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Novel Mutations in Reverse Transcriptase of Human Immunodeficiency Virus Type 1 Reduce Susceptibility to Foscarnet in Laboratory and Clinical Isolates

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Foscarnet (phosphonoformic acid) is a pyrophosphate analog that inhibits the replication of human immunodeficiency virus type 1 (HIV-1) in vitro and in patients with AIDS. HIV-1 resistance to foscarnet has not been reported despite long-term foscarnet therapy of AIDS patients with cytomegalovirus disease. We therefore attempted to select foscarnet-resistant HIV-1 in vitro by serial endpoint passage of virus in 400 μM foscarnet. After 13 cycles of passage in MT-2 cells, virus exhibiting ≥8.5-fold foscarnet resistance was isolated. The reverse transcriptase (RT) from resistant virions exhibited a similar level of foscarnet resistance in enzyme inhibition assays (~10-fold resistance). Foscarnet-resistant virus showed increased susceptibility to 3′-azido-3′-deoxythymidine (90-fold) and to the HIV-1-specific RT inhibitors TIBO R82150 (30-fold) and nevirapine (20-fold). DNA sequence analysis of RT clones from resistant virus revealed the coexistence of two mutations in all clones: Gln-161 to Leu (CAA to CTA) and His-208 to Tyr (CAT to TAT). Sequence analysis of six clinical HIV-1 isolates showing reduced susceptibility to foscarnet revealed the Tyr-208 mutation in two, the Leu-161 mutation in one, and a Trp-88–to–Ser or –Gly mutation in four isolates. Site-specific mutagenesis and production of mutant recombinant viruses demonstrated that the Leu-161, Ser-88, and Tyr-208 mutations reduced HIV-1 susceptibility to foscarnet 10.5-, 4.3-, and 2.4-fold, respectively, in MT-2 cells. In the crystal structure of HIV-1 RT, the Gln-161 residue lies in the αE helix beneath the putative deoxynucleoside triphosphate (dNTP) binding site. The Gln-161–to–Leu mutation may affect the structure of the dNTP binding site and its affinity for foscarnet. The location of the Trp-88 residue in the β5a strand of HIV-1 RT suggests that the Ser-88 mutation affects template-primer binding, as do several mutations that affect RT susceptibility to nucleoside analogs.

Foscarnet (trisodium phosphonoformic acid) is a pyrophosphate analog that inhibits the polymerases of diverse DNA and RNA viruses, including herpes simplex viruses, varicella-zoster virus, cytomegalovirus (CMV), hepatitis B virus, influenza virus, human immunodeficiency virus type 1 (HIV-1), and other retroviruses (for a review see reference 26). Foscarnet is licensed and widely prescribed for the treatment of CMV retinitis in patients with AIDS. It is also the current drug of choice for acyclovir- or ganciclovir-resistant herpesvirus infections (6). Several clinical trials have demonstrated that foscarnet has antiretroviral activity in vivo (5, 7, 12, 29). In an early trial of foscarnet for the treatment of CMV retinitis, Reddy et al. (29) observed sustained reductions in serum HIV-1 p24 antigen levels for a median of 16 weeks after initiation of foscarnet therapy. In a more recent study of foscarnet as primary therapy of HIV-1, reductions in serum p24 antigen were observed in all patients who received at least 1 week of foscarnet therapy (7). This direct antiretroviral effect of foscarnet has been cited as an explanation for the survival advantage observed with foscarnet in a recent comparative trial of foscarnet versus ganciclovir for the treatment of CMV retinitis (36).

Resistance of clinical HIV-1 isolates to foscarnet has not been reported despite its long-term administration to patients with AIDS (39). Moreover, there are no published reports of isolation of foscarnet-resistant HIV-1 variants by in vitro selection. This is notable, given that HIV-1 has developed resistance to all other selective reverse transcriptase (RT) inhibitors in clinical use (for a review see reference 4). The absence of such reports prompted the present study, in which we sought to isolate foscarnet-resistant HIV-1 variants in vitro and to determine whether resistance can develop in treated patients.

MATERIALS AND METHODS

Chemicals. Nevirapine (11-cyclopropyl-5,-11-dihydro-4-methyl-6H-dipyridol [3,2-b:2′,3′-e]diazepin-6-one) was provided by Boehringer-Ingelheim, Inc. (Ridgefield, Conn.). TIBO R82150 [(+)-(5S)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5,1-jk]benzodiazepin-2(1H)-thione] was obtained from K. Parker (Brown University, Providence, R.I.). 2′,3′-Dideoxycytidine was purchased from Pharmacia, Inc. (Piscataway, N.J.). 2′,3′-Dideoxyinosine and 2′,3′-didehydro-3′-deoxythymidine were provided by Bristol-Myers Squibb (Wallingford, Conn.). Foscarnet (phosphonoformic acid) and all other chemicals were purchased from Sigma Chemical Company, St. Louis, Mo. Stock solutions (10 mM) of the antiviral compounds were prepared in sterile water or dimethyl sulfoxide, stored at −20°C, and diluted in medium to the desired concentration immediately before use.

