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Selection And Characterization of Drug-Resistant Variants of Human Immunodeficiency Virus (96MM6759)

Abstract

A major concern in the pursuit of effective therapies for human immunodeficiency virus (HIV) infection is the potential for HIV to develop drug resistance. The therapeutic efficacy of all currently licensed antiretrovirals is limited by the emergence of drug resistant HIV strains. Several promising new inhibitors of HIV reverse transcriptase (RT) and combinations of protease inhibitors are entering clinical trial, but inadequate information is available about the potential for HIV resistance to these therapies. The isolation and molecular characterization of drug resistant HIV variants that emerge in cell culture with drug selection has helped predict both the likelihood of resistance and the types of resistant mutants that appear in treated patients. Thus, the overall goal of this one year project was to continue to evaluate RT inhibitors for the development of HIV resistance.

Specific aims of the project were to: 1) determine whether foscarnet resistance mutations reverse the phenotypic effects of AZT resistance mutations, 2) complete the molecular characterization of (-)-β-D-dioxolane guanosine (DXG) resistant laboratory HIV-1 isolates, 3) determine whether the K65R RT mutation in DXG-resistant virus reverses the phenotypic effects of AZT resistance mutations, 4) determine the cross-resistance of DXG-resistant variants to structurally related and unrelated inhibitors, 5) produce molecularly cloned, drug-resistant viruses that encode resistance to DDI (L74V) and DDC (K65R), and 6) attempt to isolate HIV-1 variants that are resistant to the new HIV-1 RT inhibitor (-)-β-L-5-fluoro-2',3'-dideoxy, didehydro-5-fluorocytidine (L-D4FC).

Results from this one year project indicate that 1) various foscarnet resistance mutations reverse the phenotypic effects of AZT resistance mutations, 2) DXG resistant laboratory isolates encode the K65R or L74V resistance mutation in RT, 3) the K65 R mutation reverses the phenotypic effects of the AZT resistance mutations D67N, K70R, T215Y, K219Q, 4) DXG-resistant virus is cross-resistant to other dioxolane derivatives as well as DDI and DDC, 5) molecular clones of HIV-1 encoding L74V or K65R mutations constructed by site-specific mutagenesis are resistant to DXG, and 6) variants resistant to L-D4FC have been isolated in MT-2 cells and encode the M184I or M184V mutation.

These accomplishments during the one year period of funding have expanded our knowledge of HIV drug resistance and helped identify novel drug combinations, such as AZT/foscarent and AZT/DXG, for evaluation in clinical trials.

FOREWORD

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5.0 INTRODUCTION

5.1 Importance of HIV-1 Drug Resistance

A major concern in the pursuit of new therapies for human immunodeficiency virus type 1 (HIV-1) infection is the potential for HIV-1 to develop drug resistance. HIV-1 variants resistant to 3'-azido-3'-deoxythymidine (AZT), 2',3'-dideoxyinosine (DDI) or 2',3'-dideoxycytidine (DDC) have been isolated from patients receiving long term monotherapy with these drugs (1-4). Mounting clinical evidence indicates that drug resistance is a predictor of poor clinical outcome in both children and adults (5-7). The rapid development of HIV-1 resistance to nonnucleoside reverse transcriptase inhibitors (NNRTIs) has also been reported both in cell culture and in human clinical trials (8-12). In the case of the NNRTI L'697,661, drug-resistant HIV-1 emerged within 2-6 weeks of initiating therapy in association with the return of viremia to pretreatment levels (12). Breakthrough viremia associated with the appearance of drug-resistant strains has also been noted with other classes of HIV-1 inhibitors, including protease inhibitors (13). This experience has led to the realization that the potential for HIV-1 drug resistance must be assessed early on in the preclinical evaluation of all new therapies for HIV-1.

5.2 Value of *In Vitro* Studies To Identify Drug-resistant HIV-1

The characterization of drug-resistant HIV-1 strains that emerge in cell culture with drug selection has helped predict both the likelihood of resistance and the types of resistant mutants that breakthrough in treated patients (8-12,14). For example, the rapid development of clinical resistance to the NNRTIs nevirapine and L-697,661, resulting from a tyrosine to cysteine mutation at residue 181 of HIV-1 reverse transcriptase (RT), was predicted by *in vitro* selection studies (8-12). Hence, a major goal of this project is to evaluate the potential of HIV-1 to develop resistance to new inhibitors of HIV-1 RT *in vitro* drug selection studies in relevant cell culture systems.

5.3 Known HIV-1 Drug Resistance Mutations

An increasingly complex number of mutations in HIV-1 that confer resistance to RT and protease inhibitors have been identified through a combination of *in vitro* selection studies and human clinical trials. These have been summarized in a comprehensive table prepared by the investigators and his colleagues (**copy in Appendix**). Several important points can be drawn from this table of resistance mutations. In many instances, the resistance mutations that were identified by *in vitro* drug selection studies were predictive of the mutants that emerged in treated patients. Second, the addition of a new resistance mutation in RT may suppress the effect of a pre-existing resistance mutation. For example, the phenotypic effects of certain AZT resistance mutations are reversed by

mutations that cause DDI resistance (L74V) (2), (-)-FTC/3TC resistance (M184V) (15) or NNRTI resistance (Y181C) (16). This suggests that simultaneous resistance of HIV-1 to several drugs may not be possible without compromise of RT function. This was initially thought to be the case for simultaneous resistance to AZT, DDI, and nevirapine (17), although this has been disproved (18, 19). Nevertheless, further identification of antagonistic interactions between resistance mutations in RT may lead to the design of more effective combination chemotherapy.

5.4 Combination Antiretroviral Therapy to Prevent Drug Resistance

It is now recognized that combinations of antiretroviral drugs have the potential to delay or prevent the emergence of drug-resistant HIV-1 strains (20). Unfortunately, strains of HIV-1 that are simultaneously resistant to three or more RT inhibitors have been identified (18,19), and clinical trials with certain drug combinations, including nucleosides and NNRTIs, have failed to prevent the emergence of resistant variants (21). Additional studies are needed to determine whether specific combinations of HIV-1 inhibitors can prevent or delay the emergence of resistant variants. Potential antagonistic interactions between resistance mutations will be sought by sequential selection of virus in different drugs. The phenotypic effects of resistance to a second drug on pre-existing resistance to the first drug will be determined. Site-specific mutagenesis will be performed to confirm the interactive effects of mutations in RT on HIV-1 susceptibility.

5.5 The Need for a Reference Panel of Drug-resistant Isolates

The DOD/ACTG assay has become a useful tool for determining the drug susceptibility of clinical isolates of HIV-1 (22). Studies on the frequency and significance of AZT resistance have been facilitated by the availability of a panel of AZT-resistant reference isolates for use as standards in the DOD/ACTG assay. However, similar reference isolates are not available for resistance to other antiretrovirals (e.g. DDI, DDC). This is due, in part, to the problem that many of the infectious molecular clones of HIV-1 that have been constructed to encode specific drug resistance mutations do not replicate consistently in peripheral blood mononuclear cells (PBMC) and thus are not useful in the PBMC-based ACTG/DOD assay. Stable infectious molecular clones of HIV-1 that encode resistance to single or multiple drugs and replicate consistently in PBM cells are needed for future clinical studies of resistance to newer antiretroviral agents. For this project, drug-resistant infectious molecular clones of HIV-1_{LAI} (formerly HIV-1_{BRU} [23]) were constructed for testing as standard reagents in the DOD/ACTG assay. The HIV_{LAI} clone was selected because virus derived from this plasmid replicates consistently to high titer (>10^4 $TCID_{50}$/ml) in PBM cells from random donors. Many drug-resistant molecular clones have already been constructed and tested (**see 5.6.2 below**).

5.6 Work Accomplished Prior To This Project

In prior studies, the prinicipal investigator's laboratory has determined the molecular basis for HIV-1 resistance to several different classes of nucleoside and nonnucleoside inhibitors of HIV-1 RT (10, 11, 14, 24, 25). Work relevant to this project is described below:

5.6.1 Comprehensive Analysis of HIV-1 Resistance to Foscarnet

The results of these studies have been published in two manuscripts (25,26): Mellors et al., Antimicrob Agents Chemother 1995; 39: 1087-1092 and Tachedjian et al., Virology 1995; 212: 58-68 (**copies in Appendix**).

Foscarnet (phosphonoformate) inhibits HIV-1 replication in cell culture and lowers circulating levels of p24 antigen and HIV-1 RNA in treated patients. In collaboration with investigators at WRARI (D. Mayers, J. Weir) and the MacFarlane Burnet Research Centre (G. Tachedjian, J. Mills), Fairfield, Australia, we investigated whether foscarnet resistant variants of HIV-1 could be selected in vitro or isolated from patients after receiving long term foscarnet therapy for cytomegalovirus retinitis. HIV-1 variants exhibiting ~8-fold foscarnet resistance were selected in cell culture by serial passage of virus in drug. In addition, five isolates showing reduced susceptibility to foscarnet (2- to 5-fold) were isolated from patients treated with foscarnet for greater than 3 months in the SOCA trial.

DNA sequencing of the RT gene from these viruses identified seven novel mutations in RT associated with foscarnet resistance: W88S, W88G, E89K, L92I, S156A, Q161L and H208Y. Four of these substitutions - W88S, W88G, Q161L, and H208Y - were identified in one or more clinical isolates. The most common mutation observed in the clinical isolates was W88S/G. To evaluate the relative effects of these mutations on foscarnet susceptibility, infectious molecular clones containing these mutations were constructed using a new proviral vector xxHIV-1_{LAI}. This vector was engineered to contain unique, silent XmaI and XbaI restriction in the 5' and 3' ends of RT, respectively, that greatly facilitate cloning of mutant RT genes into the provirus. Mutant recombinant viruses were tested for susceptibility to foscarnet, AZT and nevirapine in HeLa-CD4/LacZ cells. Foscarnet susceptibilities are shown below:

Genotype	Foscarnet IC_{50}, μM	Fold-resistance
wild-type	38 ± 3	-
W88S	105 ± 12	2.8
E89K	>600	>16
L92I	298 ± 10	7.9
S156A	169 ± 43	4.5

Genotype	Foscarnet IC_{50}, μM	Fold-resistance
Q161L	203 ± 40	5.4
Q161L/H208Y	336 ± 52	8.9
H208Y	67 ± 2	1.8

The E89K mutant was most resistant to foscarnet, but this mutation was not observed in clinical isolates. The H208Y mutation alone had little effect on foscarnet susceptibility, but this mutation increased the level of resistance of the Q161L mutation. None of the mutants showed cross-resistance to nucleoside analogs (DDI or DDC); however, the Q161L/H208 double mutant showed increased susceptibility to AZT (7.2-fold). The increased susceptibility to AZT is of interest because several of the foscarnet resistant clinical isolates were AZT susceptible despite having up to four AZT resistance mutations. **Further studies to examine the interactions between foscarnet and AZT resistance mutations were completed in the current project and are described below in Section 6.21.**

5.6.2 Production of Drug-Resistant Molecular HIV-1 Clones

We have constructed several resistant infectious proviral clones of HIV-1 for use as standards in drug susceptibility assays. Their construction has been facilitated by the introduction of unique silent restriction sites in the HIV-1_{LAI} proviral clone (23) that allow efficient subcloning of the mutant viral gene of interest (RT or protease) into the provirus. The resultant clone is termed xxHIV-1_{LAI}. These silent restriction sites are located at nucleotides 2172 (Xma I) and 4602 (Xba I) of HIV-1_{LAI}. These sites allow nucleotides 40-1470 of RT (Xma I-Xba I fragment) or the entire protease gene (native Apa I-Xma I fragment) to be readily subcloned into HIV-1_{LAI} from a mutagenesis vector. The desired mutations are introduced into the RT or protease gene by standard oligonucleotide-directed mutagenesis using the pAlter-1 vector (Promega). After subcloning the mutated fragment into xxHIV-1LAI, the recombinant provirus (10 μg) is electroporated into T-cells (e.g. MT-2 cells) and culture supernatant are harvested at peak cytopathic effect (~7 days). The presence of the desired genotype is confirmed in all mutated clones by DNA sequencing and the viral phenotype is assessed in T-cell lines and PBMC. The HIV-1_{LAI} proviral clone was initially chosen because it consistently replicates to high-titer (>10^5 $TCID_{50}$/ml) in T-cell lines and PBMC. Introduction of the two silent restriction sites into HIV-1_{LAI} to make xxHIV-1_{LAI} did not alter its replication competency as measured by p24 antigen production, infectious virus production and kinetics of syncytium formation.

Before the current project we had constructed the following drug-resistant clones:

1) Foscarnet resistant - W88S, E89G, E89K, L92I, S156A, Q161L, or Q161L/H208Y
2) NNRTI resistant - Y181C or Y188C
3) 3TC/(-)-FTC resistant - M184V or M184I
4) AZT resistant - M41L/T215Y or D67N/K70R/T215Y/K219Q

The foscarnet, NNRTI, and 3TC resistant virus have been shown to be phenotypically resistant in MT-2 cell lines as well as in PBMC using the ACTG/DOD consensus susceptibility assay. **At the request of DOD investigators, DDI (L74V) and DDC (K65R) resistant clones as well as many other clones were constructed as part of the current project (Section 6.25).**

5.6.3 Selection of HIV-1 Resistance to β-D-Dioxolane G (DXG)

(-)-β-D-dioxolane guanosine (DXG) is a potent purine nucleoside analog RT inhibitor of HIV-1 and HBV polymerase (27-29). As part of the preclinical evaluation of DXG, we attempted to select DXG-resistant HIV-1 variants by serial passage of virus in increasing concentrations of drug in MT-2 cells. The starting drug concentration was 2.5 μM (5 to 10 times the IC_{50}), and the starting virus population was HIV-1_{LAI} that had been passaged for ten cycles in the absence of drug. After 11 passages, reaching a selection pressure of 20 μM DXG, the virus population exhibited ~15-fold resistance to DXG, with the IC_{50} in MT-2 cells increasing from 0.28 μM to 4.5 μM. All comparisons were made between HIV-1_{LAI} that had been selected in DXG and control HIV-1_{LAI} that had been passaged in parallel without drug.

The full-length coding region of RT has been PCR amplified from DXG-resistant and control virus, cloned into the PCRII TA cloning vector (Invitrogen), and analyzed by automated DNA sequencing (ABI 373 Prism). Preliminary analysis of sequences from two resistant and two control clones has revealed a K65R substitution in resistant clones. This substitution in the IKKK motif of RT has been previously reported to confer ~5-fold HIV-1 resistance to DDC and DDI and thus probably is responsible, at least in part, for resistance to DG. Additional sequencing and site-specific mutagenesis are necessary to confirm this initial finding. **These studies were completed during this project period. In addition, interactions between K65R and AZT resistance mutations were investigated and are described below in Sections 6.22 - 6.24.**

5.6 Purpose of Present Work

The specific aims of this one year project were to:

1. Determine whether foscarnet resistance mutations reverse the phenotypic effects of AZT resistance mutations.

2. Complete the molecular characterization of the Dioxolane-Guanosine (DXG)-resistant laboratory HIV-1 isolates.

3. Determine whether the K65R mutation reverses the phenotypic effects of AZT resistance mutations.

4. Determine the cross-resistance pattern of DXG-resistant variants to structurally related and unrelated inhibitors.

5. Continue to produce a reference panel of molecularly cloned, drug-resistant viruses that replicate well in PBMC for use as standards in the DOD/ACTG HIV-1 drug susceptibility assay. Specific clones that will be produced include those encoding resistance to DDI (L74V) and DDC (K65R), as well as others of interest to DOD investigators or that are needed to complete the above aims.

6. Attempt to isolate HIV-1 variants that are resistant to new HIV-1 inhibitors in vitro drug selection in T-cell lines (MT-2). Determine the genetic basis for inhibitor resistance by cloning and sequencing the appropriate viral gene (RT, protease) from resistant variants to identify associated mutations. Introduce the mutation(s) identified above into an infectious proviral clone to define the role of each mutation in inhibitor resistance.

5.7 MILITARY SIGNIFICANCE

Effective treatment and prevention of HIV-1 infection is a goal of the Medical Protection Against AIDS Program of the USAMRMC. The emergence of drug-resistant HIV-1 has proven to be a significant obstacle to long term effective chemotherapy of HIV-1 infection. Several new classes of HIV-1 inhibitors are entering clinical trials, but information is lacking on the likelihood that resistance will develop to many of these candidate drugs. The work proposed in this application will address this deficiency. Characterization of the phenotype and genotype of drug-resistant variants that arise *in vitro* will improve our understanding of the molecular mechanisms of resistance and lead to more rapid detection of resistant variants in clinical samples (e.g., by using the polymerase chain reaction to detect the mutant viral genotype). In addition, the proviral clones encoding specific drug resistance mutations will be useful as standard reagents in susceptibility assays of clinical isolates. Collectively, this project should expand our knowledge of HIV-1 drug resistance and help prioritize new therapies for evaluation in clinical trials.

6.0 BODY

6.1 Experimental Methods

The experimental methods used are described in detail in each of the publications that have been included in the Appendix. Brief summaries of the key methods are provided below.

6.11 Selection of Drug Resistant Viruses

Drug-resistant variants were selected by serial *in vitro* passage of HIV-1_{LAI} in the presence of increasing drug concentrations as described (11, 15, 32). Initial drug concentrations were 2-10 times the EC_{50} (the EC_{50} had been determined in the MT-2

cells used for selection). MT-2 cells were pre-incubated with drug for 2 hours before virus challenge. A large initial viral inoculum (10^6 $TCID_{50}$ or greater) was used to increase the likelihood that rare drug-resistant variants are present. MT-2 cells were infected at a multiplicity of 0.1 $TCID_{50}$/cell. Viral progeny that "breakthrough" drug were collected after 7 days and used to initiate a new cycle of infection. After each cycle, the infectivity and drug susceptibility of the breakthrough virus was determined in comparison with the starting parental virus. When resistance (<10% inhibition) was observed at the initial drug concentration, the selective pressure (drug concentration) was increased 2 to 10-fold until the level of resistance plateaued or exceeded 100-fold.

6.13 Characterization of Drug-resistant Strains

6.131 Drug Susceptibility Phenotype

The phenotype of resistant viruses was characterized in detail by repeated dose-response assays in T-cell lines and PBMC. Resistance was defined as a 3-fold or greater increase in IC_{50} compared with parental virus. Phenotypic studies also included cross-resistance to structurally related as well as unrelated compounds.

6.132 Genotypic Analyses

To define the genetic basis for resistance, HIV-1 RT was amplified by PCR and the product sequenced directly or after cloning using a ABI 373 Prism automated sequencer. Direct sequencing of the PCR product quickly identified the dominant mutations in the resistant virus population, whereas sequencing of individual plasmid clones could identify less frequent mutations associated with resistance.

The role of specific mutations in resistance was further defined by site-specific mutational analysis. Specific mutations were introduced into a wild-type clone of the viral target by oligonucleotide-directed mutagenesis (37). The mutated viral gene was subcloned into the $HIV\text{-}1_{LAI}$ infectious proviral clone (22) to produce mutant virus for susceptibility testing as described (24, 25).

6.2 RESULTS

6.21 Project Aim #1: Determine whether foscarnet resistance mutations reverse the phenotypic effects of AZT resistance mutations

In a continuing collaboration with scientists at the MacFarlane Burnet Research Center (G. Tachedjian, J. Mills), we have investigated whether foscarnet resistance mutations influence the AZT susceptibility of viruses that encode different AZT resistance mutations. A comprehensive series of experiments was performed as part of this project to evaluate the effects of all known foscarnet resistance mutations on AZT susceptibility encoded by different sets of AZT resistance mutations. These experiments are described in detail in the attached

publication by Tachedjian G, Mellors JW, Bazmi HZ, et al. J Virol 1996; 70: 7171-7181 (See Appendix). To summarize, these experiments showed that introduction of each of the foscarnet resistance mutations W88G, E89K, L92I, or Q161L into AZT resistant viruses (M41L/D67N/K70R/T215Y or D67N/K70R/T215Y/K219Q) completely restored susceptibility to AZT. Two additional foscarnet resistance mutations, W88S and S156A partially restored AZT susceptibility. Restoration of AZT susceptibility by W88G/S, E89K, L92I and S156A was associated with loss of foscarnet resistance. These data suggested that resistance to foscarnet and AZT might be mutually exclusive. This hypothesis was further supported by the inability to isolate AZT and foscarnet co-resistant virus by serial passage in vitro. These important findings have led to the synthesis and preclinical evaluation of prodrugs of foscarnet-AZT dimers, in collaboration with Dr. Karl Hostetler, University of California, San Diego (38).

