Zidovudine Resistance Is Suppressed by Mutations Conferring Resistance of Human Immunodeficiency Virus Type 1 to Foscarnet

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Both foscarnet (PFA) and zidovudine (AZT) select for drug-resistant variants of human immunodeficiency virus type 1 (HIV-1), but the interactions between the mutations causing such resistance are unknown. The introduction of the previously identified PFA resistance mutation W to G at codon 88 (W88G), E89K, L92I, or Q161L into an HIV-1 strain having the four known AZT resistance mutations completely reversed high-level AZT resistance. Two additional PFA resistance mutations, W88S and S156A, partially suppressed AZT resistance. Phenotypic reversion of AZT resistance by W88S, W88G, E89K, L92I, and S156A was associated with a concomitant suppression of PFA resistance. The degree to which PFA resistance mutations reversed AZT resistance was directly correlated with each mutation's ability to confer high-level PFA resistance (≥5.0-fold) and AZT hypersusceptibility in a wild-type genetic background. Highly PFA-resistant HIV-1 strains were hypersusceptible to AZT; conversely, AZT-resistant strains with M41L and T215Y; M41L, L210W, and T215Y; or M41L, D67N, K70R, and T215Y mutations were 2.2- to 2.5-fold hypersusceptible to PFA. Prolonged in vitro selection of wild-type or AZT-resistant HIV-1 strains with the combination AZT and PFA failed to generate coresistant viruses, indicating that dual resistance was relatively difficult to achieve. Strains selected by passage in PFA plus AZT were phenotypically PFA resistant and AZT susceptible despite multiple reverse transcriptase mutations known to confer AZT resistance. These data show that PFA resistance mutations can phenotypically reverse AZT resistance and that AZT and PFA resistance might be mutually exclusive. The reciprocal interactions between AZT and PFA resistance-conferring mutations have implications for structure-function studies of the HIV-1 reverse transcriptase.

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with AIDS receiving long-term PFA therapy for cytomegalovirus reactivitits (35). The RT substitutions W88G, W89G, Q161L, and H208Y were observed in these clinical isolates (35). In vitro selection readily generates PFA-resistant strains of HIV-1 (35, 57) which are usually associated with single (E89K, L92I, or S156A) (57) or double (Q161L and H208Y) (35) amino acid substitutions in the HIV-1 RT.

Because we have noted that PFA-resistant strains of HIV-1 emerged readily in cell cultures but were not found in HIV-1 isolates from several patients with AIDS on prolonged combination therapy with PFA and AZT (56, 58), we hypothesized that there was an interaction between AZT and PFA resistance mutations. This hypothesis was supported by a detailed analysis of sequential HIV-1 isolates from one patient, suggesting that simultaneous therapy with AZT may have retarded or prevented the emergence of PFA-resistant HIV-1 (58).

Here we provide evidence that there are antagonistic interactions between the RT mutations which lead to either AZT or PFA resistance such that exposure to both agents simultaneously retards the development of coresistant strains.

MATERIALS AND METHODS

Cells. MT-2 cells (13) were cultured in RPMI 1640 medium (Gibco, Gaithersburg, Md.)–10% heat-inactivated fetal calf serum as previously described (59). Human peripheral blood mononuclear cells (PBMCs) were obtained from HIV-1-seronegative donors and purified from whole blood by density centrifugation (39). Mononuclear cells were incubated for 3 days in RPMI medium containing phytohemagglutinin at 10 μg/ml and then transferred to medium containing interleukin-2 (59) at the time of infection with HIV-1. HTLacZ-1 cells (45) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and G418 (Gibco) at 400 μg/ml.

Plasmids. The construction of the pKPHXB2 infectious HIV-1 molecular clone, which contains the XhoI fragment of HIV-1 provirus from pHXB2-D, has been described previously (57). The derivation of the pXHHIV-1 LAI infectious molecular clone has been reported previously (40). pXH/HOM contains a 4.3-кб HindIII fragment of HXB2 encompassing the complete pol gene (coordinates 1258 to 5578) cloned into the HindIII site of pTZ19U (Bio-Rad Laboratories, Inc., North Ryde, Australia) (15). Constructs pXH/H41+215 and pXH/3X are derived from pHX/HOM by the introduction of the AZT resistance mutations M41L and T215Y and M41L, L210W, and T215Y, respectively, by site-directed mutagenesis (15). pM.Q and pM were obtained by subcloning a 1.7-kб Xbal/EcoRI pol fragment from the M13mp18c18 clones HIVRTATG and HIVRTXVE, respectively (gifts from Brendan A. Larder) (30), into the Xbal/EcoRI site of pTZ19U (Bio-Rad Laboratories, Inc., North Ryde, Australia) and pTZ19U (Bio-Rad Laboratories, Inc.). HIVRTM, HIVRTXVE encode the AZT resistance mutations M41L and T215Y and M41L, D67N, K70R, and T215Y, respectively (22). DNA labeled with [γ-32P]ATP was used for hybridization of plasmids to gel-purified pKPHXB2-D (gift from Brendan A. Larder) has been described previously (54). pPXXHIV-1 LAI incorporates most of the HBX2 sequence except for a 1.96-kб deletion of the HIV-1 RT gene (coordinates 2168 to 4099) (15, 57). pXHIV-1 LAI-MCYC has the AZT resistance mutations M41L and T215Y and K219Q introduced into pXHIV-1 LAI by site-directed mutagenesis (35).