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Cells. MT-2 cells (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health; contributed by D. Richman) were cultured in RPMI 1640 (Whittaker M. A. Bioproducts, Walkersville, Md.) with 50 IU of penicillin per ml, 50 μg of streptomycin per ml, 2 mM L-glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, and 10% fetal bovine serum (JRH Biosciences, Lenexa, Kan.). HT4LacZ-1 cells (kindly provided by J.-F. Nicolas, Pasteur Institute, Paris, France) were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum, antibiotics, and 400 μg of geneticin (Gibco, Grand Island, N.Y.) per ml. Human peripheral blood mononuclear cells (PBMC), isolated from healthy HIV-1-seronegative donors, were activated with phytohemagglutinin (10 μg/ml; Difco Labs, Detroit, Mich.) for 3 days before HIV-1 infection. PBMC were maintained after infection in RPMI 1640 supplemented with 10% interleukin-2 (Cellular Products, Buffalo, N.Y.), 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics.

Viruses. Stock preparations of HIV-1_{LAI} (formerly HIV-1_{BRU}) were prepared by electroporation of MT-2 cells (10^7) with 10 μg of plasmid DNA encoding the HIV-1_{LAI} infectious proviral clone (27) as described previously (25). Culture supernatants were harvested at the peak of viral cytopathic effect, which occurred 5 to 7 days after transfection. This plasmid-derived virus was passaged for 10 weekly cycles as cell-free virus in MT-2 cells before the selection of foscarnet-resistant virus was begun. The infectivity of all virus preparations was determined by threefold endpoint dilution in MT-2 cells (six cultures per dilution). The 50% tissue culture infective doses ($TCID_{50}$) was calculated with the Reed and Muench equation (30). Repeated titrations of the same virus stock are reproducible to within ± 0.2 $\log_{10}$ $TCID_{50}$/ml.

Selection of resistant viruses. Selection of resistant virus was performed by endpoint dilution passage of virus in foscarnet as follows. MT-2 target cells were pretreated for 2 h with 400 μM foscarnet, distributed into 96-well tissue culture plates at a density of 10^4 cells per well, and cultured in 200 μl of medium with drug. Individual culture wells were inoculated with 10 μl of serial threefold dilutions of HIV-1_{LAI} and examined daily for the development of viral cytopathic effect (giant syncytium formation). The lowest viral inoculum (highest virus dilution) that produced syncytia in 400 μM foscarnet was considered the endpoint. Supernatant from the endpoint well(s) was harvested, centrifuged ($800 \times g$ for 10 min), and added to 10^6 MT-2 cells pretreated with 400 μM foscarnet to expand the breakthrough virus. Supernatant from the expansion culture was harvested at the peak of viral cytopathic effect (5 to 7 days), clarified by centrifugation ($800 \times g$ for 10 min), and used to initiate a new cycle of endpoint dilution passage. Expansion of the breakthrough virus was necessary for the first six endpoint passages; without expansion the breakthrough virus could not be successfully passaged in 400 μM foscarnet. After each passage, virus was evaluated for resistance by determining the reduction in viral infectivity by 400 μM foscarnet (22, 23).

Patient HIV-1 isolates. HIV-1 clinical isolates were obtained from patients enrolled in the Study of Ocular Complications in AIDS trial (36). This trial was a double-blind comparison of foscarnet and ganciclovir for the treatment of CMV retinitis. HIV-1 isolates were obtained after 3 or more months of therapy. HIV-1 was isolated at the University of Minnesota HIV Laboratory (by K. Sannerud, A. Erice, and H. Balfour, Jr.) by coculture of patient PBMC samples with phytohemagglutinin-activated normal donor PBMC as described previously (10). No pretreatment HIV-1 isolates were available for comparison. Twelve isolates from patients with no history of foscarnet therapy were used as controls.

Antiviral susceptibility determinations. Antiviral susceptibility of laboratory HIV-1 strains was determined in MT-2 and HT4LacZ-1 cells. Testing of clinical isolates was performed in PBMC.

(i) MT-2 cells. Drug inhibition of HIV-1 cytopathic effect and drug inhibition of p24 antigen production were quantitated in separate assays. For cytopathic effect inhibition assays, cells were inoculated at a multiplicity of infection (MOI) of 0.1 $TCID_{50}$ per cell and distributed into triplicate wells of 96-well plates (10^4 cells per well) containing serial twofold dilutions of drug. Complete killing of control cells that were not drug treated occurred by day 7 of infection. Cell viability was quantitated on day 7 by the MTT (3-[4,5-diamethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)-dye reduction method (16). For p24 inhibition assays, MT-2 cells were inoculated at an MOI of 0.01 $TCID_{50}$ per cell, washed, and distributed into triplicate wells of 48-well plates at a density of 5×10^4 cells per well in 0.5 ml of medium containing drug dilutions. After 7 days, culture supernatants were harvested and assayed for p24 antigen by a commercial enzyme immunoassay (Dupont, NEN Products, Wilmington, Del.).

