Comparison of levels of HIV-1 resistance to protease inhibitors by recombinant versus conventional virus phenotypic assay and two genotypic interpretation procedures in treatment-naive and HAART-experienced HIV-infected patients

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Objectives: To compare genotypic and phenotypic HIV-1 drug resistance assays.

Methods: Protease inhibitor (PI) susceptibility was phenotypically analysed in HIV-1 isolates and recombinant viruses expressing proteases from viral isolates. Two genotypic interpretation methods were carried out in parallel.

Results: Entirely concordant resistance levels were shown in 5/10 (50%) highly active anti-retroviral therapy (HAART)-experienced patients, whereas minor discrepancies were observed in the remaining five patients.

Conclusions: The four assays provide comparable results. The recombinant virus phenotypic assay may provide the most accurate evaluation of resistance; however, genotypic interpretation procedures are helpful for daily therapeutic decisions.

Introduction

Selection of human immunodeficiency virus 1 (HIV-1) variants with decreased drug susceptibility caused by mutations in the viral reverse transcriptase and protease genes represents the major cause of highly active anti-retroviral therapy (HAART) failure.1 Thus, the use of tests for evaluation of antiviral drug resistance is now recommended in view of selecting appropriate rescue therapies.2 Both genotypic and phenotypic methods for drug resistance determination are now available;3 however, these assays lack standardization and often present discrepancies between determination of HIV-1 genotypic and phenotypic drug resistance. This is particularly applicable for the determination of protease inhibitor (PI) resistance. In particular, prediction of PI resistance level on the basis of protease gene mutations alone still appears to be a controversial issue.4 This may be caused by cross-resistance phenomena and the complex interactions among different resistance mutations. Here, we compare two phenotypic and two genotypic methods for determination of PI resistance.

Materials and methods

Samples from 18 HIV-1-infected individuals (eight treatment-naive and 10 patients failing antiretroviral combination treatments including an HIV-1 PI) were analysed. HIV-1 protease nucleic acid sequence was amplified from isolate supernatants by RT–PCR and from peripheral blood mononuclear cells (PBMC) samples by PCR as reported.5 PCR products were cloned in pCR 2.1 vector (TA Cloning Kit; Invitrogen, Groningen, The Netherlands) and single positive clones were sequenced directly (ABI PRISM 377XL

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DNA Sequencer; Applied Biosystems, Foster City, CA, USA). The distribution of PI resistance-associated mutations was evaluated in 10 protease gene clones from each viral isolate and 10 protease gene clones from each of the relevant PBMC samples of both treatment-naive and HAART-experienced patients.

The conventional drug susceptibility assay was carried out using HIV-1 isolates recovered by culturing PBMC from patients with PBMC from healthy donors as reported. For each drug, the degree of inhibition of viral replication (measured by determination of the p24 concentration in the cell culture supernatant) was expressed as the fold increase in the IC50 with respect to the relevant mean IC50 value of the eight treatment-naive patients. Drug resistance values were expressed as susceptible, low-, intermediate- and high-resistance (≤3.0-, >3.0–6.0-, >6.0–10.0- and >10.0-fold change, respectively).

In order to carry out the recombinant drug susceptibility assay, HIV-1 protease PCR products were cloned into the pΔProΔenv vector to reconstitute a non-replicative HIV-1 vector. Ten positive clones with the proper insert were obtained from each of 10 cell culture supernatants relevant to viral isolates recovered from each patient. In addition, 10 recombinant clones from each of five treatment-naive patients, respectively, were chosen as relative controls. Recombinant clones showing the widest number of mutations were selected for the susceptibility assay. Plasmid DNA was then transfected into 30% confluent HeLa cells. After 6 h, supernatant was removed and replaced with four-fold dilutions of PI. No-drug controls were included for each drug dilution. After 48 h incubation, HIV-1 p24 concentration in the supernatants was quantified (NEN Research Product, Boston, MA, USA). The degree of inhibition of p24 expression was determined as described above.