Viruses. PD-1 was an HIV-1 strain isolated from PBMCs of a patient with AIDS who had never been treated with antiretroviral agents (57). Strain HX was derived by transfection of the molecular clone pKHXB2 into MT-2 cells. Clonal strains of HX/41+215, HX/3X, HX88X, and HX88G were generated by cotransfection of pHX/H41+215, pHX/3X, pHX88X, or pHX88G with pPXXHIV-1 LAI into MT-2 cells. Recombinant HIV-1 strains MQ, MQ88S, MQ88G, MQ92I, MQ92I, MQ156A, MN88S, and MN88G were recovered by cotransfection of MT-2 cells with the constructs pMQ, pMQ88S, pMQ88G, pMQ92I, pMQ156A, pMN88S, and pMN88G with pHVARTBSTieil. Strains LAI, LAIMCYC, LAIM161L, and LAIMCY161L were obtained by electroporation of MT-2 cells with the plasmids pXHIV-1 LAI-MCYC, pXHIV-1 LAI-MCY161L, and pXHIV-1 LAI-MCY161L, respectively (35).

The cotransfection in MT-2 cells was performed with 5 μg of MscI-linearized pKHXB2-D or BstEII-linearized pHVARTBSTieil with either Xbal- and EcoRI-linearized (pM-Q and pM-N derived constructs) or HindIII-linearized (pXHHOM constructs) phagemids with DOTPER (Boehringer, Mannheim, Germany) following the manufacturer’s recommendations. The vector sequence released by enzyme digestion was not removed prior to transfections. Infectious HIV strain F2, F3, F4, F5, and F6 were isolated by further purification of the pol gene derived from plasmids pF2, pF3, pF4, pF5, and pF4A, respectively, by gel electrophoresis of BamHII-EcoRI-digested phagemids with BstEII-digested pHVARTBSTieil. Cultures were maintained until the maximum cytotoxic effects were observed (7 to 21 days), at which time the culture supernatants were clarified and stored at −70°C. The RT regions (codons 1 to 500) of recombinant strains (MN88S, MN88G, MQ88S, MQ89G, MQ92K, MQ92L, MQ92L) were sequenced. Changes that differed from the expected sequence were not noted.

Drugs. PFA (Fluka Biochimica, Buchs, Switzerland) and AZT (Sigma Chemical Company, St. Louis, Mo.) were prepared as 10-mg/ml stocks in sterile water and dimethyl sulfoxide, respectively.

In vitro selection. Selection experiments were performed in MT-2 cells in the presence of increasing concentrations of the appropriate drug(s) as previously described (57). MT-2 cells (300,000 to 400,000/ml) were inoculated with 2,500 to 10,000 cells per well and incubated with 30 to 300 μM PFA and 0.2 to 0.5 μM AZT. Following the third terminal dilution, PFA330/AZT0.2p25 was amplified once in the absence of drugs.

Drug susceptibility assays. (i) In MT-2 cells. Assays were performed as previously described (57). Briefly, 250 to 500% tissue culture infective doses of each virus were used to infect 150,000 to 200,000 MT-2 cells in the presence of serial drug dilutions in duplicate wells of a 24-well tray (Greiner, Krefzminster, Germany) and the level of virus replication was measured by virion-associated RT activity (58).

(ii) In HTLacZ-1 cells. Drug inhibition of blue synctium formation was performed as previously described (45). Cells were seeded into 24-well plates (2.5 to 10,000 cells per well) and infected with 30 to 300 μM synctium-inducing units (45) of HIV-1 in 220 μl of Dulbecco’s modified Eagle’s medium–5% fetal calf serum containing 10 μg of DEAE-dextran (Amrad Pharmacia Biotech) per ml at 37°C. After 1 to 1.5 h, the inoculum was removed and 1 ml aliquot of medium containing the appropriate concentrations of drug was added to duplicate wells. After 3 days of incubation, the cells were fixed and stained as previously described (45) and synctia containing three or more blue nuclei were counted.