(ii) HT4LacZ-1 cells. Drug inhibition of syncytium formation was performed as described previously (32) with modification. Cells were seeded into 96-well plates (3×10^4 cells per well) and allowed to adhere overnight. Fresh medium containing twofold drug dilutions was added, and each well was inoculated with 50 to 100 syncytium-forming units of virus. After 72 h, cells were fixed with 0.5% gluteraldehyde, washed with phosphate-buffered saline, and stained with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal) as described previously (32). Syncytia containing five or more blue nuclei were counted in six separate wells per drug dilution.

(iii) PBMC. Clinical HIV-1 isolates were expanded and assayed for drug susceptibility in phytohemagglutinin-stimulated PBMC according to the consensus protocol developed by the AIDS Clinical Trials Group and the Department of Defense (13).

TABLE 1. Progressive in vitro resistance to foscarnet

Passage no.[a]	HIV-1 infectivity ($\log_{10}$ $TCID_{50}$/ml)[b]		
	Without foscarnet	With foscarnet (400 μM)	$\log_{10}$ reduction[c]
0	6.1	2.6	3.5
1	4.2	2.7	1.5
2	4.6	3.7	0.9
3	4.3	3.7	0.6
13	5.8	5.2	0.6

[a] Number of passages in 400 μM foscarnet. Passage 0 was the starting preparation of HIV-1_{LAI}.

[b] Infectivity was determined by serial threefold endpoint dilutions in MT-2 cells (six cultures per dilution). Standard deviations for multiple titrations of the same virus were ≤ 0.2 $\log_{10}$ $TCID_{50}$/ml.

[c] Calculated by subtracting the infectivity titer in the absence of foscarnet from that in the presence of 400 μM foscarnet.

For all susceptibility assays, the drug concentration that inhibited viral replication by 50% (EC_{50}) was calculated by linear regression analysis of $\log_{10}$-linear plots of drug concentration versus percent inhibition of viral cytopathic effect, syncytium formation, or p24 antigen production.

RT assays. Virus was pelleted from cell culture supernatants and lysed to release RT as described previously (35). RT assays were performed with a reaction mixture containing 100 mM Tris HCl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 0.05 U of poly(rA)$_n$-oligo(dT)$_{12-18}$ template-primer per ml, and 1 μM [^{3}H]dTTP (specific activity, 28.5 Ci/mmol). Bovine serum albumin at a final concentration of 100 μg/ml was used in the RT assay mixture to stabilize the viral enzyme. RT assays were performed in the presence and absence of serial dilutions of foscarnet.

Cloning and DNA sequencing of HIV-1 RT. For laboratory strains, the full-length coding sequence of HIV-1 RT was PCR amplified from infected cell lysates as described previously (22, 23). The 1.7-kb PCR product was ligated into the PCRII TA cloning vector (Invitrogen, San Diego, Calif.) and transfected into *Escherichia coli* INVαF′. Transformants were screened for the 1.7-kb insert by digestion with *Eco*RI. Plasmid DNA from appropriate clones was purified (Qiagen Inc., Chatsworth, Calif.) and sequenced by dideoxynucleotide chain termination with Sequenase kit no. 70770 (U.S. Biochemical, Cleveland, Ohio). A set of six primers was used to sequence the entire RT gene (23).

For clinical isolates, DNA was extracted from infected PBMC cultures and an 810-bp DNA segment encompassing codons 0 to 250 of RT was amplified by PCR with the following primers: +, 5′-CTGTTGACTCAGATTGGCTGC ACT-3′, and −, 5′-TCATTGACAGTCCAGCTGTC-3′ (20). The PCR product was purified by using Elutip columns (Schleicher and Schuell, Keene, N.H.) and cloned into the PCRII vector as described above. Sequencing was performed with fluorescent dye terminators (Applied Biosystems, Foster City, Calif.) and *Taq* polymerase. At least two separate clones were sequenced per isolate.

Production of mutant recombinant HIV-1. Oligonucleotide-directed mutagenesis and cloning of mutant RT genes into the pXXHIV-1_{LAI} proviral clone were performed as described previously (25). pXXHIV-1_{LAI} contains two unique silent restriction sites in the 5′ and 3′ ends of RT to facilitate cloning of mutated RT genes into the provirus. Infectious recombinant virus was produced by electroporation of MT-2 cells with 10 μg of proviral DNA as described previously (25). Culture supernatants were harvested at peak cytopathic effect, which occurred 5 to 7 days after electroporation. The presence of the desired mutations was verified by direct sequencing of PCR-amplified RT from infected cell lysates (Promega fmol DNA sequencing kit no. 70770).