As for genotypic drug resistance analysis, protease sequences from recombinant HIV-1 isolates were fed into the Stanford software system (http://hiv-4.stanford.edu/cgi-test/hivtest-web.pl), which assigns a score to each mutation based on the correlation between genotype and phenotype, genotype and treatment history, and genotype and clinical outcome, as inferred from the published literature. A second method utilizing a simplified score calculation system based on the reported IC50 fold increase associated with each mutation modified to obtain four levels of resistance instead of three (susceptible, <3; low, 3–6; moderate, 7–9; high, ≥10 IC50 fold increase) was carried out in parallel.

Results

At the time of analysis, median HIV RNA copies in plasma of treatment-naive and HAART-experienced patients were 39,136 copies/mL (range 8702–125,539) and 19,693 copies/mL (range 4922–193,953), whereas median CD4+ T cell counts were 404 cells/mm3 (range 77–1218) and 161.5 cells/mm3 (range 30–649), respectively. As shown in Table 1, HAART-experienced patients had been treated with 2–4 PI for a median time of 9.5 months (range 1–22), in combination with different reverse transcriptase inhibitors. Sequencing of the cloned protease gene from RNA of HIV-1 viral isolates from treatment-naive patients did not show drug resistance-associated mutations. In contrast, sequencing of protease gene clones from viral isolates recovered from HAART-experienced patients showed major and secondary drug resistance-associated mutations in six patients (nos 4–9). Interestingly, major PI resistance-associated mutations were often present only in a minority of clones (Table 1). It must be noted that the comparative analysis of protease mutations in clones from PBMC and viral isolate samples demonstrated that HAART-experienced patients 1, 2, 3 and 10, who did not display major PI resistance mutations in clones from viral isolates, harboured protease mutations conferring high levels of PI resistance (D30N, M46I; D30N; V82A, L90M; and M46I, I84V, L90M, respectively) in PBMC clones (data not reported).

Mean IC50 values of each PI for eight HIV-1 isolates (saquinavir, 0.005 ± 0.002 μM; ritonavir, 0.02 ± 0.01 μM; indinavir, 0.02 ± 0.01 μM; nelfinavir, 0.02 ± 0.03 μM; amprenavir, 0.01 ± 0.01 μM) and five protease recombinant HIV-1 strains (saquinavir, 0.007 ± 0.002 μM; ritonavir, 0.12 ± 0.05 μM; indinavir, 0.029 ± 0.01 μM; nelfinavir, 0.068 ± 0.07 μM; amprenavir, 0.11 ± 0.04 μM) from treatment-naive patients were found to be comparable (Mann–Whitney U-test for non-parametric data, P > 0.05).

Comparison of the four drug resistance assays in HAART-experienced patients demonstrated that resistance levels obtained in 5/10 (50%) patients were concordant with all four assays. In fact, HIV-1 isolates from 3/10 (30%) patients (nos 1–3) were found to retain full sensitivity to PI, whereas HIV-1 isolates from 2/10 (20%) patients (nos 4 and 8) showed complete resistance to all PIs independent of the assay used (data not reported). In contrast, in the other five patients (nos 5, 6, 7, 9 and 10) slightly discrepant resistance levels were obtained for the five drugs tested (total = 25 combinations) using the four assays (Table 2). In detail, when comparing results with those obtained by the isolate phenotypic assay, the recombinant virus phenotypic assay provided a discrepant resistance level in 4/25 (16%) combinations, whereas the Stanford genotypic interpretation method and our simplified score calculation system provided different results in 9/25 (36%) and 6/25 (24%) combinations, respectively (Table 2). The differences among assays were restricted to a shift from one to the next superior or inferior class of resistance. In most cases, discrepancies were relevant to detection of a degree of resistance higher by one class with respect to that detected by the isolate phenotypic assay.
### Table 1. Characteristics of the 10 HAART-experienced patients examined and distribution of PI resistance-associated mutations in HIV-1 protease clones from viral isolates and in the relevant recombinant viruses