For each virus, the percentage inhibition of either RT activity (MT-2 assay) or synctium formation (HTLacZ-1 assay) in drug-treated cultures was calculated relative to that in untreated infected cultures and the 50% inhibitory concentration (IC50) was derived from plots of the percentage inhibition versus the log 10 concentration of inhibitor (45, 58). PFA resistance was defined as HIV-1 with a greater-than-twofold increase in IC50 compared with that of the corresponding wild-type strain. In HTLacZ-1 assays, viral strains for which the IC50s were ≤0.05 μM were considered AZT-sensitive; those for which the IC50s were >0.05 and ≤1.0 μM were considered partially resistant; and those for which the IC50s were ≥1.0 μM were considered highly resistant (21). The statistical significance of differences between IC50 values was determined by the Wilcoxon rank-sum test (2).

PCR amplification. HIV-1 strains were grown in phytomagglutinin-stimulated PBMCs (2.5 × 105 cells per ml) for 4 to 7 days. Genomic DNA was prepared from infected cells with the QIAamp kit (Qiagen, Hilden, Germany). For the in vitro-selected strain PFA330/AZT0.2p25, the RT region of HIV-1 provirus was amplified by PCR using primers described previously (45, 58). HIV genomic DNA was amplified from infected PBMCs with the freshy added drug(s) at the original concentration and incubated for 4 to 7 days. PCR products were purified from agarose gels (Axygen) and sequenced. Changesthat differed from the expected sequence were not found.

Preparation of molecular clones of the HIV-1 RT region. Molecules were prepared by PCR amplification of the pol gene derived from purified chromosomal DNA from PBMCs infected with strains PFA660/AZT0.2p29 and

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The IC50 of PFA for this strain was determined in a different assay series in which the IC50 of PFA for HX was 22 ± 0.05. Statistically significant differences in the IC50 of PFA were noted for HX and HX88S (P < 0.012), HX and HX88G (P = 0.036), and MQ88S and MQ88G (P < 0.05). Statistically significant differences in the IC50 of PFA were noted for HX and HX88S (P < 0.01), HX and HX88G (P = 0.012), MN and MN88S (P = 0.008), MN and MN88G (P = 0.003), MN88S and MN88G (P = 0.014), MQ and MQ88S (P = 0.001), MQ and MQ88G (P = 0.014), MQ88S and MQ88G (P = 0.014). IC50 for mutant strain divided by IC50 for wild-type HX. Values of >1 and <1 indicate resistance and hypersusceptibility, respectively.

The IC50 of AZT for this strain was one-fifth that of wild-type HX; therefore, there was a fivefold increase in susceptibility compared with that of HX.

The IC50 of PFA for this strain was determined in a different assay series in which the IC50 of PFA for HX was 22 ± 0.05. Accordingly, fold resistance has been calculated with the IC50 of PFA for HX.
We determined the capacity of previously PFA-resistant viruses (strain MN88G and MQ88G, respectively) to result in PFA resistance (strain MN88S and MQ88S, respectively) to become fully PFA-resistant in the AZT-resistant background (strain MN and MQ, respectively). In contrast, no change in PFA resistance was observed when HX88G was compared with MN88G (P = 0.05), while HX88S was compared with MN88G (P = 0.05) and MQ88S (P = 0.05) (Table 1). In contrast, no change in PFA resistance was observed when Q161L was introduced into the AZT-resistant background (Table 2). Q161L also reversed AZT resistance in the LAIMC/Y background (Table 2). In contrast, introduction of S156A into the MQ background (strain MQ156A) resulted in only partial suppression of AZT resistance (Table 2). MQ92I and LAIMC/Y161L retained significant resistance to PFA, while MQ92I showed only a twofold increase in PFA resistance compared with wild-type HX and MQ156A was fully PFA susceptible (Table 2).

The addition of AZT resistance mutations to cloned strains with preexisting PFA resistance mutations (compare HX89K with MQ89K, HX92I with MQ92I, and HX156A with MQ156A [Tables 2 and 3]) consistently suppressed the preexisting PFA resistance (P = 0.05). A similar pattern was observed when HX88S was compared with MN88S (P = 0.05) and MQ88S (P = 0.014) as well as when HX88G was compared with MN88G (P = 0.05) and MQ88G (P = 0.05) (Table 1). In contrast, no change in PFA resistance was observed when Q161L was introduced into the AZT-resistant back-