6.22 Project Aim #2: Complete the molecular characterization of dioxolane-guanosine (DXG) resistant HIV-1

As described above, an initial selection of DXG-resistant virus *in vitro* resulted in the isolation of a variant exhibiting 7.6-fold resistance to DXG. PCR amplification, cloning and DNA sequencing of HIV-1 RT from this resistant variant identified the K65R (AAA to AGA) mutation in all HIV-1 RT clones (10 of 10). This mutation is in the IKKK motif of RT has been previously reported to confer low level (<10-fold) resistance to DDC, DDI and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) in laboratory or clinical laboratory HIV-1 isolates. A second selection of DXG-resistant virus was performed by the same procedure and resulted in the isolation of a variant exhibiting 12.2-fold DXG resistance. Eight of 10 HIV-1 RT clones from this variant encoded the L74V mutation that has been reported previously to cause resistance to DDI. None of the clones from the second selection had the K65R mutation. To confirm the role of both the K65R and L74V mutations in DXG resistance, these were introduced separately into the xxHIV-1LAI molecular clone. The presence of the desired mutations was confirmed by DNA sequencing. The K65R and L74V recombinant mutants were tested for DXG susceptibility and found to exhibit 8.7- and 4.4-fold resistance to DXG, respectively (mean of 3 experiments), thus confirming their effect on DXG susceptibility. Structural modeling of DXG and DDI, in collaboration with Dr. C.K. Chu at the Medical College of Georgia, demonstrated that the two molecules have similar conformations, which is consistent with both selecting for the K65R and L74V resistance mutations in RT. **The above studies are described in further detail in a manuscript that is being submitted for publication by Bazmi et al. (See Appendix).**

6.23 Project Aim #3: Determine whether the K65R mutation reverses the phenotypic effects of AZT resistance mutations.

Although the K65R mutation in HIV-1 RT has been described previously, its potential interaction with other mutations had not been investigated. We therefore introduced the K65R mutation into wild-type and AZT resistant xxLAI proviral

clones. The AZT resistant clone encoded four AZT resistance mutations: D67N, K70R, T215Y and K219Q. The AZT and DXG susceptibilities of the resulting viruses were tested in MT-2 cells. As detailed in Table I in the attached manuscript by Bazmi et al., the K65R mutation completely reversed the phenotypic effects of the four AZT resistance mutations, restoring AZT susceptibility ~50-fold to wild-type levels (AZT IC_{50} of 0.004 μM). The degree of DXG resistance conferred by the K65R mutation was decreased slightly from 8.7-fold in the wild-type genetic background to 5.0-fold in the AZT resistant background. Similar studies with L74V showed that this mutation only partially reversed AZT resistance from 48.0-fold to 6.3-fold. In addition, the L74V mutation conferred ~4-fold DDI resistance in both wild-type and AZT resistant genetic backgrounds. These studies are the first to identify the antagonistic interaction between K65R and AZT resistance mutations and suggest that the combination of DXG with AZT may have advantages.

6.24 Project Aim #4: Determine the cross-resistance pattern of DXG-resistant variants to structurally related and unrelated inhibitors

Extensive studies of the cross-resistance patterns of DXG-resistant virus were performed (3 independent dose-response for each drugs). The results of these studies are shown in Table 2 in the attached manuscript by Bazmi et al. To summarize, the recombinant DXG-resistant virus encoding the K65R mutation exhibited cross-resistance to DDI (3-fold), DDC (3-fold), D4T (3-fold), 3TC (>14-fold), PFA (3-fold), PMEA (>4-fold) and the dioxolane derivatives (-)-β-D-2-amino-6-chloropurine dioxolane [DCAPD] (3.7-fold), (-)-β-D-aminopurine dioxolane [DAPD] (4.5-fold) and D-dioxolany-5-flurocytosine [DDFOC] (7.7-fold). In contrast, DXG resistant virus showed increased susceptibility to AZT (3-fold), which is consistent with the antagonistic interactions between DXG and AZT resistance mutations described above.

6.25 Project Aim #5: Continue to produce a reference panel of moleculary, cloned drug resistant variants

As part of these investigations the following drug resistant molecular clones have been produced:

1) AZT resistant; M41L/T215Y and D67N/K70R/T215Y/K219Q (4XAZT)
2) Foscarnet/AZT: W88S/4XAZT, E89G/4XAZT, E89K/4XAZT, L92I/4XAZT S156A/4XAZT, Q161L/4XAZT and Q161L/H208Y/4XAZT
3) DXG resistant: K65R and L74V
4) DXG/AZT: K65R/4XAZT, L74V/4XAZT

These viruses replicate well in PBMC and CD4+T-cell lines and are available to DOD investigators on request.

6.26 Project Aim #6: Isolate HIV-1 variants resistant to new RT inhibitors

Because of the limited duration of the project (1 year) and the extensive work accomplished above, we were able to evaluate only one new compound, (-)L-5-fluoro-dideoxydidehydrocytidine [L-FD4C], for *in vitro* resistance. This compound has the same L- enantiomeric configuration as 3TC and (-)-FTC, which are known to rapidly select for the M184I/V mutations in RT, as described by our laboratory (14) and others (15). We hypothesized that the L-configuration would be more important than the sugar or base composition in determining the HIV mutants that arose. Using our standard selection procedure in MT-2 cells, HIV exhibiting high-level resistance (>30-fold; IC50 >10 μM) to L-FD4C was isolated after 4 serial passages in increasing drug concentration. DNA sequence analysis of passage 4 revealed a mixture of and M184I (dominant) and M184V mutations. Sequencing of passage 10 virus showed only the M184V mutation and disappearance of the M184I mutant. This is consistent with prior reports of 3TC and (-)-FTC resistance, in which the M184I variants arise first but is replaced by the more fit and more resistant M184V variant. Testing of L-FD4C against recombinant HIV-1 encoding M184V confirmed that this mutation confers high-level resistance. These studies support the hypothesis that the stereochemistry of certain nucleoside analogs is the dominant determinant of the resistance mutations that are selected.

7.0 CONCLUSIONS

During the 12 month period of funding, significant progress has been made on each of the specific aims of the project and most of the aims have been completed. We have performed and published a comprehensive analyses of the interactions between foscarnet and AZT resistance mutations (Tachedjian et al., J Virol 1996; 70:7171-7181 - **copy in Appendix**). These studies support the hypothesis that resistance to AZT and foscarnet are mutually exclusive and suggest a new strategy for combination antiretroviral therapy using prodrugs of AZT/foscarnet dimer that is being pursued (38).

We have also completed studies characterizing HIV-1 variants that are resistant to Dioxolane Guanosine (DXG). Resistance to DXG can result from a K65R or L74V mutation in RT. Cross-resistance studies indicate that DXG resistant exhibits resistance to DDI, DDC and other dioxolane derivatives but not AZT. Mutational interaction studies led to the discovery that the K65R mutation reverses the phenotypic effects of multiple AZT resistance mutations. These observations suggest that DXG should be used in combination with AZT but not other dideoxynucleosides such as DDI, DDC or PMEA that exhibit cross-resistance. These findings are summarized in a manuscript to be submitted by Bazmi et al. (**copy in Appendix**).

In completing these studies we have expanded the panel of drug-resistant molecular clones that can be used as standards in drug-susceptibility assays such as the PBMC-based ACTG/DOD consensus assay or in discovering drugs against resistant viruses.

In conclusion, significant accomplishments during the one year period of funding has expanded our knowledge of HIV drug resistance and helped identify novel drug combinations, such as AZT/foscarnet and AZT/DXG, for evaluation in clinical trials.

8.0 BIBILIOGRAPHY OF PUBLICATIONS AND MEETING ABSTRACTS

Articles

1. Mellors JW, Bazmi H, Weir J, Arnold E, Schinazi R, Mayers D. Novel mutations in reverse transcriptase of human immunodeficiency virus type-1 reduce susceptibility to foscarnet in laboratory and clinical isolates. Antimicrob Agents Chemother 1995; 39:1087-1092.

2. Tachedjian G, Mellors JW, Bazmi H, Birch C, Mills J. Zidovudine resistance is suppressed by mutations conferring resistance of human immunodeficiency virus type 1 to foscarnet. J Virol 1996; 70:7171-7181.

3. Rosowky A, Fu H, Pai N, Mellors JW, Richman DD, Hostetler KY. Synthesis and in vitro activity of long-chain 5'-0-[(alkoxycarbonyl)phosphinyl]-3'-azido-3'-deoxythymidines against wild-type and AZT- and foscarnet-resistant strains of HIV-1. J Med Chem 1997; 40:2482-2490.

4. Tachedjian G, Mellors JW, Bazmi H, Mills J. Impaired fitness of foscarnet-resistant strains of human immunodeficiency virus type 1. AIDS Res and Human Retroviruses 1998 (In Press).

Abstracts

1. Schinazi RF, Boudinot FD, Manouilov KK, Mellors JW, McMillan A, Schueter-Wiertz S, Lloyd R Jr, Korba BE, Tennant B, Chu CK. Anti-HBV and anti-HIV activities of dioxolane-purine nucleosides. Ninth International Conference on Antiviral Research, Urbandai, Fukushima, Japan, 1996.

2. Schinazi RF, Boudinot FD, Mellors JW, Korba BE, Tennant B, Chu CK. Dioxolane-purine nucleosides as Antiviral Agents. Tenth International Congress of Virology, Jerusalem, Israel, 1996.

3. Mellors JW, Bazmi H, Chu CK, Schinazi RF. K65R mutation in HIV-1 reverse transcriptase causes resistance to (-)-b-dioxolane-guanosine and reverses AZT resistance. Fifth International Workshop, Whistler, Canada, 1996.

4. Tachedjian G, Mellors JW, Bazmi H, Birch C, Mills J. Zidovudine resistance is suppressed by mutations conferring resistance of HIV-1 to foscarnet. Fifth International Workshop, Whistler, Canada, 1996.

5. Tachedjian G, Mellors JW, Bazmi H, Birch C, Mills J. Foscarnet resistance conferring mutations phenotypically reverse zidovudine resistance. Australian Society for HIV Medicine, Inc., Sydney, Australia, November 1996.

9.0 PERSONNEL PAID BY PROJECT

The following personnel received salary support from the project:

RESEARCH SUPERVISOR: Ms. Hengameh Z. Bazmi, M.S. Hyg, worked full-time on the project under the supervision of the PI. She selected resistant viruses in cell culture to DXG, and performed phenotypic and genotypic analyses of mutant strains, including generation of the many site-specific recombinant mutants described in this progress report.

RESEARCH ASSISTANT: Timothy Sturgeon, B.S., commited 50% effort to the project under the supervision of the PI and Ms. Bazmi. He helped perform the many the phenotyic analyses of resistant described in this project.

PRINCIPAL INVESTIGATOR: Although the PI, John W. Mellors, M.D., Associate Professor of Medicine at the University of Pittsburgh School of Medicine, and a Staff Physician in the Division of Infectious Diseases at the Veterans Affairs Medical Center (University Drive) in Pittsburgh, PA, did not receive salary support, he committed 30% of his VA effort to the project. Dr. Mellors took overall responsibility for the project including scientific direction, coordination of experiments, and preparation of this progress report.

10. CITED REFERENCES

1. Larder BA, Darby G, Richman DD. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science 1989;243:1731-4.

2. Larder BA, Kemp SD. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). Science 1989;246:1155-8.

3. St Clair MH, Martin JL, Tudor WG, et al. Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. Science 1991;253:1557-9.

4. Fitzgibbon JE, Howell RM, Haberzettl CA, Sperber SJ, Gocke DJ, Dubin DT. Human immunodeficiency virus type 1 pol gene mutations which cause decreased susceptibility to 2',3'-dideoxycytidine. Antimicrob Agents Chemother 1992;36:153-7.

5. Tudor-Williams G, St Clair MH, McKinney RE, et al. HIV-1 sensitivity to zidovudine and clinical outcome in children. Lancet 1992;339:15-9.

6. Ogino MT, Dankner WM, Spector SA. Development and significance of zidovudine resistance in children infected with human immunodeficiency virus. J Pediatr 1993;123:1-8.

7. D'Aquila RT, Johnson VA, Welles SL, et al. Zidovudine resistance and HIV-1 disease progression during antiretroviral therapy. AIDS Clinical Trials Group Protocol 116B/117 Team and the Virology Committee Resistance Working Group. Annals of Internal Medicine 1995;122(6):401-8.

8. Nunberg JH, Schleif WA, Boots EJ, et al. Viral resistance to human immunodeficiency virus type 1-specific pyridinone reverse transcriptase inhibitors. J Virol 1991;65(9):4887-92.

9. Richman D, Shih CK, Lowy I, et al. Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture. Proc Natl Acad Sci (USA) 1991;88 :11241-5.

10. Mellors JW, Dutschman GE, Im GJ, Tramontano E, Winkler SR, Cheng YC. *In vitro* selection and molecular characterization of human immunodeficiency virus-1 resistant to non-nucleoside inhibitors of reverse transcriptase. Mol Pharm 1992;41:446-51.

11. Mellors JW, Im GJ, Tramontano E, et al. A single conservative amino acid substitution in the reverse transcriptase of human immunodeficiency virus-1 confers resistance to (+)-(5S)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)-thione (TIBO R82150). Molecular Pharmacology 1993;43(1):11-6.

12. Saag MS, Emini EA, Laskin OL, et al. A short-term clinical evaluation of L-697,661, a non-nucleoside inhibitor of HIV-1 reverse transcriptase. N Engl J Med 1993;329:1065-1072.

13. Condra, JH, Schleif, WA, Blahy OM, et al. Nature 1995; 374: 569-71.

14. Schinazi RF, Lloyd RJ, Nguyen MH, et al. Characterization of human immunodeficiency viruses resistant to oxathiolane-cytosine nucleosides. Antimicrob Agents Chemother 1993;37:875-81.

15. Tisdale M, Kemp SD, Parry NR, Larder BA. Rapid *in vitro* selection of human immunodeficiency virus type 1 resistant to 3'-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. Proc Natl Acad Sci (USA) 1993;90:5653-6.

16. Larder BA. 3'-Azido-3'-deoxythymidine resistance suppressed by a mutation conferring human immunodeficiency virus type 1 resistance to nonnucleoside reverse transcriptase inhibitors. Antimicrob Agents Chemother 1992;36:2664-9.

17. Chow YK, Hirsch MS, Merrill DP, et al. Use of evolutionary limitations of HIV-1 multidrug resistance to optimize therapy. Nature 1993;361:650-4.

18. Emini EA, Graham DJ, Gotlib L, Condra JH, Byrnes VW, Schleif WA. HIV and Multidrug Resistance. Nature 1993;364:679.

19. Larder BA, Kellam P, Kemp SD. Convergent combination therapy can select viable multidrug resistant HIV-1 *in vitro*. Nature 1993;365:451-453.

20. Schinazi RF. Combined therapeutic modalities for viruses - rationale and clinical potential. In: Chou TC, Rideout DC, ed. Molecular mechanisms of chemotherapeutic synergism, potentiation, and antagonism. Orlando, Fl: Academic Press, 1991: 110-181.

21. Richman DD. Resistance of clinical isolates of human immunodeficiency virus to antiretroviral agents. Antimicrob Agents Chemother 1993;37:1207-1213.

22. Japour AJ, Mayers DL, Johnson VA, et al. Standardized peripheral blood mononuclear cell culture assay for determination of drug susceptibilities of clinical human immunodeficiency virus type 1 isolates. Antimicrob Agents Chemother 1993;37:1095-101.

23. Peden K, Emerman M, Montagnier L. The characterization of infectious molecular clones of HIV-1_{LAI}, HIV-1_{MAL}, and HIV-1_{ELI}: Changes in growth properties on passage in tissue culture. Virology 1991;185:661-672.

24. Nguyen M-H, Schinazi RF, Shi C, Goudagon NM, McKenna P, Mellors JW. Resistance of human immunodeficiency virus type-1 to acyclic 6-phenylselenenyl- and 6-phenylthiopyrimidines. Antimicrob Agents Chemother 1994; 38:2409-2414.

25. Mellors JW, Bazmi HZ, Schinazi RF, et al. Novel mutations in reverse transcriptase of human immunodeficiency virus type 1 reduce susceptibility to foscarnet in laboratory and clinical isolates. Antimicrob Agents Chemother 1995;39;1087-1092.

26. Tachedjian G, Hooker DJ, Gurusinghe AD, et al. Characterization of foscarnet-resistant strains of human immunodeficiency virus type-1. Virology 1995; 212: 58-68.

27. Kim, H O, Schinazi, RF, Nampalli, S, et al. 1,3-Dioxolane-purine nucleosides (2R,4R) and (2R,4S) with selective anti-HIV-1 activity in human lymphocytes. J. Med. Chem. 36:30-37, 1993.

28. Schinazi, RF, McClure, HM, Boudinot, FD, Jxiang, Y, Chu, CK. Development of (-)-β-D-2,6-diaminopurine dioxolane as a potential antiviral agent. Antiviral Res. 23:81, 1994.

29. Chen, H, Boudinot, FD, Chu, CK, McClure, HM, and Schinazi, RF. Pharmacokinetics of (-)-β-D-2-aminopurine dioxolane and (-)-β-D-2-amino-6-chloropurine dioxolane, and their antiviral metabolite (-)--D-dioxolane guanine in rhesus monkeys. Antimicrob. Agents Chemother 1996; 40: 2332-2336.

30. Schuurman R, Nijhuis M, van Leeuwen R, et al. Rapid changes in human immunodeficiencyvirus type 1 RNA load and appearance of drug-resistant virus populations in persons treated with lamivudine (3TC). J Infect Dis 1995; 171:1411-9.

31. Prasad VR, Lowy I, de los Santos T, Chiang L, Goff SP. Isolation and characterization of a dideoxyguanosine triphosphate-resistant mutant of human immunodeficiency virus reverse transcriptase. Proc Natl Acad Sci USA 1991;88:11363-11367.

32 Gosselin, G, Schinazi, RF, Sommadossi, et. al. Anti-human immunodeficiency viruses activities, cross-resistance profiles, andcytotoxicities, of the β-L-enantiomer of 2',3'-dideoxycytidine and its 5-fluoro derivative. Antimicrob. Agents Chemother. 1994; 38:1292-1297.

33. Schinazi, RF, Gosselin, G, Faraj, A, et al. Pure nucleoside enantiomers of β-2',3'-dideoxycytidine analogues are selective inhibitors of hepatitis B virus and human immunodeficiency virus in vitro. Antimicrob. Agents Chemother., 38:2172-2174, 1994.

34. Wilson, LJ, Choi, W-B, Spurling, T, et al. The synthesis and anti-HIV activity of pyrimidine dioxolanyl nucleosides. Bioorg. Med. Chem. Lett. 3:169-174, 1993.

35. Roberts NA, Martin JA, Kinchington D, et al. Rational design of peptide-based HIV proteinase inhibitors. Science 1990; 248:358-361.

36 Kempf DJ, Marsh KC, Denissen JF, et al. ABT-538 is a potent inhibitor of human immunodeficiency virus protease and has high oral bioavailability in humans. Proc Natl Acad Sci USA 1995; 92:2482-8

37. Kunkel TA, Roberts JD, Zakour RA. Rapid and efficient site-specific mutagenesis without phenotype selection. Methods Enz 1987;154:367-382.

38. Rosowky A, Fu H, Pai N, Mellors JW, Richman DD, Hostetler KY. Synthesis and in vitro activity of long-chain 5'-0-[(alkoxycarbonyl)phosphinyl]-3'-azido-3'-deoxythymidines against wild-type and AZT- and foscarnet-resistant strains of HIV-1. J Med Chem 1997; 40:2482-2490.

11.0 APPENDIX

The following reprints/preprints are attached in the Appendix:

1. Schinazi R, Larder BA, Mellors JW. Mutations in retroviral genes associated with drug resistance. International Antiviral News 1997; 5:129-142.

2. Mellors JW, Bazmi H, Weir J, Arnold E, Schinazi R, Mayers D. Novel mutations in reverse transcriptase of human immunodeficiency virus type-1 reduce susceptibility to foscarnet in laboratory and clinical isolates. Antimicrob Agents Chemother 1995; 39:1087-1092.

3. Tachedjian G, Mellors JW, Bazmi H, Birch C, Mills J. Zidovudine resistance is suppressed by mutations conferring resistance of human immunodeficiency virus type 1 to foscarnet. J Virol 1996; 70:7171-7181.

4. Tachedjian G, Mellors JW, Bazmi H, Mills J. Impaired fitness of foscarnet-resistant strains of human immunodeficiency virus type 1. AIDS Res and Human Retroviruses 1998 (in press).

5. Bazmi HZ, Hammond J, Chu CK, Schinazi RS, Mellors JW. K65R mutation in HIV-1 reverse transcriptase causes resistance to (-)-β-D-dioxolane guanosine and reverses AZT resistance (in preparation)

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RESISTANCE TABLE

Mutations in retroviral genes associated with drug resistance

Raymond F Schinazi, Brendan A Larder and John W Mellors

The emergence of drug-resistant variants of HIV continues to be of prime interest in the fields of HIV disease pathogenesis and antiretroviral chemotherapy. Drug resistance is the inevitable consequence of incomplete suppression of HIV replication. The rapid replication rate of HIV and its inherent genetic variation lead to the generation of a seemingly limitless number of viral variants that exhibit drug resistance. The growing number of drug resistance mutations listed in this revised table stands as a testimony to the genetic flexibility of HIV. When the first resistance table was published in 1994 (*International Antiviral News* 2(5):72-75), only 42 different mutations were listed. This new update lists 144 mutations, a 250% increase over a 3 year period. The revised table includes resistance profiles for 80 different compounds. Sections include nucleoside and non-nucleoside reverse transcriptase resistance, pyrophosphate analogue resistance, binding/fusion inhibitor resistance, multidrug resistance and drug resistance mutations that arise in other retroviruses (FIV and SIV).

The reader should be reminded that the mutations described are predominantly found in clade B virus and not in other HIV genotypes. We will continue to improve and update the table and provide it in a searchable mode on the World Wide Web in the very near future. We urge the readership to provide amendments or additions to the table. These may be sent to any of the authors. Data formatted as in the table with an appropriate reference (abstract or paper) would be welcomed.