RESULTS

In vitro selection of foscarnet-resistant HIV-1. To determine whether HIV-1 variants with reduced susceptibility to foscarnet could be selected in vitro, HIV-1_{LAI} was repeatedly passaged in MT-2 cells in the presence of 400 μM foscarnet. After each passage, virus was screened for altered foscarnet susceptibility by determining the $\log_{10}$ reduction in viral infectivity by 400 μM foscarnet. Table 1 shows that viral susceptibility to foscarnet decreased with each passage for the first 3 passages but then did not decline further with 10 additional passages. Separate passage of virus in higher foscarnet concentrations (500 and 600 μM) also did not increase the level of resistance (data not shown). Susceptibility testing of virus in MT-2 cells after 13 passages in 400 μM foscarnet showed that the EC_{50} of

TABLE 2. Susceptibility of foscarnet-resistant HIV-1 to other antiretroviral agents in MT-2 cells

Compound	EC_{50} for HIV-1_{LAI} (μM)[a]		Difference (fold)[c]
	Parental	Foscarnet resistant[b]	
Foscarnet	70.6 ± 10.0	≥600	≥8.5
AZT	0.9 ± 0.22	0.01 ± 0.01	0.01
2′,3′-Didehydro-3′-deoxythymidine	13.4 ± 2.3	7.4 ± 0.1	0.50
2′,3′-Dideoxyinosine	15.4 ± 1.7	13.5 ± 5.2	0.90
2′,3′-Dideoxycytidine	4.0 ± 1.7	2.6 ± 0.1	0.60
Nevirapine	0.2 ± 0.05	0.01 ± 0.01	0.05
TIBO R82150	0.06 ± 0.02	0.002 ± 0.001	0.03

[a] Drug susceptibilities were determined in MT-2 cells as described in Materials and Methods. Target cells were infected at an MOI of 0.05. Data shown are means ± standard errors for at least three separate determinations performed in triplicate.
[b] After 13 passages in 400 μM foscarnet.
[c] EC_{50} for resistant virus divided by EC_{50} for parental HIV-1_{LAI}.

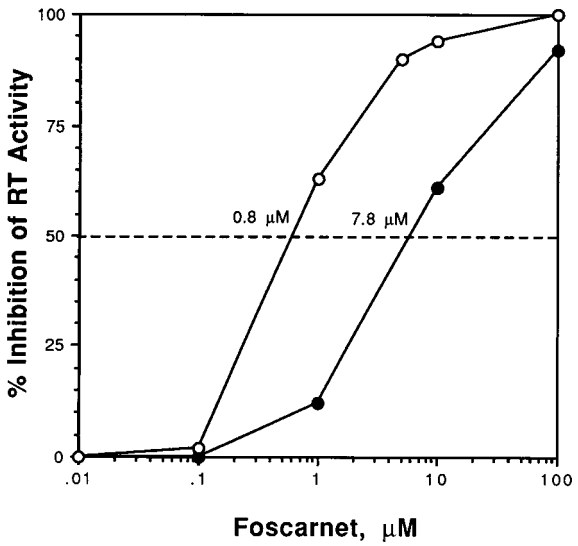

FIG. 1. Foscarnet susceptibility of virion-associated RT from parental (open circles) and resistant (solid circles) HIV-1. RT inhibition assays were performed as described in Materials and Methods. Mean values for duplicate determinations are shown (standard deviations averaged <15% of the mean values). The dashed line indicates 50% inhibition.

foscarnet had increased to ≥600 μM (Table 2). This was a ≥8.5-fold increase in EC_{50} compared with control HIV-1_{LAI}. Foscarnet concentrations above 600 μM could not be tested because of inhibition of MT-2 cell growth. The control HIV-1_{LAI} used in these comparisons had been passaged in parallel for 13 cycles in the absence of foscarnet.

The replication competency of foscarnet-resistant virus was compared with that of control HIV-1_{LAI}. MT-2 cells were infected with the viruses (MOI = 0.01) in the presence and absence of 300 μM foscarnet, and p24 antigen production was measured every 2 to 3 days. In the absence of foscarnet, resistant virus and control HIV-1_{LAI} replicated equally well: p24 antigen levels on days 5, 7, 9, and 12 postinfection were 7.3, 32.6, 55.3, and 49.8 ng/ml, respectively, for resistant virus, compared with 9.5, 17.0, 22.4, and 41.8 ng/ml, respectively, for HIV-1_{LAI}. In the presence of foscarnet, replication of resistant virus was inhibited only partially (peak p24 antigen level = 24.7 ng/ml), whereas inhibition of control HIV-1_{LAI} was >98% (peak p24 antigen level = 0.77 ng/ml).

