

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

GILDA TACHEDJIAN,¹ JOHN MELLORS,² HENGEMEH BAZMI,² CHRIS BIRCH,^{1,3} AND JOHN MILLS^{1*}

National Centre in HIV Virology Research, Macfarlane Burnet Centre for Medical Research,¹ and Victorian Infectious Diseases Reference Laboratory, Fairfield Hospital,³ Fairfield, Australia 3078, and Department of Medicine, University of Pittsburgh, and Veterans Administration Medical Centers, Pittsburgh, Pennsylvania 15213²

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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 virus, 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.

Treatment of human immunodeficiency virus type 1 (HIV-1) infection with antiretroviral agents selects for strains of HIV-1 that exhibit drug resistance (28, 44, 54). Clinical studies with several classes of antiretroviral agents have shown that the development of drug-resistant variants is associated with drug failure, as indicated by the return of the viral load and the CD4 cell numbers to pretreatment levels (4, 8, 19, 25, 36, 49, 63). The development of drug resistance is driven by the lack of fidelity of the HIV-1 reverse transcriptase (RT) and the extremely high rate of viral replication *in vivo* (5, 14, 65).

Better strategies to delay or prevent the emergence of drug-resistant strains of HIV-1 are needed. Combination drug therapy is being advocated with the premise that enhanced inhibition of viral replication achieved by antiretroviral combinations can limit viral diversity and delay the appearance of drug-resistant variants. Other desirable features of drug combinations include synergistic inhibition of virus replication, lack of cross-resistance, and nonoverlapping toxicity profiles.

More recently an important interaction between certain drug resistance mutations that may predict highly effective drug combinations has been identified. This interaction is termed a suppressor mutation or phenotypic reversal and is defined as occurring when resistance to one drug reverses the effect of resistance mutations to another drug. Such drug combinations may exert constraints on the mutability of the target enzyme which prevent or delay coresistance. Previously reported suppressor mutations in the HIV-1 RT include the

L-to-V mutation at codon 74 (L74V), Y181C, and M184V, which individually suppress zidovudine (3'-azidothymidine) (AZT) resistance while conferring resistance to didanosine (ddI), nevirapine, and lamivudine, respectively (27, 54, 62). Treatment with combinations of AZT and lamivudine or ddI has generally been associated with reductions in levels of viral RNA in plasma and increases in CD4 cell counts greater and more sustained than those associated with monotherapy (6, 31, 43). However, the eventual emergence of HIV-1 strains coresistant to these inhibitors in treated patients has been described previously (26, 41, 50, 51).

We have directed our attention to the HIV-1 RT inhibitors AZT and foscarnet (phosphonoformic acid) (PFA), which have distinct mechanisms of action and are already in widespread clinical use. AZT is a nucleoside analog that has been used for the treatment of HIV-1-infected individuals for nearly a decade, even though long-term monotherapy with AZT is well known to cause the emergence of drug-resistant strains (28). AZT resistance is mediated by the accumulation of up to six mutations, including M41L, D67N, K70R, L210W, T215Y/F, and K219Q (15, 21, 30), in the RT.

In contrast to AZT, the PP_i analog PFA inhibits reverse transcription by blocking PP_i exchange (7, 42). This drug is used to treat infections due to cytomegalovirus (55) and acyclovir-resistant varicella-zoster and herpes simplex viruses (3, 10, 46, 47, 55). PFA inhibits HIV-1 replication *in vitro* (48) and *in vivo* (18, 20), and the antiretroviral effect of PFA was one suggested explanation for the improved survival of PFA-treated patients in a clinical study which compared PFA with ganciclovir for treatment of cytomegalovirus retinitis in patients with AIDS (55).

PFA-resistant strains of HIV-1 have developed in patients

* Corresponding author. Mailing address: Macfarlane Burnet Centre for Medical Research, P.O. Box 254, Fairfield, Australia 3078. Phone: (61) 3 9282 2123. Fax: (61) 3 9282 2126. Electronic mail address: mills@burnet.mbcmr.unimelb.edu.au.

with AIDS receiving long-term PFA therapy for cytomegalovirus retinitis (35). The RT substitutions W88G, W88S, 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, Grand Island, N.Y.)–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 μ g/ml and then transferred to medium containing interleukin-2 (59) at the time of infection with HIV-1. HT4LacZ-1 cells (45) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and G418 (Gibco) at 400 μ g/ml.