Resistance Tally ( ) = mutation not unique to this drug class

HIV-1: 144 distinct mutations

Nucleosides	NNRTI	Pyrophosphate analogues	Protease Inhibitors	Fusion/Binding Inhibitors	MDR	SIV	FIV
M41L	L74V	W88G	R8Q	R22A	A62V	K65R	V47I
K65R	L74I	W88S	R8K	I84S	(V75I)	M184V	D3H
D67N	V75L	E89G	L10F	S113N	F77L	Q151M	P156S
T69D	(V75I)	E89K	L10I	S134N	F116Y	**3 total**	M183T
K70E	A98G	L92I	L10V	F145L	Q151M		**4 total**
K70R	K101E	S156A	L10R	N188K	R211K		
L74V	K101I	Q161L	K20M	K269E	L214F		
V75T	K101Q	H208Y	K20R	N270S	G333D		
Y115F	L100I	**8 total**	L23I	R272T	G333E		
M184I	K103N	**all unique**	L24I	S274R	**9 total**		
M184T	K103Q	**to this class**	L24V	Q278H	**8 unique to**		
M184V	K103R	*No. drugs: 1*	D30N	I288V	**this class**		
P119S	K103T		V32I	N293D			
Y215C	V106A		L33F	N293H			
T215F	V106L		M36I	A297T			
T215Y	V106I		K45I	N323S			
K219E	V108I		M46F	G332E			
K219Q	E138K		M46I	N351D			
L210W	T139I		M46L	P385L			
19 total	G141E		M46V	R387I			
all unique	V179D		I47V	Q410E			
to this class	V179E		G48V	S433P			
No. drugs: 12	Y181C		I50V	V457I			
	Y181I		I54L	A550T			
	Y188C		I54V	N633D			
	Y188L		D60E	L762S			
	Y188H		L63P	**26 total**			
	H188L		A71T	**all unique**			
	V189I		A71V	**to this class**			
	G190A		G73S	*No. drugs: 5*			
	G190E		V75I				
	G190Q		V77I				
	G190T		P81T				
	P225H		I82T				
	E233V		V82A				
	P236L		V82F				
	K238T		V82I				
	37 total		V82S				
	36 unique		V82T				
	to this class		I84A				
	No. drugs: 41		I84V				
			N88D				
			N88S				
			L90M				
			L97V				
			45 total				
			all unique				
			to this class				

Total number of compounds: 80

RESISTANCE TABLE

Compound	Amino acid change	Codon change	*In vitro*	*In vivo*	-Fold resistance	Cross-resistance (-fold)	Comments	Reference
HIV-1 NUCLEOSIDE RT INHIBITORS								
AZT (zidovudine)	M41L	ATG to TTG	?	Y	4		M41L/T215Y: 60-70-fold;	(1-3)
		or CTG	?	Y			K67N/K70R/T215Y/K219Q: 120-fold; M41L/K67N/	
	D67N	GAC to AAC	Y	Y			K70R/T215Y: 180-fold. Effect of T215Y is reversed	(1-3)
	K70R	AAA to AGA	Y	Y			by a ddI mutation (L74V), NNRTI mutations	(1-3)
							(L100I;Y181C) or (-)-FTC/3TC mutations (M184I/V)	
	L210W	TTG to TGG	Y	Y	Nil		Mutation arises after prolonged AZT therapy in the context of mutations M41L and T215Y. Enhanced resistance (4-fold) with M41L/D67N/K70R/T215Y	(4-7)
	T215Y	ACC to TAC	Y	Y			M41L/D67N/K70R/T215Y or	(1-3,8-10)
	T215F	ACC to TTC	?	Y			M41L/K70R/L210W/T215Y/K219Q and	(1-3)
	K219Q	AAA to CAA	?	Y			M41L/D67N/T215Y/K219Q: AZT-resistance-associated	(1-3)
	K219E	AAA to GAA	Y	N			mutations arising on ddI or d4T monotherapy, respectively	(1-3)
ddI (didanosine)	K65R	AAA to AGA	Y	Y	4-10	ddC	Infrequently observed in patients receiving ddI or ddC	(11)
	L74V	TTA to GTA	N	Y	5-10	ddC	Can reverse effect of T215Y AZT mutation	(12)
	V75T	GTA to ACA	Y	Y		ddC; d4C		(13)
	M184V	ATG to GTG	Y	Y	2-5	ddC; 3TC;(-)-FTC	Rarely observed in patients receiving ddI	(14)
ddC (zalcitabine)	K65R	AAA to AGA	Y	Y	4-10		Observed in patients receiving ddI or ddC	(11,15)
	T69D	ACT to GAT	N	Y	5			(16)
	L74V	TTA to GTA		Y	5-10			(12)
	V75T	GTA to ACA	Y	Y	5			(13,17)
	M184V	ATG to GTG	Y	Y	2-5	ddI; 3TC; (-)-FTC		(14)
	Y215C	TTC to TGC	N	Y	4		Arises on background of T215Y AZT resistance	(18)
d4T (stavudine)	V75T	GTA to ACA	Y	Y	7	ddI; ddC; d4C; (-)-FTC	Observed with d4T selection *in vitro*, rarely in patients receiving d4T	(13,17)
DXG	K65R	AAA to AGA	Y	?	8	ddC; PMEA; 3TC; other dioxolane derivatives	Reverses AZT resistance in D67N/K70R/T215Y/ K219Q background	(19,20)
	L74V	TTA to GTA	Y	?	12			(20)
L-FddC	M184V	ATG to GTG	Y	?	> 100	3TC; (-)-FTC		(21)
FddA	P119S	CCC to TCC	Y	?	4	F-ddI (1.4)		(22)
(-)-FTC	M184V	ATG to GTG	Y	?	> 100	3TC	M184V can suppress effects of AZT mutations	(23,24)
PMEA	K65R	AAA to AGA	Y	?	7-25	3TC (19-40); ddC (4-15); ddI (3-10); 3TC (7); PFA: 2-fold hypersusceptibility	K65R suppresses resistance to AZT	(25,26)
	K70E	AAA to GAA	Y	Y	9			(27,28)
PMPA	K65R	AAA to AGA	Y	?	3-5			(29)
1592U89	K65R	AAA to AGA	Y	N	3	ddI; ddC;		(30,31)
	L74V	TTA to GTA	Y	N	4	ddI; ddC		(30,31)
	Y115F	TAT to TTT	Y	N	2			(30,31)
	M184V	ATG to GTG	Y	N	2-5	3TC; ddI; ddC	K65R/L74V and/or Y115F with M184V: 10 fold K65R/M184V: 8-fold; L74V/M184V: 9-fold resistance; L74V/Y115F/M184V: 11-fold	(32) (30,31)
3TC (lamivudine)	M184V	ATG to GTG	Y	Y	> 100	ddI; ddC; (-)-FTC	M184V and M184I can suppress effects of AZT resistance mutations; GTA seen in MT-2 cells in culture	(23,24,33)
		or GTA	Y	Y				
	M184T	ATG to ACG	Y	?			Reduced replication capacity and RT activity for all variants	(34,35)
	M184I	ATG to ATA	Y	Y				(23,24,33,34, 36)

RESISTANCE TABLE

Compound	Amino acid change	Codon change	*In vitro*	*In vivo*	-Fold resistance	Cross-resistance (-fold)	Comments	Reference
HIV-1-SPECIFIC RT INHIBITORS								
AAP-BHAP (U-104489)	G190E	GGA to GAA	Y	?				(37,38)
BHAP U-87201E (atevirdine)	K101E	AAA to GAA	N	Y			K103N and Y181C observed with monotherapy;	(39,40)
	K103N	AAA to AAC	N	Y			K101E, Y188H, E233Y and K238T observed with	(39)
	Y181C	TAT to TGT	N	Y			U-87201E/AZT combination therapy	(39)
	Y188H	TAT to CAT	N	Y				(39)
	E233V	GAA to GTA	N	Y				(39)
	P236L	CCT to CTT	Y	N				(41)
	K238T	AAA to ACA	N	Y				(39)
BHAP U-88204E	L100I	TTA to ATA	Y	?				(42,43)
	V106A	GTA to GCA	Y	?				(43)
	Y181C	TAT to TGT	Y	?				(43)
	Y181I	TGT to ATT	Y	Y			Appeared after treatment of Y181C-mutated virus with BHAP; high-level resistance to BHAP, nevirapine and TIBO; observed in one nevirapine-treated patient	(44)
BHAP U-90152 (delavirdine)	K103N	AAA to AAC	?	Y			K103N/Y181C seen separately and in combination in	(45)
	K103T	AAA to ACA	?	Y			patients	(45)
	Y181C	TAT to TGT	?	Y				
	P236L	CCT to CTT	Y	Y			Sensitizes RT ~10-fold to nevirapine, TIBO R82913 and L-697,661	(41)
BM+51.0836	Y181C	TAT to TGT	Y	?				(46)
Calanolide A	T139I	ACA to ATA	Y	?	> 70	Not other NNRTIs		(47)
8-Chloro-TIBO K(R91767)	K101E	AAA to GAA	?	Y				(48)
	K103N	AAA to AAC	?	Y				(48)
DMP 266 (L-743,726)	L100I	TTA to ATA	Y	?	8-11		Combination of sequential mutations needed for high-level resistance	(49-51)
	K101E	AAA to GAA	Y	?	< 8			(51)
	K103N	AAA to AAC	Y	?	67			
	V108I	GTA to ATA	Y	?			L100I/V108I: 1,000-fold	(49,50)
	V179D	GTT to GAT	Y	?			L100I/V179D/Y181C: 1,000-fold	(49,50)
	Y181C	TAT to TGT	Y	?	4			(49-51)
	Y188L	TAT to TTA	Y	?	1,000			(51)
E-EBU	Y181C	TAT to TGT	Y	?				(52)
E-EBU-dM	V106A	GTA to GCA	Y	?				(52)
E-EPSeU	Y181C	TAT to TGT	Y	?	> 50		Y188C is the predominant mutation for E-EPSeU;	(53)
	Y188C	TAT to TGT	Y	?	> 250		Y188C confers greater resistance than Y181C	
E-EPU	Y181C	TAT to TGT	Y	?	> 95			
	Y188C	TAT to TGT	Y	?	> 250		Y188C confers greater resistance than Y181C	(53)
HBY 097	L74V	TTA to GTA	Y	?			For V75I, a compensatory effect on RT activity was found	(54)
	L74I	TTA to ATA	Y	?				
	V75I	GTA to ATA	Y	?				
	V75L	GTA to TTA	Y	?				
	V106I	GTA to ATA	Y				Appears under lowered drug concentration selection	(55)
	V106L	GTA to TTA	Y					(56)
	V189I	GTA to ATA	Y	?	2	Other NNRTIs (2-6)		(54)
	G190E	GGA to GAA	Y	?		Other NNRTIs	Reduces enzymatic activity of RT and viral replication competency	(57)
	G190Q	GGA to CAA	Y	?		Other NNRTIs	Appears exclusively in connection with a V179D change	(54)
	G190T	GGA to ?					Appears under lowered drug concentration selection	(55)
HEPT	P236L	CCT to CTT	Y	?				(58)
	Y188C	TAT to TGT	Y	?				(52)
L-697,593	K103N	AAA to AAC	Y	?	20		K103N/Y181C: > 1,000-fold	(59)
	Y181C	TAT to TGT	Y	?	> 100			(59)

RESISTANCE TABLE

Compound	Amino acid change	Codon change	*In vitro*	*In vivo*	-Fold resistance	Cross-resistance (-fold)	Comments	Reference
HIV-1-SPECIFIC RT INHIBITORS continued								
L-697,661	A98G	GCA to GGA	N	Y	8			(60)
	L100I	TTA to ATA	Y	N	2			(60)
	K101E	AAA to GAA	N	Y	8			(60)
	K103N	AAA to AAC	Y	Y	8		K103N and Y181C most common with monotherapy	(60,61)
	K103Q	AAA to CAA	N	Y	8			(61)
	V108I	GTA to GCA	Y	Y	4			(60)
	V179D	GTT to GAT	N	Y	4			(60)
	V179E	GTT to GAG	N	Y	8			(60)
	Y181C	TAT to TGT	Y	Y	> 30			(60,61)
Loviride (R89439, α-APA)	K103N	AAA to AAC	Y	Y				(62)
	V108I	GTA to ATA	Y	?				(62)
	Y181C	TAT to TGT	?	Y				(63)
	Y188H/L	TAT to CAT/ CTT	?	Y				(63)
	G190A	GGA to GCA	?	Y				(64)
α-APA R18893 (loviride analogue)	Y181C	TAT to TGT	Y	?				(65)
MKC442 (I-EBU)	K103N	AAA to AAC	Y	Y				(66)
	K103R	AAA to ?	Y	Y			Most commonly seen *in vivo*	(67)
	V108I	GTA to GCA	Y	Y				
	E138K	GAG to AAG	Y	N			Obtained in the concomitant presence of low 3TC concentrations	(68)
	Y181I	TAT to ATT	Y	N	1,000	All NNRTIs		(68)
	Y181C	TAT to TGT	?	Y				(67)
Nevirapine	A98G	GCA to GGA	N	Y				(69)
	L100I	TTA to ATA	N	Y				(70)
	K103N	AAA to AAC	N	Y				(70)
	V106A	GTA to GCA	Y	Y	~ 100		No effect on AZT resistance	(42,69-71)
	V108I	GTA to ATA	N	Y				(70)
	Y181C	TAT to TGT	Y	Y	> 100	Other NNRTIs	Can suppress effects of AZT mutations	(59,69,72,73)
	Y181I	TGT to ATT	N	Y	High-level	Observed in one patient		(74)
	Y188C	TAT to TGT	N	Y				(70)
	G190A	GGA to GCA	N	Y				(69)
NSC 648400 (E-BPTU)	Y181C	TAT to TGT	Y	?	160	Other NNRTIs		(58)
S-2720 (Quinoxaline)	G190E	GGA to GAA	Y	?			Mutation decreases RT activity and viral replication competency	(75)
	V106A	GTA to GCA	Y	?			Also seen with L101I and Y181C	(76)
	P225H	CCT to CAT	Y	?			Double or triple mutant resistant to MKC442	(76)
TIBO R82150	L100I	TTA to ATA	Y	?	> 100		Can reverse effects of AZT mutations	(77-79)
TIBO R82913	L100I	TTA to ATA	Y	?				(71)
	K103N	AAA to AAC	Y	?	> 100			(42)
	V106A	GTA to GCA	Y	?	~ 100			(71)
	V108I	GTT to GAT	N	Y	> 100	R82150 (> 100)		(80)
	E138K	GAG to AAG	Y	?			Found in combination with L100I	(78)
	V179D	GTT to GAT	N	Y	20	R82150 (20)	Untreated patient	(81)
	Y181C	TAT to TGT	Y	?	> 100		K103N/Y181C: > 1,000-fold	(71)
	Y188H	TAT to CAT	Y	?				(78)
	Y188L	TAT to TTA	N	Y				(81)
Trovirdine	K101Q	AAA to CAA	Y	?			Found in combination with V108I	(82,83)
	K103R	AAA to AGA	Y	?		Nevirapine; 9-chloro-TIBO	K103R/V179D: 500-fold	(82,83)
	V108I	GTA to ATA	Y	?				(82,83)
	V179D	GTT to GAT	Y	?			Found in combination with K103R or Y181C	(82,83)
	Y181C	TAT to TGT	Y	?		Nevirapine; 9-chloro-TIBO	V179D/Y181C: > 1,000-fold	(82,83)

RESISTANCE TABLE

Compound	Amino acid change	Codon change	*In vitro*	*In vivo*	-Fold resistance	Cross-resistance (-fold)	Comments	Reference
HIV-1-SPECIFIC RT INHIBITORS continued								
TSAO	E138K	GAG to AAG	Y	?	> 100		E138A (GAG to GCG) in TSAO-naive patients confers TSAO viral resistance	(84-86)
UC-10	K101E	AAA to GAA	Y	?			K101E/Y181C: 200-fold	(87)
(645129)	K103N	AAA to AAC	Y	N	5			(88)
	V179D	GTT to GAT	Y	?	16			(88)
	Y181C	TAT to TGT	Y	?				(87)
UC-16	K101I	AAA to ATA	Y	N	10		K101I/G141E: 10-fold	(88)
	G141E	GGG to GAG	Y	N				(88)
UC-32 (645542)	Y181C	TAT to TGT	Y	?	38			(87)
UC-38 (88, 89)	K101E	AAA to GAA	Y	N			K101E/G190E: > 100-fold; cross resistance to: TSAO-m3T,	
(629243)	G190E	GGA to GAA	Y	N			Nev, TIBO R82913, BHAP U88204; susceptible to L697,661 L697,661	
	Y181C	TAT to TGT	Y	?	8-149	Other NNRTIs		(87, 90)
UC-42	K103T	AAA to ACA	Y	N	100			(88)
UC-57	K101E	AAA to GAA	Y	?			K101E/Y181C: 58-fold	(87)
(647014)	Y181C	TAT to TGT	Y	?				(87)
UC-68	L100I	TTA to ATA	Y	?	70			(88)
(638532)	Y181C	TAT to TGT	Y	?	5			(87)
UC-69	V106A	GTA to GCA	Y	?			V106A/V181C: 166-fold	(87)
(646989)	Y181C	TAT to TGT	Y	?				(87)
UC-70 (638534)	L100I	TTA to ATA	Y	?	758			(87)
UC-80 (639475)	Y181C	TAT to TGT	Y	?	18			(87)
UC-81	K103N	AAA to AAC	Y	?	40			
(615727)	Y181C	TAT to TGT	Y	?	53			(88, 91)
UC-82	E138K	GAG to AAG	Y	?	5		Activity of UC-82 versus L100I, K103N, V106A, E138K,	(92, 93)
	V106A	GTA to GCA	Y	?	13		Y181C and Y188L reduced by 2-, 6-, 1.5-, 2-, 4- and 200-fold, respectively, compared to wild type	(92)
UC-84	L100I	TTA to ATA	Y	?	> 40, > 33	Other NNRTIs		(47, 87)
(615985)	E138K	GAG to AAG	Y	?	> 100	TSAOs		(88, 94)
	Y181C	TAT to TGT	Y	?	> 118			(87)
UC-781	L100I	TTA to ATA	Y	?	20		Activity of UC-781 versus L100I, K103N, V106A, E138K, Y181C and Y188L reduced by 2-, 7-, 1.5-, 1.5-, 5- and 150-fold, respectively, compared to wild type	(92, 93)
PROTEASE INHIBITORS								
A-77003	R8Q	CGA to CAA	Y	?	10			(95, 96)
	R8K	CGA to AAA	Y	?	10			(95)
	V32I	GTA to ATA	Y	?	7 (enzyme resist.)		V32I appears first; progression to V32I/M46V and V32I /M46V/A71V/V82A occurs even in the absence of drug	(96, 97)
	M46I	ATG to ATA	Y	?			No effect on susceptibility but improves replication competency of R8Q mutant	(95)
	M46L	ATG to TTC	Y	?	2-3 (enzyme resist.)			(96)
	M46F	ATG to TTC	Y	?	4 (enzyme resist.)			(96)
	M46V	ATG to GTG	Y	?				(97)
	G48V	GGG to GTG	Y	?			R8K/M46I/G48V: 20-fold	(98)
	A71V	GCT to GTT	Y	?				(97)
	V82I	GTC to ATC	Y	?			No resistance alone but V32I and V82I are synergistic mutations yielding 20-fold enzyme resistance	(96)
	V82A	GTC to GCC	Y	?			Rare; seen with M46F	(97, 99)

