35.8–88.1) was significantly higher than those whose treatment did not select NNRTI resistance-associated mutations (median CV 8.3% IQR 3.8–10.1; P=0.008, two-tailed). These results remained invariable after changing the theoretical drug level cutoff by 25%. Moreover, even after excluding from the analysis the null plasma drug concentration values or the CV values based on only two samples, the results retained their significance (P=0.016).

Table 1. Frequencies of drug-resistant mutations in NNRTI-treated patients undergoing treatment interruption, determined by population-based and clonal sequencing

<table>
<thead>
<tr>
<th>Patient</th>
<th>Treatment</th>
<th>TI cycle, n</th>
<th>Retrospective mutations*</th>
<th>Proviral DNA</th>
<th>Plasma RNA (first TI)</th>
<th>Plasma RNA (second TI)</th>
<th>Plasma RNA (third TI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ddI, 3TC, EFV</td>
<td>3</td>
<td>None</td>
<td>None</td>
<td>Y188C (3/28)†</td>
<td>Y181C (P)</td>
<td>K103N (P)</td>
</tr>
<tr>
<td>B</td>
<td>ddI, 3TC, ABC, EFV</td>
<td>3</td>
<td>T215F (P)</td>
<td>None</td>
<td>V106I (2/45)</td>
<td>K103N (P)</td>
<td>K103E (1/33)</td>
</tr>
<tr>
<td>C</td>
<td>ZDV, 3TC, EFV</td>
<td>3</td>
<td>None</td>
<td>None</td>
<td>Y188C (1/31)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>D</td>
<td>ddI, d4T, NVP</td>
<td>2</td>
<td>None</td>
<td>None</td>
<td>Y106A (1/26)</td>
<td>V106A (1/46)</td>
<td>NA</td>
</tr>
<tr>
<td>E</td>
<td>ddI, d4T, NVP</td>
<td>2</td>
<td>None</td>
<td>None</td>
<td>ND</td>
<td>K103N + Y181C (P)</td>
<td>NA</td>
</tr>
<tr>
<td>F</td>
<td>3TC, NVP, NVP</td>
<td>3</td>
<td>None</td>
<td>None</td>
<td>Y181H (1/35)</td>
<td>None</td>
<td>Y188C (1/33)</td>
</tr>
<tr>
<td>G</td>
<td>ddI, TDF, NVP</td>
<td>1</td>
<td>None</td>
<td>None</td>
<td>V106M + G190A (P)</td>
<td>V179D (35/35)</td>
<td>NA</td>
</tr>
<tr>
<td>H</td>
<td>ddI, d4T, NVP</td>
<td>1</td>
<td>None</td>
<td>None</td>
<td>K103N + Y181C (P)</td>
<td>K103R (4/31)</td>
<td>NA</td>
</tr>
<tr>
<td>I</td>
<td>ddI, 3TC, NVP</td>
<td>1</td>
<td>Y181C (P)</td>
<td>L210W (P)</td>
<td>K103Q (1/25)</td>
<td>Y181C (P)</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Mutations detected in plasma RNA before starting highly active antiretroviral therapy. †Number of mutated clones or total sequenced clones. *Mutations detected by population-based sequencing. ABC, abacavir; ddI, didanosine; d4T, stavudine; EFV, efavirenz; NA, not applicable; NVP, nevirapine; NNRTI, non-nucleoside reverse transcriptase inhibitor; NVP, nevirapine; TDF, tenofovir; TI, treatment interruption; ZDV, zidovudine; 3TC, lamivudine.
Figure 1. Selection of NNRTI-resistant variants in four NNRTI-treated patients who underwent sequential treatment interruption cycles

Plasma viraemia and drug plasma levels are shown during the 96-week study period. The light grey areas represent time off therapy and the drug regimen is shown beside the patient identification. The dark grey, dotted areas represent the non-nucleoside reverse transcriptase inhibitor (NNRTI) levels detected in plasma during the treatment periods. The solid line shows the plasma viraemia level. The broken line shows the lowest therapeutic cutoff established for efavirenz (EFV) and nevirapine (NVP). The arrow indicates the time point where the NNRTI mutation was detected. Minority populations of NNRTI-associated resistant variants are represented in small font and the bulk population is in large font and boxed. ABC, abacavir; ddI, didanosine; d4T, stavudine; HIV-1, HIV type-1; TI, treatment interruption; ZDV, zidovudine; 3TC, lamivudine.
A greater variability of NNRTI plasma concentrations before treatment withdrawal was significantly associated with the presence of major NNRTI-associated mutations (K103N, Y181C and G190S) after treatment was interrupted. However, in the absence of drug pressure, major HIV-1 variants did not appear but alternative minor variants with different drug resistance mutation patterns containing the substitutions V106I/A, K103R/E, Y181H and Y188C/D emerged (Figure 1).