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>CD4 cells (cells/mm³)</th>
<th>HIV RNA copies/mL plasma</th>
<th>Ongoing therapy</th>
<th>Previously administered PI (months of exposure)</th>
<th>Mutations conferring PI resistance in HIV isolates (no. of clones with the indicated mutation)</th>
<th>Protease mutations in selected recombinant viruses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>134</td>
<td>193 953</td>
<td>d4T+ABC+NVP</td>
<td>SQV(12), IDV(4)</td>
<td>M36I(10), L63P(4), A71V(10)</td>
<td>M36I, L63P, A71V</td>
</tr>
<tr>
<td>2</td>
<td>151</td>
<td>28 305</td>
<td>3TC+d4T</td>
<td>SQV(10), RTV(10), IDV(18)</td>
<td>M36I(1)</td>
<td>M36I</td>
</tr>
<tr>
<td>3</td>
<td>649</td>
<td>16096</td>
<td>3TC+ABC+d4T</td>
<td>SQV(22), RTV(1), IDV(13), NVP(1)</td>
<td>L63P(10)</td>
<td>L63P</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>23 290</td>
<td>EFV+d4T+NFV</td>
<td>RTV(8), IDV(2), NFV(5)</td>
<td>L10I(10), M46I(10), I54V(10), L63P(10), V82A(10), L90M(10)</td>
<td>L10I, M46I, I54V, L63P, V82A, L90M</td>
</tr>
<tr>
<td>5</td>
<td>172</td>
<td>55 298</td>
<td>EFV+d4T+NFV</td>
<td>SQV(8), IDV(10), NFV(12)</td>
<td>L10I(10), K20R(3), M36I(10), G48V(3), L63P(10), G73S(5), V82I(5), L90M(5)</td>
<td>L10I, M46I, V77I, L90M</td>
</tr>
<tr>
<td>6</td>
<td>226</td>
<td>4922</td>
<td>d4T+RTV+IDV</td>
<td>RTV(2), IDV(2), NFV(14)</td>
<td>L10I(10), M46I(10), I54V(10), L63P(10), A71V(7), G73S(3), V77I(2), V82A(7), L90M(5)</td>
<td>L10I, M46I, M36I, G73S, V82I, L90M</td>
</tr>
<tr>
<td>7</td>
<td>146</td>
<td>10 201</td>
<td>3TC+RTV+IDV</td>
<td>RTV(5), IDV(19), NFV(10)</td>
<td>L10I(10), K20I(3), V32I(7), M36I(6), M46I(10), L63P(10), A71V(7), G73S(3), V77I(2), V82A(7), L90M(5)</td>
<td>L10I, M46I, L63P, A71V, V82A, L90M</td>
</tr>
<tr>
<td>8</td>
<td>276</td>
<td>6484</td>
<td>EFV+d4T+NFV</td>
<td>SQV(18), RTV(8), NFV(9)</td>
<td>M46I(10), G48V(3), L63P(10), G73S(10), V77I(10), V82A(4), I84V(10), L90M(10)</td>
<td>M46I, L63P, G73S, V77I, V82A, I84V, L90M</td>
</tr>
<tr>
<td>9</td>
<td>298</td>
<td>15 632</td>
<td>EFV+d4T+NFV</td>
<td>SQV(10), IDV(5), NFV(15)</td>
<td>L10I(10), G48V(10), I54V(2), L63P(10), A71V(10), V77I(10), V82A(10)</td>
<td>L10I, G48V, I54V, L63P, A71V, V77I, V82A</td>
</tr>
<tr>
<td>10</td>
<td>66</td>
<td>64 328</td>
<td>DDI+3TC+d4T+EFV</td>
<td>SQV(4), IDV(2), NFV(15)</td>
<td>L10I(10), L63P(3), V77I(10)</td>
<td>L10I, L63P, V77I</td>
</tr>
</tbody>
</table>

d4T, stavudine; ABC, abacavir; NVP, nevirapine; 3TC, lamivudine; EFV, efavirenz; DDI, didanosine; SQV, saquinavir; RTV, ritonavir; IDV, indinavir; NFV, nelfinavir.
Discussion

The benefits of HIV-1 drug resistance evaluation have been investigated in retrospective and prospective clinical studies. However, no consensus has been reached on which assay, either genotypic or phenotypic, should be used in clinical research and medical practice.\textsuperscript{2-4}

We compared the results of the recombinant virus phenotypic assay with those of the conventional isolate phenotypic assay, showing that in 4/25 determinations the latter indicated lower resistance levels. This could be explained by the fact that the isolate phenotypic test evaluates the mean resistance level of a pool of viral variants obtained from the patient’s PBMC, whereas the recombinant phenotype test documents the level of resistance of the viral variant carrying the most mutated protease gene.