Plasmids. The construction of the pKPHXB2 infectious HIV-1 molecular clone, which contains the *Xba*I fragment of HIV-1 provirus from pHXB2-D, has been described previously (57). The derivation of the pXXHIV-1_{LAI} infectious molecular clone has been reported previously (40). pHX/HOM contains a 4.3-kb *Hind*III fragment of HXB2 encompassing the complete *pol* gene (coordinates 1258 to 5578) cloned into the *Hind*III site of pTZ19U (Bio-Rad Laboratories, Inc., North Ryde, Australia) (15). Constructs pHX/41+215 and pHX/3X were 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). pMQ and pMN were obtained by subcloning a 1.7-kb *Xba*I-*Eco*RI fragment from the M13mptac18.1 clones HIVRT_{MO} and HIVRT_{MN}, respectively (gifts from Brendan A. Larder) (30), into the *Xba*I-*Eco*RI site of pT7T319U (AMRAD Pharmacia Biotech, North Ryde, Australia) and pTZ19U (Bio-Rad Laboratories, Inc.). HIVRT_{MN} and HIVRT_{MO} encode the AZT resistance mutations M41L and T215Y and M41L, D67N, K70R, and T215Y, respectively (22). The derivation of pHIV Δ RTBstEII (a gift from Brendan A. Larder) has been described previously (54). pKPHXB2 Δ RT possesses most of the HXB2 sequence except for a 1.96-kb deletion of the HIV-1 RT gene (coordinates 2168 to 4099) (15, 57). pXXHIV-1_{LAI}MC/Y has the AZT resistance mutations D67N, K70R, T215Y, and K219Q introduced into pXXHIV-1_{LAI} by site-directed mutagenesis (35).

Viruses. PD 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 pKPHXB2 into MT-2 cells. Clonal strains of HX/41+215, HX/3X, HX88S, and HX88G were generated by cotransfection of pHX/41+215, pHX/3X, pHX88S, or pHX88G with pKPHXB2 Δ RT into MT-2 cells. Recombinant HIV-1 strains MQ, MQ88S, MQ88G, MQ89K, MQ92I, MQ156A, MN88S, and MN88G were recovered by cotransfection of MT-2 cells with the constructs pMQ, pMQ88S, pMQ88G, pMQ89K, pMQ92I, pMQ156A, pMN88S, or pMN88G with pHIV Δ RTBstEII. Strains LAI, LAIMC/Y, LAI161L, and LAIMC/Y161L were prepared by electroporation of MT-2 cells with pXXHIV-1_{LAI}, pXXHIV-1_{LAI}MC/Y, pXXHIV-1_{LAI}161L, and pXXHIV-1_{LAI}MC/Y161L, respectively (35).

The cotransfection in MT-2 cells was performed with 5 μ g of *Msc*I-linearized pKPHXB2 Δ RT or *Bst*EII-linearized pHIV Δ RTBstEII with either *Xba*I- and *Eco*RI-linearized (pMQ- and pMN-derived constructs) or *Hind*III-linearized (pHX/HOM constructs) phagemids with DOTAP (Boehringer, Mannheim, Germany) following the manufacturer's recommendations. The vector sequence released by enzyme digestion was not removed prior to transfections. Infectious HIV-1 strains F2, F3, F4, F5, and FA4 containing the *pol* gene derived from plasmids pF2, pF3, pF4, pF5, and pFA4A, respectively, were generated by cotransfection of *Bam*HI-*Eco*RI-digested phagemids with *Bst*EII-digested pHIV Δ RTBstEII. Cultures were maintained until the maximum cytopathic 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 300) of recombinant

strains (MN88S, MN88G, MQ88S, MQ88G, MQ89K, MQ92I, MQ156A) were sequenced. Changes that differed from the expected sequence were not found.

Drugs. PFA (Fluka Biochemika, 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 400,000/4 ml) were inoculated with 2,500 50% tissue culture infective doses of virus and cultured in the presence of either PFA or a combination of PFA and AZT. When HIV-specific cytopathic effects involved 75 to 100% of the cells, culture fluids were clarified by centrifugation for 10 min at 600 \times g and 0.5 to 1 ml of the supernatant virus suspension was used to infect fresh MT-2 cells in the presence of 1.3- to 2.5-fold higher concentrations of the appropriate drug(s). If after 7 days the cytopathic effects did not involve at least 75% of the cells, the cell suspension was diluted 1 to 4 in medium containing the freshly added drug(s) at the original concentration and incubated further. Each such subculture was considered a single passage.

Biological cloning. Strain PFA330AZT0.2p25 was biologically cloned by three terminal dilutions in MT-2 cells in the presence of 330 μ M PFA and 0.2 μ M AZT. Following the third terminal dilution, PFA330AZT0.2p25 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 50% tissue culture infective doses of each virus was 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, Kremmsmünster, Austria) and the level of virus replication was measured by virion-associated RT activity (58).

(ii) In HT4LacZ-1 cells. Drug inhibition of blue syncytium formation was performed as previously described (45). Cells were seeded into 24-well plates (2.5 \times 10⁴ cells per well) and allowed to adhere overnight. Cells were then infected with 30 to 300 syncytium-inducing units (45) of HIV-1 in 220 μ l of Dulbecco's modified Eagle's medium–5% fetal calf serum containing 10 μ g of DEAE-dextran (AMRAD Pharmacia Biotech) per ml at 37°C. After 1 to 1.5 h, the inoculum was removed and 1-ml aliquots of medium containing the appropriate concentrations of drug were added to duplicate wells. After 3 days of incubation, the cells were fixed and stained as previously described (45) and syncytia containing three or more blue nuclei were counted.

