A Novel Approach to Assessing the Drug Susceptibility and Replication of Human Immunodeficiency Virus Type 1 Isolates

Robert M. Jellinger, Robert W. Shafer, and Thomas C. Merigan

Division of Infectious Diseases and Geographic Medicine, Stanford University Medical Center, Stanford, California

Human immunodeficiency virus type 1 (HIV-1) drug susceptibility testing is often curtailed because such testing is expensive and time consuming. A colorimetric tetrazolium dye method previously used for high-throughput antiviral drug screening was adapted to assess the susceptibility of 16 HIV-1 isolates to zidovudine, didanosine, lamivudine, and nevirapine in MT-2 cells. Cell viability was assessed colorimetrically, and all measurements and calculations were automated. Each HIV-1 isolate was tested in ≥5 assays to determine the reproducibility of the assay in HIV-1 isolates with known reverse-transcriptase mutations. The drug susceptibility of several mutant HIV-1 strains whose drug susceptibilities had not previously been well defined was also determined. Data on HIV-1 replication from the susceptibility assays indicated that some mutant HIV-1 isolates may have been less cytopathic in MT-2 cells than wild type HIV-1 isolates.

Methods

Virus isolates. We studied wild type HIV-1 isolates derived from clones λHXB2 and pNL4-3 and a multiply passaged syncyti- um-inducing clinical isolate, H112-2, obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD) (table 1). Mutant HIV-1 included the following groups of isolates (table 1): (1) 3 HIV-1 strains created by site-directed mutagenesis (Medical Research Council AIDS Reagent Program, London); (2) 2 zidovudine-resistant clinical isolates (NIH AIDS Research and Reference Reagent Program; the reverse-transcriptase [RT] sequence of these isolates was determined in our laboratory); (3) 3 multinucleoside-resistant isolates created by site-directed mutagenesis of pNL4-3 [6]; (4) an isolate containing Y181C (HIV-1mt), created by passing NL4-3 in the presence of increasing concentrations of nevirapine, and 2 isolates containing mutations at codon 184 (M184I and M184V; both designated HIV-1184) created by passaging NL4-3 in the presence of increasing concentrations of lamivudine; (5) 3 HIV-1 isolates created by homologous recombination of polymerase chain reaction (PCR)–amplified RT fragments from patient plasma HIV-1, with an RT-deleted noninfectious proviral HIV-1 clone (Δ2–262; provided by W. Keulen, Academic Medical Center, University of Amsterdam) [7, 8]. Each of these 3 HIV-1 isolates was derived using PCR-amplified RT fragments from non-syncyti-um-inducing strains.

Susceptibility assay. MT-2 cells were used for propagating virus and for susceptibility testing. The cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% l-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL). Medium with phenol red was used for virus propagation, and phenol red–deficient medium was used for susceptibility testing.

One flat-bottomed 96-well low evaporation plate (Costar, Cambridge, MA) was used for each susceptibility assay. MT-2 cells (104) were added to each well in a final volume of 200 μL. Twenty wells were uninfected control wells. Sixteen wells were used to titrate HIV-1; these wells were drug-free and contained 8 serial 3-fold dilutions of virus stock in duplicate. The remaining 60 wells of the plate contained the 6 most concentrated virus stock dilutions tested against 5 different drug concentrations in duplicate.

For zidovudine susceptibility assays, the drug concentrations were 0.02, 0.1, 0.5, 2.5, and 12.5 μM. For didanosine (dDI) susceptibility assays, the drug concentrations were 2, 4, 8, 16, and 32 μM. For lamivudine susceptibility assays, the drug concentrations...
were 0.032, 0.16, 0.8, 4, and 20 μM. For nevirapine susceptibility assays, the drug concentrations were 0.032, 0.16, 0.8, 4, and 20 μM. To examine for the possibility of cytotoxicity, the 20 virus-free control wells contained each of the 5 serial dilutions of drug (4 wells each).