Patients G, H and I underwent a single interruption and NNRTI-related mutations (mainly Y181C, K103N, G190A or V106M) appeared immediately. After 30 weeks without antiretroviral treatment, major NNRTI mutations gave way to minority viral populations with substitutions at residue 188. Of the two patients whose treatment selected Y181C, one also had K103N in the population-based genotype, and both cases presented a shift from the polymorphism K103R to K103E at the clonal level.

Discussion

Efavirenz and nevirapine have a long plasma half-life and a relatively low genetic barrier to HIV-1 resistance [14]. Hence, it is reasonable to presume that simultaneously stopping all drugs in a combination antiretroviral regimen could result in temporary NNRTI monotherapy, thus favouring the selection of drug-resistant variants over the wild type [14]. However, treatment strategies that withdraw NNRTI before HAART is stopped fail to prevent the selection of NNRTI resistance mutations in virological responders [4]. The results presented here suggest that the selection of major NNRTI-related mutations could be favoured if the NNRTI plasma concentrations were highly variable during the treatment periods before interruption. The most probable cause of this variability could be irregular adherence to NNRTI in these patients, despite the fact that the proportion of patients with self-reported adherence above 95% at the end of the study was 80% [3]. Poor adherence is known to play a role in low efavirenz concentrations and viral failures, and therapeutic drug monitoring could represent a valuable tool to encourage adherence [15]. However, other factors such as drug disposition or metabolism could also be implicated.

Furthermore, we observed a transient and changeable selection of NNRTI-associated mutations in patients who underwent several interruption cycles. It is noteworthy that all study participants resuppressed their pVL upon restarting treatment, suggesting either that minority NNRTI-resistant variants were not archived in proviral DNA or that, if they were archived, they were not involved in drug resistance mechanisms when treatment was restarted. Plasma viraemia rebound following treatment discontinuation results in T-cell activation [16] and accelerated apoptosis, thus potentially limiting the number of infected CD4+ T-cells containing NNRTI-resistant variants in proviral DNA. Only one patient had a low number of detectable clones with an NNRTI-associated mutation in proviral DNA.

Table 2. Description of NNRTI plasma concentrations and the coefficient of variation in the different periods of treatment and their association with the presence of NNRTI-related mutations

<table>
<thead>
<tr>
<th>Patient identifier</th>
<th>Treatment interval, weeks</th>
<th>NNRTI included in the treatment</th>
<th>Time below the cutoff*, %</th>
<th>Coefficient of variation during treatment, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of NNRTI-related mutations†</td>
<td>A 12–44 Efavirenz</td>
<td>31.3</td>
<td>92.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A 60–76 Efavirenz</td>
<td>0</td>
<td>26.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 12–32 Efavirenz</td>
<td>0</td>
<td>45.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D 12–30 Nevirapine</td>
<td>16.7</td>
<td>57.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E 6–68 Nevirapine</td>
<td>61.3</td>
<td>83.7</td>
<td></td>
</tr>
<tr>
<td>Absence of NNRTI-related mutations†</td>
<td>B 54–80 Efavirenz</td>
<td>100</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C 17–32 Efavirenz</td>
<td>0</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C 64–80 Efavirenz</td>
<td>0</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F 10–28 Nevirapine</td>
<td>0</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F 48–68 Nevirapine</td>
<td>0</td>
<td>9.1</td>
<td></td>
</tr>
</tbody>
</table>

P-value‡ 0.524 0.008

*Suggested therapeutic threshold for efavirenz (trough concentration [C_{trough}] >1.1 mg/l) or nevirapine (C_{trough} >3.0 mg/l). †Emergence of major non-nucleoside reverse transcriptase inhibitor (NNRTI)-related mutations during the consecutive treatment interruption cycle by population-based sequencing. ‡The Mann–Whitney U test was used to determine the association between the distribution of the coefficient of variation (or the percentage of time below the cutoff) and the presence of NNRTI-related mutations. Exact P-value (two-tailed) is shown.