For each virus, the percentage inhibition of either RT activity (MT-2 assay) or syncytium formation (HT4LacZ-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 log₁₀ 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 HT4LacZ-1 assays, viral strains for which the IC₅₀s were \leq 0.05 μ M were considered AZT-sensitive; those for which the IC₅₀s were $>$ 0.05 and $<$ 1.0 μ M were considered partially resistant; and those for which the IC₅₀s were \geq 1.0 μ M were considered highly resistant (21). The statistical significance of differences between IC₅₀s was determined by the Wilcoxon rank-sum test (2).

PCR amplification. HIV-1 strains were grown in phytohemagglutinin-stimulated PBMCs (2.5 \times 10⁶ cells per 10 ml) for 4 to 7 days. Purified genomic DNA was prepared from infected cells with the QIAamp kit (Qiagen, Hilden, Germany). For the in vitro-selected strain PFA330AZT0.2p25, the RT region of HIV-1 proviral DNA was amplified by two rounds of PCR using nested primers to yield a 2.2-kb DNA product. This product was used as the DNA template for the nucleotide sequencing analysis of the HIV-1 RT region. The outer primers were 5'V3 and 3'V2, and the inner primers were 5'V2 and GT3'V1 (5' GGG AAT TCC AAA TTC CTG CTT G 3' [complementary to positions 4180 to 4200 of the sense strand]). The positions of the primers were based on the sequence of HXB2R (37). The sequences of 5'V3, 5'V2, and 3'V2 and the PCR amplification conditions were as previously described (57).

To confirm the RT sequence of strains recovered by transfection in MT-2 cells, the RT region was amplified by two rounds of PCR. The first round used outer primers 5'V3 and 3'V2. This was followed by two separate second-round amplifications using the M13 forward- and reverse primer pairs M13 5'V2 (5' TGT AAA ACG ACG GCC AGT CCT ACA CCT GTC AAC ATA ATT GGA AG 3' [coordinates 2033 to 2052]) and M13Rcomb3 (5' CAG GAA ACA GCT ATG ACC ATA GGC TGT ACT GTC CAT TTA TCA GG 3' [complementary to positions 2801 to 2826 of the sense strand]) or M13 5'V4 (5' TGT AAA ACG ACG GCC AGT CAA AAA ACA TCA GAA AGA ACC TCC 3' [coordinates 2749 to 2772]) and M13R 3'V6 (5' CAG GAA ACA GCT ATG ACC ATC TGG TTG TGC TTG AAT GAT TC 3' [complementary to positions 3605 to 3628 of the sense strand]). These procedures yielded 793- and 849-bp fragments containing RT codons 1 to 244 and 218 to 511, respectively. PCR amplification conditions were as previously described for primer pairs 5'V3-3'V2 and 5'V2-3'V1 (57) with the exception of a 1-min extension for the second-round primers. PCR amplimers were initially purified with Qiaquick kit (Qiagen), and this was followed by further purification and concentration involving the dilution of DNA to 500 μ l with water and concentration in Microcon-100 microconcentrators (Amicon Inc., Beverly, Mass.) according to the manufacturer's instructions.

Preparation of molecular clones of the HIV-1 RT region. Molecular clones were prepared by PCR amplification of a 2.2-kb *pol* fragment from purified chromosomal DNA from PBMCs infected with strains PFA660AZT0.2p29 and

TABLE 1. Phenotypic reversal of AZT resistance by PFA resistance mutations at codon 88

Strain	Amino acid at indicated RT codon ^a					Susceptibility to indicated drug			
	<u>41</u>	<u>67</u>	<u>70</u>	88	<u>215</u>	PFA		AZT	
						Mean IC ₅₀ ± SD (μM) ^b	Resistance (fold) ^c	Mean IC ₅₀ ± SD (μM)	Resistance (fold)
HX	M	D	K	W	T	27 ± 6.6	1	0.019 ± 0.012	1
HX88S	—	—	—	S	—	63 ± 20	2.3	0.016 ± 0.02	0.8
HX88G	—	—	—	G	—	207 ± 36	7.7	0.004 ± 0.001	0.2 ^d
MN	L	—	—	—	Y	10 ± 2.6	0.5 ^e	0.62 ± 0.36	33
MN88S	L	—	—	S	Y	27 ± 9	1	0.25 ± 0.16	13.2
MN88G	L	—	—	G	Y	112 ± 45	4.2	0.025 ± 0.02	1.3
MQ	L	N	R	—	Y	11.2 ± 4.0	0.4 ^e	2.55 ± 1.34	134
MQ88S	L	N	R	S	Y	34 ± 5.0	1.3	0.21 ± 0.16	11.1
MQ88G	L	N	R	G	Y	122 ± 21	4.5	0.01 ± 0.003	0.53 ^f

^a RT amino acid residues shown are numbered as for HX (HXB2 sequence). Mutations at codon 88 to either serine or glycine were introduced into the wild-type HX (HXB2 backbone) or into the AZT-resistant clones pMN (carrying M41L and T215Y mutations) or pMQ (carrying M41L, D67N, K70R, and T215Y mutations). Infectious virus was recovered by transfection of MT-2 cells as described in Materials and Methods. Underlined codons and those in boldface type denote those associated with AZT and PFA resistance, respectively. —, no change from HX.

