Evolution of resistance to drugs in HIV-1-infected patients failing antiretroviral therapy

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Background and objective: The optimal time for changing failing antiretroviral therapy (ART) is not known. It involves balancing the risk of exhausting future treatment options against the risk of developing increased drug resistance. The frequency with which new drug-resistance mutations (DRM) developed and their potential consequences in patients continuing unchanged treatment despite persistent viremia were assessed.

Design: A retrospective study of consecutive sequence samples from 106 patients at one institution with viral load (VL) of more than 400 copies/ml, with no change in ART for more than 2 months despite virologic failure.

Methods: Two consecutive pol sequences, CD4 cell counts and VL were analyzed to quantify the development of new DRM and to identify changes in immunologic and virologic parameters. Genotypic susceptibility scores (GSS) and viral drug susceptibilities were calculated by a computer program (HIVDB). Poisson log-linear regression models were used to predict the expected number of mutations at the second time point.

Results: After a median of 14 months of continued ART, 75% (80 of 106) of patients acquired new DRM and were assigned a significantly lower GSS, potentially limiting the success of future ART. The development of new DRM was proportional to the time between the two sequences and inversely proportional to the number of DRM in the first sequence. However, the development of DRM was not associated with significant changes in CD4 or VL counts.

Conclusions: Despite stable levels of CD4 and VL over time, maintaining a failing therapeutic regimen increases drug resistance and may limit future treatment options.

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Introduction

The identification of virologic failure during antiretroviral therapy (ART) raises complex management issues: when to switch to a different regimen, and which elements of the regimen to change. Prospective and retrospective clinical trials among patients failing ART demonstrate that using genotypic resistance testing to guide treatment may improve short-term virologic outcome. Some reports indicate that regimens

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containing more drugs to which plasma virus is susceptible have a greater likelihood of achieving durable suppression of virus replication than regimens containing fewer active drugs [1–9]. However, methods to identify fully and partially active drugs following resistance testing, and strategies for altering drug treatment are not well defined in those reports.

Prompt intervention is recommended in patients failing ART, to minimize emergence of cumulative drug resistance and cross-resistance, and to preserve future drug options [10]. However, observational studies and clinical trials suggest that a significant fraction of subjects continuing a failing regimen might have immunologic and virologic benefits. These benefits include the selection of virus with decreased replicative capacity and the maintenance of stable CD4+ cell counts [11–13].

Thus, it is unclear how rapidly an alternative treatment regimen should be instituted when virologic failure has been detected: Is it better to change a failing regimen immediately or to wait until either viral load (VL) or CD4 cell counts reach a certain threshold? Changing treatment due to virologic failure in patients whose CD4 cell count is stable or rising may prematurely exhaust treatment options, and particularly when there are few alternative treatment options continuing the failing regimen might be beneficial. On the other hand, continuing a failing regimen may lead to increased resistance and cross-resistance.

To assess the extent to which maintaining ART leads to the selection of increased drug resistance, we analyzed consecutive reverse transcriptase (RT) and protease sequences in patients continuing a regimen associated with persistent plasma viremia. Genotypic changes and the evolution of new drug resistance mutations (DRM) were used to calculate drug susceptibility and determine whether continued therapy with a failing regimen might limit future treatment options.

Material and methods

Study population
Between 1997 and 2003, genotypic testing was performed at Stanford University Hospital on 754 HIV-infected patients with known treatment history from one institution (Kaiser Permanente, Northern California, USA); 372 of these patients had two sequences performed. We identified those patients who had two genotypic resistance tests separated by more than 2 months without an intervening change in their drug regimen. Those who had a change in drug regimen within 2 months prior to the first sequence were excluded from the analysis. Treatment history was obtained by chart review. Maintenance treatment regimens were defined as antiretroviral drugs received during the time between the first and second sequences. Previous treatment regimens were defined as drugs to which patients had been exposed prior to the maintenance regimen (Fig. 1). We analyzed CD4 cell counts and VL levels collected at the start of the maintenance regimen, and at the first and second sequence time points.