When comparing the isolate phenotypic assay with the genotypic interpretation method and our score calculation system, discrepant results were observed in 9/25 (36%) and 6/25 (24%) combinations, respectively. These results might be explained by the fact that phenotypic assays are based on the replicative capacity in the presence of PIs, whereas the genotypic interpretation methods are based on historical clinical and phenotypic data that may now be superseded.\textsuperscript{10}

This study focused on the analysis of PI resistance of HIV-1 isolates from HAART-experienced patients using four different methods. The results showed that even using a single biological material as a source for HIV-1 sequences some discrepancies between the four assays could be detected. Thus, it seems conceivable that additional factors (such as the presence of drug-resistant variants in different biological samples) might affect the prediction of treatment failure to an even greater extent. In this respect, it is interesting that cloning of viral sequences from PBMC of HAART-experienced patients nos 1, 2, 3 and 10, who did not display PI resistance mutations in clones from viral isolates, revealed the presence of HIV-1 strains with PI resistance-associated mutations.\textsuperscript{6}

\begin{table}[h]
\centering
\caption{Results of four HIV-1 susceptibility assays to PIs in five HAART-experienced patients with discrepant results}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Patient no. & Drug & Isolate phenotypic assay & Recombinant virus phenotypic assay & Genotypic interpretation \\
\hline
 & & fold increase\textsuperscript{a} & resistance level & fold increase & resistance level\textsuperscript{b} & Stanford method\textsuperscript{b} & our simplified score\textsuperscript{b} \\
\hline
5 & SQV & 20 & H & 17.1 & H & M & H \\
RTV & 6 & L & 4.16 & L & M & M \\
IND & 6 & L & 6.9 & M & M & M \\
NFV & 20 & H & 12.5 & H & H & H \\
APV & 5 & L & 3.18 & L & M & M \\
6 & SQV & 20 & H & 500 & H & M & H \\
RTV & 40 & H & >100 & H & M & M \\
IND & 30 & H & 120 & H & M & M \\
NFV & 20 & H & 127.9 & H & H & H \\
APV & 7 & M & 60.9 & H & M & M \\
7 & SQV & 10 & M & 100 & H & H & H \\
RTV & 160 & H & 60 & H & H & H \\
IND & 25 & H & 241 & H & H & H \\
NFV & 20 & H & 61.7 & H & H & H \\
APV & 14 & H & 16.3 & H & H & H \\
8 & SQV & 160 & H & 1828 & H & H & H \\
RTV & 81 & H & >100 & H & H & H \\
IND & 50 & H & >551 & H & H & H \\
NFV & 46 & H & >217 & H & H & H \\
APV & 14 & H & 77.2 & H & M & H \\
9 & SQV & <1 & S & 5 & L & S & S \\
RTV & <1 & S & 0.75 & S & S & S \\
IND & 1 & S & 0.68 & S & S & S \\
NFV & <1 & S & 0.29 & S & S & S \\
APV & 1 & S & 0.09 & S & S & S \\
10 & SQV & <1 & S & 5 & L & S & S \\
RTV & <1 & S & 0.75 & S & S & S \\
IND & 1 & S & 0.68 & S & S & S \\
NFV & <1 & S & 0.29 & S & S & S \\
APV & 1 & S & 0.09 & S & S & S \\
\hline
\end{tabular}
\begin{flushright}
SQV, saquinavir; RTV, ritonavir; IDV, indinavir; NFV, nelfinavir; APV, amprrenavir; S, susceptible; L, low; M, moderate; H, high.
\end{flushright}
\begin{flushright}
\textsuperscript{a}Fold increase in IC\textsubscript{50} relative to the mean IC\textsubscript{50} for treatment-naïve patients.
\end{flushright}
\begin{flushright}
\textsuperscript{b}Recombinant phenotypic and genotypic resistance levels that were found to be different from those obtained by the conventional isolate phenotypic assay are indicated in bold type.
\end{flushright}
\end{table}
In conclusion, a combined (phenotypic and genotypic) analysis of a large number of recombinant plasmid clones for each patient (particularly if derived from PBMC and plasma HIV-1 sequences) would provide the best prediction of the resistance level. However, this approach cannot be applied to wide clinical settings because of its complexity. In contrast, available genotypic interpretation methods remain, at present, a useful laboratory tool for routine application.

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