### Table 2. Role of non-codon 88 mutations conferring PFA resistance in phenotypic reversal of AZT resistance

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amino acid at indicated RT codon</th>
<th>Susceptibility to indicated drug</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>D</td>
</tr>
<tr>
<td>HX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MQ</td>
<td>L</td>
<td>N</td>
</tr>
<tr>
<td>MQ89K</td>
<td>L</td>
<td>N</td>
</tr>
<tr>
<td>MQ92I</td>
<td>L</td>
<td>N</td>
</tr>
<tr>
<td>MQ156A</td>
<td>L</td>
<td>N</td>
</tr>
<tr>
<td>LAI</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>LAIMC/Y161L</td>
<td>—</td>
<td>N</td>
</tr>
</tbody>
</table>

RT amino acid residues are as defined in footnote a to Table 1. The mutations at codons 89, 92, and 156 were introduced into the AZT-resistant clone pMQ, while the mutation at codon 161 was introduced by site-directed mutagenesis into pNXHV-1LAIMC/Y. Infectious virus was recovered by transfection of MT-2 cells as described in Materials and Methods.

### Table 3. AZT and PFA susceptibilities of PFA-resistant recombinant strains of HIV-1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amino acid at indicated RT codon</th>
<th>Susceptibility to indicated drug</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>89</td>
<td>92</td>
</tr>
<tr>
<td>HX</td>
<td>E</td>
<td>L</td>
</tr>
<tr>
<td>HX89K</td>
<td>K</td>
<td>—</td>
</tr>
<tr>
<td>HX92I</td>
<td>—</td>
<td>I</td>
</tr>
<tr>
<td>HX156A</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LAI</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LAI161L</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

RT amino acid residues are as defined in footnote a to Table 1. The IC₅₀ of this strain was 3-to-3.4-fold lower than that for the wild-type strain, indicating that it was 3-to-3.4-fold hypersusceptible.
The results show that mutations which suppress AZT resistance generally also diminish PFA resistance.

Inverse correlation between degrees of AZT and PFA resistance of recombinant HIV-1 strains. We have previously reported that strains of HIV-1 that are PFA resistant because of a variety of RT mutations have increased susceptibility (hypersusceptibility) to AZT compared with wild-type strains (35, 57). The present study extends this observation to the PFA-resistant strains HX/88G, HX/89K, and HX/92I (Tables 1 and 3). PFA resistance mutations which did not confer either increased hypersusceptibility or resistance to AZT (88S or 156A) (Tables 1 and 3) did not confer PFA resistance in the AZT-resistant genetic backgrounds examined. We also determined whether AZT-resistant strains were hypersusceptible to PFA. The PFA susceptibilities of four AZT-resistant strains, HX/41+215 (MN), HX/3X, MQ, and LAIMC/Y, were assessed in the HT4LacZ-1 assay (Table 4). AZT-resistant strains HX/41+215, HX/3X, and MQ were hypersusceptible to PFA (2.2- to 2.5-fold more susceptible than the wild type) ($P = 0.05$).

Taking all the data together (Tables 1 to 4), individual RT mutations had a clear inverse relationship between AZT and PFA susceptibility (Fig. 1) (linear regression analysis excluding wild-type strains HX and LAI: $r = -0.901$, slope $= -1.81$, standard error for slope $= 0.22$, $P < 0.0001$).

An HIV-1 strain fully resistant to both AZT and PFA could not be generated by in vitro selection. The data above clearly demonstrate that PFA resistance mutations suppress AZT resistance and vice versa. To examine the biological consequence of these interactions we determined whether an HIV-1 strain coresistant to both AZT and PFA could be produced by in vitro selection. The wild-type HIV-1 strain PD, previously used to select PFA-resistant strains (57), was passaged in the presence of increasing concentrations of PFA and AZT in MT-2 cells (results not shown). Following 25 passages (164 days in culture), we selected strain PFA330AZT0.2p25, which replicated in the presence of 330 $\mu$M PFA and 0.2 $\mu$M AZT. This strain was PFA resistant (7.8-fold increase in IC$_{50}$) but fully AZT susceptible compared with a wild-type PD that had undergone 23 passages in MT-2 cells in the absence of drug (results not shown).

Nucleotide sequence analysis of the entire RT region of PFA330AZT0.2p25 revealed three substitutions not present in wild-type PD (K70R, V75I, and K219R). These mutations were different from the single-amino-acid substitution E89K or L92I observed when this strain was exposed to PFA alone (57).