Susceptibility of virion RT. To assess the foscarnet susceptibility of RT derived from resistant virus, concentrated virions from culture supernatant were disrupted and assayed for RT activity in the presence of increasing concentrations of foscarnet. Figure 1 demonstrates that the RT from foscarnet-resistant virions was ~10-fold less susceptible to inhibition by foscarnet than control RT from HIV-1_{LAI}. This degree of RT resistance was similar to that observed for the resistant virus, indicating that the enzyme and viral phenotypes correlated.

Cross-resistance to other antiretroviral agents. Table 2 summarizes the activities of various nucleoside and nonnucleoside RT inhibitors against foscarnet-resistant HIV-1 in comparison with control HIV-1_{LAI}. Resistant virus showed increased susceptibilities to 3′-azido, 3′-deoxythymidine (~90-fold), nevirapine (~30-fold), and TIBO R82150 (~20-fold). Susceptibilities to 2′,3′-didehydro-3′-deoxythymidine, 2′,3′-dideoxyinosine, and 2′,3′-dideoxycytidine were not affected.

Genetic analyses. To investigate the genetic basis for foscarnet resistance, the full-length coding sequence of RT was cloned from cells infected with resistant virus (passage 13) or control HIV-1_{LAI}. DNA sequencing demonstrated that all seven RT clones derived from resistant virus encoded two mutations: glutamine to leucine at codon 161 (CAA to CTA) and histidine to tyrosine at codon 208 (CAT to TAT). These changes were not detected in any RT clones (0 of 7) from control HIV-1_{LAI}. In addition, the Gln-161 and His-208 residues have been conserved in all previously reported HIV-1 isolates (24). Three additional amino acid variations were found in only single RT clones: Phe-87 to Ile, Phe-346 to Ser, and Gly-436 to Glu.

Foscarnet susceptibility of clinical isolates. Six HIV-1 isolates from patients enrolled in the Study of Ocular Complications in AIDS trial were tested for foscarnet susceptibility. No pretherapy isolates from these patients were available. For comparison, 12 control isolates from patients who had no history of foscarnet therapy were assayed. As shown in Table 3, the average EC_{50} for control isolates was 58 μM. The six patient isolates exhibited variable reductions in foscarnet susceptibility, with EC_{50}s ranging from 128 to 303 μM (two- to fivefold higher than that of controls). The polymerase domain of RT from these isolates was sequenced to determine if any of the mutations observed in vitro were present or whether other common mutations could be detected. The Tyr-208 mutation was found in two isolates, and the Leu-161 mutation was found

TABLE 3. RT mutations in clinical HIV-1 isolates with reduced foscarnet susceptibility

Isolate(s)	EC_{50} of foscarnet (μM)[a]	RT amino acid residue[b]:		
		88	161	208
Controls[c]	58 ± 20	Trp	Gln	His
1	137 ± 43	Ser	wt	wt
2	280 ± 21	wt	wt	Tyr
3	177 ± 36	Ser	wt	Tyr
4	217 ± 29	Gly	wt	wt
5	128 ± 27	Ser	wt	wt
6	303 ± 60	wt	Leu	wt

[a] Foscarnet susceptibilities were determined in phytohemagglutinin-stimulated PBMC. Data are mean values ± standard errors for two to five separate determinations performed in quadruplicate.
[b] wt, wild-type (i.e., same as for control isolates).
[c] Controls consisted of 12 isolates from patients with no history of foscarnet therapy.

TABLE 4. Foscarnet susceptibility of mutant recombinant HIV-1

Mutation	Result in:			
	MT-2 cells[a]		HT4LacZ-1 cells[b]	
	EC_{50} (μM)[c]	Resistance (fold)	EC_{50} (μM)[d]	Resistance (fold)
Wild type	28 ± 10		38 ± 2	
Trp-88 to Ser	120 ± 10	4.3	105 ± 7	2.8
Glu-89 to Gly	399 ± 19	14.3	504 ± 12	13.3
Gln-161 to Leu	295 ± 15	10.5	203 ± 23	5.3
His-208 to Tyr	68 ± 4	2.4	67 ± 1	1.8
Leu-161 + Tyr-208	213 ± 23	7.6	336 ± 30	8.8

[a] Determined by inhibition of p24 antigen production as described in Materials and Methods. Cells were infected at an MOI of 0.01.
[b] Determined by inhibition of syncytium formation as described in Materials and Methods.
[c] Data are means ± standard errors for two to three separate determinations performed in triplicate.
[d] Data are means ± standard errors for three separate determinations performed in sextuplicate.

in one (Table 3). The tryptophan at position 88 was substituted by a serine or glycine in four isolates (Table 3). One or more zidovudine (AZT) resistance mutations at codon(s) 41, 67, 70, 210, 215, and/or 219 were also found in all six isolates (data not shown). No other mutations that were common to more than one isolate were identified.