RESISTANCE TABLE

Compound	Amino acid change	Codon change	*In vitro*	*In vivo*	-Fold resistance	Cross-resistance (-fold)	Comments	Reference
PROTEASE INHIBITORS continued								
	I82T	ATC to ACC	Y	?			G48V/I82T: 100-fold (82T was derived from *in vitro* passage of 82I)	(99)
A-75925	V32I	GTA to ATA	Y	?	40			(100)
ABT-538 (ritonavir)	K20R	AAG to AAA	N	Y			82A/ 54V/I/ 36I/ 20K/R: 41-fold	(101-104)
	L33F	TTA to TTC	N	Y				
	M36I	ATG to ATA	N	Y			82T/ 54V/ 71V/ 36I: 8-fold	
	M46I	ATG to ATA	Y	Y				
	I54L	ATC to ?	N	Y				
	I54V	ATC to GTC	N	Y			82T/ 54V: 9-fold	
	A71V	GCT to GTT	Y	Y			82T/ 54V/ 71V/A/ 36M/I/ 20K/R: 28-fold	
	V82F	GTC to TTC	Y	Y			V82F/I84V: 8- to 10-fold	(101)
	V82A	GTC to GCC	N	Y	2		*In vivo*, V82 occurs first, often followed by changes at I54, A71 and M36; V82T has reduced replication efficacy in natural background	
	V82T	GTC to ACC	N	Y	3			
	V82S	GTC to TCC	N	Y	6		M46I/L63P/A71V/V82F/I84V: 27-fold	(101)
	I84V	ATA to GTA	Y	Y			82A/ 54V/I/ 71V/ 90L/M: 7-fold	
	L90M	TTG to ATG	N	Y				
AG1343 (nelfinavir)	D30N	GAT to AAT	Y	Y			D30N/A71V: 7-fold	(105, 106)
	M36I			Y			M46I/L63P/A71V/I84V: 30-fold	
	M46I	ATG to ATA	Y	?			D30N and N88D are most common *in vivo* after 24 weeks of therapy; they do not cause cross-resistance to other protease inhibitors	
	L63P	CTC to CCC		Y				
	A71V	GCT to GTT	Y	?	5			
	V77I		Y	Y				
	I84V	ATA to GTA		?				
	N88D		Y	Y				
	L90M	TTG to ATG	N	Y			Rare *in vivo*	(105)
BILA 1906 BS	V32I	GTA to ATA	Y	?		Ro 31-8959 (200) L 735,524 (60)	V32I/A71V: 3-fold	(107-109)
	M46I	ATG to ATA	Y	?			V32I/A71V/M46I/I84V: 500-1,000-fold	(107-109)
	M46L	ATG to TTG	Y	?			p1/p6 cleavage site mutation (L to F (CTT to TTT) at P1'); p7(NC)/p1 cleavage site mutation (Q to R (CAG to CGG) at P3, A to V (GCT to GTT) at P2V)	(107-109)
	A71V	GCT to GTT	Y	?				(107-109)
	I84A	ATA to GCA	Y	?				(107-109)
	I84V	ATA to GTA	Y	?				(107-109)
BILA 2011 (palinavir)	V32I	GTA to ATA	Y	?	1200	BILA 1906 (1400); BILA 2185 (30); Ro 31-8959 (400); L 735,524 (50)	Other mutations found in second locus p1/p6	(109)
	A71V	GCT to GTT	Y	?				
	I84A	ATG to ATA	Y	?			I84A is the most common mutation	
	L63P	CTC to CCC	Y	?				
BILA 2185 BS	L23I	CTA to ATA	Y	?		Ro 31-8959 (50); L-735,524 (80)	p1/p6 cleavage site mutation (L to F (CTT to TTT) at P1'); p7(NC)/p1 cleavage site mutation (Q to R (CAG to CGG) at P3, A to V (GCT toGTT) at P2V); L23I/V32I/M46I/I47V/I54M/A71V/I84V:1300-fold	(107)
BMS 186,318	A71T	GCT to ACT	Y	?			A71T/V82A: 15-fold	(110, 111)
	V82A	GTC to GCC	Y	?		A-77003 (4)	G48V/A71T/V82A *in vitro* breakthrough with sequential treatment with saquinavir	(111, 112)
DMP 450	L10F	CTC to TTC	Y	?			Probably compensatory	(113, 114)
	M46I	ATG to ATA	Y	?			Probably compensatory	(113, 114)
	D60E	GAT to GAA	Y	?			Probably compensatory	(113, 114)
	I84V	ATA to GTA	Y	?			S1 subsite	(113, 114)
KNI-272	V32I	GTA to ATA	Y	?	2		V32I/M46I/I84V (SDM): 37-fold; V32I/L33F/K45I/F53L/A71V/I84V/L89M (SDM): 130-fold	(115)
MK-639 (L-735,524, indinavir)	L10I	CTC to ATC	?	Y				(116)
	L10R	CTC to CGC	N	Y		XM-323 (15); A-80987 (4); VX-478 (8); Ro-31-8959 (8); SC-52151 (8)	M46I/L63P/V82T: 4-fold;	(117)
	L10V	CTC to GTC	?	Y				(116)
	K20M	AAG to ATG	?	Y				(116)
	K20R	AAG to AAA	?	Y				(116)
	L24I	TTA to ATA	?	Y				(116)
	V32I	GTA to ATA	Y	?			V32I/M46L/V82A: 3-fold	(98)
	M46I	ATG to ATA	N	Y			L10R/M46I/L63P/V82T: 4-fold	(117-119)
	M46L	ATG to TTG	Y	Y			V32I/M46L/A71V/V82A: 14-fold	(98)
	I54V	ATC to GTC	?	Y				(116)

RESISTANCE TABLE

Compound	Amino acid change	Codon change	*In vitro*	*In vivo*	-Fold resistance	Cross-resistance (-fold)	Comments	Reference
PROTEASE INHIBITORS continued								
	L63P	CTC to CCC	N	Y			L10R/M46I/L63P/V82T/I84V: 8-fold	(117)
	A71T	GCT to ACT	?	Y				(116)
	A71V	GCT to GTT	Y	Y				(98)
	G73S	GGT to GCT	?	Y			Found *in vivo* in all post-indinavir treatment sequences	(120)
	V82A	GTC to GCC	Y	Y				(98, 121)
	V82F	GTC to TTC	?	Y				(116)
	V82T	GTC to ACC	N	Y				(117)
	I84V	ATA to GTA	N	Y				(117)
	L90M	TTG to ATG	?	Y			Most common mutation *in vivo*	(116, 120)
P9941	V82A	GTC to GCC	Y	?	6-8			(122)
Ro 31-8959	L10I	CTC to ATC		Y			Found in combination with G48V *in vivo*	(123)
(saquinavir)	G48V	GGG to GTG	Y	Y			G48V/L90M/I54V: > 50-fold (subtype B or O)	(124, 125)
							No back mutation seen in absence of drug at passage 26	
							Seen at high frequency in patients receiving higher monotherapy doses	(126)
	I54V	ATC to GTC	Y				In subtype B	(124, 125)
	I54V	ATA to GTA	Y				In subtype O	
	G73S	GGT to AGT	?	Y			Found in combination with L90M, A71V or L101	(160)
	V82A	GTC to GCC	?	Y			Seen only in the presence of G48V	(127, 128)
	I84V	ATA to GTA	Y	?				(98)
	L90M	TTG to ATG	Y	Y			G48V/L90M: >100-fold enzyme resistance; double mutant rare *in vivo*; L90M most common in vivo; G48V/I84V/L90M: 30-fold	(124) (98)
RPI-312	I84V	ATA to GTA	Y	?	5			(129)
SC-52151	L24V	TTA to GTA	Y	?	10-20	SC55389A;	G48V/V82A, G48V/L63P/V82A or I54T: 10-	(130, 131)
	G48V	GGG to GTG	Y	?		Ro 31-8959;	to 20-fold	(130, 131)
	A71V	GCT to GTT	Y	?		not L-735,524	A71V/V75I/P81T: 20- to 30-fold	(130, 131)
	V75I	GTA to ATA	Y	?			L24V/G48V/A71V/V75I/P81T: 1000-fold	(130, 131)
	P81T	CCT to ACT	Y	?				
	V82A	GTC to GCC	Y	?				
	N88D	AAT to GAT	Y	?			N88D or I11V/M46I/F53L/A71V/N88D:10- to 20-fold	
SC-55389A	L10F	CTC to CGC	Y	?		Not SC-52151		(130, 131)
	N88S	AAT to AGT	Y	?	20	Not SC-52151	N88S/L10F: 10-fold	(130, 131)
SKF108842	V82T	GTC to ACC	Y	?				(132)
	I84V	ATA to GTA	Y	?				
SKF108922	V82A	GTC to GCC	Y	?				
	V82T	GTC to ACC	Y	?				
VB 11,328	L10F	CTC to GGC	Y	?			L10F/I84V: 8-fold	(133)
	M46I	ATG to ATA	Y	?			I50V/M46I/I47V: 20-fold	(98, 133)
	I47V	ATA to CTA	Y	?				
	I50V	ATT to GTT	Y	?	3			(98)
	I84V	ATA to GTA	Y	?				
VX-478	L10F	CTC to CGC	Y	?				(134)
(141W94)	M46I	ATG to ATA	Y	?	Nil			
	I47V	ATA to CTA	Y	?	Nil			
	I50V	ATT to GTT	Y	?	3			(135)
	I84V	ATA to GTA	Y	?				
XM323	L10F	CTC to CGC					L10F/V82A: 2-fold	(136)
	K45I	AAA to ATA					L10F/K45I/I84V: 50-fold	(98)
	M46L	ATG to CTG	Y	?				(136)
	V82A	GTC to GCC	Y	?			V82A/M46L: 7-fold; V82A/M46L/L97V: 11-fold	(136)
	V82I	GTC to ATC	Y	?	< 2			(136)
	V82F	GTC to TTC	Y	?				(136)
	I84V	ATA to GTA	Y	?	12	P9941; not A-77003 or Ro 31-8959	V82F/I84V: 92-fold	(98, 136)
	L97V	TTA to GTA	Y	?			No resistance alone; V82A/L97V: 3-fold	(136)

RESISTANCE TABLE

Compound	Amino acid change	Codon change	*In vitro*	*In vivo*	-Fold resistance	Cross-resistance (-fold)	Comments	Reference
PYROPHOSPHATE ANALOGUE RT INHIBITORS								
Foscarnet (PFA)	W88G	TGG to GGG	Y	Y	5	Hypersusceptibility to AZT	Observed in isolates exposed to both AZT and PFA; completely suppresses effects of AZT mutations in genetic backgrounds M41L/D67N/K70R/T215Y and M41L/K70R/T215Y while conferring 3.5- to 4.7-fold PFA resistance	(137, 138)
	W88S	TGG to TCG	N	Y	2-4	Wild-type susceptibility to AZT	Partially suppresses (by 8-fold) effects of AZT mutations in genetic background M41L/D67N/K70R/T215Y while losing resistance to PFA	(137, 139)
	E89G	GAA to GGA	Y	N	14		Isolated by screening RT clones for ddGTP resistance	(140)
	E89K	GAA to GGA	Y	N	> 16			(138)
	L92I	TTA to ATA	Y	N	8			(138)
	S156A	TCA to GCA	Y	N	4.5			(138)
	Q161L	CAA to CTA	Y	Y	5			(137)
	H208Y	CAT to TAT	Y	Y	2		Q161L/H208Y: 9-fold; increased susceptibility to AZT (100-fold), nevirapine (20-fold) and TIBO R82150 (30-fold); Q161L/H208Y reverses effects of AZT mutations D67N, K70R, T215Y and K219Q	(137)
FUSION/BINDING INHIBITORS								
Dextran sulphate (DS)	S113N	AGT to AAT	Y	?			S113N/S134N/K269E/Q278E/N293D/N323S/R387I: 250-fold; 113 is in the V1 loop region	(141, 142)
	S134N	AGC to AAC	Y	?			V2 loop region	
	K269E	AAA to GAA	Y	?			V3 loop region	
	Q278H	CAG to CAT	Y	?			V3 loop region	
	N293D	AAT to GAT	Y	?			V3 loop region	
	N323S	AAT to AGT	Y	?			C3 region	
	R387I	AGA to ACA	Y	?			CD4 binding region	
JM-2763	S274R	AGT to AGA	Y	?		Combination of mutations: DS (1-8); JM-3100: (5-22)	Combination of mutations: 95- to 792-fold	(143, 144)
	Q278H	CAG to CAT	Y	?				
	I288V	ATA to GTA	Y	?				
	A297T	GCA to ACA	Y	?				
	P385L	CCA to CTA	Y	?				
JM-3100 (AMD-3100)	F145L	TTC to TTA	Y	?		Combination of mutations: JM-2763 (> 500); DS (> 7 to 6,667)	Combination of mutations: 2- to 100-fold	(143, 144)
	N270S	AAT to AGT	Y	?				
	R272T	AGA to ACA	Y	?				
	S274R	AGT to AGA	Y	?				
	Q278H	CAG to CAC	Y	?				
	I288V	ATA to GTA	Y	?				
	N293H	AAT to CAT	Y	?				
	A297T	GCA to ACA	Y	?				
	P385L	CCA to CTA	Y	?				
	Q410E	CAA to GAA	Y	?				
	S433P	TCC to CCC	Y	?				
	V457I	GTA to ATA	Y	?				
RPR103611 (145)	R22A	AGG to ?	Y	?			No changes in gp120; R22A and I84S seen only in gp41	(145)
	I84S	ATC to ?	Y	?				
Siamycin I	N188K	AAT to AAA	Y	?			N188K/G332E/N351D/A550T/N633D/L762S: 9-fold	(146)
	G332E	GGA to GAA	Y	?				
	N351D	AAT to GAT	Y	?				
	A550T	GCC to ACC	Y	?				
	N633D	AAT to GAT	Y	?				
	L762S	TTG to TCG	Y	?				
MULTIPLE DRUG RESISTANCE (COMBINATIONS)								
AZT + ddI/ddC	A62V	GCC to GTC	N	Y	Nil		Associated with 75, 77, 116 & 151	(147, 148)
	V75I	GTA to ATA	N	Y	Nil		Associated with 77, 116 & 151	(147, 148)
	F77L	TTC to CTC	N	Y	Nil		Associated with 75, 116 & 151	(147, 148)
	F116Y	TTT to TAT	N	Y	Nil		Associated with 75, 77 & 151	(147, 148)

RESISTANCE TABLE

Compound	Amino acid change	Codon change	*In vitro*	*In vivo*	-Fold resistance	Cross-resistance (-fold)	Comments	Reference
MULTIPLE DRUG RESISTANCE (COMBINATIONS) continued								
	Q151M	CAG to ATG	N	Y		AZT (10)	Pivotal MDR mutation (first to occur and is then found in association with various of the other four mutations);	(147-149)
						ddI/ddC (5)	A62V/V75I/F77L/F116Y/Q151M: AZT 190-fold; ddI 50-fold; ddC 20-fold; d4T > 10-fold	
AZT + 3TC	R211K	AGG to AAG	?	Y			M184V/R211K/L214F confers high level resistance to both AZT and 3TC	(17,150,151)
	L214F	CTT to TTT	?	Y				
	G333D	GGC to ?	Y	Y			Standard AZT mutations + R211K + L214F	
	G333E	GGC to ?	Y	Y			Standard AZT mutations + G333E/D	(151)
SIV								
NUCLEOSIDE RT INHIBITORS								
AZT	Q151M	CAG to ATG	?	Y	> 100	ddI; ddC; d4T; 3TC		(161)
(-)-FTC	M184V	ATG to GTG	Y	?				(21)
PMPA	K65R	AAA to AGA	?	Y	5	3TC (80); ddI; ddC; d4T; PMEA	K65R appears first, followed by N69S and I118V: 5-fold. Observed changes at N69S, N69T, R82K, I118V, A158S and S211N do not result in increased resistance.	(152-154)
FIV								
NUCLEOSIDE RT INHIBITORS								
d4T	V47I	GTA to ATA	Y	?	4-6	PFA (> 50); AZT; ddI; PMEA		(155)
ddC	D3H	GAT to CAT	Y	?	4	ddI; PFA		(156)
(-)-FTC	M183T	ATG to ACG	Y	?	10	ddC	Corresponds to 184 in HIV; M183V made by SDM: 10-fold resistance to 3TC or (-)-FTC	(157,158)
3TC	P156S	CCA to TCA	Y	?	7	AZT (4), AZT + 3TC (6)		(159)

Abbreviations

Amino acids: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenyalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.
1592U89: (1*S*,4*R*)-4-[(2-amino-6-cyclopropyl-amino)-9H-purin-9-yl]-2-cyclopentene-1-methanol succinate (a carbovir analogue; GlaxoWellcome); **3TC**: (-)—L-2′,3′-dideoxy-3′-thiacytidine (GlaxoWellcome); **α-APA R18893**: a-nitro-anilino-phenylacetamide; **A-77003, A-75925 and A-80987**: C_2 symmetry-based protease inhibitors (Abbott Laboratories); **AAP-BHAP**: bisheteroarylpiperazine analogue (Pharmacia & Upjohn); **ABT-538**: C2 symmetry-based protease inhibitor (Abbott Laboratories); **AZdU**: 3′-azido-2′,3′-dideoxyuridine; **AZT**: 3′-azido-3′-deoxythymidine (GlaxoWellcome); **AZT-p-ddI**: 3′-azido-3′-deoxythymidilyl-(5′,5′)-2′,3′-dideoxyinosinic acid (Ivax); **BHAP**: bisheteroarylpiperazine; **BILA 1906**: *N*-{1*S*-[[[3-[2*S*-[(1,1-dimethylethyl)amino]carbonyl)-4*R*-]3-pyridinylmethylthio]-1-piperidinyl]-2*R*-hydroxy-1*S*-(phenylmethyl)propyl]amino]carbonyl]-2-methylpropyl)-2-quinolinecarboxamide (Bio-Mega/Boehringer Ingelheim); **BILA 2185**: *N*-(1,1-dimethylethyl)-1-[2*S*-[[2-2,6-dimethyphenoxy)-1-oxoethyl]amino]-2*R*-hydroxy-4-phenylbutyl]4*R*-pyridinylthio)-2-piperidine-carboxamide (Bio-Mega/Boehringer Ingelheim); **BM+51.0836**: thiazolo-isoindolinone derivative; **BMS 186,318**: aminodiol derivative HIV-1 protease inhibitor (Bristol-Myers Squibb); **d4API**: 9-[2,5-dihydro-5-(phosphonomethoxy)-2-furanel]adenine (Gilead Sciences); **d4C**: 2′,3′-didehydro-2′,3′-dideoxycytidine; **d4T**: 2′,3′-didehydro-3′-deoxythymidine (Bristol-Myers Squibb); **ddC**: 2′,3′-dideoxycytidine (Roche); **ddI**: 2′,3′-dideoxyinosine (Bristol-Myers Squibb); **DMP 266**: a 1,4-dihydro-2*H*-3,1-benzoxazin-2-one; **DMP 450**: [[4*R*-(4-a,5-a,6-8,7-b)]-hexahydro-5,6-bis(hydroxy)-1,3-bis(3-amino)phenyl]methyl)-4,7-bis(phenylmethyl)-2H-1,3-diazepin-2-one]-bismesylate (Avid Therapeutics); **DXG**: (-)-β-D-dioxolane-guanosine; **EBU-dM**: 5-ethyl-1-ethoxymethyl-6-(3,5-dimethylbenzyl)uracil; **E-EBU**: 5-ethyl-1-ethoxymethyl-6-benzyluracil; **DS**: dextran sulphate; **E-EPSeU**: 1-(ethoxymethyl)-(6-phenylselenyl)-5-ethyluracil; **E-EPU**: 1-(ethoxymethyl)-(6-phenyl-thio)-5-ethyluracil; FIV: feline immunodeficiency virus; **FddA:**2′-thiacytidine (Triangle Pharmaceuticals); HBY 097: (*S*)-4-isopropoxycarbonyl-6-methoxy-3-(methylthio-methyl)-3,4-dihydroquinoxalin-2(1*H*)-thione; **HEPT**: 1-[(2-hydroxyethoxy)methyl]6-(phenylthio)thymine; **HIV-1**: human immunodeficiency virus type 1; **JM2763**: 1,1′-(1,3-propanediyl)-bis-1,4,8,11-tetraazacyclo-tetradecane (Johnson Matthey); **JM3100 (Also known as AMB3100)**: 1,1′-[1,4-phenylenebis-(methylene)]bis-(1,4,8,11-tetraazacyclotetradecane)octahydrochloride dihydrate (Johnson Matthey); **KNI-272**: (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid-containing tripeptide; **L-697,593**: 5-ethyl-6-methyl-3-(2-phthalimido-ethyl)pyridin-2(1*H*)-one; **L-697,661**: 3-[[(-4,7-dichloro-1,3-benzoxazol-2-yl)methyl]amino]-5-ethyl-6-methylpyridin-2(1*H*)-one; **L-FDDC**: (-)-β-L-5-fluoro-2′,3′-dideoxy-cytidine; **L-FDOC**: (-)-β-L-5-fluoro-dioxolane cytosine; **MK-639**: hydroxy-aminopentane amide HIV-1 protease inhibitor (Merck & Co); **MKC442**: 6-benzyl-1-ethoxymethyl-5-isopropyluracil (I-EBU; Triangle Pharmaceuticals/Mitsubishi); **nevirapine**: 11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyridol[3,2-b:2′,3′-e] diazepin-6-one (Boehringer Ingelheim); **NNRTI**: non-nucleoside reverse transcriptase inhibitor; **NSC648400**: 1-benzyloxymethyl-5-ethyl-6-(alpha-pyridylthio)uracil (E-BPTU); **P9941**: [2-pyridylacetyl-IlePheAla-y(CHOH)]2 (Dupont Merck); **PFA**: phosphonoformate (foscarnet; Astra); **PMEA**: 9-(2 phosphonylmethoxyethyl)adenine (Gilead Sciences); **PMPA**: (*R*)-9-(2-phosphonyl-methoxypropyl)adenine (Gilead Sciences); **Ro 31-8959**: hydroxyethylamine derivative HIV-1 protease inhibitor (Roche); **RPI-312**: 1-[(3*S*)-3-(*n*-alpha-benzyloxycarbonyl)-l-asparginyl)-amino-2-hydroxy-4-phenyl-butyryl]-*n*-tert-butyl-l-proline amide (peptidyl protease inhibitor); **RT**: reverse transcriptase; **S-2720**: 6-chloro-3,3-dimethyl-4-(isopropenyl-oxycarbonyl)-3,4-dihydro-quinoxalin-2(1*H*)thione; **SC-52151**: hydroxyethylurea isostere protease inhibitor (Searle); **SC-55389A**: hydroxyethyl-urea isostere protease inhibitor (Searle); **SDM:** Site-directed mutagenesis; **SIV**: simian immunodeficiency virus; **TIBO R82150**: (+)-(5*S*)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)-imidazo[4,5,1-jk][1,4]-benzodiazepin-2(1*H*)-thione (Janssen); **TIBO 82913**: (+)-(5*S*)-4,5,6,7,-tetrahydro-9-chloro-5-methyl-6-(3-methyl-2-butenyl)-imidazo-[4,5,1-jk]-[1,4]benzo-diazepin-2(1*H*)-thione (Janssen); **TSAO-m³T**: [2′,5′-bis-O-(tert-

pentofuranosyl-N^4-methylthymine; **U-90152**: 1-[3-[(1-methylethyl)-amino]-2-pyridinyl]-4-[[5-[(methylsulphonyl)-amino]-1H-indol-2yl]carbonyl]-piperazine; UC: thiocarboxanilide derivatives (Uniroyal Chemical Co): **UC-781**: N-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furan-carbothioamide; **UC-82**: *N*-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-thiophene-carbothioamide; **VB 11,328**: hydroxyethyl-sulphonamide protease inhibitor (Vertex Pharmaceuticals); **VX-478**: hydroxyethylsulphonamide protease inhibitor (Vertex Pharmaceuticals); **XM 323**: cyclic urea protease inhibitor (DuPont Merck).

