Antiretroviral Drug Resistance in HIV-2: Three Amino Acid Changes Are Sufficient for Classwide Nucleoside Analogue Resistance

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Genotypic surveys suggest that human immunodeficiency virus type 1 (HIV-1) and HIV-2 evolve different sets of mutations in response to nucleoside reverse-transcriptase inhibitors (NRTIs). We used site-directed mutagenesis, culture-based phenotyping, and cell-free assays to determine the resistance profiles conferred by specific amino acid replacements in HIV-2 reverse transcriptase. Although thymidine analogue mutations had no effect on zidovudine sensitivity, the addition of Q151M together with K65R or M184V was sufficient for high-level resistance to both lamivudine and zidovudine in HIV-2, and the combination of K65R, Q151M, and M184V conferred classwide NRTI resistance. These data suggest that current NRTI-based regimens are suboptimal for treating HIV-2 infection.

HIV-2 infection is endemic in West Africa and has achieved a limited prevalence in southern Africa, India, Brazil, and parts of western Europe [1]. As with HIV type 1 (HIV-1) infection, drug resistance in HIV-2 is a major barrier to sustained antiretroviral therapy. HIV-2 is intrinsically resistant to nonnucleoside reverse-transcriptase inhibitors (NNRTIs) and the fusion inhibitor T-20 (enfuvirtide) [2], and some HIV-2 isolates also exhibit reduced susceptibility to certain protease inhibitors [2, 3]. In contrast, wild-type (WT) HIV-1 and HIV-2 exhibit comparable sensitivities to nucleoside reverse-transcriptase inhibitors (NRTIs) [4]. Although regimens that include 2 NRTIs and a protease inhibitor can initially suppress viral RNA levels in treatment-naive patients infected with HIV-2 [5], the emergence of drug-resistant variants in response to therapy [5–7] is a major obstacle to clinical treatment because most of the inhibitors that are active against HIV-2 in vitro are not widely available in West Africa and other developing regions.

Currently, efforts to identify the genetic changes responsible for drug resistance in HIV-2 in vivo are limited to a handful of small-scale studies. One potentially important trend observed in HIV-2–infected patients is the frequent emergence of mutations that encode the K65R and Q151M substitutions in reverse transcriptase (RT) [5–8] (figure A1 in appendix A, which appears only in the electronic version of the Journal). These changes are substantially more common in HIV-2 than in HIV-1 and often appear together with M184V in bulk sequences obtained from individuals who have received NRTI therapy. In contrast, the thymidine analogue mutations (TAMs) that provide the principal route to nucleoside analogue resistance in HIV-1 [9] are rarely observed in HIV-2. Although it is appropriate to be cautious in interpreting the limited amount of genotypic data available for HIV-2, existing studies suggest that HIV-1 and HIV-2 evolve NRTI resistance by different mutational pathways. This inference relies on the assumption that known genotype-phenotype relationships for HIV-1 are directly applicable to HIV-2, because the resistance phenotypes conferred by specific mutations in HIV-2 RT have not been quantified in a defined molecular clone and because only limited data from biochemical and culture–based studies are available [10–12]. To obtain a better understanding of the evolution of drug resistance in HIV-2, we directly tested the effects of clinically observed substitutions in HIV-2 RT on NRTI sensitivity using site-directed mutagenesis, cell culture-based phenotypic assays, and cell-free polymerase reactions.

Materials and methods. Site-directed mutants of HIV-2rod were constructed in the pROD9 molecular clone, which was kindly provided by Michael Emerman (Fred Hutchinson Cancer Research Center, Seattle, Washington) (see appendix B, which appears only in the electronic version of the Journal). Mutants of HIV-1sc-las were constructed in pNL4-3 or in a modified version of pBR, as described elsewhere [13]. Each WT or mutant molecular clone was transfected into 293T–17 (293tsA1609neo) cells for the production of virus, and drug susceptibilities were measured using our established HeLa-CD4 (MAGIC-5A) indicator cell assay [4, 13] (see appendix B, which appears only in the electronic version of the Journal).
assay quantifies the dose-dependent inhibition of HIV replication in a single cycle of infection, enabling head-to-head comparisons of HIV-1 and HIV-2 in the same cell type. Zidovudine (AZT), didanosine (ddI), and stavudine (d4T) were obtained commercially (Sigma-Aldrich), as were tenofovir (PMPA) and abacavir (ABC) (Moravek Biochemicals). Lamivudine (3TC) and emtricitabine (FTC) were kindly provided by Raymond Schinazi (Emory University, Atlanta, Georgia) or were obtained commercially (Moravek Biochemicals).

To examine the effects of amino acid replacements in HIV-2 RT on nucleoside analogue monophosphate incorporation, we expressed WT and mutant forms of HIV-1 and HIV-2 RT in Escherichia coli and purified the heterodimeric forms of the enzymes by column chromatography. Detailed descriptions of the RT-expressing plasmids, conditions for bacterial growth, purification procedures, and assays used to quantify analogue triphosphate susceptibility are provided in appendix B, which appears only in the electronic version of the Journal.