Drug susceptibility plates were incubated at 37°C in 5% CO₂. After 6 days, 50 μL of a solution containing 1.5 μM XTT (Polysciences, Warrington, PA) and 125 μM phenazine methosulfate (Sigma, St Louis) were added to all 96 wells. Plates were sealed with tape, agitated to evenly distribute the XTT, and incubated for 4 h. After incubation, the plates were placed in a V-Max spectrophotometer ( Molecular Devices, Menlo Park, CA). The optical densities (ODs) of each well were read at a test wavelength of 450 nm and a reference wavelength of 650 nm and imported into a spreadsheet program.

Wells were considered positive for HIV-1 infection if the OD for the well was less than the lowest OD of the 20 control wells and <2 SD below the mean OD of control wells. This cutoff was a conservative indicator of viral infection because, in preliminary experiments, 3.2-fold more virus stock was required to meet this cutoff than to cause HIV-1-induced syncytium formation (95% confidence interval [CI]: 2.5–4.4-fold). In addition, this cutoff was a conservative indicator of viral infection because, in preliminary experiments, 3.2-fold more virus stock was required to meet this cutoff than to cause HIV-1-induced syncytium formation (95% confidence interval [CI]: 2.5–4.4-fold).

After establishing the cutoff for a positive well and using this value to determine which wells contained a TCID₅₀ of 27–45, we assessed the effects of different drug concentrations on cell viability. The drug required to prevent a reduction in OD compared with the uninfected control wells. The drug concentration required to prevent a 50% reduction in OD was defined as the IC₅₀ and was calculated using the mean effect equation [10].

**HIV-1 RT sequencing.** Cell pellets saved at the time of stock creation were digested with proteinase K, and the resulting lysate was run on nested PCR using primer pairs RT18/RT21 and RT19/RT20 (described in [11]). Direct sequencing of PCR product was done using overlapping internal primers, Taq polymerase, and dye-labeled dideoxy-terminators (Applied Biosystems, Foster City, CA). The RT sequence between codons 28 and 246 was examined.

**Statistical analysis.** All statistical descriptions of groups of IC₅₀s were done on the log-transformed IC₅₀s (i.e., geometric means were used instead of arithmetic means). Student’s t test was used to assess the statistical significance of differences in susceptibility between wild type and mutant HIV-1 isolates. For purposes of analysis, IC₅₀s ≥ 100 μM were assigned an IC₅₀ equal to 100 μM. In addition, to assess differences in susceptibility nonparametrically, each IC₅₀ result on mutant HIV-1 isolates was compared with the highest IC₅₀ obtained during tests of wild type HIV-1 isolates.

### Results

**HIV-1 drug susceptibility test results.** Table 2 shows the IC₅₀s of laboratory and clinical HIV-1 isolates with known drug resistance mutations that were tested for susceptibility to zidovudine, ddI, lamivudine, and nevirapine. There was a statistically significant increase in the IC₅₀ of every isolate with a known drug resistance mutation compared with wild type
Table 2. Drug susceptibilities of wild type and mutant HIV-1 isolates.

<table>
<thead>
<tr>
<th>Drug*</th>
<th>Reverse-transcriptase mutations</th>
<th>No. of tests</th>
<th>IC₅₀*</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zidovudine</td>
<td>HIV-1WT</td>
<td>None</td>
<td>16</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>HIV-1215</td>
<td>T215Y</td>
<td>9</td>
<td>0.4</td>
</tr>
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<td></td>
<td>HIV-1151</td>
<td>Q151M</td>
<td>7</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>HIV-141215</td>
<td>M41L, T215Y</td>
<td>5</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>HIV-17577,151</td>
<td>V75I, F77L, Q151M</td>
<td>5</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>HIV-141210,215</td>
<td>M41L, L210W, T215Y</td>
<td>7</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>HIV-17577,116,151</td>
<td>V75I, F77L, F116Y, Q151M</td>
<td>7</td>
<td>79</td>
</tr>
<tr>
<td>Didanosine</td>
<td>HIV-1WT</td>
<td>None</td>
<td>25</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>HIV-174</td>
<td>L74V</td>
<td>8</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>HIV-1151</td>
<td>Q151M</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>HIV-17577,151</td>
<td>V75I, F77L, Q151M</td>
<td>8</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>HIV-1G908-88</td>
<td>V75I, F77L, F116Y, Q151M</td>
<td>5</td>
<td>99</td>
</tr>
<tr>
<td>Lamivudine</td>
<td>HIV-1WT</td>
<td>None</td>
<td>5</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>HIV-17577,151</td>
<td>V75I, F77L, Q151M</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>HIV-1184I</td>
<td>M184I (and M184V)</td>
<td>8</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Nevirapine</td>
<td>HIV-1WT</td>
<td>None</td>
<td>5</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>HIV-1181C</td>
<td>Y181C</td>
<td>5</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>HIV-1181C</td>
<td>Y181C</td>
<td>5</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