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References

1. Larder BA & Kemp SD. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* 1989; **246**:1155-1158.
2. Larder BA, Coates KE & Kemp SD. Zidovudine-resistant human immunodeficiency virus selected by passage in cell culture. *Journal of Virology* 1991; **65**:5232-5236.
3. Kellam P, Boucher CA & Larder BA. Fifth mutation in human immunodeficiency virus type 1 reverse transcriptase contributes to the development of high-level resistance to zidovudine. *Proceedings of the National Academy of Sciences, USA* 1992; **89**:1934-1938.
4. Gurusinghe AD, Land SA, Birch C, McGavin C, Hooker DJ, Tachedjian G, Doherty R & Deacon NJ. Reverse transcriptase mutations in sequential HIV-1 isolates in a patient with AIDS. *Journal of Medical Virology* 1995; **46**:238-243.
5. Arnold E, Tantillo C, Boyer P, Ding J, Roy BM, Clark Jr AD, Pauwels R, Andries K, Janssen PAJ, Mellors J, Deacon N & Hughes SH. Implications of the three-dimensional structure of HIV-1 reverse transcriptase for resistance to antiviral drugs. *Third International Workshop on HIV Drug Resistance, 1994, Kauai, Hawaii, USA;* Abstract no. 30.
6. Harrigan PR, Kinghorn I, Bloor S, Kemp SD, Najera I, Kohli A & Larder BA. Significance of amino acid variation at human immunodeficiency virus type 1 reverse transcriptase residue 210 for zidovudine susceptibility. *Journal of Virology* 1996; **70**:5930-5934.
7. Hooker DJ, Tachedjian G, Solomon AE, Gurusinghe AD, Land S, Birch C, Anderson JL, Roy BM, Arnold E & Deacon NJ. An *in vivo* mutation from leucine to tryptophan at position 210 in human immunodeficiency virus type 1 reverse transcriptase contributes to high-level resistance to 3'-azido-3'-deoxythymidine. *Journal of Virology* 1996; **70**: 8010-8018.
8. Demeter TM, Nawaz T, Morse G, Dolin R, Dexter A, Gerondelis P, & Reichman, RC. Development of zidovudine resistance mutations in patients receiving prolonged didanosine therapy. *Journal of Infectious Diseases* 1995; **172**: 1480-1485.
9. Winters MA, Shafer RW, Jellinger RA, Mamtora G, Gingeras T & Merigan TC. Human immunodeficiency virus type 1 reverse transcriptase genotype and drug susceptibility changes in infected individuals receiving dideoxyinosine monotherapy. *Antimicrobial Agents and Chemotherapy* 1997; **41**: 757-762.
10. Lin PF, Samanta H, Rose RE, Patick AK, Trimble J, Bechtold CM, Revie DR, Khan NC, Federici ME, Li H, Lee A, Anderson R & Colonno RJ. Genotypic and Phenotypic Analysis of Human Immunodeficiency Virus Type I from Patients on Prolonged Stavudine Therapy. *Journal of Infectious Diseases* 1994; **170**: 1157-1164.
11. Zhang D, Caliendo AM, Eron JJ, DeVore KM, Kaplan JC, Hirsch MS & D'Aquila RT. Resistance to 2',3'-dideoxycytidine conferred by a mutation in codon 65 of the human immunodeficiency virus type 1 reverse transcriptase. *Antimicrobial Agents and Chemotherapy* 1994; **38**: 282-287.
12. St. Clair MH, Martin JL, Tudor-Williams G, Bach MC, Vavro CL, King DM, Kellam P, Kemp SD & Larder BA. Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. *Science* 1991; **253**: 1557-1559.
13. Lacey SF & Larder BA. Novel mutation (V75T) in human immunodeficiency virus type 1 reverse transcriptase confers resistance to 2',3'-didehydro-2',3'-dideoxythymidine in cell culture.
14. Gu Z, Gao Q, Li X, Parniak MA & Wainberg MA. Novel mutation in the human immunodeficiency virus type 1 reverse transcriptase gene that encodes cross-resistance to 2',3'-dideoxyinosine and 2',3'-dideoxycytidine. *Journal of Virology* 1992; **66**: 7128-7135.
15. Gu Z, Gao Q, Fang H, Salomon H, Parniak MA, Goldberg E, Cameron J & Wainberg MA. Identification of a mutation at codon 65 in the IKKK motif of reverse transcriptase that encodes human immunodeficiency virus resistance to 2',3'-dideoxycytidine and 2',3'-dideoxy-3'-thiacytidine. *Antimicrobial Agents and Chemotherapy* 1994; **38**:275-281.
16. Fitzgibbon JE, Howell RM, Haberzettl CA, Sperber SJ, Gockle DJ & Dubin DT. Human immunodeficiency virus type 1 *pol* gene mutations which cause decreased susceptibility to 2',3'-dideoxycytidine. *Antimicrobial Agents and Chemotherapy* 1992; **36**:153-157.
17. Schinazi RF, Stuyver L, Wyseur A, Lloyd RMJ, Hough L, Rombout A, Rossau R & Rimland D. Proviral and plasma virus genotyping using a line probe assay in nucleoside treated HIV infected Veterans Affairs (VA) patients. *Fifth International Workshop on HIV Drug Resistance, 1996, Whistler, Canada*; Abstract no: 65.
18. Slade DE, Vavro CL, Stapelton JT, Swack N & St Clair MH. A cysteine at codon 215 of HIV RT confers resistance to ddC. *Second International HIV-1 Drug Resistance Workshop, 1993, Noordwijk, The Netherlands.*
19. Schinazi RF, Boudinot FD, Manouilov KK, Mellors JW, McMillan A, Schlueter-Wirtz S, Lloyd R Jr., Korba BE, Tennant B & Chu CK. Anti-HBV and anti-HIV activities of dioxolane-purine nucleosides. *Ninth International Conference on Antiviral Research, 1996, Urabandai, Fukushima, Japan.*
20. Mellors JW, Bazmi H, Chu CK & Schinazi RF. K65R mutation in HIV-1 reverse transcriptase causes resistance to (-)β-D-dioxolane-guanosine and reverses AZT resistance. *Fifth International Workshop on HIV Drug Resistance, 1996, Whistler, Canada;* Abstract no: 7.
21. Schinazi RF, Lloyd RM Jr. McMillan A, Gosselin G, Imbach J-L & Sommadossi J-P. Development of HIV-1 and SIV resistant to β-L-2',3'-dideoxycytidine analogues. *Fourth International Workshop on HIV Drug Resistance, 1995, Sardinia, Italy*; Abstract no: 10.
22. Tanaka M, Srinivas RV, Ueno T, Kavlick MF, Hui FK, Fridland A, Driscoll JS & Mitsuya H. *In vitro* induction of human immunodeficiency virus type 1 variants resistant to 2'-β-fluoro-2',3'-dideoxyadenosine. *Antimicrobial Agents and Chemotherapy* 1997; **41**:1313-1318.
23. Tisdale M, Kemp SD, Parry NR & Larder BA. Rapid *in vitro* selection of human immunodeficiency virus type 1 resistant to 3'-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. *Proceedings of the National Academy of Sciences, USA* 1993; **90**:5653-5656.
24. Schinazi RF, Lloyd RM Jr., Nguyen M-H, Cannon DL, McMillan A, Ilksoy N, Chu CK, Liotta DC, Bazmi HZ & Mellors JW. Characterization of human immunodeficiency viruses resistant to oxathiolane-cytosine nucleosides. *Antimicrobial Agents and Chemotherapy* 1993; **37**:875-881.
25. Foli A, Sogocio KM, Anderson B, Kavlick M, Saville MW, Wainberg MA, Gu Z, Cherrington JM, Mitsuya H & Yarchoan R. *In vitro* selection and molecular characterization of human immunodeficiency virus type 1 with reduced sensitivity to 9-[2-(phosphonomethoxy)-ethyl]adenine (PMEA). *Antiviral Research* 1996; **32**:91-98.
26. Gu Z, Salomon H, Cherrington JM, Mulato AS, Chen MS, Yarchoan R, Foli A, Sogocio KM & Wainberg MA. K65R mutation of human immunodeficiency virus type 1 reverse transcriptase encodes cross-resistance to 9-(2-phosphonylmethoxyethyl)adenine. *Antimicrobial Agents and Chemotherapy* 1995; **39**:1888-91.
27. Cherrington JM, Mulato AS, Fuller MD & Chen MS. A novel mutation (K70E) in human immunodeficiency virus type 1 reverse transcriptase confers decreased susceptibility to 9-[2-(phosphonomethoxy)ehtyl]adenine *in vitro*. *Antimicrobial Agents and Chemotherapy* 1996; 40:2112-2216.
28. Mulato A, Lamy P, Li W, Miller M & Cherrington J. Genotypic chracterization of HIV-1 variants isolated from AIDS patients treated with adefovir dipivoxil (bis-POM PMEA). *Sixth International Workshop on HIV Drug Resistance, St Petersburg, USA, 25-28 June 1997*; Abstract 24 .
29. Cherrington J, Chandok R, Mulato A, Lamy P, Mitsuya H & Wainberg M. In vitro selection and characterization of HIV-1 variants with reduced susceptibility to PMPA. *Sixth International Workshop on HIV Drug Resistance, 25-28 June 1997; St. Petersburg, USA*: Abstract 26.
30. Tisdale M, Parry NR, Cousens D, St Clair MH & Boone LR. Anti-HIV actvity of 1*S*,4*R*-[2-amino-6-cyclopropylamino)-9*H*-purin-9-yl]-2-cyclopentene-1-methanol (1592U89). 34th *Interscience Conference on Antimicrobial Agents and Chemotherapy, 1994, Orlando, FL, USA*; Abstract no: 92.
31. Tisdale M, Alnadaf T & Cousens D. Combination of mutations in human immunodeficiency virus type 1 reverse transcriptase required for resistance to the carbocyclic nucleoside 1592U89. *Antimicrobial Agents and Chemotherapy* 1997; **41**:1094-1098.
32. Harrigan PR, Tisdale M, Najera I, Cousens D, St. Clair M, Stone C, Kohli A, Myers R & Larder BA. Antiretroviral activity and resistance to 1592U89, a novel HIV RT inhibitor. *Fifth International Workshop on HIV Drug Resistance, 1996, Whistler, Canada*; Abstract no: 16.
33. Gao Q, Gu Z, Parniak MA, Cameron J, Cammack N, Boucher C & Wainberg MA. The same mutation that encodes low-level human

2',3'-dideoxycytidine confers high-level resistance to the (-) enantiomer of 2',3'-dideoxy-3'-thiacytidine. *Antimicrobial Agents and Chemotherapy* 1993; **37**:1390-1392.

34. Keulen W, Back NK, van Wijk A, Boucer CA & Berkhout B. Initial appearance of the 184Ile variant in lamivudine-treated patients is caused by the mutational bias of human immunodeficiency virus type 1 reverse transcriptase. *Journal of Virology* 1997; **71**:3346-3350.
35. Back N, Nijhuis M, Keulen W, Boucher C, Oude Essink B, van Kuilenburg A, van Gennip A & Berkhout B. Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme. *EMBO Journal* 1996; **15**:4040-4049.
36. Larder BA, Kemp SD & Harrigan PR. Potential mechanism for sustained antiretrovial efficacy of AZT-3TC combination therapy. *Science* 1995; **269**:696-699.
37. Sharma SK, Fan N, Bank KB, Kopta LA, Olmsted RA, Poppe SM, Slade DE, Thomas RC & Tarpley WG. A drug resistance mutation (G190E) in the inhibitor binding pocket impairs both RNA-dependent DNA polymerase and ribonuclease H activities of HIV-1 reverse transcritase. *Fourth International Workshop on HIV Drug Resistance, 1995, Sardinia, Italy; Abstract* no: 2.
38. Olmsted RA, Slade DE, Kopta LA, Poppe SM, Poel TJ, Newport SW, Rank KB, Biles C, Morge RA, Dueweke TJ, Yagi Y, Romero DL, Thomas RC, Sharma SK & Tarpley WG. (Alkylamino) piperidine bis(heteroaryl)piperizine analogs are potent, broad-spectrum nonnucleoside reverse transcriptase inhibitors of drug-resistant isolates of human immunodeficiency virus type 1 (HIV-1) and select for drug-resistant variants of HIV-1IIIB with reduced replication phenotypes. *Journal of Virology* 1996; **70**: 3698-3705.
39. Demeter L, Resnick L, Nawaz T, Timpone JG Jr., Batts D & Reichman RC. Phenotypic and genotypic analysis of atevirdine (ATV) susceptibility of HIV-1 isolates obtained from patients receiving ATV monotherapy in a phase I clinical trial (ACTG 187): comparison to patients receiving combination therapy with ATV and zidovudine. *Third Workshop on Viral Resistance, 1993, Gaithersburg, MD, USA.*
40. Fan N, Rank KB, Evans DB, Thomas RC, Tarpley WG & Sharma SK. Simultaneous mutations at Tyr-181 and Tyr-188 in HIV-1 reverse transcriptase prevents inhibition of RNA-dependent DNA polymerase activity by the bisheteroarylpiperazine (BHAP) U-90122s. *FEBS Letters* 1995; **370**:59-62.
41. Dueweke TJ, Pushkarskaya T, Poppe SM, Swaney SM, Zhao Q, Chen SY, Stevenson M & Tarpley WG. A mutation in reverse transcriptase of bis(heteroaryl)piperazine-resistant human immunodeficiency virus type 1 that confers increased sensitivity to other nonnucleoside inhibitors. *Proceedings of the National Academy of Sciences, USA* 1993; **90**:4713-4717.
42. Balzarini J, Karlsson A, Pérez-Pérez M-J, Camarasa M-J, Tarpley WG & De Clercq E. Treatment of human immunodeficiency virus type 1 (HIV-1)-infected cells by combinations of HIV-1-specific inhibitors results in a different resistance pattern than does treatment with single-drug therapy. *Journal of Virology* 1993; **67**:5353-5359.
43. Vasudevachari MB, Battista C, Lane HC, Psallidopoulos MC, Zhao B, Cook J, Palmer JR, Romero DL, Tarpley WG & Salzman NP. Prevention of the spread of HIV-1 infection with nonnucleoside reverse transcriptase inhibitors. *Virology* 1992; **190**:269-277.
44. Balzarini J, Karlsson A, Sardana VV, Emini EA, Camarasa MJ & De Clercq E. Human immunodeficiency virus 1 (HIV-1)-specific reverse transcriptase (RT) inhibitors may suppress the replication of specific drug-resistant (E138K) RT HIV-1 mutants or select for highly resistant (Y181C— C181I) RT HIV-1 mutants. *Proceedings of the National Academy of Sciences, USA* 1994; **91**:6599-6603.
45. Demeter LM, Shafer RW, Para M, Morse G, Freimuth W, Merigan TC & Reichman RC. Delavirdine (DLV) susceptibility of HIV-1 isolates obtained from patients (PTS) receiving DLV monotherapy (ACTG 260). *Fourth International Workshop on HIV Drug Resistance, 1995, Sardinia, Italy*; Abstract no: 23.
46. Maass G, Immendoerfer U, Koenig B, Leser U, Mueller B, Goody R & Pfaff E. Viral resistance to the thiazolo-iso-indolinones, a new class of nonnucleoside inhibitors of human immunodeficiency virus type 1 reverse transcriptase. *Antimicrobial Agents and Chemotherapy* 1993; **37**:2612-2617.
47. Buckheit J R W, Fliakas-Boltz V, Decker WD, Roberson JL, Stup TL, Pyle CA, White EL, McMahon JB, Currens MJ, Boyd MR & Bader JP. Comparative anti-HIV evaluation of diverse HIV-1-specific reverse transcriptase inhibitor-resistant virus isolates demonstrates the existence of distinct phenotypic subgroups. *Antiviral Research* 1995; **26**:117-132.
48. Moeremans M, De Raeymaeker M, Van den Broeck R, Stoffels P, De Brabander M, De Cree J, Hertogs K, Pauwels R, Staszewski S & Andries K. Virological analysis of HIV-1 isolates in patients treated with the non-nucleoside reverse transcriptase inhibitor RO91767, 8-chloro-TIBO. *Fourth International Workshop on HIV Drug Resistance, 1995, Sardinia, Italy*; Abstract no: 33.
49. Winslow DL, Garber S, Reid C, Scarnati H, Korant B, Emini E & Anton ED. Development of high-level resistance to DMP 266 requires multiple mutations in the reverse transcriptase gene. *Fourth International Conference on HIV Drug Resistance, 1995, Sardinia, Italy*; Abstract no: 13.
50. Winslow DL, Reid C, Garber S, Scarnati H, Rayner M & Anton E. Selection conditions affect the evolution of specific mutations in the reverse transcriptase gene associated with resistance to DMP 266. *Whistler, Canada*; Abstract no: 10.
51. Young SD, Britcher SF, Tran LO, Payne LS, Lumma WC, Lyle TA, Huff JRA, Olsen DB, Carroll SS, Pettibone DJ, O'Brien JA, Ball RG, Balani SK, Lin JH, Chen IW, Schleif WA, Sardana VV, Long WJ, Byrenes VW & Emini EA. L-743, 726 (DMP-266): a novel, highly potent nonnucleoside inhibitor of the human immunodeficiency virus type 1 reverse transcriptase. *Antimicrobial Agents and Chemotherapy* 1995;**39**:2602-2609.
52. Balzarini J, Karlsson A & De Clercq E. Human immunodeficiency virus type 1 drug-resistance patterns with different 1-[(2-hydroxyethoxy)-methyl]-6-(phenylthio)thymine derivatives. *Molecular Pharmacology* 1993; **44**:694-701.
53. Nguyen MH, Schinazi RF, Shi C, Goudgaon NM, McKenna PM & Mellors JW. Resistance of human immunodeficiency virus type 1 to acyclic 6-phenylselenenyl- and 6-phenylthiopyrimidines. *Antimicrobial Agents and Chemotherapy*1994; **38**:2409-2414.
54. Kleim JP, Rosner M, Winkler I, Paessens A, Kirsch R, Hsiou Y, Arnold E & Riess G. Selective pressure of a quinoxaline nonnucleoside inhibitor of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) on HIV-1 replication results in the emergence of nucleoside RT-inhibitor-specific (RT Leu-74-Val or Ile and Val-75-Leu or Ile) HIV-1 mutants. *Proceedings of the National Academy of Sciences, USA* 1996; **93**:34-8.
55. Kleim JP, Winkler I, Rosner M, Kirsch R, Rubsamen-Waigmann H, Paessens A & Reiss G. Different mutational pathways of the HIV-1 RT gene are defined by alternative experimental protocols applied to generate *in vitro* resistance of HIV-1 to HBY 097. *Fifth International Workshop on HIV Drug Resistance, 1996, Whistler, Canada*; Abstract no: 15.
56. Kleim JP, Winkler I, Rosner M, Kirsch R, Rubsamen-Waigmann H, Paessens A & Reiss G. *In vitro* selection for different mutational patterns in the HIV-1 reverse transcriptase using high and low selective pressure on the nonnucleoside reverse transcriptase inhibitor HBY 097. *Virology* 1997; **231**:112-118.
57. Kleim J-P, Bender R, Kirsch R, Meichsner C, Paessens A, Rösner M, Rübsamen-Waigmann H, Kaiser R, Wichers M, Schneweis KE, Winkler I & Riess G. Preclinical evaluation of HBY 097, a new nonnucleoside reverse transcriptase inhibitor of human immunodeficiency virus type 1 replication. *Antimicrobial Agents and Chemotherapy* 1995; **39**:2253-2257.
58. Buckheit RW Jr., Fliakas-Boltz V, Yeagy-Bargo S, Weislow O, Mayers DL, Boyer PL, Hughes SH, Pan BC, Chu SH & Bader JP. Resistance to 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine derivatives is generated by mutations at multiple sites in the HIV-1 reverse transcriptase. *Virology* 1995; **210**:186-193.
59. Nunberg JH, Schleif WA, Boots EJ, O'Brien JA, Quintero JC, Hoffman JM, Emini EA & Goldman ME. Viral resistance to human immunodeficiency virus type 1-specific pyridinone reverse transcriptase inhibitors. *Journal of Virology* 1991; **65**:4887-4892.
60. Byrnes VW, Sardana VV, Schleif WA, Condra JH, Waterbury JA, Wolfgang JA, Long WJ, Schneider CL, Schlabach AJ, Wolanski BS, Graham DJ, Gotlib L, Rhodes A, Titus DL, Roth E, Blahy OM, Quintero JC, Staszewski S & Emini EA. Comprehensive mutant enzyme and viral variant assessment of human immunodeficiency virus type 1 reverse transcriptase resistance to nonnucleoside inhibitors. *Antimicrobial Agents and Chemotherapy*1993; **37**:1576-1579.
61. Saag MS, Emini EA, Laskin OL, Douglas J, Lapidus WI, Schleif WA, Whitley RJ, Hildebrand C, Byrnes VW, Kappes JC, Anderson KW, Massari FE & Shaw GM. A short-term clinical evaluation of L-697,661, a non-nucleoside inhibitor of HIV-1 reverse transcriptase. L-697,661 Working Group. *New England Journal of Medicine* 1993; **329**:1065-72.
62. Staszewski S, Miller V, Kober A, Colebunders R, Vandercam B, Delescluse J, Clumeck N, Van Wanzeele F, De Brabander M, De Cree J, Moeremans M, Andries K, Boucher C, Stoffels P & Janssen PAJ. Evaluation of the efficacy and tolerance of RO18893, RO89439 (loviride) and placebo in asymptomatic HIV-1-infected patients. *Antiviral Therapy* 1996; **1**:42-50.
63. Staszewski S, Miller V, Rehmet S, Stark T, De Cree J, De Brabander M, Peeters M, Andries K, Moeremans M, De Raeymaeker M, Pearce G, Van Den Broeck RM, Verbiest W & Stoffels P. Virological and immunological analysis of a triple combination pilot study with loviride, lamivudine and zidovudine in HIV-1-infected patients. *AIDS* 1996; **10**:F1-F7.
64. Moeremans M, De Raeymaeker M, Van den Broeck R, Stoffels P & Andries K. Genotypic analysis of HIV-1 isolates from patients receiving loviride alone or in combination with nucleoside reverse transcriptase inhibitor. *Fourth International Workshop on HIV Drug Resistance, 1995, Sardinia, Italy*; Abstract no: 34.
65. de Béthune M-P, Pauwels R, Andries K, Vandamme A-M, Peeters M, Colebunders R, Stoffels P, De Clercq E & Desmyter J. AZT resistance reversal by the non-nucleoside reverse transcriptase inhibitor α-APA R18893 in a symptomatic HIV-infected individual. *Second International HIV-1 Drug Resistance Workshop, 1993, Noordwijk, The Netherlands.*
66. Seki M, Sadakata Y, Yuasa S & Baba M. Isolation and characterization of human immunodeficiency virus type-1 mutants resistant to the non-nucleotide reverse transcriptase inhibitor MKC-442. *Antiviral Chemistry and Chemotherapy* 1995; **6**:73-79.
67. Borroto-Esoda K, Noel DS, Moxham CP & Furman PA. Preliminary genotypic analysis of HIV-1 in plasma from volunteers receiving repeated multiple doses of MKC-422. *6th International Workshop on*

HIV Drug Resistance, 25-28 June 1997; St. Petersburg, USA: Abstract 22.