The drug concentrations required to inhibit focus formation in cell culture by 50% (EC50 values; data are means ± SDs of data from ≥3 independent experiments) were obtained in cultured MAGIC-5A cells; see Materials for details. Values in bold type differ significantly from values for wild-type (WT) HIV-1NL4-3 (mutant Q151M).

### Table 1. Susceptibilities of HIV-1NL4-3 and HIV-2ROD strains to nucleoside analogue reverse-transcriptase inhibitors.

<table>
<thead>
<tr>
<th>Strain, variant</th>
<th>AZT</th>
<th>ddi</th>
<th>d4T</th>
<th>PMPA</th>
<th>3TC</th>
<th>FTC</th>
<th>ABC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1NL4-3</td>
<td></td>
<td></td>
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<tr>
<td>WT</td>
<td>0.16 ± 0.07 (1)</td>
<td>4.7 ± 2.9 (1)</td>
<td>5.5 ± 1.6 (1)</td>
<td>7.2 ± 2.0 (1)</td>
<td>0.87 ± 0.28 (1)</td>
<td>0.25 ± 0.09 (1)</td>
<td>7.3 ± 3.2 (1)</td>
</tr>
<tr>
<td>Q151M</td>
<td>0.65 ± 0.25 (4)</td>
<td>10 ± 3.7 (2)</td>
<td>20 ± 7.9 (4)</td>
<td>7.8 ± 3.5 (1)</td>
<td>1.4 ± 0.4 (2)</td>
<td>0.41 ± 0.31 (2)</td>
<td>21 ± 7.1 (3)</td>
</tr>
<tr>
<td>Q151M+4</td>
<td>7.0 ± 3.4 (44)</td>
<td>45 ± 17 (10)</td>
<td>79 ± 42 (14)</td>
<td>26 ± 11 (4)</td>
<td>3.8 ± 2.8 (4)</td>
<td>0.79 ± 0.10 (3)</td>
<td>40 ± 24 (5)</td>
</tr>
<tr>
<td>HIV-2ROD</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>0.12 ± 0.06 (1)</td>
<td>8.7 ± 6.3 (1)</td>
<td>7.1 ± 3.7 (1)</td>
<td>8.4 ± 5.3 (1)</td>
<td>1.8 ± 1.2 (1)</td>
<td>0.60 ± 0.31 (1)</td>
<td>6.5 ± 2.1 (1)</td>
</tr>
<tr>
<td>Q151M</td>
<td>5.2 ± 2.3 (43)</td>
<td>42 ± 21 (5)</td>
<td>40 ± 14 (7)</td>
<td>2.9 ± 0.8 (0.3)</td>
<td>3.2 ± 0.9 (2)</td>
<td>2.7 ± 1.6 (5)</td>
<td>14 ± 8.3 (2)</td>
</tr>
<tr>
<td>M184V</td>
<td>0.073 ± 0.04 (1)</td>
<td>19 ± 14 (2)</td>
<td>2.1 ± 0.9 (0.1)</td>
<td>6.0 ± 2.1 (1)</td>
<td>&gt;400 (&gt;200)</td>
<td>&gt;400 (&gt;200)</td>
<td>6.2 ± 2.3 (1)</td>
</tr>
<tr>
<td>Q151M and M184V</td>
<td>3.5 ± 1.4 (29)</td>
<td>72 ± 11 (8)</td>
<td>17 ± 3.8 (2)</td>
<td>6.8 ± 4.6 (1)</td>
<td>&gt;400 (&gt;200)</td>
<td>&gt;400 (&gt;200)</td>
<td>14 ± 5.1 (2)</td>
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<tr>
<td>K65R</td>
<td>0.14 ± 0.03 (1)</td>
<td>37 ± 35 (4)</td>
<td>11 ± 2.1 (2)</td>
<td>8.7 ± 3.1 (1)</td>
<td>68 ± 29 (38)</td>
<td>51 ± 32 (85)</td>
<td>10 ± 4.1 (2)</td>
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<tr>
<td>K65R and Q151M</td>
<td>6.7 ± 1.6 (56)</td>
<td>55 ± 30 (6)</td>
<td>86 ± 46 (12)</td>
<td>18 ± 13 (2)</td>
<td>134 ± 50 (74)</td>
<td>152 ± 40 (250)</td>
<td>35 ± 32 (8)</td>
</tr>
<tr>
<td>K65R, Q151M, and M184V</td>
<td>7.9 ± 4.8 (66)</td>
<td>373 ± 71 (43)</td>
<td>29 ± 14 (4)</td>
<td>39 ± 23 (5)</td>
<td>&gt;400 (&gt;200)</td>
<td>&gt;400 (&gt;200)</td>
<td>&gt;100 (&gt;10)</td>
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</table>

**NOTE.** Drug concentrations required to inhibit focus formation in cell culture by 50% (EC50 values; data are means ± SDs of data from ≥3 independent experiments) were obtained in cultured MAGIC-5A cells; see Materials for details. Values in bold type differ significantly from values for wild-type (WT) HIV-1NL4-3 (mutant Q151M).