^b IC₅₀s and standard deviations were determined in drug susceptibility assays performed in HT4LacZ-1 cells and were calculated from at least three independent assays. The differences in IC₅₀s of AZT for HX and HX88S were not statistically significant ($P > 0.115$). In contrast, statistically significant differences in the IC₅₀s of AZT were noted for HX and HX88G ($P = 0.009$), MN and MN88S ($P = 0.033$), MN and MN88G ($P = 0.033$), MN88S and MN88G ($P = 0.014$), MQ and MQ88S ($P < 0.01$), MQ and MQ88G ($P = 0.036$), and MQ88S and MQ88G ($P < 0.05$). Statistically significant differences in the IC₅₀s 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 ($P = 0.008$), MN88S and MN88G ($P = 0.014$), MQ and MQ88S ($P = 0.018$), MQ and MQ88G ($P < 0.01$), and MQ88S and MQ88G ($P = 0.05$).

^c IC₅₀ for mutant strain divided by IC₅₀ for wild-type HX. Values of >1 and <1 indicate resistance and hypersusceptibility, respectively.

^d 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.

^e The IC₅₀ of PFA for this strain was determined in a different assay series in which the IC₅₀ of PFA for HX was 22 μM. Accordingly, fold resistance has been calculated with this value.

^f The IC₅₀ of AZT for this strain was not significantly different from that for wild-type HX.

PFA660p12 by using KlenTaqLA-16 (1). Two rounds of PCR amplification were performed with the outer primers 5'V3 and 3'V2 and the inner primers 5'V2 (with the *Bam*HI site) and GT3'V1 (with the *Eco*RI site), respectively. The first-round amplification conditions involved one denaturation cycle (94°C for 3 min) followed by 35 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s), and extension (68°C for 3 min) and ending with one extension cycle (68°C for 7 min). The second-round conditions were the same as for the first round except that annealing was performed at 62°C. Amplifications were performed in 50-μl volumes containing 2 μl of purified DNA in the presence of 0.4 μl of KlenTaqLA-16, 300 μM (each) deoxynucleoside triphosphate (dNTP), 0.2 μM (each) primer, and 1.5 mM MgCl₂. Five 50-μl reaction mixtures were pooled, purified by phenol-chloroform extraction, and ethanol precipitated. DNA was digested with *Bam*HI-*Eco*RI and subcloned into *Bam*HI-*Eco*RI-digested pT7T319U. The six molecular clones obtained from PFA660p12 and PFA660AZT0.2p29 were designated pF1 to pF6 and pFA1 to pFA6, respectively.

Nucleotide sequence analysis of the HIV-1 RT region. The nucleotide sequence of the RT region was determined by automated sequencing with the PRISM ready reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) and the T3 and T7 *Taq* dye primer cycle sequencing kits (Applied Biosystems). The sequencing primers and reaction conditions used for the dye terminator reactions were as previously described (15, 57). The nucleotide sequence analysis of M13 tailed amplimers was performed with the Applied Biosystems PRISM dye primer cycle sequencing ready reaction kit with AmpliTaq DNA polymerase FS with either -21M13 or M13Rev dye primers (Perkin-Elmer, Foster City, Calif.). The resolution of the sequencing products and sequence alignments were as previously described (57).

Generation of mutant strains. The phagemid clone pHX/HOM was used to introduce PFA resistance mutations in a wild-type genetic background. The mutations W88S (TGG to TCG), W88G (TGG to GGG), E89K (GAA to AAA), and S156A (TCA to GCA) were introduced in the RT gene by using mutagenic oligonucleotides complementary to the sense strand to generate the constructs pHX88S, pHX88G, pHX89K, and pHX156A, respectively. pHX92I was constructed as previously described (57). pMQ was used as a template to introduce the RT mutations W88S, W88G, E89K, L92I, and S156A. Recombinant viruses with these changes were designated MQ88S, MQ88G, MQ89K, MQ92I, and MQ156A, respectively. pMQ and pHX/HOM (15) were mutagenized with the Transformer site-directed mutagenesis kit (Clontech Laboratories Inc., Palo Alto, Calif.) with modifications as previously described (60). The selection primer used in this procedure was GTSacII (5' CTT GCA TGC CCG CGG GTC GAC TCT 3') which altered a unique *Pst*I site in the plasmids to *Sac*II. pXX-HIV-1_{LA}161L and pXXHIV-1_{LA}MC/Y161L, both with the RT mutation