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**TABLE 4.** PFA susceptibility of AZT-resistant recombinant strains of HIV-1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Susceptibility to indicated drug</th>
<th>Resistance (fold)$^b$</th>
<th>Mean IC$_{50}$ ± SD (µM)$^a$</th>
<th>Resistance (fold)$^b$</th>
<th>Mean IC$_{50}$ ± SD (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HX</td>
<td>22 ± 5</td>
<td>1</td>
<td>0.019 ± 0.012</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HX/41+215 (MN)$^c$</td>
<td>10 ± 2.6</td>
<td>0.5</td>
<td>0.62 ± 0.36</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>HX/3X$^d$</td>
<td>8.9 ± 4.6</td>
<td>0.4</td>
<td>2.23 ± 1.7</td>
<td>117</td>
<td></td>
</tr>
<tr>
<td>MQ$^e$</td>
<td>11.2 ± 4.0</td>
<td>0.4</td>
<td>2.55 ± 1.34</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td>LAI</td>
<td>37 ± 7.0</td>
<td>1</td>
<td>0.026 ± 0.006</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>LAIMC/Y$^f$</td>
<td>45 ± 7.0</td>
<td>1.2</td>
<td>0.76 ± 0.3</td>
<td>29</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ IC$_{50}$ and standard deviations were determined in drug susceptibility assays performed in HT4LacZ-1 cells and were calculated from at least two independent assays. The differences between IC$_{50}$ of PFA for HX/41+215 (MN), HX/3X, and MQ were significantly different compared with that for HX ($P \leq 0.05$). IC$_{50}$ of AZT for HX/41+215 (MN), HX/3X, and MQ were statistically different compared with that for HX ($P = 0.008$). IC$_{50}$ of AZT for LAI and LAIMC/Y were significantly different ($P = 0.014$).

$^b$ The fold increase in resistance was calculated by dividing the IC$_{50}$ of the mutant strain by the IC$_{50}$ of the corresponding wild-type strain.

$^c$ AZT-resistant strain with mutations M41L and T215Y.

$^d$ AZT-resistant strain with mutations M41L, D67N, K70R, and T215Y.

$^e$ AZT-resistant strain with mutations M41L, D67N, K70R, and T215Y.

$^f$ AZT-resistant strain with mutations M41L, D67N, K70R, T215Y, and K219Q.

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**FIG. 1.** Effect of HIV-1 RT mutations on relative susceptibility to AZT and PFA. Fold changes in PFA or AZT resistance were calculated relative to the corresponding wild-type strain (HX or LAI), from the data in Tables 1, 2, 3, and 4. Symbols depict different RT genetic backbones and are labelled with the associated PFA resistance-conferring mutation.
One of these changes (K70R), has been shown to be associated with an eightfold increase in AZT resistance (29). Changes at codon 219 have also been associated with AZT resistance; however, the mutation in PFA330AZT0.2p25 (K219R) was different from the K219Q change usually observed (30). V75I has been previously reported in HIV-1 variants resistant to PFA (57), indicating that preexisting AZT resistance did not hinder the ability of the virus to become PFA resistant. Although strain PFA660p12 was moderately PFA resistant (IC50 increased by 3.5-fold), it had reverted to being fully AZT-susceptible (Table 5).

Sequencing of the RT region of six molecular clones (pf1 to pf6) derived from this PFA-resistant strain showed four mutations common to all six clones (Table 5). Three of these were the original AZT resistance-associated mutations at codons 41, 70, and 215. The fourth was W88G, which has been previously reported for a PFA-resistant clinical isolate (35). Other changes were also observed in single clones at codons 91, 172, 217, 211, 326, and 386 and in two clones at codon 217 (Table 5). Of these, R172K and R211K were previously described changes (37), while the other mutations included in Table 5 represent new polymorphic substitutions.

TABLE 5. Amino acid sequences of RT region and PFA and AZT susceptibilities of HIV-1 strain PFA660p12 and molecular clones derived from this strain

<table>
<thead>
<tr>
<th>Strain or clone</th>
<th>Amino acid at indicated RT codon</th>
<th>Susceptibility to indicated drug</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>D</td>
</tr>
<tr>
<td>HX PFA660p12</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>62</td>
</tr>
<tr>
<td>pf1</td>
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<td>pf2</td>
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<td>R</td>
</tr>
<tr>
<td>pf5</td>
<td>L</td>
<td>R</td>
</tr>
<tr>
<td>pf6</td>
<td>L</td>
<td>R</td>
</tr>
</tbody>
</table>

* RT amino acid residues shown are numbered as for HX (HXB2 sequence) and are those that differ from the sequence for isolate HX, as predicted from the observed nucleotide sequence codons 1 to 414. Underlined codons and those in boldface type denote those associated with AZT and PFA resistance, respectively. Mean IC50 and standard deviations were determined in drug susceptibility assays performed in HT14LacZ-1 cells and were calculated from at least three independent experiments. Statistically significant differences in IC50 of PFA were observed for HX and F3 or PFA660p12 (P = 0.05). The difference in IC50 of AZT for HX and F3 was not statistically significant (P = 0.2).