* a Viruses produced by full-length plasmid clones of HIV-1NL4-3 (pNL4-3 or pR9A[Apa]) or HIV-2ROD (pROD9).

* b Nos. in parentheses are the n-fold change in EC50 compared with the corresponding WT value.

The frequency with which K65R, Q151M, and M184V appear together in HIV-2 RT suggests that these replacements define a genetic pathway leading to escape from NRTI-based regimens [5–8] (figure A1 in appendix A, which appears only in the electronic version of the Journal). We therefore examined the individual and combined effects of K65R, Q151M, and M184V on NRTI sensitivity in HIV-2 (table 1). We initially introduced point mutations encoding the Q151M replacement into HIV-2ROD and tested the susceptibility of the resulting variant to AZT. Surprisingly, the dose-response profile of Q151M HIV-2ROD was comparable to that of the highly resistant Q151M/A62V/V75I/F77L/F116Y (mutant Q151M+4, which is resistant to multiple nucleoside reverse-transcriptase inhibitors) HIV-1NL4-3 have been reported elsewhere [4]. ABC, abacavir; AZT, zidovudine; ddi, didanosine; d4T, stavudine; FTC, emtricitabine; PMPA, tenofovir; 3TC, lamivudine.

To determine whether this outcome was specific to the Q151M variant of HIV-2ROD, the Q151M variant of HIV-2ROD was comparable to that of the highly resistant Q151M/A62V/V75I/F77L/F116Y (mutant Q151M+4) HIV-1NL4-3 mutant (figure A2 in appendix A, which appears only in the electronic version of the Journal). Over multiple trials, Q151M HIV-2ROD exhibited a mean EC50 for AZT that was 43-fold greater than that for WT HIV-2ROD, whereas the mean EC50 for Q151M HIV-1NL4-3 was only 4-fold greater than that for WT HIV-1NL4-3 (table 1). To determine whether this outcome was specific to the ROD isolate of HIV-2, we replaced the RT-encoding region of prOD9 (RT codons 14–542) with an equivalent region from the HIV-2UC2– encoding plasmid pUC2 and introduced mutations encoding Q151M into the resultant molecular clone. As observed for Q151M HIV-2ROD, the Q151M variant of HIV-2ROD/UC2 was highly resistant to AZT (a 30-fold increase in EC50 compared with that of WT HIV-2ROD/UC2; the mean EC50 values [±SD] were 0.071 ± 0.2 and 2.1 ± 0.2 μmol/L for WT and Q151M HIV-2ROD/UC2, respectively). Taken together, these data demonstrate that Q151M alone is sufficient to produce high-level AZT resistance in HIV-2.

In HIV-1 RT, TAMs confer AZT resistance by increasing the primer-unblocking activity of the polymerase, which results in enhanced removal of AZT-5'-monophosphate from the nascent DNA strand [9]. We constructed HIV-1NL4-3 and HIV-2ROD variants encoding 2 pivotal replacements in the TAM series (M41L and T215Y) and compared their sensitivities to AZT. In contrast
to HIV-1NL4-3, HIV-2ROD mutants that harbored M41L, T215Y, or both substitutions showed no detectable resistance to AZT in the single-cycle assay (<2-fold increase in EC50 relative to WT HIV-2ROD) (figure A3 in appendix A, which appears only in the electronic version of the Journal). This result is consistent with the infrequent occurrence of TAMs in HIV-2 sequences obtained from patients who had received antiretroviral therapy (figure A1 in appendix A, which appears only in the electronic version of the Journal).

To assess the effects of other replacements that are commonly associated with Q151M in HIV-1, we constructed an A62V/F77L/F116Y/Q151M (Q151M+3) variant of HIV-2ROD. This mutant is genetically equivalent to Q151M+4 HIV-1NL4-3 because HIV-2ROD encodes an isoleucine at RT codon 75 that is highly conserved in HIV-2 sequences from treatment-naive patients [14]. Although A62V, F77L, and Y116F increase the level of Q151M-mediated AZT resistance in HIV-1 [15], the Q151M and Q151M+3 variants of HIV-2ROD exhibited comparable sensitivity to the drug (mean EC50 [±SD], 5.2 ± 2.3 vs. 3.1 ± 0.3 μmol/L, for Q151M and Q151M+3, respectively), indicating that A62V, F77L, and Y116F do not enhance AZT resistance in HIV-2.

We also measured the susceptibilities of Q151M-, K65R-, and M184V-containing variants of HIV-2ROD to other NRTIs used in antiretroviral therapy. Q151M had no statistically significant effect on viral sensitivity to PMPA, 3TC, or ABC, but it imparted 5–7-fold resistance to d4T, ddI, and FTC, compared with that of the WT (table 1). K65R imparted 38-fold resistance to 3TC and 85-fold resistance to FTC, as well as 4-fold resistance to ddI. The M184V substitution conferred >200-fold resistance to both 3TC and FTC. Importantly, the addition of either K65R or M184V together with Q151M conferred robust resistance to 3TC and FTC without substantially reducing the level of Q151M-mediated AZT resistance; the HIV-2ROD mutants that contained K65R and Q151M and the mutants that contained Q151M and M184V showed >70-fold resistance to 3TC and FTC, as well as 29-fold and 56-fold resistance to AZT, respectively (table 1). This result differs substantially from the outcome observed for HIV-1, in which M184V suppresses TAM-mediated AZT resistance by impairing the primer-unblocking activity of RT [9].