Q161L (CAA to CTA), were prepared as previously described (35). pMN88S and pMN88G were constructed by digestion of pHX88S and pHX88G with *Bpm*I (HXB2 coordinate, 2253) and *Eco*RV (HXB2 coordinate, 2525) to release a 272-bp fragment containing RT codon 88. pMN was digested with *Bpm*I and *Eco*RV where 272-, 1609-, and 2628-bp fragments were generated. The 272-bp fragments of pHX88S and pHX88G were ligated to DNA fragments 1609 and 2628 of pMN to construct pMN88S and pMN88G, respectively. The presence of the desired mutations in these clones and those generated by site-directed mutagenesis was verified by nucleotide sequencing of the entire RT gene.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this article have been deposited in the GenBank database under accession numbers as follows: PDBC, U28648; PFA 165BC (PD165BC), U28649; PFA 330BC (PD660BC), U28652; PFA330AZT0.2p25, U53869; PFA660p12 (clone pF3), U53870; and PFA660AZT0.2p29 (clone pFA4), U53871.

RESULTS

PFA resistance codon 88 substitutions suppress AZT resistance. RT mutations W88S, W88G, Q161L, and H208Y have been observed in PFA-resistant HIV-1 clinical isolates from patients receiving long-term therapy with PFA (≥3 months) for cytomegalovirus retinitis (35). In addition to these strains, mutations at codon 88 were also observed in clinical isolates from patients who have received AZT and PFA (56). Since the substitutions W88S and W88G were the most frequently observed in these HIV-1 clinical isolates, their effect on AZT susceptibility was observed by inserting them into wild-type and AZT-resistant genetic backgrounds and examining the recombinant viruses for their AZT and PFA susceptibilities (Table 1). Introduction of W88S into the wild-type background of HXB2 (HX88S) resulted in a strain with low-level PFA resistance but full susceptibility to AZT (Table 1). Insertion of W88G into the wild-type HXB2 background (HX88G) yielded a highly PFA-resistant strain which was hypersusceptible to AZT (Table 1).

In contrast, introduction of the PFA resistance mutation

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

Strain	Amino acid at indicated RT codon ^a										Susceptibility to indicated drug			
	41	67	70	88	89	92	156	161	215	219	PFA		AZT	
											Mean IC ₅₀ ± SD (μM) ^b	Resistance (fold) ^c	Mean IC ₅₀ ± SD (μM) ^b	Resistance (fold)
HX	M	D	K	W	E	L	S	Q	T	K	27 ± 6.6	1	0.019 ± 0.012	1
MQ	L	N	R	—	—	—	—	—	T	—	11.2 ± 2.0	0.4 ^d	2.55 ± 1.34	134
MQ89K	L	N	R	—	K	—	—	—	Y	—	137 ± 31	5.1	0.024 ± 0.006	1.3
MQ92I	L	N	R	—	—	I	—	—	Y	—	54 ± 9.6	2	0.014 ± 0.002	0.7
MQ156A	L	N	R	—	—	—	A	—	Y	—	33 ± 5.6	1.2	0.06 ± 0.02	3.2
LAI	—	—	—	—	—	—	—	—	—	—	37 ± 7.0	1	0.026 ± 0.006	1
LAIMC/Y	—	N	R	—	—	—	—	—	Y	Q	45 ± 7.0	1.2	0.76 ± 0.3	29
LAIMC/Y161L	—	N	R	—	—	—	—	L	Y	Q	179 ± 58	4.8	0.05 ± 0.01	1.9

^a 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 pXXHIV-1_{LAI}MC/Y. Infectious virus was recovered by transfection of MT-2 cells as described in Materials and Methods.

^b As in footnote *b* to Table 1 except that PFA susceptibility data for LAI strains were derived from two independent assays. The differences in IC₅₀s of AZT were not statistically significant for HX and MQ89K ($P > 0.2$), and HX and MQ92I ($P > 0.2$) but were significant for HX and MQ156A ($P = 0.05$), MQ and MQ89K ($P = 0.012$), MQ and MQ92I ($P = 0.012$), MQ and MQ156A ($P = 0.012$), LAI and LAIMC/Y ($P = 0.029$), and LAI and LAIMC/Y161L ($P = 0.029$). The IC₅₀ of PFA for HX and MQ156A was not statistically different ($P > 0.114$). In contrast, IC₅₀s of PFA were significantly different for HX and MQ89K ($P = 0.029$), HX and MQ92I ($P = 0.029$), MQ and MQ89K ($P = 0.018$), MQ and MQ92I ($P = 0.018$), and MQ and MQ156A ($P = 0.018$).

^c The fold increase in resistance was calculated by dividing the IC₅₀ of the mutant strain by the IC₅₀ of the corresponding wild-type strain HX or LAI. Resistance was defined as described in footnote *c* to Table 1.