68. Balzarini J, Pelemans H, Pérez-Pérez M-J, San-Félix A, Camarasa M-J, De Clercq E & Karlsson A. Marked inhibitory activity of non-nucleoside reverse transcriptase inhibitors against human immunodeficiency virus type 1 when combined with (-)2',3'-dideoxy-3'-thiacytidine. *Molecular Pharmacology* 1996; **49**:882 .
69. Richman DD, Havlir D, Corbeil J, Looney D, Ignacio C, Spector SA, Sullivan J, Cheeseman S, Barringer K, Pauletti D, Shih C-K, Myers M & Griffin J. Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. *Journal of Virology* 1994; **68**:1660-1666.
70. Richman DD. Resistance of clinical isolates of human immunodeficiency virus to antiretroviral agents. *Antimicrobial Agents and Chemotherapy* 1993; **37**:1207-1213.
71. Larder BA. 3'-Azido-3'-deoxythymidine resistance suppressed by a mutation conferring human immunodeficiency virus type 1 resistance to nonnucleoside reverse transcriptase inhibitors. *Antimicrobial Agents and Chemotherapy* 1992; **36**:2664-2669.
72. Mellors JW, Dutchman GE, Im JG-J, Tramontano E, Winkler SR & Cheng Y-C. *In vitro* selection and molecular characterization of human immunodeficiency virus-1 resistant to non-nucleoside inhibitors of reverse transcriptase. *Molecular Pharmacology* 1992; **41**:446-451.
73. Richman D, Shih C-K, Lowy I, Rose J, Prodanovich P, Goff S & Griffin J. Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture. *Proceedings of the National Academy of Sciences, USA* 1991; **88**:11241-11245.
74. Shaw G, Wei X, Johnson V, Taylor M, Decker J, Kilby M, Lifson J, Hahn B & Saag M. Nucleotide sequence analysis of HIV-1 RNA and DNA from plasma and PBMCs of patients treated with ZDV, ddI, and nevirapine: rapid turnover and resistance development *in vivo*. *Third International Workshop on HIV Drug Resistance, 1994, Kauai, Hawaii, USA*.
75. Kleim J-P, Bender R, Billhardt U-M, Meichsner C, Riess G, Rösner M, Winkler I & Paessens, A. Activity of a novel quinoxaline derivative against human immunodeficiency virus type 1 reverse transcriptase and viral replication. *Antimicrobial Agents and Chemotherapy* 1993; **37**:1659-1664.
76. Balzarini J, Pelemans H, Esnouf R, Dunkler A, Parniak MA, Vandamme A-M, Karlsson A, De Clerq E & Kleim J-P. Significance of the 225 Pro/His mutation in HIV-1 reverse transriptase. *6th International Workshop on HIV Drug Resistance, 25-28 June 1997; St. Petersburg, USA*: Abstract 21.
77. Mellors JW, Im G-J, Tramontano E, Winkler SR, Medina DJ, Dutchman GE, Bazmi HZ, Piras G, Gonzalez CJ & Cheng Y-C. A single conservative amino acid substitution in the reverse transcriptase of human immunodeficiency virus-1 reverse transcriptase confers resistance to TIBO R82150. *Molecular Pharmacology* 1993; **43**:11-16.
78. Balzarini J, Karlsson A, Pérez-Pérez M-J, Vrang L, Walbers J, Zhang H, Öberg B, Vandamme A-M, Camarasa M-J & De Clercq E. HIV-1 specific reverse transcriptase inhibitors show differential activity against HIV-1 mutant strains containing different amino acid substitutions in the reverse transcriptase. *Virology* 1993; **192**:246-253.
79. Byrnes V, Blahy O, Condra J, Gotlib L, Graham D, Long W, Quintero J, Rhodes A, Roth E, Sardana V, Schlabach A, Schleif W, Schneider C, Titus D, Wolanski B, Wolfgang J & Emini E. Phenotypic susceptibility of human immunodeficiency virus type 1 RT containing substitutions which engender resistance to nucleoside and non-nucleoside inhibitors. *Third Workshop on Viral Resistance, 1993, Gaithersburg, MD, USA*.
80. Vandamme A-M. Polymerase chain reaction (PCR) as a diagnostic tool in HIV infection. *Verh K Acad Geneeskd Belg* 1994; **56**:231-265.
81. Vandamme A-M, Debyser Z, Pauwels R, De Vreese K, Goubau P, Youle M, Gazzard B, Stoffels PA, Cauwenbergh GF, Anne J, Andries K, Janssen PAJ, Desmyter J & De Clercq E. Characterization of HIV-1 strains isolated from patients treated with TIBO R82913. *AIDS Research and Human Retrovirology* 1994; **10**:39-46.
82. Zhang H, Vrang L, Backbro K, Lind P, Sahlberg C, Unge T & Öberg B. Inhibition of human immunodeficiency virus type 1 wild-type and mutant reverse trasncriptases by the phenyl ethyl thiazolyl thiourea derviatives trovirdine and MSC-127. *Antiviral Research* 1995;**28**:331-342.
83. Vrang L, Rydergard C, Ahgren C, Engelhardt P, Hogberg M, Johansson NG, Kangasmetsa J, Lind P, Noreen R, Sahlberg C, Zhou X-X, Karlsson A, Lopez C, Morin JM Jr., Pernansky RJ, Bell FW, Jordan CL, Kinnick MD, Palkowitz JA, Parrish CA, Pranc P, Vasileff RT, West SJ & Öberg B. Comparative rates of *in vitro* resistance development of HIV-1 to non-nucleoside analog RT inhibitors. *Antiviral Research* 1993; **20**(Supplement 1):77.
84. Balzarini J, Velazquez S, Sanfelix A, Karlsson A, Pérez-Pérez M-J, Camarasa M-J & De Clercq E. Human immunodeficiency virus type-1 specific purine analogues show a resistance spectrum that is different from that of the human immunodeficiency virus type-1-specific non-nucleoside analogues. *Molecular Pharmacology* 1993;**43**:109-114.
85. Balzarini J, Karlsson A, Vandamme AM, Perez-Perez MJ, Zhang H, Vrang L, Öberg B, Backbro K, Unge T, San-Felix A, *et al.* Human immunodeficiency virus type 1 (HIV-1) strains selected for resistance against the HIV-1-specific [2',5'-bis-O-(tert-butyldimethylsilyl)-3'-spiro-5'-(4'-amino-1',2'-oxathiole-2',2'-dioxide)]-β-D-pentofuranosyl (TSAO) nucleoside analogues retain sensitivity to HIV-1-specific nonnucleoside inhibitors. *Proceedings of the National Academy of Sciences, USA* 1993; **90**:6952-6956.
86. Vandamme A-M, Schmit JC, Balzarini J, Van Laethem K, Witvrouw M, Hermans P, Sprecher S, Martinez-Picado J, Clotet B, Peetermans W, Desmyter J & De Clercq E. Presence of TSAO-resistant virus strains in non-experienced patients. *Fifth International Workshop on HIV Drug Resistance, 1996, Whistler, Canada*; Abstract no: 47.
87. Buckheit RW, Kinjerski TL, Fliakas-Boltz V, Russell JD, Stup TL, Pallansch LA, Brouwer WG, Dao DC, Harrison WA, Schultz RJ, Bader JP, & Yang SS. Structure-activity and cross-resistance evaluations of a series of human immunodeficiency virus type 1-specific compounds related to oxathiin carboxanilide. *Antimicrobial Agents and Chemotherapy* 1995; **39**:2718-2727.
88. Balzarini J, Pérez-Pérez M-J, Vélazquez S, San-Félix A, Camarasa M-J, De Clercq E & Karlsson A. Suppression of the breakthrough of human immunodeficiency virus type 1 (HIV-1) in cell culture by thiocarboxanilide derivatives when used individually or in combination with other HIV-1-specific inhibitors (i.e., TSAO derivatives). *Proceedings of the National Academy of Sciences, USA* 1995; **92**:5470-5474.
89. Balzarini J, Brouwer WG, Felauer EE, De Clercq E & Karlsson A. Activity of various thiocarboxanilide derivatives against wild-type and several mutant human immunodeficiency virus type 1 strains. *Antiviral Research* 1995; **27**:219-236.
90. Kinjerski TL, Pallansch LA & Buckheit RW, Jr. Isolation and characterization of HIV-1 isolates resistant to oxathiin carboxanilide analogues: evaluation of variables in the drug resistance selection process. *Antiviral Chemistry and Chemotherapy* 1996; **7**:261-269.
91. Yang SS, Pattabiraman N, Gussio R, Pallansch L, Buckheit RW Jr. & Bader JP. Cross-resistance analysis and molecular modeling of non-nucleoside reverse transcriptase inhibitors targeting drug-resistance. *Leukemia* 1996; in press.
92. Balzarini J, Pelemans H, Aquaro S, Perno C-F, Witvrouw M, Schols D, De Clercq E & Karlsson, A. Highly favorable antiviral activity and resistance profile of the novel thiocarboxanilide pentenyloxy ether derivatives UC-781 and UC-82 as inhibitors of human immunodeficiency virus type 1 (HIV-1) replication. *Molecular Pharmacology* 1996; **50**:394-401.
93. Balzarini J, Brouwer WG, Dao DC, Osika EM & De Clercq E. Identification of novel thiocarboxanilide derivatives that suppress a variety of drug-resistant mutant human immunodeficiency virus type 1 strains at a potency similar to that for wild-type virus. *Antimicrobial Agents and Chemotherapy* 1996; **40**:1454-1466.
94. Balzarini J, Jonckheere H, Harrison WA, Dao DC, Anné J, De Clercq E & Karlsson A. Oxathiin carboxanilide derivatives: a class of non-nucleoside HIV-1-specific reverse transcriptase inhibitors (NNRTIs) that are active against mutant HIV-1 strains resistant to other NNRTIs. *Antiviral Chemistry and Chemotherapy* 1995; **6**:169-178.
95. Ho DD, Toyoshima T, Mo H, Kempf DJ, Norbeck D, Chen C-M, Wideburg NE, Burt SK, Erickson JW & Singh MK. Characterization of human immunodeficiency virus type 1 variants with increased resistance to a C_2-symmetric protease inhibitor. *Journal of Virology* 1994; **68**:2016-2020.
96. Kaplan AH, Michael SF, Wehbie RS, Knigge MF, Paul DA, Everitt L, Kempf DJ, Norbeck DW, Erickson JW & Swanstrom R. Selection of multiple human immunodeficiency virus type 1 variants that encode viral proteases with decreased sensitivity to an inhibitor of the viral protease. *Proceedings of the National Academy of Sciences, USA* 1994; **91**:5597-5601.
97. Borman AM, Paulous S & Clavel F. Continued accumulation of protease inhibitor resistance mutations in culture in the absence of the drug. *Fourth International Workshop on HIV Drug Resistance, 1995, Sardinia, Italy*; Abstract no: 93.
98. Tisdale M, Myers R, Parry NR, Oliver N, Machera B & Blair E. Comprehensive analysis of HIV-1 variants individually selected for resistance to six HIV protease inhibitors. *Third International Workshop on HIV Drug Resistance, 1994, Kauai, Hawaii, USA*.
99. Swanstrom R, Smith T, Petit S, Irlbeck D, Shao W, Wehbie R, Sawhney R, Everitt L & Erickson I. Multiple sequence changes within HIV-1 protease confer reduced sensitivity to a symmetric protease inhibitor. *Third International Workshop on HIV Drug Resistance, 1994, Kauai, Hawaii, USA*.
100. Maschera B, Blance C, Brown D & Blair E. Mutations conferring resistance to HIV-1 protease inhibitors can lie outside, as well as within, the enzyme active site and binding pockets. *Third International Workshop on HIV Drug Resistance, 1994, Kauai, Hawaii, USA*.
101. Kempf D, Markowitz M, Marsh K, Denissen J, Mo H, Bhat T, Park C, Kong X-P, Stewart K, McDonald E, Vasavanonda S, Flentge C, Wideburg N, Robins T, Hsu A, Leonard J, Ho D & Norbeck D. Pharmacokinetic and *in vitro* selection studies with ABT-538, a potent inhibitor of HIV protease with high oral bioavailability. *34th Interscience Conference on Antimicrobial Agents and Chemotherapy, 1994, Orlando, FL, USA*.
102. Molla A, Korneyeva M, Gao Q, Vasavanonda S, Schipper PJ, Mo H-M, Markowitz M, Chernyavskiy T, Niu P, Lyons N, Hsu A, Granneman R, Ho DD, Boucher CAB, Leonard JM, Norbeck DW & Kempf DJ. Ordered accumulation for mutation in HIV protease confers resistance to ritonavir. *Nature Medicine* 1996; **2**:760-766.

103. Schmit JC, Ruiz L, Clotet B, Van Remoortel B, Witvrouw M, Raventos A, Tor J, Desmyter J, De Clercq E & Vandamme AM. Resistance-related multiple mutations in the HIV-1 protease gene of patients treated with the protease inhibitor ritonavir. *Fifth International Workshop on HIV Drug Resistance, 1996, Whistler, Canada*; Abstract no: 33.
104. Nijhuis M, Schipper P, de Jong D, van Bommel T, de Groot T, Molla A, Schuurman R, Danner S & Boucher C. The relative contribution of resistance and replication efficacy on the accumulation of mutations during ritonavir therapy. *Fifth International Workshop on HIV Drug Resistance, 1996, Whistler, Canada*; Abstract no: 94.
105. Patick A, Mo H, Markowitz M, Appelt K, Wu B, Musick L, Kalish V, Kaldor S, Reich S, Ho D & Webber S. Antiviral and resistance studies of AG1343, an orally bioavailable inhibitor of human immunodeficiency virus protease. *Antimicrobial Agents and Chemotherapy* 1996; **40**:292-297; **40**:1575 (erratum).
106. Patick AK, Duran M, Cao Y, Ho T, Pei A, Keller MR, Peterkin J, Chapman S, Anderson B, Ho D & Markowitz M. Genotypic and phenotypic characterization of HIV-1 variants isolated from *in vitro* selection studies and from patients treated with the protease inhibitor, nelfinavir. *Fifth International Workshop on HIV Drug Resistance, 1996, Whistler, Canada*; Abstract no: 29.
107. Doyon I, Croteau G, Thibeault D, Poulin F, Pilocle L & Lamarre D. Second locus involved in human immunodeficiency virus type 1 resistance to protease inhibitors. *Journal of Virology* 1996; **70**:3763-3769.
108. Lamarre D, Croteau G, Pilote L, Rousseau P & Doyon L. Molecular characterization of HIV-1 variants resistant to specific viral protease inhibitors. *Third International Workshop on HIV Drug Resistance, 1994, Kauai, Hawaii, USA; Abstract no: 10.*
109. Lamarre D, Doyon L, Croteau G, Pilote L & Thibeault D. Molecular basis of HIV-1 resistance to protease inhibitors. Structural flexibility of the protease and second-site compensatory mutations in cleavage sites. *Fourth International Workshop on HIV Drug Resistance, 1995, Sardinia, Italy*; Abstract no: 62.
110. Patick AK, Rose R, Greytok J, Bechtol CM, Hermsmeier MA, Chen PT, Barrish JC, Zahler R, Colonno RJ & Lin PF. Characterization of a human immunodeficiency virus type 1 variant with reduced sensitivity to an aminodiol protease inhibitor. *Journal of Virology* 1995; **69**:2148-2152.
111. Rose B, Greytok J, Bechtold C, Alam M, Terry B, Gong Y-F, DeVore K, Patrick A, Colonno R & Lin P-F. Combination therapy with two protease inhibitors as an approach to antiviral therapy. *Third International Workshop on HIV Drug Resistance, 1994, Kauai, Hawaii, USA.*
112. Rose RE, Gong YF, Greytok JA, Bechtold CM, Terry BJ, Robinson BS, Alam M, Colonno RJ & Lin PF. Human immunodeficiency virus type 1 viral background plays a major role in development of resistance to protease inhibitors. *Proceedings of the National Academy of Sciences, USA* 1996; **93**:1648-1653.
113. Otto M, Reid C, King R, Garber S, Baker D, Anton E & Winslow D. Exposure of chronically infected PBMCs to DMP 450 can completely suppress virus replication or select resistant variants depending upon the dose of compound. *Second National Conference on Human Retroviruses and Related Infections, 1995, Washington, USA.*
114. Winslow DL, Garber S, Reid C, Anton E & Otto MJ. DMP 450, a new cyclic urea inhibitor of HIV protease with potent *in vitro* antiviral activity. *Eighth International Conference on Antiviral Research, 1995, Santa Fe, New Mexico, USA.*
115. Gulnik SV, Suvorov LI, Liu B, Yu B, Anderson B, Mitsuya H & Erickson JW. Kinetic characterization and cross-resistance patterns of HIV-1 protease mutants selected under drug pressure. *Biochemistry* 1995; **34**:9282-9287.
116. Condra JH, Holder DJ, Schleif WA, Blahy OM, Danovich RM, Gabryelski LJ, Graham DJ, Laird D, Quintero JC, Rhodes A, Robbins HL, Roth E, Shivaprakash M, Yang T, Chodakewitz JA, Deutsch PJ, Leavitt RY, Massari FE, Mellors JW, Squires KE, Steigbigel RT, Teppler H & Emini EA. Genetic correlates of *in vivo* viral resistance to the HIV-1 protease inhibitor indinavir. *Journal of Virology* 1996; **70**:8270-8276.
117. Emini E, Schleif W, Graham D, Deutsch P, Massari F, Teppler H, Squires K & Condra J. Phenotypic and genotypic characterization of HIV-1 variants selected during treatment with the protease inhibitor L-735, 524. *Third International Workshop on HIV Drug Resistance, 1994, Kauai, Hawaii, USA.*
118. Condra JH & Schleif WA. *In vivo* emergence of HIV-1 variants resistant to multiple protease inhibitors. *Nature* 1995; **374**:569-571.
119. Condra JH, Holder DJ, Schleif WA, Blahy OM, Danovich RM, Gabryelski LJ, Graham DJ, Laird D, Quintero JC, Rhodes A, Robbins HL, Roth E, Shivaprakash M, Yang T, Chodakewitz JA, Deutsch PJ, Leavitt RY, Massari FE & Emini EA. Genetic correlates of *in vivo* viral resistance to the HIV-1 protease inhibitor indinavir. *Fifth International Workshop on HIV Drug Resistance, 1996, Whistler, Canada*; Abstract no: 30.
120. Dulioust A, Paulous S, Guillemot L, Boue F, Galanaud P & Clavel F. Selection of saquinavir-resistant mutants by indinavir following a switch from saquinavir. *6th International Workshop on HIV Drug Resistance, 25-28 June 1997; St. Petersburg, USA*: Abstract 16.
121. Eastman PS, Gee C, Dewar R, Fyfe G, Metcalf J, Kolberg J, Urdea M, Lane HC & Falloon J. An evaluation of crixivan monotherapy and crixivan/IL-2 combination therapy subjects. *Fifth International Workshop on HIV Drug Resistance, 1996, Whistler, Canada*;
122. Otto MJ, Garber S, Winslow DL, Reid CD, Aldrich P, Jadhav PK, Patterson CE, Hodge CN & Cheng YS. *In vitro* isolation and identification of human immunodeficiency virus (HIV) variants with reduced sensitivity to C-2 symmetrical inhibitors of HIV type 1 protease. *Proceedings of the National Academy of Sciences, USA* 1993; **90**:7543-7537.
123. Schapiro JM, Winters MA, Vierra M, Jacobsen H, Mous J & Merigan TC. Resistance mutations in patients receiving saquinavir: simultaneous appearnace in lymph nodes, peripheral blood mononuclears (PBM) and plasma. *Fifth International Workshop on HIV Drug Resistance, 1996, Whistler, Canada*; Abstract no: 28.
124. Jacobsen H, Brun-Vezinet F, Duncan I, Hanggi M, Ott M, Vella S, Weber J & Mous J. Genotypic characterization of HIV-1 from patients after prolonged treatment with proteinase inhibitor saquinivir. *Third International Workshop on HIV Drug Resistance, 1994, Kauai, Hawaii, USA.*
125. Eberle J, Bechowsky B, Rose D, Hauser U, Von Der Helm K, Gurtler L & Nitschki H. Resistance of HIV type 1 to proteinase inhibitor Ro 31-8959. *AIDS Research and Human Retroviruses* 1995; **11**:671-676.
126. Winters MA, Schapiro JM, Lawerence J & Merigan TC. Genotypic and phenotypic analysis of the protease in HIV-1-infected patients that failed long-term saquinavir therapy and switched to other protease inhibitors. *6th International Workshop on HIV Drug Resistance, 25-28 June 1997; St. Petersburg, USA:* Abstract 17.
127. Eastman PS, Duncan IB, Gee C & Race E. Acquisition of genotypic mutations associated with reduced susceptibility to protease inhibitors saquinavir monotherapy. *6th International Workshop on HIV Drug Resistance, 25-28 June 1997; St. Petersburg, USA*: Abstract 30.
128. Schapiro JM, Winters M, Lawrence J, Norris J & Merigan TC. Clinical and genotypic cross-resistance between the protease inhibitors saquinavir and indinavir. *6th International Workshop on HIV Drug Resistance, 25-28 June 1997; St. Petersburg, USA*: Abstract 87.
129. el-Farrash MA, Kuroda MJ, Kitazaki T, Masuda T, Kato K, Hatanaka M & Harada S. Generation and characterization of a human immunodeficiency virus type 1 (HIV-1) mutant resistant to an HIV-1 protease inhibitor. *Journal of Virology* 1994; **68**:233-239.
130. Potts KE, Smidt ML, Stallings W, Clare M, Pillay D, Richman DD & Bryant ML. *In vitro* selection and characterization of human immunodeficiency virus type 1 (HIV-1) variants with decreased sensitivity to hydroxyethylurea isostere containing protease inhibitors. *Third International Workshop on HIV Drug Resistance, 1994, Kauai, Hawaii, USA.*
131. Pillay D, Smidt ML, Potts KE, Bryant ML & Richman DD. *In vitro* selection of protease inhibitors resistant human immunodeficiency virus type 1 (HIV-1) strains. *34th Interscience Conference on Antimicrobial Agents and Chemotherapy, 1994, Orlando, FL, USA*; Abstract no: 7.
132. Shao WTS & Swanstrom R. Selection and analysis of HIV-1 variants with increased resistance to SKF108842 and SKF108922, two protease inhibitors. *Fourth International Workshop on HIV Drug Resistance, 1995, Sardinia, Italy*; Abstract no: 65.
133. Partaledis JA, Yamaguchi K & Byrn RA. *In vitro* selection and characterization of HIV-1 viral isolates with reduced sensitivity to inhibitors of HIV protease. *Third International Workshop on HIV Drug Resistance, 1994, Kauai, Hawaii, USA.*
134. Tisdale M, Myers R, Najera I, Kohli A, Kemp S & Larder BA. Analysis of resistance interactions with 141W94 (VX-478) and other HIV-1 protease inhibitors. *Fifth International Workshop on HIV Drug Resistance, 1996, Whistler, Canada*; Abstract no: 27.
135. Rao BG, Dwyer MD, Thomson JA, Baker CT, Deininger DD, Murcko MA, Tung RD, Navia MA & Kim EE. Structural and modeling analysis of the basis of viral resistance to VX-478. *Fifth International Workshop on HIV Drug Resistance, 1996, Whistler, Canada*; Abstract no: 22.
136. King RW, Garber S, Winslow DL, Reid C, Bacheler LT, Anton E & Otto MJ. Multiple mutations in the human immunodeficiency virus protease gene are responsible for decreased susceptibility to protease inhibitors. *Antiviral Chemistry and Chemotherapy* 1995; **6**:80-88.
137. Mellors J, Bazmi H, Schinazi RF, Roy B, Hsiou Y, Arnold E, Weir J & Mayers D. Novel mutations in the reverse transcriptase of human immunodeficiency virus type 1 reduce susceptibility to foscarnet in laboratory and clinical isolates. *Antimicrobial Agents and Chemotherapy* 1995; **39**:1087-1092.
138. Tachedjian G, Hooker DJ, Gurusinghe AD, Bazmi H, Deacon NJ, Mellors J, Birch C & Mills J. Characterisation of foscarnet-resistant strains of human immunodeficiency virus type 1. *Virology* 1995; **212**:58-68.
139. Tachedjian G, Mellors J, Bazmi H, Birch C & Mills J. Zidovudine resistance is suppressed by mutations conferring resistance of human immunodeficiency virus type 1 to foscarnet. *Journal of Virology* 1996; **70**:7171-7181.
140. Prasad VR, Lowy I, de los Santos T, Chiang L & Goff SP. Isolation and characterization of a dideoxyguanosine triphosphate-resistant mutant of human immunodeficiency virus reverse transcriptase. *Proceedings of the National Academy of Sciences, USA* 1991; **88**:11363-11367.
141. Este JA, Van Laethem K, Vandamme A-M, Desmyter J & De Clercq E. Resistant phenotype of human immunodeficiency virus type 1 to dextran sulfate is conferred by specific amino acid substitutions in the gp120 molecule. *Fifth International Workshop on HIV Drug Resistance, 1996, Whistler, Canada*; Abstract no: 80.