HIV-1 RT and protease sequencing
Sequencing was performed using a previously described method [14]. Briefly, RNA was extracted from 0.2 ml of plasma using the guanidine–thiocyanate lysis reagent

Fig. 1. Study timeline. Median duration of exposure to antiviral therapy prior to the maintenance regimen (gray square), and during the maintenance regimen (black square), and how these relate to the time of the first and second sequences (bottom arrows). CD4 (cells × 10^6/l) and viral load (log_{10} copies/ml) at the three time-points are shown.
Resistance evolves with failing therapy

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Mutations

Mutations were defined as amino acid changes from the consensus B (http://hiv-web.lanl.gov). Positions containing mixtures of wild-type and mutant residues were classified as mutant. In agreement with International AIDS Society – USA drug-resistance testing guidelines [15], primary protease inhibitor (PI) resistance mutations were defined as mutations at protease positions 30, 46, 48, 50, 82 (but not V82I), 84 and 90; secondary PI resistance mutations were defined as mutations at protease positions 10, 20, 24, 32, 33, 36, 47, 53, 54, 71, 73, 77 and 88. Nucleoside RT inhibitor (NRTI) resistance mutations were defined as mutations at RT positions 100, 103, 106, 108, 181, 188, 190, 225, 230 and 236. Non-nucleoside RT inhibitor (NNRTI) resistance mutations were defined as mutations at RT positions 10, 20, 24, 32, 33, 36, 47, 53, 54, 71, 73, 77 and 88. Nucleoside RT inhibitor (NRTI) resistance mutations were defined as mutations at RT positions 41, 44, 62, 65, 67, 69, 70, 74, 75, 77, 115, 116, 118, 151, 184, 210, 215 and 219. Non-nucleoside RT inhibitor (NNRTI) resistance mutations were defined as mutations at RT positions 100, 103, 106, 108, 181, 188, 190, 225, 230 and 236.

Data analysis

For each patient, RT and protease DRM were identified in sequences isolated at two different times during the maintenance drug regimen. Mutations were defined as ‘acquired’ if they were found in the second sequence and not the first. Mutations were defined as ‘no longer detected’ if they were identified in the first sequence and not the second. RT (positions 1–240) and protease (positions 1–99) sequences were aligned and analyzed using the programs LAP (http://www.hgmp.mrc.ac.uk/Registered/Webapp/lap) and HIVSEQ [16].

Personal characteristics examined included: age, ART history, drug therapy duration, time between first and second sequences, CD4 cell counts, VL, DRM, and the predicted drug susceptibility and genotypic susceptibility score (GSS).

Drug susceptibility was scored by HIVDB, a computer program that uses publicly available mutation scoring tables that are based on published literature to assign levels of resistance to 17 approved RT and protease inhibitors [17]. Each drug resistance mutation is assigned a drug penalty score, and a total drug score is derived by adding the scores of each mutation associated with resistance to that drug. The program then reports a level of inferred drug resistance: susceptible (score 0–9), potential low-level resistance (10–14), low-level resistance (15–29), intermediate resistance (30–59) and high-level resistance (> 60).

For the GSS calculation each of the 17 drugs was assigned a value of 1, and the individual drug GSS was estimated as [1 – (drug score)/60]. Negative numbers (total drug score > 60) were assigned a value of 0. The ‘maintenance regimen’ GSS was calculated by summing up scores of drugs included in the maintenance regimen, with a range of 0 (most resistant) to 5 (least resistant). The ‘other drugs’ GSS was calculated by summing up scores of drugs not included in the maintenance regimen, with a range of 0 (most resistant) to 14 (least resistant).

Phylogenetic trees of paired sequences from each study patient confirmed the absence of laboratory cross-contamination. Subtypes were determined by inspecting phylogenetic relationships of each sequence segment to aligned segments of reference sequences, using bootscanning [18].

Comparisons between the results from the first and second sequences were performed using the Wilcoxon Signed-Ranks test. For each personal characteristic, measured at the first sequence time point, Poisson log-linear regression models were used to predict the expected number of DRM at the second time point. The main effect of the characteristic and its interaction with the time between sequences were assessed, adjusting for CD4, VL and number of previous drugs at the first time point.