Susceptibilities of mutant recombinant viruses. To define the roles of the mutations identified above in resistance to foscarnet, mutant recombinant viruses encoding Gln-161 to Leu, His-208 to Tyr, both mutations, or Trp-88 to Ser were constructed. For comparison, a mutant virus encoding Glu-89 to Gly was prepared and tested. The Glu-89–to–Gly mutation has been reported previously to cause RT resistance to ddGTP and viral resistance to foscarnet in vitro (28). The relative susceptibilities of these viruses to foscarnet were determined in MT-2 cells (by inhibition of p24 antigen production) and in HT4LacZ-1 cells (by inhibition of syncytium formation).

Table 4 shows that the Gln-161 and Tyr-208 mutations together conferred 7.6- and 8.8-fold foscarnet resistance in MT-2 and HT4LacZ-1 cells, respectively. This degree of resistance was similar to that observed with the foscarnet-resistant virus selected in MT-2 cells. Of the two mutations, the Leu-161 change was more important (Table 4), while the Tyr-208 substitution alone had only a minor effect on foscarnet susceptibility (1.8- to 2.5-fold increase in EC_{50}). In HT4LacZ-1 cells, the Tyr-208 mutation increased foscarnet resistance from 5.3-fold with the Leu-161 mutation alone to 8.8-fold with both mutations. This effect of the Tyr-208 substitution was not observed in MT-2 cells, however.

The Trp-88–to–Ser mutation observed in the clinical isolates reduced foscarnet susceptibility 2.8- to 4.3-fold. Virus with the Glu-89–to–Gly mutation was the most resistant of the viruses tested, showing a 13.3- to 14.3-fold increase in EC_{50}. None of the mutations studied altered the infectivity, replication kinetics (p24 antigen production), or cytopathicity (syncytium formation) of the recombinant viruses in MT-2 cells in comparison with control HIV-1_{LAI} (data not shown).

Virus with both the Leu-161 and Tyr-208 mutations or the Leu-161 mutation alone was hypersusceptible to AZT, nevirapine, and TIBO R82150 (data not shown). The degree of AZT hypersusceptibility was greater for the double mutant (45-fold) than for Leu-161 alone (11-fold). Similarly, the double mutant showed greater hypersusceptibility to nevirapine (20-fold) and TIBO R82150 (18-fold) than the Leu-161 mutant (6-fold for both compounds).

FIG. 2. Locations of foscarnet resistance mutation sites in the crystal structure of HIV-1 RT bound with a double-stranded DNA template-primer. The wild-type amino acid residues Trp-88 (in β5a), Gln-161 (in αE), and His-208 (in αF) are shown as light brown ball-and-stick models. The catalytically essential Asp-110, Asp-185, and Asp-186 residues, which are the putative site of foscarnet binding, are shown in yellow. The backbone of HIV-1 RT is represented as a solid ribbon with the p66 and p51 finger subdomains shown in blue, the p66 palm in red, and the p66 thumb in green. The double-stranded DNA is indicated in purple. The foscarnet resistance mutations may affect the conformation of the foscarnet binding site indirectly through changes in protein and/or nucleic acid structure.

Locations of mutations in crystal structure of RT. The crystal structure of the p66/51 heterodimer bound with a double-stranded DNA template-primer (11) was examined to identify the sites of the Ser-88, Leu-161, and Tyr-208 mutations. As shown in Fig. 2, the Ser-88 mutation is located on the β5a strand of p66 adjacent to the template strand of the duplex region of the template-primer. The Gln-161 mutation lies in the αE helix of p66 just underneath the putative deoxynucleoside triphosphate (dNTP) binding site, whereas the Tyr-208 mutation is located on helix αF of the p66 palm subdomain away from the dNTP and template-primer binding sites.

DISCUSSION

The development of viral resistance to foscarnet has been reported previously for herpes simplex viruses, varicella-zoster virus and CMV (1, 6, 14, 33, 34, 37). Genetic analyses of these resistant herpesviruses have identified point mutations in the viral DNA polymerase gene that probably alter the affinity of the enzyme for foscarnet (3, 6, 8). For patients with AIDS, isolation of foscarnet-resistant herpes simplex virus type 2 from genital lesions has been associated with clinical resistance to foscarnet therapy (34).

In this report, we demonstrate that HIV-1 variants with reduced susceptibility to foscarnet can be isolated both in cell culture and from patients after prolonged therapy. The resistant virus selected in vitro encodes two point mutations in RT altering the predicted amino acids at residues 161 (Gln to Leu) and 208 (His to Tyr). Site-specific mutagenesis and production of recombinant HIV-1 demonstrated that the Leu-161 mutation conferred the majority of the foscarnet resistance, while the Tyr-208 substitution had only a minor effect. The Leu-161 and Tyr-208 mutations were detected in at least one clinical

HIV-1 isolate exhibiting reduced in vitro susceptibility to foscarnet, but substitution of Trp-88 by Ser or Gly was more common in these isolates (four of six). Mutagenesis confirmed that the Ser-88 mutation reduced HIV-1 susceptibility to foscarnet approximately 3- to 4-fold.