^d See footnote *e* in Table 1.

W88S into the AZT-resistant backgrounds MN and MQ (strains MN88S and MQ88S, respectively) resulted in fully PFA-susceptible virus in which the phenotypic effect of the preexisting AZT resistance mutations was suppressed by 2.5- and 12-fold, respectively (Table 1). Likewise, insertion of the W88G mutation into either of the MN or MQ genetic backbones (MN88G and MQ88G, respectively) resulted in PFA-resistant strains (Table 1) which were fully susceptible to AZT, despite retaining the preexisting AZT-resistant genotype.

Effect of other PFA resistance mutations on suppression of AZT resistance. We determined the capacity of previously reported PFA resistance mutations E89K, L92I, S156A, and Q161L (35, 57) to suppress phenotypic AZT resistance. When mutations E89K and L92I were introduced into the highly AZT-resistant MQ background, the resultant recombinant strains (strains MQ89K and MQ92I, respectively) became fully AZT susceptible (Table 2). Q161L also reversed AZT resis-

tance 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). MQ89K 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 3. AZT and PFA susceptibilities of PFA-resistant recombinant strains of HIV-1

Strain	Amino acid at indicated RT codon ^a				Susceptibility to indicated drug			
	89	92	156	161	PFA		AZT	
					Mean IC ₅₀ ± SD (μM) ^b	Resistance (fold) ^c	Mean IC ₅₀ ± SD (μM)	Resistance (fold)
HX	E	L	S	Q	21 ± 5.0	1	0.016 ± 0.002	1
HX89K	K	—	—	—	248 ± 40	12	0.0053 ± 0.001	0.33 ^d
HX92I	—	I	—	—	145 ± 13	7	0.0046 ± 0.004	0.29 ^d
HX156A	—	—	A	—	85 ± 13	4	0.0125 ± 0.007	0.8
LAI	—	—	—	—	37 ± 7.0	1	0.026 ± 0.006	1
LAI161L	—	—	—	L	192 ± 50	5.2	0.0084 ± 0.0016	0.32 ^d

^a RT amino acid residues are defined in footnote *a* to Table 1. Mutations at codons 89, 92, and 156 were introduced into pHX/HOM, and codon 161 was changed in pXXHIV-1_{LAI} by site-directed mutagenesis. Infectious virus was recovered by transfection in MT-2 cells as described in Materials and Methods.

^b As in footnote *b* to Table 2. Differences between IC₅₀s of AZT for HX89K and HX92I were significantly different from that for HX ($P \leq 0.05$), while IC₅₀s of AZT for LAI and LAI161L were of borderline significance ($P = 0.067$). IC₅₀s of PFA for HX89K, HX92I, and HX156A were significantly different from that for HX ($P = 0.05$).

^c As in footnote *c* to Table 1.

^d 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.

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

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

^a IC₅₀s 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 IC₅₀s of PFA for HX/41+215 (MN), HX/3X, and MQ were significantly different compared with that for HX ($P \leq 0.05$). IC₅₀s of AZT for HX/41+215 (MN), HX/3X, and MQ were statistically different compared with that for HX ($P = 0.008$). IC₅₀s of AZT for LAI and LAIMC/Y were significantly different ($P = 0.014$).

^b The fold increase in resistance was calculated by dividing the IC₅₀ of the mutant strain by the IC₅₀ of the corresponding wild-type strain.

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

^d AZT-resistant strain with mutations M41L, L210W, and T215Y.

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

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

ground of LAIMC/Y (Table 2). These 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 HX88G, HX89K, and HX92I (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 corestant 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 μM PFA and 0.2 μM AZT. This strain was PFA resistant (7.8-fold increase in IC₅₀) 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).