Antiviral Therapy 13.7 949
that was different from the mutations that emerged during the interruption. A potential explanation for this variability could be the existence of residual viremia (pVL > 2.5 copies/ml) recently reported in patients with undetectable pVL (<50 copies/ml) who had been treated with HAART [17]. Similarly, even though our patients were on a virologically suppressive regimen, it seemed likely that minor NNRTI-associated resistance variants could be replicating in plasma at very low levels, and could only be detected by clone sequencing or other ultra-sensitive assays [18–20].

The transient selection of these mutations differs from that observed in other studies, in which persistence of NNRTI-related mutations was found even years after NNRTI treatment was stopped [7,21]. However, the patients analysed in those studies experienced treatment failures, sometimes because of the presence of multidrug-resistant viral variants. The de novo emergence of NNRTI-related mutations during TI in patients on a virologically suppressive regimen has already been reported [2,22]. In this study, we also report clonal data from patients who underwent a single long-term treatment discontinuation period, suggesting that NNRTI-associated mutations do not persist in the long term in those patients who discontinued successful antiretroviral regimens.

A persistent suboptimal NNRTI plasma level induces the selection and subsequent evolution of drug-resistant HIV-1 variants. Patient E had persistent subtherapeutic nevirapine levels that resulted in the selection of substitution K103N and the subsequent accumulation of Y181C 20 weeks later. Single mutants have been reported to be fitter than double mutants [23]. However, fitness is not always directly related to the level of drug resistance [24] and, as we can observe under the irregular pharmacological conditions of patient D, the selection of the double mutant K103N plus Y181C seems to confer a high level of drug resistance, rather than better fitness. Recent studies show that there is a fragile balance between viral suppression and selection of drug resistance. Thus, moderate levels of adherence (>50%) to an NNRTI regimen are sufficient to suppress viral replication, but when these levels are too low to suppress this replication (<50%) selection of drug resistance mutations dramatically increases [25].

The replacement of the NNRTI-associated resistant variant K103N or Y181C by minor viral variants harbouring substitutions Y188C/H, V106I/A or K103R/E after weeks of no drug pressure could be explained by their greater replicative capacity in the absence of drug pressure [26]. Variants at HIV-1 reverse transcriptase codon 103, other than K103N, should be considered for their potential to alter susceptibility to NNRTI [27]. The shift from K103N to K103R and K103E observed at the clonal level in the absence of drug is more likely the result of polymorphic changes derived from wild-type viral strains than changes from K103N. This feature could indicate that the selection and maintenance of these polymorphisms is easier for the virus than K103N and could represent a drug resistance reservoir for future NNRTI challenges.

In conclusion, patients receiving NNRTI regimens who underwent TIs harboured different and transient NNRTI-related mutations (K103N, Y181C and G190S/A) during different interruption cycles, despite restarting treatment. The emergence of these mutations in the dominant viral population is de novo and seems to be associated with a high level of variation of NNRTI plasma levels during the period before interruption. Minor NNRTI-associated resistant variants containing substitutions V106I/A, K103R/E or Y188C/D/H can be detected even when drug levels are suboptimal, suggesting that minor NNRTI-associated resistant variants archived at different compartments re-emerge when treatment pressure is withdrawn. Thus, even in patients with suppressive antiretroviral regimens, high oscillations in NNRTI plasma concentrations should be reduced by encouraging treatment adherence. Moreover, the presence of hidden minor NNRTI-associated resistant variants with different patterns of mutations should be considered for its potential interference with the efficacy of future NNRTI-containing regimens in virological responders. Our data provide proof of concept. Additional studies with a greater number of patients and a more rigorous assessment of adherence would strengthen our findings.

Acknowledgements

LD is supported by the Spanish Ministry of Education and Science through the Juan de la Cierva Programme. Work in the laboratory of JM-P is supported by the Spanish Ministry of Education and Science through grant SAF2007-64696, the Spanish AIDS network Red Temática Cooperativa de Investigación en SIDA (RD06/0006) and the Fundación para la Investigación y Prevención del Sida en España (FIPSE) through grants 36356/05, 36523/05 and 36621/06.

Disclosure statement

The authors declare no competing interests.

References