The Ser-88 mutation did not alter HIV-1 susceptibility to AZT, whereas the Leu-161 mutation alone or together with the Tyr-208 mutation increased susceptibility to AZT. This "sensitizing" effect of Leu-161 may help to explain why the Ser-88 mutation was detected more commonly in clinical isolates. The majority of the patients receiving foscarnet in the Study of Ocular Complications in AIDS trial were also taking concomitant AZT (36). In this setting of foscarnet and AZT coselection, the Ser-88 mutation may have been preferred, since it has no effect on AZT susceptibility, whereas the Leu-161 mutation would be selected against because of AZT hypersusceptibility. A similar observation has been observed with resistance to AZT and nonnucleoside RT inhibitors. Monotherapy with the nonnucleoside RT inhibitor nevirapine rapidly selects for resistant mutants encoding a Tyr-181–to–Cys mutation, but when nevirapine is given in combination with AZT, the Cys-181 mutation does not appear (31). This is probably explained by the in vitro observation that when the Cys-181 mutation is introduced into a virus encoding AZT resistance mutations (Leu-41 and Tyr-215), viral resistance to AZT is reversed (15). Thus, specific mutations such as Cys-181 or Leu-161 may be less favored under AZT selective pressure because they restore or increase HIV-1 susceptibility to AZT.

Mutations that affect HIV-1 susceptibility to foscarnet have been reported previously (9, 17–19, 28). These mutants were identified by means other than selection for viral resistance to foscarnet. Larder et al. performed site-specific mutagenesis of conserved domains of HIV-1 RT and characterized the functional activities and drug susceptibilities of the mutant enzymes (17, 18). Mutations at residues 113 (Asp to Glu or Gly), 114 (Ala to Ser), and 115 (Tyr to Asn or His) reduced both enzyme activity (20 to 90%) and susceptibility to foscarnet. Proviruses encoding the mutations at residue 113 (Asp to Gly) or 114 (Ala to Ser) replicated slowly and exhibited ~5-fold-higher resistance to foscarnet, but virus with the Tyr-115–to–Asn substitution did not replicate (17).

By screening bacterial clones expressing HIV-1 RT, Prasad et al. (28) identified an RT mutant that was resistant to ddGTP and cross resistant to foscarnet. The RT mutant encoded a nonconservative amino acid substitution at residue 89 from Glu to Gly. When the Glu-89–to–Gly mutation was introduced into a proviral clone, the resultant virus was foscarnet resistant but not ddG resistant. Our experiments confirm that the Glu-89–to–Gly mutation reduces HIV-1 susceptibility to foscarnet. In fact, HIV-1 encoding this mutation was the most resistant of the recombinant viruses that we constructed (Table 4).

Im et al. (9) reported a spontaneously arising mutant of HIV-1 RT that was resistant to foscarnet and several dideoxynucleotide triphosphates. This mutant RT contained a valine-to-alanine substitution at position 90. However, when this mutation was introduced into a proviral clone, only low-titer virus could be produced ($<10^3$ $TCID_{50}$/ml), indicating that the Ala-90 substitution reduced viral replication competency (21).

In the present study, we did not detect mutations at residues 89, 90, 113, 114, or 115 in any of the laboratory or clinical isolates analyzed. This does not preclude their detection in subsequent studies, since only a small sample of isolates have been examined to date. Indeed, Tachedjian et al. (38) recently described a Glu-89–to–Lys mutation in a foscarnet-resistant variant that was selected in vitro.

Examination of the crystal structure of RT shows that several foscarnet mutations lie in a region of the β5a strand of p66 involved in binding of the nucleic acid template-primer. These mutations include Ser-88, Gly-89, and Ala-90. In addition, Tachedjian et al. (38) reported a Leu-92–to–Ile mutation in a foscarnet-resistant variant selected in vitro. It is unclear how these substitutions alter foscarnet susceptibility, but recent studies by Boyer et al. (2) suggest that alterations in binding of the template-primer resulting from dideoxynucleoside resistance mutations in the β5a strand affect the ability of the template-primer-enzyme complex to accept or reject an incoming dideoxynucleoside triphosphate. A similar mechanism may be operative for foscarnet.

In contrast to the mutations at residues 88 to 90, the Leu-161 mutation is located in the αE helix, which is distinct from the β5a strand. The Gln-161 residue lies below the active site of HIV-1 RT, and its substitution by Leu may have a more direct effect on foscarnet binding by altering the conformation of the dNTP binding site and its affinity for foscarnet. In wild-type HIV-1 RT, foscarnet, which is a pyrophosphate analog, probably binds to the active-site Asp-110, Asp-185, and Asp-186 residues via Mg^{2+} ions, which are required for catalysis. The location of the Tyr-208 mutation on the αF helix away from the dNTP and template-primer binding sites is consistent with its having a relatively minor effect on foscarnet susceptibility.