NOTE. NA, not applicable; CI, confidence interval.
* Tests on HIV-1WT included isolates H112-2, HXB2, and NL4-3; 8 tests on HIV-141215 included 5 on M184I and 3 on M184V.

IC₅₀ is geometric mean of IC₅₀s of replicate tests. Highest drug concentrations in assays were 12.5 μM for zidovudine, 32 μM for didanosine, and 20 μM for lamivudine and nevirapine. IC₅₀s higher than highest drug concentration represents extrapolations using median-effect equation. Extrapolated IC₅₀ results are less precise than IC₅₀ results that are within range of drug concentrations used.

(HIV-1WT; P < .05, Student’s t test), as shown by the nearly complete absence of overlap between the 95% CI of the estimated geometric mean IC₅₀ of wild type and mutant HIV-1 isolates (table 2).

Zidovudine IC₅₀s for HIV-1215 and HIV-1151 were ~5-fold greater than that of wild type HIV-1. HIV-141,215 and HIV-17577,151 had zidovudine IC₅₀s ~40-fold greater than those of wild type, and HIV-141,67,70,215,219, HIV-141,210,215, and HIV-17577,116,151 had zidovudine IC₅₀s ~600-fold greater than those of wild type.

HIV-174 and HIV-1151 had ddI IC₅₀s ~3-fold greater than HIV-1WT, HIV-17577,151 had a ddI IC₅₀ ~6-fold greater than wild type, and HIV-17577,116,151 had a ddI IC₅₀ ~30-fold greater than wild type. HIV-17577,116,151 had a ddI IC₅₀ ~30-fold greater than that of wild type.

HIV-141215 had lamivudine IC₅₀ >100-fold greater than wild type, and HIV-17577,151 had a lamivudine IC₅₀ ~10-fold greater than wild type. HIV-181C had a nevirapine IC₅₀ >100-fold greater than wild type. HIV-1181C, a recombinant virus derived from a patient treated with nevirapin, had a nevirapine IC₅₀ >100-fold greater than wild type.

Reproducibility of drug susceptibility testing. Each isolate was tested in ≥ 5 assays to obtain reproducibility data and to improve our estimates of the mean IC₅₀. Wild type HIV-1 strains were tested for zidovudine susceptibility in 16 assays and for ddI susceptibility in 25 assays. Resistance could then be defined as having an IC₅₀ greater than the highest IC₅₀ obtained in all assays of wild type HIV-1 isolates.

The zidovudine IC₅₀ of HIV-1215 was greater than the highest IC₅₀ of a wild type HIV-1 isolate (0.3 μM) in 7 (78%) of 9 assays. In the 2 assays in which HIV-1215 was not resistant, its IC₅₀ was at the upper limit of the range for wild type isolates (0.27 and 0.30 μM). The other isolates with zidovudine resistance mutations (HIV-1151, HIV-141,215, HIV-17577,151, HIV-141,210,215, HIV-141,67,70,215,219, and HIV-17577,116,151) were resistant to zidovudine in 37 of 37 assays.

HIV-174 was resistant to ddI in 6 (86%) of 7 assays. The remaining isolates with ddI resistance mutations (HIV-1151,
HIV-1_H739 [L74V + M184V], HIV-1_{75,77,151}, HIV-1_{75,77,116,151}, and HIV-1_{GW908-88} [V75I, F77I, F116Y, Q151M] were resistant to ddI in 34 of 34 tests.