To further examine the potential for dual AZT-3TC resistance in HIV-2, we quantified the effects of K65R and Q151M on the incorporation of analogue-5’-monophosphate by purified HIV-2 RT. In comparison with WT HIV-2 RT, Q151M HIV-2 RT exhibited a 15-fold increase in the IC50 for AZT-5’-triphosphate. The combination of K65R and Q151M conferred high-level resistance to AZT-5’-triphosphate (an 83-fold increase) and substantial resistance to 3TC-5’-triphosphate (a 7-fold increase) (table 2). Taken together with our cell culture data (table 1), these results demonstrate that the combination of Q151M with either K65R or M184V is sufficient to produce dual resistance to AZT and 3TC in HIV-2.

Finally, in the HIV-2ROD mutant that contained K65R, Q151M, and M184V we observed greater-than-additive increases in the levels of resistance to ddI and ABC, compared with the resistance levels of the single- or double–amino acid variants (table 1). As a result, the HIV-2ROD mutant that contained K65R, Q151M, and M184V showed 40-fold resistance to AZT, ddI, 3TC, and FTC; >10-fold resistance to ABC; and 4–5-fold resistance to d4T and PMPA. These findings demonstrate that, in HIV-2ROD, the combination of K65R, Q151M, and M184V confers classwide NRTI resistance, with high-level resistance to AZT, ddI, 3TC, FTC, and ABC.

**Discussion.** To our knowledge, this is the first study showing the individual contributions of clinically observed amino acid replacements in HIV-2 RT to nucleoside analogue resistance. Our analysis provides 2 important insights that help to explain why the mutations that emerge in HIV-2 during therapy differ from those typically seen in HIV-1. First, in contrast to HIV-1, a single Q151M replacement in HIV-2 RT confers high-level phenotypic resistance to AZT as well as substantial cross-resistance to other nucleoside analogues (table 1). Second, 2 key replacements in the TAM pathway (M41L and T215Y) have no effect on AZT susceptibility in HIV-2 in culture (figure A3 in appendix A, which appears only in the electronic version of the Journal). This result is consistent with the outcome of a recent study showing that, compared with WT HIV-1 RT, WT HIV-2 RT exhibits a substantially lower level of primer–unblocking activity [10]. Taken together, these data demonstrate that equiva-
lent substitutions in HIV-1 and HIV-2 RT can have different effects on NRTI susceptibility and that the genetic algorithms used to predict drug resistance phenotypes in HIV-1 RT are not necessarily applicable to HIV-2.

The dual AZT-3TC resistance phenotypes observed in HIV-2 in cell culture (table 1) and in cell-free RT assays (table 2) are of particular concern because fixed-dose formulations of AZT and 3TC are still widely used in West Africa and other developing regions where HIV-2 infection is endemic. In HIV-1, high-level resistance to both AZT and 3TC typically requires the combination of multiple TAMs, M184V, and additional replacements in RT [9]. In contrast, in HIV-2, only 2 amino acid changes (K65R and Q151M or Q151M and M184V) are required for high-level resistance to both of these inhibitors (table 1). These findings strongly suggest that the mutational barrier to dual AZT-3TC resistance is lower in HIV-2 than it is in HIV-1. Although other NRTIs retained substantial antiviral activity against the HIV-2 variants tested in our study (i.e., PMPA and ABC; see table 1), we cannot presently exclude the possibility that, in HIV-2, high-level resistance to these drugs can also be acquired via a relatively small number of mutational steps. Additional studies are required to address this issue.

In a recent analysis of patient-derived HIV-2 isolates, Damond et al. concluded that Q151M alone only confers resistance to d4T and ABC and that resistance to other NRTIs involved the coselection of a V111I change in RT [11]. Although the strains examined in their study were comprised of mixed populations of variants, further examination of their data indicates that, in fact, Q151M confers broad-spectrum NRTI resistance both in the presence and in the absence of the V111I substitution. In our experiments, we introduced the Q151M change into both an HIV-2ROD-based and an HIV-2UC2-based RT background; both of these strains encode a valine at position 111 of RT. Our findings lead us to conclude that Q151M alone is sufficient to produce high-level AZT resistance in HIV-2 and that the V111I substitution is not required for broad-spectrum NRTI resistance.