Obtained by passaging a mixture of AZT-resistant cloned strains (HX/41+215, HX/3X, and MQ) in MT-2 cells in the presence of increasing concentrations of PFA (Fig. 2).

† Infectious virus with RT region of pf3 (F3) generated by cotransfection of MT-2 cells with pf3 and pHIVAR1stBl-EII.
could have been genuine or could have been introduced during PCR amplification.

Two attempts to recover infectious virus containing the RT regions of pF2, pF3, pF4, and pF5 were made. Of these, only pF3 yielded infectious virus following cotransfection with pHIV\textsuperscript{\textregistered}RTBstEII in MT-2 cells, suggesting that Q91L, D177A, P217S, I326M, or mutations in other parts of the 2.2-kb pol amplimers may have been lethal. Examination of the drug susceptibilities of strain F3 showed that it was PFA resistant and AZT susceptible as was observed for the original uncloned strain PFA660p12.

In contrast to passage in PFA alone, passage of the AZT-resistant virus mixture in the presence of PFA in conjunction with 0.2 μM AZT markedly delayed the emergence of an HIV-1 strain with the ability to replicate in the presence of 660 μM PFA (Fig. 2). This strain (PFA660AZT0.2p29) was observed after 29 passages (170 days in culture) and was phenotypically PFA resistant (2.3-fold) and AZT susceptible (IC\textsubscript{50} ≤ 0.05 μM) (Table 6).

Nucleotide sequence analysis of the RT region of PFA660AZT0.2p29 revealed seven substitutions common to each of the six molecular clones examined (pFA1 to pFA6) (Table 6). Four of these were the AZT resistance mutations at codons 41, 67, 70, and 215 which were collectively present in the starting mixture and which together confer high-level resistance to AZT (134-fold [strain MQ in Table 4]). W88S and S68N changes were also observed in all six clones, and it is noteworthy that W88S has been previously reported for three out of six PFA-resistant clinical isolates (34, 35). Infectious virus containing the RT region of pFA4 (which had an additional nonpolymorphic mutation, N175S) was recovered. This strain (pFA4) was PFA resistant and AZT susceptible as was observed for the original uncloned strain PFA660AZT0.2p29.

To confirm that HIV-1 core resistant to other inhibitors could be generated by the selection system described, the cloned AZT-resistant strain HX/41 + 215 was exposed to increasing concentrations of the nonnucleoside RT inhibitor nevirapine (11) in the presence of 0.2 μM AZT. After 10 passages (36 days in culture) HIV-1 coreistant to AZT and nevirapine was generated (increase in AZT and nevirapine resistance, 31- and 162-fold, respectively) (56).

**DISCUSSION**

The data presented in this work show that we were unable to generate strains in HIV-1 resistant to both PFA and AZT by prolonged in vitro passage. Furthermore, the pattern of resistance to AZT and PFA in drug-selected and recombinant HIV-1 strains (generated by site-directed mutagenesis) was mutually exclusive, in that resistance to either AZT or PFA, but not both, was observed. Mutagenesis studies revealed that PFA resistance mutations suppressed AZT resistance, with most showing a concomitant loss in the level of PFA resistance normally conferred by these individual changes in a wild-type genetic background. The analysis of all recombinant strains examined in this study showed a clear inverse correlation between phenotypic PFA and AZT resistance (Fig. 1). This data has led to the hypothesis that there may be constraints on the HIV-1 RT such that a more complicated evolutionary pathway would be required for the enzyme to adopt conformations in the PFA and AZT triphosphate (AZT-TP) binding sites consistent with coreistance to PFA and AZT.

Invariably, RT inhibitors that select mutations conferring phenotypic suppression of AZT resistance, including ddI-selected L74V, nonnucleoside reverse transcriptase inhibitor-selected Y181C, and lamivudine-selected M184V, have achieved coreistance in vivo by complicated escape routes (26, 41, 50, 51). Similarly, we expect that multiple mutations would be required.
to generate a strain coresistant to AZT and PFA. Given the reciprocal interactions between AZT and PFA resistance mutations, not previously reported with other AZT-resistance suppressor mutations, and the assumption that no mutation is considered neutral (5), it is possible that a strain coresistant to AZT and PFA may have an impaired replication capacity. HIV-1 strains from patients receiving long-term AZT and PFA would need to be examined to confirm this notion.