Results

Patients and treatments

One hundred and six patients had two genotypic resistance tests separated by more than 2 months without an intervening change in drug regimen from at least 2 months prior to the first sequence until the second sequence. Median age was 45 years [interquartile range (IQR), 40–50 years] and 94% (100 of 106) were males. Median duration of drug therapy prior to the maintenance regimen was 36 months (IQR, 16–55 months). The maintenance regimen was the first regimen for 12 (11%) patients, the second to fourth regimen for 58 (55%), and the fifth to tenth regimen for 36 (34%) patients. Median duration of the maintenance regimen prior to the first sequence was 29 months (IQR, 19–48 months). The median time period between the two sequences was 14 months.
Mens were stavudine received only NRTIs. The most commonly used regimen was nelfinavir or indinavir (n = 13). The most commonly used NNRTIs were efavirenz (n = 18) and nevirapine (n = 10).

**Sequences and mutations**

All RT and protease sequences were subtype B, and in every case the two sequences from each individual were phylogenetically closer to one another than to sequences from other patients (data not shown). Seventy-five percent of patients (80 of 106) acquired new DRM between the two sequence time points, including PI-resistance mutations in 48 of 78 (62%) patients receiving PIs, NRTI-resistance mutations in 47 of 106 (44%) receiving NRTIs, and NNRTI-resistance mutations in nine of 31 (29%) receiving NNRTIs. The most commonly acquired mutations and the number of these mutations in the first sequence are shown in Figure 2. Twenty-seven (25%) patients acquired only a PI mutation, 20 (19%) acquired PI + NRTI mutations, 20 (19%) acquired only NRTI mutations, five (3%) acquired only NNRTI mutations, four (4%) acquired NRTI + NNRTI mutations, two (2%) acquired PI + NNRTI mutations and two (2%) acquired mutations rendering resistance to all three drug classes. In 21% of patients (22 of 106) one or more drug resistance mutations were no longer detected in the second sequence.

New mutations at RT and protease positions not associated with drug resistance developed in 80 of 106 (75%) patients: 63 of 106 (59%) acquired a RT mutation and 43 of 77 (56%) acquired a protease mutation. Most of these acquired RT mutations were at positions 135 (n = 8), 43 (n = 7), 122 (n = 6), 39, 60, 123, and 138 (n = 5 each), and 20, 200 and 214 (n = 4 each). Most of these acquired protease mutations were at positions 13 (n = 10), 62 (n = 5), and 35, 37, 43 and 89 (n = 4 each).

Table 1 shows comparisons between the first and second sequences. After continued treatment, a significantly greater number of DRM were identified in sequences from the second time point than from the first time point (a median of 10 versus eight mutations per pol gene sequence, P < 0.001), resulting in a lower median GSS both for the maintenance regimen (0.5 versus 0.3, P < 0.001) and for other drugs (7.7 versus 7.1, P < 0.001). Moreover, a greater number of drugs were predicted to be inactive by the second time point (median of six versus four at the first time point, P = 0.003).

**Virus load and CD4 cell changes**

Twenty-one patients (20%) experienced an increase in their CD4 cell counts and a decrease in their VL in the interval between the two time points. In 15 patients (15%) both CD4 and VL decreased, in 31 patients (30%) both CD4 and VL increased, and in 36 patients (35%) CD4 counts decreased and VL levels increased (Fig. 3a–d). Despite these differences, a similar proportion of each of these four subgroups acquired new DRM (67, 87, 74 and 75% respectively, P = 0.6, chi square test). There was no significant difference in the development of new mutations among the patients with rising or falling CD4 counts (71 versus 78%, P = NS) or among those with increasing or decreasing VL (74 versus 75%, P = NS). The ‘maintenance regimen’ GSS was significantly lower at the second time point than it had been at the first time point in each of the four groups. The ‘other drugs’ GSS declined in all groups except for the 31 patients in whom both CD4 and VL increased.

The results of the regression models are shown in Table 2. The presence of fewer mutations and a higher ‘other drugs’ GSS at the first time point were predictive of a greater frequency of developing new DRM at the second time point. The interaction of both of these characteristics with the time between sequences was also significant, although smaller in magnitude compared to the main effect of each of these variables. Longer intervals between the two time points increased the main predictive effect of the major protease mutations and decreased that of the ‘other drugs’ GSS.