From a therapeutic perspective, resistance to multiple NRTIs is particularly challenging in HIV-2 because naturally occurring resistance mutations in HIV-2 appear to accelerate the development of high-level resistance to multiple protease inhibitors [2, 3]. Thus, strict adherence to therapy may be particularly important for delaying the outgrowth of drug-resistant HIV-2 variants. Our in vitro analyses of HIV-2 suggest that the emergence of NRTI-resistant mutants during first-line treatment, particularly in patients treated with AZT and 3TC, will likely result in poor responsiveness to subsequent nucleoside-based regimens. Our findings support initiatives to provide a broader array of antiretrovirals to HIV-2–infected patients in resource-limited settings and emphasize the need to identify drug combinations that inhibit HIV-2 strains resistant to multiple NRTIs.

Acknowledgments

We thank Drs. Salif Sow (University of Dakar, Senegal), James Mullins (University of Washington), and Nancy Kviat (University of Washington) for helpful discussions.

References

Figure A1. Frequencies with which resistance-associated substitutions were observed in HIV-1 and HIV-2 reverse transcriptase (RT). Data for HIV-1 (gray bars) are from 1779 sequences obtained from patients treated with nucleoside RT inhibitors [22]. Data for HIV-2 RT (black bars) are compiled from 110 sequences reported in 10 independent surveys of individuals who had received antiretroviral therapy [5–8, 12, 23–27]. Note that, in many cases, the individual HIV-1 and HIV-2 sequences represented by this figure contain combinations of 2 or more resistance-associated changes in RT as well as polymorphisms of unknown significance. For HIV-2 sequences that encode the combination K65R and Q151M, the combination Q151M and M184V, and the combination K65R, Q151M, and M184V, with or without additional resistance-associated substitutions in RT, the observed frequencies were 13%, 16% and 8%, respectively. Thymidine analogue mutations are the canonical mutations that emerge in HIV-1 in response to zidovudine or stavudine [28]. For the RT amino acid sites that differed between wild-type HIV-1 and HIV-2, the substitutions that emerged in HIV-2–infected patients are shown in parentheses. Drug-resistance mutations that encode K219E and V75I exist in the majority of HIV-2 sequences obtained from treatment-naive individuals [14]; therefore, these are not shown. Additional nucleoside analogue resistance mutations that encoded E44D, A62V, T69D, L74V, F77L, Y115F, F116Y, and insertions at T69 were each present in =10% of all HIV-1 sequences and ≤4% of all HIV-2 sequences compiled [5–8, 12, 23–27].
Figure A2. High-level zidovudine (AZT) resistance in Q151M HIV-2 in cell culture. A, Dose-response analysis for wild-type (WT) and Q151M mutant strains of HIV-1\textsubscript{NL4-3} and HIV-2\textsubscript{ROD}. Data points are the percentages of β-galactosidase–positive foci in AZT-treated MAGIC-5A cultures relative to solvent-only controls. Each point represents the mean of data from 6 independent dose-response experiments, with 3 determinations of β-galactosidase–positive foci per drug concentration per experiment. Curves were generated using a sigmoidal regression equation (Prism, version 4.0; GraphPad). B, AZT concentrations required to inhibit focus formation in cell culture by 50% (EC\textsubscript{50} values) for WT and Q151M strains of HIV-1\textsubscript{NL4-3} and HIV-2\textsubscript{ROD}. Bars represent means ± SDs of data from ≥10 independent dose-response experiments, including the 6 assays presented in panel A. EC\textsubscript{50} values for Q151M HIV-2 and Q151M/A62V/V75I/F116Y (Q151M\textsubscript{+4}) HIV-1 were statistically equivalent. Portions of the data for WT HIV-1\textsubscript{NL4-3}, WT HIV-2\textsubscript{ROD}, and Q151M\textsubscript{+4} HIV-1\textsubscript{NL4-3} have been reported elsewhere [4]. *P < .001 (by analysis of variance with Tukey’s multiple-comparison test) for comparison with WT HIV-1 and WT HIV-2; **P < .001 (by analysis of variance with Tukey’s multiple-comparison test) for comparison with WT HIV-1, WT HIV-2, and Q151M HIV-1.

Figure A3. Differing response to thymidine analogue mutations in HIV-1 and HIV-2. Zidovudine sensitivity was measured in MAGIC-5A cultures, as described in the legend for figure A2 and in appendix A, which does not appear in the print version of the Journal. Bars represent the mean (±SD) drug concentrations required to inhibit focus formation in cell culture by 50% (EC\textsubscript{50} values) from ≥3 independent dose-response experiments. *P < .05 (by analysis of variance with Tukey’s multiple-comparison test) for comparison with wild-type (WT) HIV-1.
**APPENDIX B**

**SUPPLEMENTARY MATERIALS AND METHODS**

**Site-directed mutagenesis.** To create site-directed mutants of HIV-2$_{ROD}$ point mutations were introduced into a pol-spanning HindIII subclone of pROD9 (nt 2013–638) using the QuickChange II Mutagenesis System (Stratagene). The mutant pol genes were then ligated back into pROD9 to generate full-length infectious molecular clones. The nucleotide changes introduced in each of the targeted RT codons were as follows: Q151M, CAG to ATT; K65R, AAA to AGA; M184V, ATG to GTG; M41L, ATG to TTG; S215Y, TCT to TAT; A62V, GCA to GTA; F77L, TTC to TAT; and F116Y, TTT to TAT. The presence of the desired nucleotide changes and the absence of additional mutations were verified by automated DNA sequencing.