We were unable to select HIV-1 strains coresistant to AZT and PFA by in vitro passage under conditions under which strains resistant either to PFA or AZT alone or to the AZT and nevirapine combination were rapidly selected. Selected strains were consistently PFA resistant and AZT susceptible, despite the presence of mutations in the RT region known to confer phenotypic resistance to AZT. While coresistant strains were not selected in our in vitro selection experiments, no in vitro system can effectively mimic the high viral turnover in HIV-1-infected individuals, the major determinant driving genetic variation observed in vivo (5, 14, 65). A similar pattern of genomic AZT and PFA resistance with phenotypic PFA resistance and AZT susceptibility was suggested in isolates obtained from six patients with AIDS who received PFA with prior or concomitant AZT therapy (34, 35). These strains had wild-type susceptibility to AZT (IC₅₀ < 0.2 μM) as assayed in PBMCs (34), despite the presence of one or more AZT resistance mutations at codon(s) 41, 67, 70, 210, 215, and/or 219, including the atypical substitutions M41V, K70E/G, T215L, and K219R. These anecdotal data suggest that genotypic AZT resistance can be phenotypically reversed by PFA resistant mutations. However, since the timing and duration of AZT and PFA therapy in these patients varied, these data cannot be used to support our hypothesis that coresistance to AZT and PFA is difficult to achieve. Therefore, analysis of further clinical isolates to confirm the assertion that the combination of AZT and PFA imposes constraints on the mutability of HIV-1 RT will be required.

We decided to investigate the role of the PFA resistance-conferring mutations W88S, W88G, and Q161L in the phenotypic reversal of AZT resistance since they comprise three of the four mutations found in PFA-resistant clinical isolates (35). In addition to seeing those HIV-1 clinical isolates published by Mellors et al. (35), we have also seen mutations at codon 88 in clinical isolates from patients who have received AZT and PFA (56), H208Y, found in two of the six PFA-resistant isolates from the Mellors et al. study (35), was shown to confer only twofold PFA resistance in a wild-type genetic background. Since many permutations and combinations of PFA and AZT resistance-conferring mutations could potentially be examined, we chose those that were the most frequently observed in PFA-resistant clinical isolates, that conferred high levels of PFA resistance, or that were observed in strains selected in vitro in the presence of PFA or AZT and PFA.

All PFA resistance mutations studied caused phenotypic reversal of genomic AZT resistance. The presence of W88G in several AZT-resistant genetic backgrounds resulted in complete suppression of AZT resistance, while W88S partially suppressed AZT resistance and conferred PFA susceptibility in strains with M41L and T215Y and M41L, D67N, K70R, and T215Y mutations. Similarly, an AZT-resistant, PFA-susceptible clinical isolate, E6, obtained from a patient with AIDS treated with long-term PFA and AZT therapy (12, 58), had W88S in the presence of M41L, D67N, K70R, L210W, and T215Y. However, PFA resistance and AZT susceptibility were observed in the genetic contexts found in three HIV-1 clinical isolates which had W88S in association with changes that included codon 219 and/or nonclassical substitutions at codons 70 and 215 (34, 35). Therefore, the effect of W88S on AZT resistance is dependent on the genetic background in which it appears, mirroring an observation we made previously with the L210W mutation (15). Mutagenesis studies demonstrated that other PFA resistance mutations, E89K, L92I, S156A, and Q161L, could also suppress AZT resistance.

PFA resistance mutations could be divided into two groups based on the PFA and AZT susceptibility patterns observed when these mutations were introduced into an AZT-resistant genetic background. One group of mutations (W88G, E89K, L92I, and Q161L) yielded PFA-resistant, AZT-susceptible phenotypes, while the other group (W88S and S156A) yielded PFA-susceptible phenotypes which were partially or completely AZT resistant. Mutations belonging to the first group conferred high-level PFA resistance (~5.0-fold) and concomitant hypersusceptibility to AZT (three- to fivefold) when present in a wild-type genetic background, while mutations in the second group conferred only low-level PFA resistance (two- to fourfold) and failed to alter AZT susceptibility. These data indicate that the magnitude of suppression of AZT resistance and the capacity to express PFA resistance directly correlate with the level of PFA resistance and AZT hypersusceptibility conferred by the individual PFA resistance mutations. As shown in Fig. 1, W88S and S156A confer differential results in viruses with AZT-resistant genetic backgrounds MN and MQ, compared with wild-type HX, which contrasts with similar profiles obtained for W88G, E89K, and L92I in MN, MQ, and HX genetic backgrounds. A possible explanation could be that conformational changes conferred by W88S or S156A on the AZT-TP binding site of the HIV-1 RT may not be as great as those conferred by W88G, E89K, and L92I to completely counteract the effects of the AZT resistance mutations in MN and MQ.