In summary, HIV-1 variants with reduced susceptibility to foscarnet can emerge under selection in cell culture and in foscarnet-treated patients. The clinical significance of this resistance is unclear, although it provides additional evidence that foscarnet exerts a selective antiretroviral effect in vivo. This antiretroviral effect may provide benefit to some HIV-infected individuals for whom standard therapy with nucleoside analogs is failing. In the present study, only a small number of clinical isolates were examined for foscarnet resistance, pretreatment isolates were not available for comparison, and the foscarnet resistance observed was low level (≤5-fold). Additional studies of the emergence of foscarnet-resistant HIV-1 in treated patients and the relationship of this resistance to viral load and clinical outcome are warranted.

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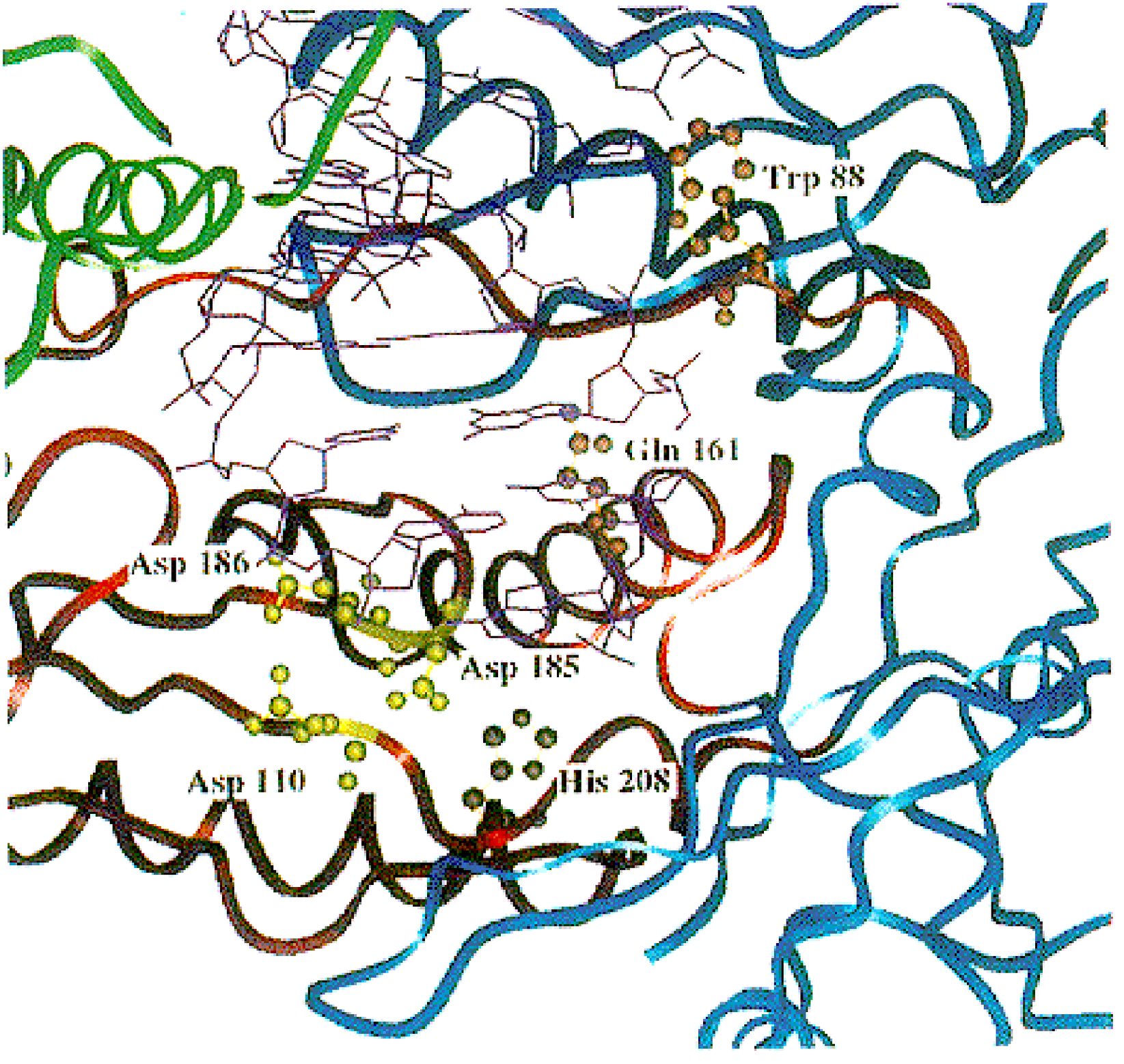
Trp 88
Gln 161
Asp 186
Asp 185
Asp 110
His 208