**Virus production and drug-sensitivity assays.** Virus stocks were prepared by chloroquine-mediated transfection of 293T-17 cells (293tsA1609neo), as described elsewhere [16]. Each of the plasmid DNA preparations used for transfection was purified from E. coli using an Endo-Free Maxiprep Kit (Qiagen). 293T-17 cells were cultured (at 37°C in 5% CO$_2$) in 10-cm plates containing 10 mL of complete Dulbecco’s modified Eagle medium (DMEM) (supplemented with 4 mmol/L L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% heat-inactivated fetal bovine serum). For transfection, 293T-17 cells were trypsinized and resuspended at a density of 1.5 × 10$^5$ cells/mL in complete DMEM. The cells were then seeded into 6-well plates (2 mL/well) and returned to the incubator for 18-20 h. To prepare CaPO$_4$-DNA coprecipitates, 2 μg of each plasmid preparation was added to 100 μL of 0.2 mol/L CaCl$_2$; the resulting suspensions were added dropwise with mixing into 100 μL of 2× HEPES-buffered saline and then incubated at room temperature for 10 min.

During this time, chloroquine was added to each well of the 293T-17 cultures to a final concentration of 25 μmol/L. The coprecipitate suspensions were then mixed by pipetting and added directly to the chloroquine-treated cultures, and the plates were placed in the incubator for 8–10 h. After this period, the culture supernatants were aspirated and replaced with 2 mL of fresh complete DMEM per well. The plates were then returned to the incubator for 30–35 h.

To harvest the virus stocks, supernatants from the transfected 293T-17 cultures were transferred to 15-mL polypropylene tubes and centrifuged at 470 g for 5 min at room temperature. The supernatants were then transferred into new 15-mL centrifuge tubes, divided into 250-μL aliquots in 1-mL cryogenic storage vials, and frozen immediately at −150°C. For HIV-1 stocks, the mean titers of infectious virus (MAGIC-5A cultures, focus-forming units [ffu]/mL) were as follows: WT, 6.0 × 10$^4$; Q151M, 1.3 × 10$^4$; Q151M+4, 3.9 × 10$^3$; M41L, 5.9 × 10$^3$; T215Y, 6.0 × 10$^3$; and M41L and T215Y, 6.3 × 10$^3$. For HIV-2 stocks, the mean titers were as follows: WT, 1.8 × 10$^5$; K65R, 3.7 × 10$^5$; Q151M, 6.6 × 10$^4$; M184V, 9.8 × 10$^4$; K65R and Q151M, 2.8 × 10$^4$; Q151M and M184V, 1.4 × 10$^5$; K65R, Q151M, and M184V, 1.9 × 10$^4$; M41L, 1.6 × 10$^4$; S215Y, 1.9 × 10$^5$; and M41L and S215Y, 5.0 × 10$^4$.

Drug sensitivities were measured by use of the MAGIC-5A indicator cell line (kindly provided by Michael Emerman, Fred Hutchinson Cancer Research Center, Seattle, Washington). These CD4$^+$CCR5$^+$ HeLa cell derivatives express β-galactosidase under the control of an HIV-1 promoter sequence. MAGIC-5A cells were seeded into 48-well plates at a density of 1.5 × 10$^4$ cells/well and incubated for 20–22 h at 37°C in 5% CO$_2$. After this incubation period, varying concentrations of nucleoside analogues were added to the cultures, and the plates were returned to the incubator for an additional 2 h. Immediately before infection, the virus stocks were diluted to 3000 ffu/mL in complete DMEM supplemented with 20 μg/mL diethylaminoethyl dextran. Supernatants from the drug-treated MAGIC-5A cultures were aspirated and replaced with 100 μL of each diluted virus stock/well, and the plates were returned to the incubator for 2 h. After this time, an additional 300 μL of complete DMEM was added, a second drug dose was added (at the same concentration as the first dose), and incubation was continued for an additional 40 h.

To score Lac$^+$ foci, 100 μL of fixative solution (1% formaldehyde and 0.2% glutaraldehyde in PBS) were added to each culture well, and the plates were incubated at 37°C for 10 min. After washing the fixed monolayers twice with 100 μL of PBS, we added 100 μL of staining solution (4 mmol/L potassium ferrocyanide [II] trihydrate, 4 mmol/L potassium ferricyanide, 2 mmol/L MgCl$_2$, and 0.4 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal] in PBS) to each well, and the plates were placed in the incubator for 1 h. The cultures were then aspirated to remove the X-Gal staining solution, rinsed with 100 μL of PBS per well, aspirated again, and stored in 200 μL of PBS per well for counting by light microscopy. Foci of infection appeared as isolated groups of 1–5 contiguous Lac$^+$ (blue) cells.