Elucidation of the crystal structure of the HIV-1 RT (17) and molecular modelling of a dNTP in the polymerase active site of the HIV-1 RT-DNA-Fab complex has revealed the likely dNTP binding site (61). PP exchange and therefore PFA binding would be expected to occur in close proximity to this site, possibly in the region flanked by the three catalytically active aspartyl residues at codons 110, 185, and 186 (38, 61). The side chains of these residues probably bind to the triphosphate moiety of the incoming dNTP via Mg²⁺ (61). Enzyme kinetic analysis has shown that PFA is a noncompetitive inhibitor with respect to dTTP, indicating that the binding sites for PFA and dTTP (and therefore AZT-TP) on HIV-1 RT are not identical (64). However, use of inhibitor combinations has shown that the inhibition of HIV-1 RT by PFA and that by AZT-TP are mutually exclusive, suggesting overlapping binding sites (53). Consistent with this finding is the reported additive inhibition of HIV-1 RT by the combination of AZT and PFA (9, 24).

Our data suggest that the majority of PFA resistance mutations induce a structural change in the PFA binding site which simultaneously alters the conformation of the AZT-TP binding site. Since the locations of most mutations conferring AZT or PFA resistance described to date are distant from the putative AZT-TP and PFA binding sites on the HIV-1 RT (35, 57, 61), the conformational changes at these sites induced by these mutations would probably be mediated by a change in the positioning or conformation of the template-primer on the surface of the HIV-1 RT enzyme.

We found that the AZT-resistant strain LAIMC/Y was not hypersusceptible to PFA and that PFA resistance caused by Q161L was not suppressed in the LAIMC/Y background. However, Q161L was able to completely suppress AZT resistance in the LAIMC/Y background. This difference could be
explained by the effects of these mutations on RT structure. Q161L is the only PFA resistance-conferring change that may directly affect the dNTP binding site (35). In addition, LAIMC/Y contains a change at codon 219 which also may interact directly with the dNTP binding site (61). Since Q161L confers increased susceptibility to AZT (35), it is possible that the inability to suppress PFA resistance in the LAIMC/Y background and the lack of PFA hypersusceptibility of LAIMC/Y are mediated by changes at codon 219 alone, or in combination with other AZT-resistance mutations observed in this genotype. Mutations at codon 219 may result in a conformational change quite distinct from those conferred by PFA resistance mutations at codons 88, 89, 92, 156, and 208 and the AZT resistance mutations at codons 41, 67, 70, 210, and 215, which are thought to mediate resistance through an indirect mechanism (35, 57, 61). It is possible that potential escape routes for HIV-1 to become resistant to AZT and PFA involve codon 219 and other changes that do not result in reciprocal changes in AZT and PFA resistance. If coreistance to AZT and PFA is an unfavorable option for the HIV-1 RT, as suggested by our data, the obvious corollary is that such a strain (if generated) would be expected to have impaired replication capacity. Evidence to support this notion comes from previous studies (32, 33) which showed that the introduction of mutations in the HIV-1 RT at codons 113, 114, 115, 151, or 154 resulted in RT with coreistance to AZT-TP and PFA but with impaired RT activity in in vitro assays compared with the wild-type enzyme. In addition, a trend whereby greater impairment of RT activity was generally associated with higher levels of coreistance to AZT-TP and PFA was observed (32). Furthermore, infectious virus could not be recovered from HIV-1 constructs with low levels of RT activity (32). Paradoxically, recombining encodons A114S and D113E, which had 80 and 71% wild-type RT activity, respectively, were phenotypically PFA resistant but AZT hypersusceptible (32), suggesting that the conformation of the HIV-1 RT within the intracellular reverse transcription complex differs from that in the cell-free assay system employed. The identification of similar discrepancies between AZT susceptibilities in cell-free and virus replication systems with other mutations selected by AZT and PFA would confirm this hypothesis and could provide clues as to why mutant RT resistance to AZT-TP in cell-free assays does not correlate with resistance in infectious virions. AZT and PFA have several desired properties required of potential drug combinations. These include demonstrable in vitro additive or synergistic antiretroviral effects (9, 24), distinct in vivo toxicity profiles, and lack of cross-resistance. This study has shown that an array of PFA resistance mutations can cause varying levels of suppression of AZT resistance and that, at least in vitro, dually resistant virus is difficult to generate. We have postulated that this is because of conformational constraints on HIV-1 RT. PFA, however, is far from being an ideal antiretroviral drug, since it requires intravenous administration and adverse reactions are common (42). As a consequence, it is unsuitable for long-term use in asymptomatic HIV-1-infected individuals. Ideally an orally bioavailable prodrug of PFA would be useful in the context of combined treatment with AZT. An orally bioavailable glycerophospholipid PFA prodrug with enhanced activity and synergy with AZT against HIV-1 in vitro has been described previously (16). This drug, or other inhibitors of PP, exchange with improved pharmacological properties, might be able to take advantage of the favorable drug resistance interactions with AZT.

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