Desmyter J & De Clercq E. Development of resistance of human immunodeficiency virus type 1 to dextran sulfate associated with the emergence of specific mutations in the envelope gp120 glycoprotein. Submitted for publication 1996.
143. De Vreese K, Reymen D, Griffin P, Steinkasserer A, Werner G, Bridger GJ, Este J, James W, Henson GW, Desmyter J, Anne J & De Clercq E. The bicyclams, a new class of potent human immunodeficiency virus inhibitors, block viral entry after binding. *Antiviral Research* 1996; 29:209-219.
144. De Vreese K, Kofler-Mongold V, Leutgeb C, Weber V, Vermeire K, Schacht S, Anne J, de Clercq E, Datema R & Werner G. The molecular target of bicyclams, potent inhibitors of human immunodeficiency virus replication. *Journal of Virology* 1996; 70:689-696.
145. Labrosse B, Pleskoff O, Sol N, Jones C, Henin Y & Alizon M. Antiviral and resistance studies of RPR103611, an inhibitor of HIV replication. *6th International Workshop on HIV Drug Resistance, 25-28 June 1997; St. Petersburg, USA;* Abstract 33.
146. Lin P, Samanta H, Bechtold CM, Deminie CA, Patick AK, Alam M, Riccardi K, Rose RE, White RJ & Colonno RJ. Characterization of siamycin 1, a human immunodeficiency virus fusion inhibitor. *Antimicrobial Agents and Chemotherapy* 1995; 40:133-138.
147. Iversen AKN, Shafer RW, Wehrly K, Winters MS, Mullins JI, Chesebro B & Merigan TC. Multidrug-resistant human immunodificiency virus type 1 strains resulting from combinaton antiretroviral therapy. *Journal of Virology* 1996; 70:1086-1090.
148. Shirasaka T, Kavlick MF, Ueno T, Gao W-Y, Kojima E, Alcaide ML, Chokekijchai S, Roy BM, Arnold E, Yarchoan R & Mitsuya H. Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides. *Proceedings of the National Academy of Sciences, USA* 1995; 92:1-5.
149. Schmit JC, Vanderlinden I, Ruiz L, Clotet B, Hermans P, Sprecher S, Arendt V, Peetermans W, Harrer T, Vaira D, Desmyter J, De Clercq E & Vandamme A-M. Prevalence of multi-drug resistance to dideoxynucleoside (ddN) analogues in patients on ddN combination therapy. *Fifth International Workshop on HIV Drug Resistance, 1996, Whistler, Canada*; Abstract no: 39.
150. Stuyver L, Wyseur A, Rombout A, Louwagie J, Scarcez T, Verhofstede V, Rimland D, Schinazi RF & Rossau R. Line probe assay for rapid detection of drug-selected mutations in the human immunodeficiency virus type 1 reverse transcriptase gene. *Antimicrobial Agents and Chemotherapy* 1997; 41:284-291.
151. Kemp S & Bloor S. Two distinct mutational pathways in HIV-1 RT confer zidovudine/lamivudine dual resistance. *Sixth International workshop on HIV Drug Resistance, 25-28 June 1997; St. Petersburg, USA*: Abstract 11.
152. Van Rompay KKA, Cherrington JM, Marthas ML, Berardi CJ, Mulato AS, Spinner A, Tarara RP, Canfield DR, Telm S, Bischofberger N & Pedersen NC. PMPA therapy of established SIV infection of infant rhesus macaques. *Antimicrobial Agents and Chemotherapy* 1996; 40:2586-2591.
153. Cherrington JM, Van Rompay KKA, Multao AS, Marthas ML, Berardi CJ, Telm S, Bischofberger N & Pedersen NC. Phenotypic and genotypic characterization of simian immunodeficiency viruses (SIV) with reduced susceptibility to PMPA isolated after PMPA therapy. *Fifth International Workshop on HIV Drug Resistance, 1996, Whistler, Canada* Abstract no: 75.
154. Van Rompay K, Cherrington J, Marthas M, Agatep E, Dehqanzada Z, Lamy P, Berardi C, Bischofberger N and Pedersen N. Therapeutic efficacy of PMPA treatment for infant macaques infected with PMPA-resistant simian immunodeficiency virus. *6th International Workshop on HIV Drug Resistance, 25-28 June 1997; St. Petersburg, USA*: Abstract 117.
155. Medlin HK, Zhu Y, Remington KM, Phillips TR and North TW. Selection and characterization of a mutant of feline immunodeficiency virus resistant to 2',3'-dideoxycytidine. *Antimicrobial Agents and Chemotherapy* 1996; 40:953-957.
156. Zhu Y-Q, Remington KM and North TW. Mutants of feline immunodeficiency virus resistant to 2',3'-dideoxy-2',3'-didehydrothymidine. *Antimicrobial Agents and Chemotherapy* 1996; 40:1983-1987.
157. Smith R, Remington K, Lloyd R, Schinazi R and North T. Mutants of feline immunodeficiency virus resistant to FTC and 3TC. *Third International Feline Retrovirus Research Symposium, 1996, Ft. Collins, CO*; Abstract no: 41.
158. Smith, RA, Remington, K, Lloyd, J, R. M., Schinazi, RF, and North, TW. A novel met-to-thr mutation in the YMDD motif of reverse transcriptase from feline immunodeficiency virus confers resistance to oxathiolane nucleosides. *Journal of Virology* 1997; 71:2357-2362.
159. Smith, RA, Remington, KM, Lloyd Jr., RM, Schinazi, RF, and North TW. A novel point mutation at position 156 of reverse transcriptase from feline immunodeficiency virus confers resistance to the combination of (-)-β-L-2',3'-dideoxy-3'-thiacytidine and 3'-azido-3'- deoxythymidine. *Journal of Virology* 1997; submitted.
160. Dulioust A, Paulous S, Guillemot L, Boue F, Galanaud P & Clavel F. Selection of saquinavir-resistant mutants by indinavir following a switch from saquinavir. *6th International Workshop on HIV Drug Resistance, 25-28 June 1997; St. Petersburg, USA*: Abstract 11.
161. Van Rompey KKA, Greenier JL, Marthas ML, Otsyula MG, Tarara RP, Miller CJ & Pedersen NC. A zidovudine resistant simian immunodeficiency virus mutant with a Q151M mutation in reverse transcriptase causes AIDS in newborn macaques. *Antimicrobial Agents and Chemotherapy* 1997; 41:278-283.

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Editorial

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An important new clinical development is the discovery that a significant number of HIV-infected individuals treated with triple combinations of anti-AIDS drugs now experience infection flares caused by hepatitis B and C virus. All these new developments emphasize the need to develop antiviral agents or combined modalities that are effective against HIV as well as HBV (and HCV). Careful prospective viral load, genotyping, and clinical monitoring of these individuals during and after antiviral therapy is critical.

Clearly the search must continue for novel compounds with anti-HIV activity and improved treatment strategies. In this issue scientists at Hybridon describe the use of the burgeoning, yet relatively new, technology of antisense oligonucleotides for targeting viral diseases. It is encouraging that a number of carefully designed oligonucleotides are already in the clinic. Antisense technology offers great promise of becoming an important approach for combating HIV and related infections. As the number of diseases defined at the genetic level increase, this technique will offer potentially highly specific ways to interrupt selectively the most vulnerable genetic targets. The use of antisense technology in combination with current antiviral agents for HIV and HBV could become an important "dead viruses don't mutate" strategy.

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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 × *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 × *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}.

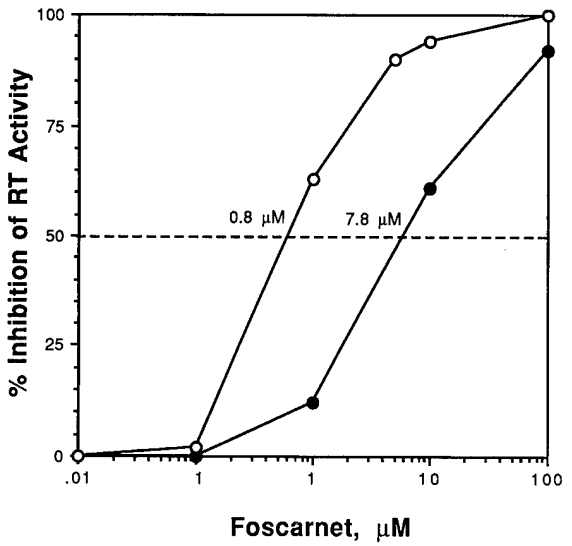

FIG. 1. Foscarnet susceptibility of virion-associated RT from parental (open circles) and resistant (solid circles) HIV-1. RT inhibition assays were performed as described in Materials and Methods. Mean values for duplicate determinations are shown (standard deviations averaged <15% of the mean values).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

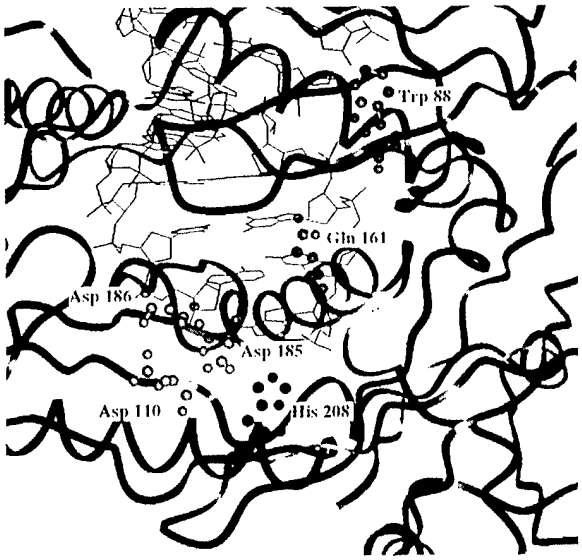

FIG. 2. Locations of foscarnet resistance mutation sites in the crystal structure of HIV-1 RT bound with a double-stranded DNA template-primer. The wild-type amino acid residues Trp-88 (in β5a), Gln-161 (in αE), and His-208 (in αF) are shown as light brown ball-and-stick models. The catalytically essential 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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REFERENCES

1. **Birch, C. J., G. Tachedjian, R. R. Doherty, K. Hayes, and I. D. Gust.** 1990. Altered sensitivity to antiviral drugs of herpes simplex virus isolates from a patient with the acquired immunodeficiency syndrome. J. Infect. Dis. **162:** 731–734.
2. **Boyer, B. L., C. Tantillo, A. Jacobo-Molina, R. G. Nanni, J. Ding, E. Arnold, and S. H. Hughes.** 1994. Sensitivity of wild-type human immunodeficiency virus type 1 reverse transcriptase to dideoxynucleotides depends on template length; the sensitivity of drug-resistant mutants does not. Proc. Natl. Acad. Sci. USA **91:**4882–4886.
3. **Coen, D. M., D. P. Aschman, P. T. Gelep, M. J. Retondo, S. K. Weller, and P. A. Schaffer.** 1984. Fine mapping and molecular cloning of mutations in the herpes simplex virus DNA polymerase locus. J. Virol. **49:**236–247.
4. **De Clercq, E.** 1994. HIV-1 resistance to reverse transcriptase inhibitors.

Biochem. Pharmacol. **47:**155–169.
5. **Farthing, C. F., A. G. Dalgleish, A. Clark, M. McClure, A. Chanas, and B. G. Gazzard.** 1987. Phosphonoformate (foscarnet): a pilot study in AIDS and AIDS related complex. AIDS **1:**21–25.
6. **Field, A. K., and K. E. Biron.** 1994. "The end of innocence" revisited: resistance of herpesviruses to antiviral drugs. Clin. Microbiol. Rev. **7:**1–13.
7. **Fletcher, C. V., A. C. Collier, F. S. Rhame, D. Bennet, M. F. Para, C. C. Beatty, C. E. Jones, and H. H. Balfour.** 1994. Foscarnet for the suppression of human immunodeficiency virus replication. Antimicrob. Agents Chemother. **38:**604–607.
8. **Hwang, B. C. C., K. L. Ruffner, and D. M. Coen.** 1992. A point mutation within a conserved region of the herpes simplex virus DNA polymerase gene confers drug resistance. J. Virol. **66:**1774–1776.
9. **Im, G. J., E. Tramontano, C. J. Gonzalez, and Y. C. Cheng.** 1993. Identification of the amino acid in the human immunodeficiency virus type 1 reverse transcriptase involved in the pyrophosphate binding of antiviral nucleoside triphosphate analogs and phosphonoformate. Implications for multiple drug resistance. Biochem. Pharmacol. **46:**2307–2313.
10. **Jackson, J. B., R. W. Coombs, K. Sannerud, F. S. Rhame, and H. H. Balfour, Jr.** 1988. Rapid and sensitive viral culture method for human immunodeficiency virus type 1. J. Clin. Microbiol. **26:**1416–1418.
11. **Jacobo-Molina, A., J. Ding, R. G. Nanni, A. D. Clark, Jr., X. Lu, C. Tantillo, R. L. Williams, G. Kamer, A. L. Ferris, P. Clark, A. Hizi, S. H. Hughes, and E. Arnold.** 1993. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. Proc. Natl. Acad. Sci. USA **90:**6320–6324.
12. **Jacobson, M. A., S. Crowe, J. Levy, F. Aweeka, J. Gambertoglio, N. McManus, and J. Mills.** 1988. Effect of foscarnet therapy on infection with human immunodeficiency virus in patients with AIDS. J. Infect. Dis. **158:** 862–865.
13. **Japour, A. J., D. L. Mayers, V. A. Johnson, D. R. Kuritzkes, L. A. Beckett, J. M. Arduino, J. Lane, R. J. Black, P. S. Reichelderfer, R. T. D'Aquila, C. S. Crumpacher, the RV-43 Study Group, and the AIDS Clinical Trials Group Virology Committee Resistance Working Group.** 1993. Standardized peripheral blood mononuclear cell culture assay for determination of drug susceptibilities of clinical human immunodeficiency virus type 1 isolates. Antimicrob. Agents Chemother. **37:**1095–1101.
14. **Knox, K. K., W. R. Drobyski, and D. R. Carrigan.** 1991. Cytomegalovirus isolate resistant to ganciclovir and foscarnet from a marrow transplant patient. Lancet **ii:**1292–1293.
15. **Larder, B. A.** 1992. 3′-Azido-3′-deoxythymidine resistance suppressed by a mutation conferring human immunodeficiency virus type 1 resistance to nonnucleoside reverse transcriptase inhibitors. Antimicrob. Agents Chemother. **36:**2664–2669.
16. **Larder, B. A., B. Chesebro, and D. D. Richman.** 1990. Susceptibilities of zidovudine-susceptible and -resistant human immunodeficiency virus isolates to antiviral agents determined by using a quantitative plaque reduction assay. Antimicrob. Agents Chemother. **34:**436–441.
17. **Larder, B. A., S. D. Kemp, and D. J. Purifoy.** 1989. Infectious potential of human immunodeficiency virus type 1 reverse transcriptase mutants with altered inhibitor sensitivity. Proc. Natl. Acad. Sci. USA **86:**4803–4807.
18. **Larder, B. A., D. J. Purifoy, K. L. Powell, and G. Darby.** 1987. Site-specific mutagenesis of AIDS virus reverse transcriptase. Nature (London) **327:**716–717.
19. **Lowe, D. M., V. Parmar, S. D. Kemp, and B. A. Larder.** 1991. Mutational analysis of two conserved sequence motifs in HIV-1 reverse transcriptase. FEBS Lett. **282:**231–234.
20. **Mayers, D. L., F. E. McCutchan, E. E. Sanders-Buell, L. I. Merritt, S. Dilworth, A. K. Fowler, C. A. Marks, N. M. Ruiz, D. D. Richman, C. R. Roberts, and D. S. Burke.** 1992. Characterization of HIV isolates arising after prolonged zidovudine therapy. J. Acquired Immune Defic. Syndr. **5:**749–759.
21. **Mellors, J. W.** Unpublished observations.
22. **Mellors, J. W., G. E. Dutschman, G.-J. Im, E. Tramontano, S. R. Winkler, and Y.-C. Cheng.** 1991. *In vitro* selection and molecular characterization of human immunodeficiency virus-1 resistance to non-nucleoside inhibitors of reverse transcriptase. Mol. Pharmacol. **41:**446–451.
23. **Mellors, J. W., G.-J. Im, E. Tramontano, S. R. Winkler, D. J. Medina, G. E. Dutschman, H. Z. Bazmi, G. Piras, C. J. Gonzalez, and Y.-C. Cheng.** 1993. A single conservative amino acid substitution in the reverse transcriptase of human immunodeficiency virus-1 confers resistance to (+)-(5S)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl) imidazo[4,5, 1-jk][1,4]benzodiazepin-2 (1H)-thione (TIBO R82150). Mol. Pharmacol. **43:**11–16.
24. **Myers, G., J. A. Berzofsky, B. Korber, R. F. Smith, and G. N. Pavlakis.** 1991. Human retroviruses and AIDS 1991: a compilation and analysis of nucleic acid and amino acid sequences, p. II–22. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, N.M.
25. **Nguyen, M. H., R. F. Schinazi, C. Shi, N. M. Goudgaon, P. M. McKenna, and J. W. Mellors.** 1994. Resistance of human immunodeficiency virus type 1 to acyclic 6-phenylselenenyl- and 6-phenylthiopyrimidines. Antimicrob. Agents Chemother. **38:**2409–2414.
26. **Oberg, B.** 1989. Antiviral effects of phosphonoformate (PFA, foscarnet sodium). Pharmacol. Ther. **40:**213–285.
27. **Peden, K., M. Emerman, and L. Montagnier.** 1991. The characterization of infectious molecular clones of HIV-1_{LAI}, HIV-1_{MAL}, and HIV-1_{ELI}: changes in growth properties on passage in tissue culture. Virology **185:**661–672.
28. **Prasad, V. R., I. Lowy, T. de los Santos, L. Chiang, and S. P. Goff.** 1991. Isolation and characterization of a dideoxyguanosine triphosphate-resistant mutant of human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. USA **88:**11363–11367.
29. **Reddy, M. M., M. H. Grieco, G. F. McKinley, D. M. Causey, C. M. van der Horst, D. M. Parenti, T. M. Hooton, R. B. Davis, and M. A. Jacobson.** 1992. Effect of foscarnet therapy on human immunodeficiency virus p24 antigen levels in AIDS patients with cytomegalovirus retinitis. J. Infect. Dis. **166:** 607–610.
30. **Reed, L. J., and H. Muench.** 1938. A simple method for estimating fifty percent end points. Am. J. Hyg. **27:**493–496.
31. **Richman, D. D., D. Havlir, J. Corbeil, D. Looney, C. Ignacio, S. A. Spector, J. Sullivan, S. Cheesman, K. Barringer, D. Pauletti, C.-K. Shih, M. Myers, and J. Griffin.** 1994. Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. J. Virol. **68:**1660–1666.
32. **Rocancourt, D., C. Bonnerot, H. Jouin, M. Emerman, and J.-F. Nicolas.** 1990. Activation of a β-galactosidase recombinant provirus: application to titration of human immunodeficiency virus (HIV) and HIV-infected cells. J. Virol. **64:**2660–2668.
33. **Safrin, S., T. G. Berger, I. Gilson, P. R. Wolfe, C. V. Wofsy, J. Mills, and K. K. Biron.** 1991. Foscarnet therapy in five patients with AIDS and acyclovir-resistant varicella zoster virus infection. Ann. Intern. Med. **115:**19–21.
34. **Safrin, S., S. Kemmerly, B. Plotkin, T. Smith, N. Weissbach, D. de Veranez, L. D. Phan, and D. Cohn.** 1994. Foscarnet-resistant herpes simplex virus infection in patients with AIDS. J. Infect. Dis. **169:**193–196.
35. **Schinazi, R. F., B. F. H. Eriksson, and S. H. Hughes.** 1989. Comparison of inhibitory activities of various antiretroviral agents against particle-derived and recombinant human immunodeficiency virus type 1 reverse transcriptases. Antimicrob. Agents Chemother. **33:**115–117.
36. **Studies of Ocular Complications of AIDS Research Group in Collaboration with the AIDS Clinical Trials Group.** 1992. Mortality in patients with the acquired immunodeficiency syndrome treated with either foscarnet or ganciclovir for cytomegalovirus retinitis. N. Engl. J. Med. **326:**213–220.
37. **Sullivan, V., and D. M. Coen.** 1991. Isolation of foscarnet-resistant human cytomegalovirus: patterns of resistance and sensitivity to other antiviral drugs. J. Infect. Dis. **164:**781–784.
38. **Tachedjian, G., A. Gurusinghe, D. Hooker, N. Deacon, J. Mills, and C. Birch.** 1994. In vitro generation and characterization of foscarnet-resistant HIV-1, p. 23. *In* Abstracts of the Third International Workshop on HIV Drug Resistance.
39. **Tachedjian, G., J. Hoy, J. Mills, and C. J. Birch.** 1994. Foscarnet therapy is not associated with the emergence of foscarnet-resistant human immunodeficiency virus type 1 in an acquired immunodeficiency syndrome patient. J. Med. Virol. **42:**207–211.