Untreated control cultures typically contained 200–500 foci/well. $EC_{50}$ values were calculated from dose-response plots by sigmoidal regression analysis (Prism; GraphPad Software). $EC_{50}$ values obtained for WT and mutant strains of HIV-1$_{NL4.3}$ were comparable to the values obtained in other single-cycle drug-sensitivity assays [17–20].

**RT expression and purification.** For recombinant RT expression, DNA sequences that encode the large and small subunits of HIV-1 and HIV-2 RT were amplified by polymerase chain reaction (PCR) from the full-length HIV-1$_{NL4.3}$ and HIV-2$_{ROD}$ clones and ligated into pET17b (Novagen). For HIV-1, nt 2550–3869 (p51) and 2550–4229 (p66) were amplified. For HIV-2, nt 2936–4384 (p58) and 2936–4612 (p68) were amplified. PCR (50 μL) was performed with 2.5 U of PfuUltra High-Fidelity DNA Polymerase (Stratagene), in accordance with the manufacturer’s recommended protocol. The primer sequences were as follows: HIV-1 RT forward, 5’-GTCCCTGACATATGCGATTCGCCGATTACCGACTGTACCC-3’; p51 reverse, 5’-CGCCG-
CAAGCTTTAGAAGTTCTGCTCC-3'; p66 reverse, 5'-CGGCAAGCTTTATATGACTTTCGATCCAGC-3'; HIV-2 forward, 5'-GTCTGCTCATATGCCGTTGCGAAGTG3'GGGCC-3'; p58 reverse, 5'-CGGCAGAGCTTTCTCACATCGAAAGGGCTTTAATTCTGC-3' and p68 reverse, 5'-CGGCCGAAGCTTTTACACCTTGCTGATACCC-3'. The italicized bases were added to the 5' end of each primer to enhance restriction enzyme activity. Bold type indicates synonymous nucleotide changes that indicate stop anticodons. Bases that are both underlined and in bold type are unique Ndel and HindIII restriction sites. PCR-amplified products were digested with Ndel and HindIII (New England Biosciences), agarose gel–purified, and ligated into Ndel–HindIII–digested pET17b plasmid DNA. Restriction mapping and automated DNA sequencing were used to confirm that each plasmid used for protein expression was correct.

We used the strategy developed in our previous study of HIV-1 [21] to establish optimal conditions for purifying HIV-2 RT. Plasmids for each HIV-1 and HIV-2 RT subunit were individually transformed into BL21(DE3)pLysS E. coli (Novagen), and the transformants were used to inoculate LB-ampicillin (100 µg/mL) cultures (1 L of culture for E. coli that expressed HIV-1 RT p66 or HIV-2 RT p68 and 4 L of culture for E. coli that expressed either HIV-1 RT p51 or HIV-2 RT p58). The cultures were incubated at 37°C until they reached an absorbance read at 600 nm (A600 nm) of ~0.6. At this time, HIV RT expression was induced by adding isopropyl-β-D-thiogalactopyranoside at a final concentration of 1 mmol/L. All cultures were incubated for an additional 7 h at 37°C except for those expressing HIV-2 RT, because optimal expression of p58 was obtained at 30°C (data not shown). The resulting cells were harvested by centrifugation, and wet cell pastes were stored at −80°C. Overexpression of each RT subunit was verified by SDS-PAGE and Coomassie brilliant blue staining. For both HIV-1 and HIV-2 RT, typical yields of wet cell paste were 3–5 g from cultures expressing the large subunit and 2.0–2.5 g from cultures expressing the small subunit.

For RT purification, cell pastes were mixed to obtain a 1:2 ratio of large to small subunits (as estimated by SDS-PAGE and Coomassie staining) to favor heterodimer formation. Combined cell pastes (~9 g) were resuspended in 30 mL of lysis buffer (50 mmol/L Tris-HCl [pH 7.0], 300 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol [DTT], 0.1% Nonidet P-40, and 10% glycerol) containing 30 mg of Complete Protease Inhibitor Cocktail (Roche Applied Science). All subsequent steps of cell lysis and protein purification were performed at 4°C or on ice. Each cell lysate was passed 2–3 times through a FRENCH Press (Thermo Scientific) at 16,000 psi and clarified by centrifugation at 21,000 g for 45 min. An additional 30 mg of Complete Protease Inhibitor Cocktail was added to the resultant supernatant, which was then transferred to Spectrum^®Spectra/Por^® 2 RC Dialysis Membrane Tubing (12,000–14,000-Da molecular weight cutoff; Fisher Scientific) and dialyzed against buffer M (50 mmol/L Tris-HCl, 1 mmol/L EDTA, 1 mmol/L DTT, 0.1% Nonidet P-40, and 10% glycerol). Unless otherwise stated, the pH of buffer M was 7.0.