HIV-1_{184} was resistant to lamivudine in 5 of 5 tests, and HIV-1_{75,77,151} was resistant to lamivudine in 4 of 5 tests. HIV-1_{184} and HIV-1_{LR-8} were resistant to nevirapine in all 10 tests.

Cytopathic effects of mutant HIV-1 isolates. To determine whether differences in the RT genotype could influence an isolate's cytopathic effect, we compared the cytopathic effects of HIV-1 isolates genetically identical, except for specific RT mutations. One set of isolates (HIV-1_{151}, HIV-1_{75,77,151}, and HIV-1_{75,77,116,151}) was derived by site-directed mutagenesis of pNL4-3. Another set of isolates (HIV-1_{74}, HIV-1_{215}, and HIV-1_{41,215}) was derived by site-directed mutagenesis of lambda-HXB2. The OD percent reduction (compared with uninfected control wells) was examined in each drug-free well with a TCID_{50} of 27–45 from each susceptibility assay of the 8 isolates.

At a mean TCID_{50} of 36, the wild type isolates, HIV-1_{NL4-3} and HIV-1_{HXB2}, caused a mean reduction in OD of 85% (95% CI, 81%–89%) and 87% (95% CI, 82%–92%), respectively. There were no significant differences in the extent of cytopathic effect caused by these 2 wild type isolates and HIV-1_{215}, HIV-1_{41,215}, and HIV-1_{151} (figure 1). However, the 3 multinucleoside-resistant isolates (HIV-1_{151}, HIV-1_{75,77,151}, and HIV-1_{75,77,116,151}) each caused lower mean OD reductions than the wild type HIV-1_{NL4-3}. This difference was significant for HIV-1_{151} and HIV-1_{75,77,151}, which at a mean TCID_{50} of 38 caused mean OD reductions of 67% (95% CI, 57%–77%; P = .01) and 72% (95% CI, 65%–79%; P = .02), respectively.

Discussion

Current methods for detecting HIV-1 drug resistance include phenotypic cell culture assays and genotypic assays for mutations known to confer drug resistance. Genotypic methods such as DNA sequencing are useful when there is a strong correlation between specific mutations and drug resistance. Phenotypic methods are needed when the genetic mechanisms of resistance to a treatment regimen have not been fully defined. This occurs when new drugs or drug combinations are studied or if the pattern of mutations associated with a treatment regimen is particularly complex.

Most phenotypic susceptibility assays use donor peripheral blood mononuclear cells (PBMC) or CD4 cell lines. Assays using donor PBMC are expensive and time consuming because HIV-1 usually does not produce visible cytopathology in donor PBMC, and viral growth must be assessed by measuring a virus product such as p24 antigen. Assays using cell lines are reproducible and inexpensive because visible cytopathology can be used to assess HIV-1 replication. However, HIV-1 replication in cell lines requires the use of strains with a syncytium-inducing phenotype.

We have described a drug susceptibility assay in which the viability of CD4 cells was assessed by the metabolic reduction

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Cytopathic effects of 4 HIV-1 isolates derived from pNL43 (HIV-1_{NL43}, HIV-1_{151}, HIV-1_{75,77,151}, and HIV-1_{75,77,116,151}) and of 4 HIV-1 isolates derived from lambda-HXB2 (HIV-1_{HXB2}, HIV-1_{215}, HIV-1_{41,215}, and HIV-1_{74}). HIV-1_{NL43} and HIV-1_{HXB2} are wild type isolates; mutant isolates contain indicated reverse transcriptase mutations (M41L, L74V, V75I, F77I, T116Y, Q151M, and T215Y). Cytopathic effect was measured as reduction in optical density (OD) in wells containing HIV-1 TCID_{50} of 27–45 vs. uninfected control wells. Mean TCID_{50} for each of 8 strains was ≈36.
of the tetrazolium salt XTT to a soluble, colored, formazan product. MT-2 cells were infected with a standardized HIV-1 inoculum in the presence of a range of drug concentrations, and after 6 days, cell viability was measured using a 96-well plate spectrophotometer. Susceptibility results were obtained using laboratory HIV-1 isolates, syncytium-inducing clinical isolates, and recombinant HIV-1 isolates. Recombinant HIV-1 isolates were created by the homologous recombination of PCR-amplified RT genes from patient plasma with a replication-deficient RT-deleted proviral HXB2 clone [7]. Because the HXB2 envelope is syncytium-inducing, the RT genes of non-syncytium-inducing isolates could be tested [7].