The interval between the two time points was also significantly related to the number of newly acquired mutations: An increased interval was associated with a greater number of new DRM. The median time between sequences was 10 months in the 35 patients who acquired one mutation and 18 months in the 45 patients who acquired more than one mutation (range 2–8 mutations, P = 0.001, Mann–Whitney test).
Drug susceptibility assessment
We used HIVDB to predict the evolution of resistance to drugs in the maintenance regimen as well as to the remaining available antiretroviral drugs. The second sequence demonstrated either new or increased resistance to at least one drug in the maintenance regimen (range, 1–5) in 37 (35%) patients, and unchanged resistance in 69 (65%). A total of 49 of 106 (46%) patients developed high-level resistance to a median of three of all the remaining available drugs (IQR, 1–5 drugs) during this time interval and 61 of 106 (58%) patients developed a higher level of resistance to a mean of four remaining available drugs (IQR, 2–6) during this interval.

Discussion
Our observations provide insight into how uninterrupted, failing ART affects the development of DRM. These data show little change in CD4 and VL, despite
the continuation of the same ‘failing’ ART for a median of 14 months. However, there was a concurrent time-dependent and significant increase in drug resistance in the majority of patients, which may limit future treatment options. These data have implications for physicians who need to decide when to change treatment in patients with viremia, despite ART.

Our cohort, which had taken a median of six drugs during a 36-month period and continued a failing regimen for a median of 29 months, had median CD4 cell counts of 336 × 10⁶ cells/l and a median VL of 3.7 log₁₀ copies/ml. Continuing the failing regimen for a median of 14 additional months resulted in unchanged median CD4 counts (339 × 10⁶ cells/l) and a small rise in median VL (0.3 log₁₀). Despite little change in these parameters, RT and protease sequences from 75% of patients accumulated RT and/or protease DRM in that same time period. The evolution of drug resistance in these patients resulted in a significantly greater number of DRM per patient and lower genotypic susceptibility scores that indicate increased resistance to more of the available antiretroviral drugs, including those in the maintenance regimen.

Table 1. Comparison of characteristics between first and second sequence time points.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>First time point</th>
<th>Second time point</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 cells (cells × 10⁶/l)</td>
<td>336 (230–454)</td>
<td>339 (195–485)</td>
<td>0.8</td>
</tr>
<tr>
<td>Viral load (log₁₀ copies/ml)</td>
<td>3.7 (3.4–4.0)</td>
<td>4.0 (3.4–4.4)</td>
<td>0.002</td>
</tr>
<tr>
<td>Number of total resistance mutations</td>
<td>8 (5–12)</td>
<td>10 (6–14)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Number of PI resistance mutations in 77 PI treated patients</td>
<td>5 (3–7)</td>
<td>6 (4–7)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Number of NRTI mutations in 106 NRTI treated patients</td>
<td>4 (3–6)</td>
<td>5 (3–7)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Number of NNRTI mutations in 31 NNRTI treated patients</td>
<td>2 (1–2)</td>
<td>2 (1–2)</td>
<td>0.2</td>
</tr>
<tr>
<td>‘Maintenance regimen’ genotypic susceptibility score</td>
<td>0.5 (0.1–1.2)</td>
<td>0.3 (0–0.9)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>‘Other drugs’ genotypic susceptibility score</td>
<td>7.7 (4.5–10.1)</td>
<td>7.1 (4.3–9.6)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

CD4 and VL for both sequence time points were available for only 103 of 106 patients. *Calculated using the Wilcoxon sum-of-ranks test. IQR, interquartile range; PI, protease inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor.

CD4 and VL for both sequence time points were available for only 103 of 106 patients. *Calculated using the Wilcoxon sum-of ranks test. IQR, interquartile range; PI, protease inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor.

Fig. 3. Mutations that were gained, ‘maintenance regimen’ genotypic susceptibility score (GSS) and ‘other drugs’ GSS according to CD4 (cells × 10⁶/l) and VL (log₁₀ copies/ml) level patterns between the two sequence time-points (solid lines). Dashed lines indicate median CD4 and VL levels between the start of the maintenance regimen and the first sequence time-point. (a) CD4 increase, VL decrease; (b) CD4 decrease, VL decrease; (c) CD4 increase, VL increase; (d) CD4 decrease, VL increase (67, 87, 74 and 75% gained resistance mutations, respectively). Data were available for 103 of 106 patients.
over, 60% of patients continuing a failing drug regimen became more resistant to a median of four drugs, which for most was a high level of resistance. Resistance mutations accumulated in all subgroups regardless of VL or CD4 trajectory.