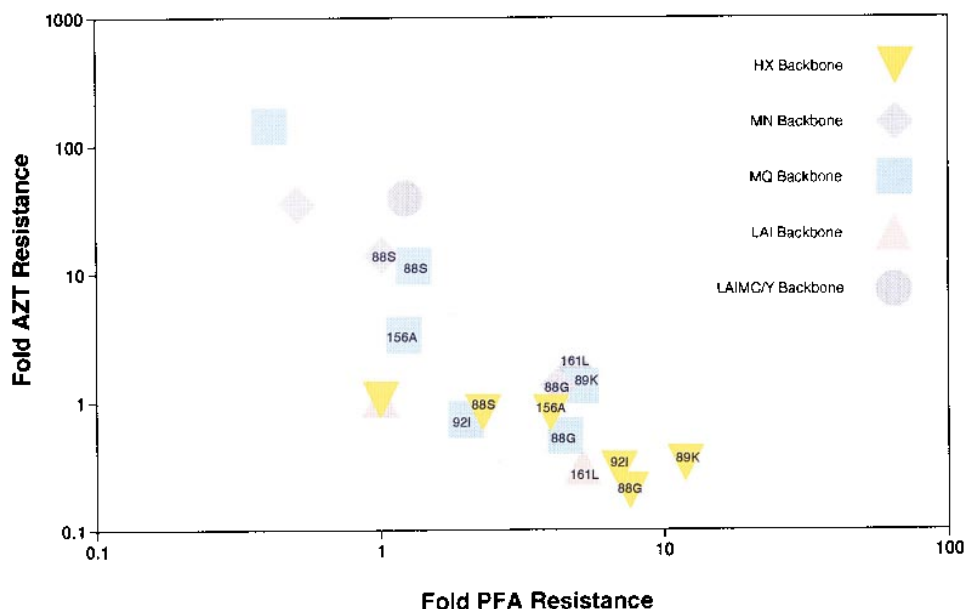


FIG. 1. Effect of HIV-1 RT mutations on relative susceptibility to AZT and PFA. Fold changes in PFA or AZT resistance were calculated relative to the corresponding wild-type strain (HX or LAI), from the data in Tables 1, 2, 3, and 4. Symbols depict different RT genetic backbones and are labelled with the associated PFA resistance-conferring mutation.

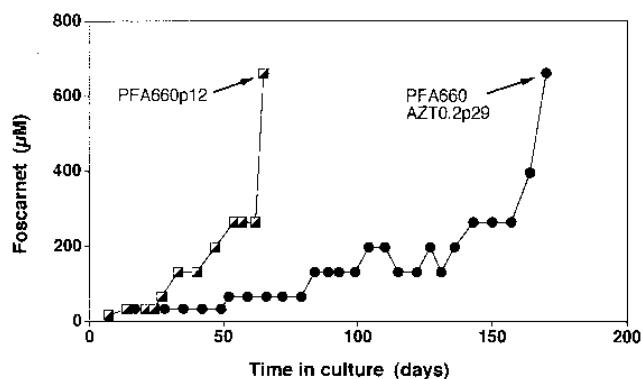


FIG. 2. Attempted selection of HIV-1 coresistant to AZT and PFA by in vitro selection in MT-2 cells starting with cloned AZT-resistant HIV-1 strains (MQ, HX/41+215, and HX/3X). See Materials and Methods for details. The time in culture is plotted against the concentration of PFA at which the isolate was able to replicate at each passage level. Squares denote the AZT-resistant mixture exposed to PFA alone, and circles represent the AZT-resistant mixture exposed to PFA in the presence of 0.2 μ M AZT.

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 multiple dideoxynucleosides recovered from patients receiving AZT and ddI or AZT and dideoxycytosine combination therapy (50, 52).

Since HIV-1 coresistant to AZT and PFA could not be generated from a wild-type strain, another strategy was used to

attempt to generate dually resistant HIV-1, taking advantage of the ability of HIV-1 to recombine (23). The starting inoculum contained a mixture of AZT-resistant, molecularly cloned strains, HX/41+215 (carrying M41L and T215Y mutations) MQ (carrying M41L, D67N, K70R, and T215Y mutations), and HX/3X (carrying M41L, L210W, and T215Y mutations), which collectively contain most of the AZT resistance-associated mutations reported to date. Selection was performed either in the presence of escalating concentrations of PFA or in increasing concentrations of PFA with 0.2 μ M AZT. AZT was included in the latter experiment to exert pressure on the virus to maintain its AZT-resistant phenotype.

Exposure of this mixture of AZT-resistant, molecularly cloned strains to PFA alone resulted in strain PFA660p12, which replicated in the presence of 660 μ M PFA after 12 passages (65 days in culture) (Fig. 2 and Table 5). The rate at which this variant was selected was similar to that observed when the wild-type HX strain was exposed to escalating concentrations of 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 (IC_{50} increased by 3.5-fold), it had reverted to being fully AZT-susceptible (Table 5).

Sequencing of the RT region of six molecular clones (pF 1 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, 177, 211, 326, and 386 and in two clones at codon 217 (Table 5). Of these, R172K and R211K were previously described polymorphic substitutions (37), while the other mutations

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

Strain or clone	Amino acid at indicated RT codon ^a												Susceptibility to indicated drug			
													PFA		AZT	
	<u>41</u>	<u>67</u>	<u>70</u>	<u>88</u>	91	172	177	211	<u>215</u>	217	326	386	Mean $IC_{50} \pm$ SD (μ M) ^b	Resistance (fold) ^c	Mean $IC_{50} \pm$ SD (μ M)	Resistance (fold)
Strains																
HX	M	D	K	W	Q	R	D	R	T	P	I	T	34 \pm 19	1	0.02 \pm 0.01	1
PFA660p12 ^d													119 \pm 20	3.5	0.026 \pm 0.02	1.3
Molecular clones derived from PFA660p12 ^e																
pF1	L	—	R	G	—	—	—	K	Y	—	M	—				
pF2	L	—	R	G	—	—	A	—	Y	S	—	—				
pF3 ^f	L	—	R	G	—	—	—	—	Y	—	A	—	165 \pm 0	4.8	0.04 \pm 0.04	2
pF4	L	—	R	G	L	K	—	—	Y	—	—	—				
pF5	L	—	R	G	—	—	—	—	Y	S	—	—				
pF6	L	—	R	G	—	—	—	—	Y	—	—	—				

^a 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. Mutations at codons 172 and 211 represent known polymorphisms. —, no change from HX.