There was a statistically significant increase in the mean IC₅₀ of every HIV-1 isolate with a known drug-resistance mutation compared with the mean IC₅₀ of the wild type control isolates. In addition, a single test reliably detected resistance in mutant HIV-1 isolates. Ninety-six percent (43/45) of tests on isolates with zidovudine-resistance mutations had a zidovudine IC₅₀ greater than the highest IC₅₀ obtained in 16 tests of wild type control strains; 98% (40/41) of tests on isolates with ddI-resistance mutations had an IC₅₀ greater than the highest IC₅₀ obtained in 25 tests of wild type strains, and all of the tests on isolates with M184I or Y181C were highly resistant to nevirapine and lamivudine, respectively.

Our drug susceptibility assay did not yield results in exactly the same range as those of two other well-established assays, the HeLa CD4 plaque assay [12] and the AIDS Clinical Trial Group—Department of Defense consensus protocol [13]. However, the relative decreases in susceptibility detected in isolates with well-characterized mutations were similar to those reported for the other assays [6, 14–16]. The assay was capable of detecting changes in susceptibility over a 2–3 log range for zidovudine, lamivudine, and nevirapine and over a 1.5 log (30-fold) range for ddI. In all cases, the highest levels of drug resistance were associated with IC₅₀s above the peak serum concentrations achievable by the RT inhibitors tested (1–25 μM) [17–19].

In addition to testing standard mutant HIV-1 isolates, we tested several isolates with patterns of mutations that had not been fully characterized phenotypically. HIV-1 [14,15] was found to be as resistant to zidovudine as HIV-1 [16,7,7,215]. The contribution of L210W to zidovudine resistance has recently been confirmed by site-directed mutagenesis [20]; nonetheless, its precise effect on phenotype may vary depending on the remainder of the HIV-1 RT sequence. A recombinant HIV-1 strain with L74V and M184V was three times less susceptible to ddI than were strains with L74V alone. Although both L74V and M184V confer ddI resistance individually, the impact of these mutations in combination has not previously been reported. HIV-1 [17,77,15] had a lamivudine IC₅₀ ≈8-fold higher than wild type, confirming a recent observation made with a PBMC assay [6].

The use of recombinant HIV-1 strains for drug susceptibility testing has several advantages over the use of cultured clinical isolates [7, 8, 15, 21, 22]. Because PCR is used to amplify the gene encoding the target of therapy, drug susceptibility can be assessed on HIV-1 strains in samples that are often negative by culture (e.g., plasma), and the selective effects of culture can be minimized. Indeed, the drug susceptibilities of HIV-1 [15] and HIV-1 [17,22] recombinant isolates derived from plasma virus, were similar to the susceptibilities of laboratory constructs containing identical mutations.

An additional advantage of using recombinant HIV-1 isolates for susceptibility testing is that all such isolates are isogenic, with the exception of their RT genes. Thus, changes in replication reflect changes in RT function resulting from the introduction of amino acid substitutions into the parent virus. In this study, the HIV-1 strain with Q151M caused significantly less cytopathic effect than the wild type parent strain, suggesting that in the absence of selective drug pressure, this mutation may cause attenuated viral replication. Whether this phenomenon can be attributed solely to the mutations or to an interaction of the mutations with the rest of the NL43 virus requires further study. Nonetheless, this assay may be useful for identifying mutant HIV-1 isolates whose RT genes should undergo further studies of replication and biochemical function.