The extent of resistance mutation acquisition may reflect the diversity of latent quasi-species within HIV-infected individuals; viral populations harboring resistance mutations from previous drug exposure may become evident after sustained drug pressure and virus replication. In the 21% of patients that appeared to have lost a drug-resistant mutation during the interval between the first and second sequences, it is likely that the mutation is retained in the population of archived virus, and was undetected due to insensitivity of population sequencing [19,20]. Alternatively, some mutations may decrease in prevalence because they either no longer contribute to drug resistance or do not provide a replicative advantage in the face of additional mutations.

The strongest predictor of the number of new DRM was the number of major protease mutations at the first sequence time point; fewer mutations were associated with the acquisition of increased numbers of new mutations by the second time point. The risk of acquiring new mutations was also higher with increasing time between the first and second sequences. This suggests a ceiling effect, in which patients with many mutations at the first time point are at decreased risk of developing new mutations. Similar analyses performed according to specific drug-class exposure did not reveal new predictors of DRM acquisition.

Longer duration of unchanged drug treatment was associated with increased selection of drug resistance regardless of the VL or immune response during virologic failure. Of the 106 patients in our study, 39 (37%) with VL less than 5000 copies/ml and 37 (35%) with CD4 counts greater than 350 × 10^6 cells/l (19 overlapping patients) acquired DRM between the first and second time points. Mutations were acquired even in 12 of 13 patients who maintained a VL of 50–999 copies/ml and by 11 of 13 patients with VL 1000–1999 copies/ml. These results agree with other studies that show loss of drug susceptibility and selection of DRM even at low levels of viral replication [3,21,22], as well as in later stages and in patients with a VL–CD4 discordance [23,24]. The level of VL, as a surrogate for replication rate during viral failure, does not appear to predict the rate of accumulation of resistance mutations.

Acquisition of RT and protease mutations at positions not usually associated with drug resistance was also observed. Many of these, including protease positions 11, 13, 22, 35, 43, 45, 55, 58, 62, 66, 72, 75, 76, 79, 85, 89 and 92; and RT positions 20, 39, 43, 203, 208, 218, 221, 223 and 228 (observed in 46 patients), have recently been described as treatment-associated mutations [25,26], which further substantiates our theory of ongoing selection pressure leading to the evolution of drug resistance. As the role of these mutations in resistance and fitness has yet to be established, these mutations were not accounted for by the algorithm used to detect drug resistance, which uses only commonly accepted mutations.
In summary, early studies of drug resistance demonstrated ordered accumulation of DRM in patients receiving ART regimens, that we now know were insufficiently potent [30–32]. Here we illustrate a time-dependent accumulation of DRM during a failing drug regimen. Persistence of viral replication despite stable CD4 cell numbers leads to the selection of viruses that are potentially more resistant, perhaps diminishing future treatment options.

References


Appendix

The GenBank accession numbers of the sequences from this study are AF514048, AF514073, AF514080, AF514082, AF514087, AF514093, AF514098, AF514125, AF514155, AF514162, AF514165, AF514169, AF514195, AF514205, AF514209, AF514219, AF514224, AF514228, AF514231–2, AF514236, AF514239, AF514251, AF544456, AF544469, AF544478, AF544487, AF544489, AF544497, AF544501, AF544518, AF544533, AF544539, AF544540, AF544542, AF544557, AF544565, AF544569, AF544579, AF544583, AF544587–9, AF544593, AF544595, AY030511, AY030548, AY030553, AY030609, AY030626, AY030643, AY030656, AY030749, AY030805, AY030878, AY030934, AY030954, AY030986, AY031133, AY031356, AY031414, AY031432, AY031454, AY031494, AY031762, AY031792, AY031799, AY031861, AY032006, AY032020, AY032030, AY032042, AY032178, AY032217, AY032264, AY032317, AY032325, AY032370, AY032452, AY032496, AY047432, AY047472, AY305908, AY305915, AY305919, AY305923–5, AY305927–8, AY305938, AY305942, AY305957, AY305967, AY305976, AY305983, AY305990, AY305993, AY305995, AY559503—AY559728.