For chromatography, dialyzed cell lysate (28 mL) was filtered through a 0.45-micron-pore syringe filter and loaded to a buffer M–equilibrated 400-mL DEAE-Sepharose column (Amersham Biosciences) that was connected to a fast-protein liquid chromatography system (Pharmacia FPLC). The resin was washed with 600 mL of buffer M at a flow rate of 4 mL/min. RT was eluted in the void volume of the DEAE-Sepharose column, as assessed by A280nm and SDS-PAGE. Peak protein-containing fractions were combined and loaded onto a 1-mL HiTrap heparin sepharose column (0.7 × 2.5 cm; Amersham Biosciences) that was equilibrated with buffer M. The resin was washed with 5 column volumes of buffer M containing 150 mmol/L NaCl and eluted with a 7–column volume linear gradient of 150–500 mmol/L NaCl in buffer M. For HIV-1 RT, excess p51 was eluted at ~210 mmol/L NaCl, followed by p66/p51 heterodimers at ~270 mmol/L NaCl. For HIV-2 RT, the p68/p58 heterodimers exhibited an elution profile at pH 7.0 that was indistinguishable from that of monomeric p58 protein (data not shown). Therefore, pooled HIV-2 RT fractions from the DEAE-Sepharose column were dialyzed against buffer M (pH 8.0), loaded onto a buffer M–equilibrated (pH 8.0) heparin column, washed, and eluted with a NaCl gradient as described above. HIV-2 heterodimers were eluted at ~410 mmol/L NaCl, and excess p58 was eluted at ~470 mmol/L NaCl. Peak fractions of heterodimers were pooled, dialyzed against buffer M, and loaded onto a buffer M–equilibrated POROS 20 S strong cation exchange column (10 × 0.46 cm) that was connected to a BioCAD SPRINT perfusion chromatography system (Applied Biosystems). The resin was washed with 5 column volumes of buffer M, and RT was eluted with a 10–column volume linear gradient of 0–1000 mmol/L NaCl in buffer M. Heterodimers were eluted at ~35 and 120 mmol/L NaCl for HIV-1 and HIV-2, respectively. Peak fractions were dialyzed against buffer M, divided into aliquots, frozen in liquid nitrogen, and stored at −80°C.

This procedure typically yielded 0.5–1.0 mg of total protein with a purity of >95% heterodimeric RT, as determined by SDS-PAGE and Coomassie brilliant blue staining (data not shown). Specific activities for WT, Q151M, and Q151M and K65R HIV-1 RT were 30, 3.3, and 15 U/µg of protein, respectively, when dTTP was used as substrate and poly(rA)-oligo(dT)12-18 (Amersham Biosciences) was used as the template-primer. For M184V HIV-1 RT, specific polymerase activity was measured using dCTP and poly(ri)(r)-oligo(dC)12 (Sigma-Aldrich) and was 3.7 U/µg; the activity of WT HIV-1 RT under these conditions was 31 U/µg. Specific activities for WT, Q151M, and Q151M and K65R HIV-2 RT were 87, 120, and 84 U/µg, respectively, when dTTP was used as substrate and poly(rA)-oligo(dT)12-18 was used as the template-primer. One unit of activity was defined as the amount of enzyme needed to incorporate 1 nmol of dNTP.
into the synthetic template–primer in 60 min at 37°C under the reaction conditions described below.

**Assays of AZT-5'-triphosphate (AZTTP) and 3TC-5'-triphosphate (3TCTP) sensitivity with purified RTs.** The susceptibility of each RT to AZTTP was determined in 30-μL reactions containing 35 mmol/L Tris–HCl (pH 8.0), 50 mmol/L KCl, 0.03% Nonidet P-40, 3 mmol/L MgCl₂, 1 mmol/L DTT, 0.05 U of poly(rA)–oligo(dT)₁₂₋₁₈ (Amersham Biosciences), 50 μmol/L (2.5 μCi) [³²P]dCTP, and 4 U of RT. 3TCTP susceptibility was measured in 30-μL reactions containing 50 mmol/L Tris–HCl (pH 8.0), 2 mmol/L MgCl₂, 0.025% Triton X-100, 0.075 U of poly(rI)–oligo(dC)₁₂ (Sigma-Aldrich), 50 μmol/L (2.5 μCi) [α-³²P]dCTP, and 0.6 U of RT. All reactions were performed at 37°C for 20 min. Under these conditions, DNA synthesis was maintained in the steady state, as ascertained by preliminary experiments in which the concentrations of RT and the reaction times were varied (data not shown). The polymerase reactions were terminated by adding 1 μL of 1 mol/L EDTA, and 25 μL of solution was removed from each reaction tube and loaded onto DE81 filter discs (Whatman). The filters were dried at 60°C for 10–15 min and transferred to a glass beaker; they were then washed with four 500-ml changes of 500 mmol/L sodium phosphate buffer (pH 7.0) and rinsed with two 200-ml changes of 95% ethanol. After a second drying step, the filters were transferred to 7-ml scintillation vials (Fisher Scientific) 200-ml changes of 95% ethanol. The resulting data were plotted against the log₁₀ inhibitor concentration to those obtained in no-drug control reactions that were performed in parallel. The resulting data were plotted against the log₁₀ inhibitor concentration, and IC₅₀ values were calculated using a sigmoidal regression analysis in Prism (version 4.0; GraphPad Software).

**References**