^b IC_{50} s and standard deviations were determined in drug susceptibility assays performed in HT4LacZ-1 cells and were calculated from at least three independent assays. Statistically significant differences in IC_{50} s of PFA were observed for HX and F3 or PFA660p12 ($P = 0.05$). The difference in IC_{50} s of AZT for HX and F3 was not statistically significant ($P = 0.2$).

^c IC_{50} of mutant strain divided by IC_{50} of wild-type HX. Values of >1 and <1 indicate resistance and hypersusceptibility, respectively.

^d 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).

^e Molecular clones obtained by PCR amplification of 2.2 kb of *pol* by using KlenqLA-16 and cloned in *Bam*HI-*Eco*RI sites in pT7T319U as described in Materials and Methods.

^f Infectious virus with RT region of pF3 (F3) generated by cotransfection of MT-2 cells with pF3 and pHIV Δ RTBstEII.

TABLE 6. Amino acid sequences of RT region and PFA and AZT susceptibilities of HIV-1 strain PFA660AZT0.2p29 and molecular clones derived from this strain

Strain or clone	Amino acid at indicated RT codon ^a												Susceptibility to indicated drug			
													PFA		AZT	
	<u>41</u>	61	<u>67</u>	68	<u>70</u>	88	175	214	<u>215</u>	260	295	322	Mean IC ₅₀ ± SD (μM) ^b	Resistance (fold) ^c	Mean IC ₅₀ ± SD (μM)	Resistance (fold)
Strains																
HX	M	F	D	S	K	W	N	L	T	L	L	S	35 ± 14	1	0.019 ± 0.013	1
PFA660-AZT0.2p29 ^d													92 ± 40	2.6	0.044 ± 0.03	2.3
Molecular clones derived from PFA660AZT0.2p29 ^e																
pFA1	L	L	N	N	R	S	—	F	Y	V	P	S				
pFA2	L	—	N	N	R	S	—	F	Y	V	—	P				
pFA3	L	—	N	N	R	S	—	F	Y	V	—	—				
pFA4 ^f	L	—	N	N	R	S	S	F	Y	V	—	—	98 ± 10	2.8	0.03 ± 0.02	1.5
pFA5	L	—	N	N	R	S	—	F	Y	—	—	—				
pFA6	L	—	N	N	R	S	—	F	Y	V	—	—				

^a 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. Unique changes at codons 1 to 323 are indicated, with the exception of pFA6 (codons 1 to 310). Underlined codons and those in boldface type denote those associated with AZT and PFA resistance, respectively. Codon 214 represents a previously described polymorphism. —, no change from HX.

^b As defined in footnote *b* to Table 5. Statistically significant differences were observed for the IC₅₀s of PFA for HX and PFA660AZT0.2p29 ($P = 0.05$) or FA4 ($P = 0.014$). The IC₅₀s of AZT for HX and PFA660AZT0.2p29 ($P = 0.2$) or FA4 ($P > 0.171$) were not significant.

^c As defined in footnote *c* to Table 5.

^d Obtained by passaging a mixture of AZT-resistant cloned strains (HX41+215, HX/3X, and MQ) in MT-2 cells in the presence of increasing concentrations of PFA and 0.2 μM AZT (Fig. 2).

^e Molecular clones obtained by PCR amplification of 2.2 kb of *pol* by using KlenTaqLA-16 and cloned in *Bam*HI-*Eco*RI sites in pT7T319U as described in Materials and Methods.

^f Infectious virus with RT region of pFA4 (FA4) generated by cotransfection of MT-2 cells with pFA4 and pHIVΔRTBstEII.

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 pHIVΔRTBstEII in MT-2 cells, suggesting that Q91L, D177A, P217S, I326M, or mutations in other parts of the 2.2-kb *pol* amplifiers may have been lethal. Examination of the drug susceptibility 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 (IC₅₀ ≤ 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 (FA4) was PFA resistant and AZT susceptible as was observed for the original uncloned strain PFA660AZT0.2p29.

To confirm that HIV-1 coresistant 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 coresistant 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 to 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). These data have 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 coresistance 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 coresistance 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 ($IC_{50} < 0.2 \mu M$) 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_i 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 Mg^{2+} (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 distal 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 coresistant to AZT and PFA involve codon 219 and other changes that do not result in reciprocal changes in AZT and PFA resistance.

If coresistance 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 coresistance 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 coresistance 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, recombinants encoding 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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