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Characterisation of Foscarnet-Resistant Strains of Human Immunodeficiency Virus Type 1

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Foscarnet is a broad-spectrum viral DNA polymerase inhibitor active *in vitro* and *in vivo* against human immunodeficiency virus type 1 (HIV-1). Strains of HIV-1 resistant to foscarnet were selected by *in vitro* passage in increasing concentrations of drug. Reduced susceptibility to foscarnet was evident at the levels of both HIV-1 replication and reverse transcriptase. Biologically cloned, foscarnet-resistant strains with distinct genotypes were hypersensitive to zidovudine, azidodeoxyuridine, nevirapine, and R82913 but had unchanged susceptibility to zalcitibine and didanosine. The reverse transcriptase of foscarnet-resistant strains had unique substitutions Glu89-Lys, Leu92-Ile, or Ser156-Ala, the third being associated with six polymorphic changes. Introduction of these mutations into wild-type HIV-1 by site-directed mutagenesis confirmed their role in foscarnet resistance. In the three-dimensional structure of the reverse transcriptase enzyme these amino acids are located close to the template strand of the template primer and far away from the putative pyrophosphate binding site, suggesting that the mechanism by which HIV-1 becomes resistant to foscarnet is indirect. Foscarnet resistance is thus likely to be mediated through an altered interaction of the mutant enzyme with the template strand of the template primer which distorts the geometry of the polymerase active site and thereby decreases foscarnet binding.

INTRODUCTION

Foscarnet (trisodium phosphonoformate, Foscavir) is a broad spectrum antiviral agent which inhibits DNA polymerases including the HIV-1 reverse transcriptase (RT). This drug inhibits pyrophosphate exchange by reversibly binding to the putative pyrophosphate binding site on these enzymes, consequently preventing DNA chain elongation (Oberg, 1989; Crumpacker, 1992). Foscarnet is used to treat acyclovir-resistant herpes simplex (Erlich *et al.*, 1989; Birch *et al.*, 1990; Safrin *et al.*, 1991b) and varicella zoster infections (Safrin *et al.*, 1991a). It also has efficacy equivalent to ganciclovir in the treatment of cytomegalovirus (CMV) retinitis in patients with acquired immune deficiency syndrome (AIDS) (SOCA, 1992). In this latter study, AIDS patients with CMV retinitis treated with foscarnet survived longer than patients receiving ganciclovir. As foscarnet has been shown to inhibit human immunodeficiency virus type 1 (HIV-1) replication *in vitro* (Sandstrom *et al.*, 1985) and *in vivo* (Jacobson *et al.*, 1988; Fletcher *et al.*, 1994), it has been suggested that one explanation for the prolonged survival of foscarnet-treated patients in this clinical study was the antiretroviral activity of foscarnet, either alone (Sandstrom *et al.*, 1985; Jacobson *et al.*, 1988) or acting in concert with other antiretrovirals (Eriksson and Schinazi, 1989; Koshida *et al.*, 1989).

Because foscarnet has antiretroviral activity and is used in the treatment of some AIDS patients, it is possible that foscarnet-resistant strains of HIV-1 might develop in these individuals. Random mutagenesis of the HIV-1 RT gene has shown that the Glu89-Gly and Val90-Ala mutations result in a foscarnet-resistant RT (Prasad *et al.*, 1991; Song *et al.*, 1992; Im *et al.*, 1993). Specific mutations introduced into the HIV-1 RT for the purpose of defining important functional sites on the enzyme have also been reported to result in foscarnet-resistant RT (Larder *et al.*, 1987, 1989; Lowe *et al.*, 1991). In these studies, mutations at codons 113, 114, 115, 151, 154, 183, and 190 yielded RT with varying levels of foscarnet resistance; however, all mutations were associated with reduced activity compared with the wild-type enzyme.

While these studies have revealed information on the structure and function of the HIV-1 RT, mutations deliberately introduced into the RT gene are unlikely to mimic

The nucleotide sequence data reported in this article have been deposited with the GenBank Database under Accession Nos. HXBC, HX330BC, HX660BC, PDBC, PD165BC, PD330BC, PD495BC, PD660BC, U28646, U28653, U28647, U28648, U28649, U28650, U28651, and U28652.

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in vivo selection pressures, and these mutations may very well not be the same as those that develop in foscarnet-treated patients. Selection of drug-resistant virus by passage in cell culture is more likely to result in mutations observed in clinical isolates, as has been reported for zidovudine (AZT) and second-site, nonnucleoside RT inhibitors (Larder *et al.*, 1991; Nunberg *et al.*, 1991; Richman *et al.*, 1991).

The three-dimensional structures of the p66 and p51 subunits of the HIV-1 RT enzyme have been elucidated (Kohlstaedt *et al.*, 1992; Jacobo-Molina *et al.*, 1993), permiting localisation of drug resistance mutations on the enzyme and allowing determination of the mechanism by which resistance occurs (Nanni *et al.*, 1993; Tantillo *et al.*, 1994). Structural studies suggest that nucleoside analogue resistance mutations generally mediate their effect by altering the geometry of the substrate binding site through an indirect conformational change. In contrast, mutations induced by second-site nonnucleoside RT inhibitors occur within their binding site, a hydrophobic pocket located in the palm subdomain close to the polymerase active site (Nanni *et al.*, 1993; Tantillo *et al.*, 1994). Because the mechanism of action of foscarnet differs from other inhibitors studied to date, it was of interest to determine the nature of resistance mutations generated by *in vitro* selection and their positions within the three-dimensional structure of the HIV-1 RT. Such studies will aid in the elucidation of the mechanism of foscarnet resistance at the enzyme level.

Here we describe the selection of foscarnet-resistant strains of HIV-1 during passage in the presence of drug, their susceptibility to other antiretrovirals, and the mutations in the RT region associated with resistance. We also propose a mechanism by which foscarnet induces resistance at the level of the HIV-1.

MATERIALS AND METHODS

Cells

MT-2 cells (Harada *et al.*, 1985) were cultured in RPMI medium containing RPMI 1640 (Gibco, Grand Island, NY) and 10% heat-inactivated foetal calf serum (Commonwealth Serum Laboratories) as previously described (Tachedjian *et al.*, 1990). Human peripheral blood mononuclear cells (PBMCs) were obtained from HIV-1 seronegative donors and purified from whole blood by density centrifugation (Neate *et al.*, 1987). Mononuclear cells were incubated for 3 days in RPMI medium containing phytohaemagglutinin (PHA) at 10 μg/ml and were then transferred to medium containing IL-2 (Tachedjian *et al.*, 1990) at the time of infection with HIV-1. HT4LacZ-1 cells (Rocancourt *et al.*, 1990) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum and G418 at 400 μg/ml.

Viruses

HIV-1 strain 237288 (hereafter termed strain PD) was isolated from the PBMCs of an AIDS patient who was antiretroviral drug naive. Strain HX was obtained by transfection into MT-2 cells of 5 μg of the *Xba*I-linearised molecular clone pKPHXB2 using the lipofectin reagent DOTAP (Boehringer Mannheim, Germany). pKPHXB2 is a construct containing the *Xba*I fragment of the HIV-1 provirus from pHXB2-D (Ratner *et al.*, 1987) which was inserted into the *Xba*I site of the low copy number vector pKP57 (a gift from Keith Peden; Peden, 1992).

Drugs

Foscarnet (Fluka Biochemika) was prepared as a 10 mg/ml stock in sterile water. AZT (a gift from Burroughs Wellcome), 3′-azido-2′,3′dideoxyuridine (AZDU; Sigma Chemicals), R82913 (TIBO; a gift from Janssen Pharmaceuticals), and nevirapine (a gift from Boehringer Ingelheim Pharmaceuticals) were prepared as 25 m*M* stocks in dimethyl sulfoxide. Zalcitibine (ddC; Sigma Chemicals) and didanosine (ddI; a gift from Brystol Myers Squibb) were prepared at a concentration of 25 m*M* in sterile water.

In vitro selection process

Foscarnet-resistant HIV-1 was produced by sequential passage of the PD or HX strains in MT-2 cells in the presence of increasing concentrations of foscarnet. Initially, MT-2 cells (400,000/4 ml) were inoculated with 2500 $TCID_{50}$ of virus and cultured in the presence of 33 μM of foscarnet. When HIV-specific cytopathic effects (CPE) involved 75–100% of the cells, culture supernatants were clarified by low-speed centrifugation and a 500-μl inoculum was used to infect fresh MT-2 cells in the presence of increasing concentrations of foscarnet (Fig. 1). Each passage included a duplicate culture at the highest concentration of foscarnet present in the previous passage. If after 7 days CPE did not involve at least 75–100% of cells, the cell suspension was diluted 1 to 4 in medium containing freshly added foscarnet at the original concentration and incubated further. Each such subculture was considered a single passage. To control for any changes resulting from repeated passage the same viruses (PD and HX) were passaged in parallel in the absence of drug.

Biological cloning

Virus suspensions were biologically cloned by three sets of terminal dilutions in MT-2 cells in the presence of the indicated concentrations of foscarnet. Wild-type strains were cloned in the absence of inhibitor. Following the third terminal dilution, isolates were amplified first once in the presence of drug and then a second time

without foscarnet to yield virus stocks not containing inhibitor.

Drug susceptibility assays

In MT-2 cells. Each virus strain at 250–500 $TCID_{50}$ was used to infect 150,000–200,000 MT2 cells in the presence of serial drug dilutions in duplicate wells of a 24-well tray (Costar, MA). Titrations of each strain were included in assays to confirm that equivalent doses of virus were tested. At the time of maximal cytopathic effect in non-drug-treated cultures (4–6 days postinfection), culture supernatants were clarified by low-speed centrifugation and virion-associated RT activity determined as described previously (Neate *et al.*, 1987). The percentage inhibition of RT activity was calculated in drug-treated cultures relative to untreated infected cultures of the relevant isolate and the 50% inhibitory concentrations (IC_{50}s) calculated from linear $\log_{10}$ plots of the percentage inhibition versus concentration of inhibitor. The statistical significance of differences between drug IC_{50}s was determined by the Wilcoxon Rank-Sum Test (Bhattacharyya and Johnson, 1977).

In HT4LacZ-1 cells. Drug inhibition of blue syncytium formation was performed as published (Rocancourt *et al.*, 1990) with modification. Cells were seeded into 96-well plates (3×10^4 cells/well) and allowed to adhere overnight. Fresh medium containing twofold drug dilutions were added, followed by inoculation of each well with 50–100 syncytium forming units of virus. After 72 hr, cells were fixed with 0.5% gluteraldehyde, washed with phosphate-buffered saline, and stained with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside as described (Rocancourt *et al.*, 1990). Syncytia containing five or more blue nuclei were counted in six separate wells per drug dilution.

RT inhibition assay

The foscarnet susceptibility profile of virion-associated RT was determined in an *in vitro* RT inhibition assay as previously described (Tachedjian *et al.*, 1994). RT was from lysed virions obtained from clarified supernates of infected MT-2 cultures. Viral lysates were directly applied to the reaction mix in these assays.

DNA preparation for polymerase chain reaction amplification (PCR)

Biologically-cloned HIV-1 strains were grown in PHA-stimulated PBMCs (2.5×10^6 cells/10 ml culture) for 3–4 days. Cells were washed with magnesium- and calcium-free phosphate-buffered saline (PBS) and then resuspended in 200 μl of PBS. Purified genomic DNA was prepared from infected cells using the QIAamp Kit (Qiagen, Germany) following the manufacturer's instructions. The RT region of HIV-1 proviral DNA was amplified by two rounds of PCR using nested primers based on the sequence of HXB2R (Myers *et al.*, 1993). The outer and inner upstream primers designated 5′V3 and 5′V2 were 5′-GTAAGACAGTATGATCAGATA-3′ (nucleotides 1964–1984) and 5′-CAGGATCCTACACCTGTCAACATAAT-3′ (nucleotides 2033–2052), respectively. The outer and inner downstream primers 3′V2 and 3′V1 were 5′-TTGTAGGGAATTCCAAATTCC-3′ (nucleotides 4206–4186) and 5′-GGGAATTCCTTATTCCTGCTTG-3′ (nucleotides 4201–4180), respectively. Amplifications were performed in 50-μl volumes containing 2 μl of purified DNA in the presence of 1.0 U *Taq* polymerase (Boehringer Mannheim), 200 μM of each dNTP, 0.2 μM of each primer, and 1.5 mM $MgCl_2$. First round amplification conditions involved one denaturation cycle (95° for 3 min) followed by 35 cycles of denaturation (95° for 1 min), annealing (50° for 1 min) and extension (72° for 2 min), and ending with one extension cycle (72° for 7 min). Second-round conditions were as for first, with the exception that annealing was performed at 55°. For preparation of large quantities of amplified product for direct nucleotide sequencing, 12 separately amplified PCR reactions were pooled, concentrated to 300 μl by butanol extraction, and purified with Promega Magic PCR-prep columns (Promega, WI).

Nucleotide sequence analysis of HIV-1 RT region

The nucleotide sequence of the entire RT region was determined by automated sequencing using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Sequencing primers spanned the entire 1.7 kb of the HIV-1 RT and included 5′V2, SP1a 5′-CTGAAAATCCATACAATAC-3′ (2250–2268), SP3 5′-GATTTGTATGTAGGATCTG-3′ (2651–2669), SP4a 5′-GGACTGTCAATGACATACAG-3′ (2850–2869), SP5 5′-CAATTAACAGAGGCAGTG-3′ (3194–3211), and SP6 5′-CACAACAAATCAGAAGACTG-3′ (3508–3526) (Hooker *et al.*, submitted for publication). The reaction involved 25 cycles of denaturation (96° for 30 sec), annealing (45° for 15 sec), and extension (60° for 4 min). Unincorporated terminators were removed by phenol–chloroform extraction as described in the manufacturer's protocol (Applied Biosystems). Sequencing reaction products were resolved on an Applied Biosystems DNA Sequencer at the Monash University Nucleotide Sequencing Service, Microbiology Department, Clayton, Australia. Sequence alignments were performed using Seq Ed version 1.0.3 (Applied Biosystems).

Site-directed mutagenesis

HXB2 genetic backbone. The phagemid clone pHX/HOM (Hooker *et al.*, submitted for publication) contains a 4.3-kb *Hin*dIII fragment of HXB2 encompassing the complete *pol* gene [coordinates 1258 to 5578 (Myers *et al.*, 1993)] cloned into the *Hin*dIII site of pT7T319U phagemid (Pharmacia). The single-stranded form of pHX/

HOM was mutagenised according to procedures in Amersham's oligonucleotide-directed *in vitro* mutagenesis system (version 2.1) based on the method of Sayers *et al.* (1988), with the modification of pT7T319U recombinants rather than M13 recombinants as the initial single-stranded templates. The mutagenesis oligonucleotide GT92 5′-GGTATTCCTATTTGAACTTCC-3′ (2379–2359) was designed to mutate Leu (TTA) to Ile (ATA) at codon 92. The resultant phagemid was designated pHX92.

LAI genetic backbone. The mutations Glu89-Lys, Glu89-Gly, Leu92-Ile, and Ser156-Ala were introduced into the pXXHIV-1_{LAI} proviral clone by site-directed mutagenesis as previously described (Nguyen *et al.*, 1994) using the mutagenic oligonucleotides 5′-CAAGACTT-CTGGAAAGTTCAATTAG-3′ (2348–2372), 5′-GACTTCT-GGGGAGTTCAATTAG-3′ (2351–2372), 5′-CTGGGAAGT-TCAAATAGGAATAC-3′ (2356–2378), and 5′-GGAAAGG-AGCACCAGCAATA-3′ (2553–2572), respectively. The recombinant viruses with these changes were designated 89LAI-Lys, 89LAI-Gly, 92LAI-Ile, and 156LAI-Ala. The presence of the desired mutations in the proviral clones in genetic backbones HXB-2 and LAI was verified by sequencing.

Transfection and homologous recombination in MT-2 cells

Infectious virus with the Leu92-Ile mutation (strain HX92) was generated by homologous recombination in MT-2 cells of molecular constructs pHX92 and pKPHXB2ΔRT. The construct pKPHXB2ΔRT possesses most of the HXB2 (Myers *et al.*, 1993) sequence except for a 1.96-kb deletion of the HIV-1 RT gene [coordinates 2168 to 4099; (Myers *et al.*, 1993)]. The complete derivation of this construct is described elsewhere (Hooker *et al.*, submitted for publication). MT-2 cells were cotransfected with 5 μg of *Msc*I-linearised pKPHXB2ΔRT and 5 μg of *Hin*dIII-digested pHX92 using DOTAP following the manufacturer's recommendations. Vector sequence released by these enzymes was not removed prior to transfection. Cultures were maintained until maximum CPE was observed (7–12 days) at which time supernates were clarified and stored at −70°C. The titre of virus was determined in MT-2 cells and the $TCID_{50}$ was calculated using the Karber formula (Hawkes, 1979).

Generation of infectious viruses 89LAI-Lys, 89LAI-Gly, 92LAI-Ile, and 156LAI-Ala was performed by electroporation of plasmid pXXHIV-1_{LAI} containing these mutations into MT-2 cells as previously described (Nguyen *et al.*, 1994).

RESULTS

Generation of foscarnet-resistant HIV-1 by *in vitro* selection

To determine whether foscarnet-resistant HIV-1 could be produced by *in vitro* selection, the HIV-1 strain PD (isolated from an AIDS patient) and the molecular clone HX (derived from HXB-2) were passaged in the presence of increasing concentrations of foscarnet in MT-2 cells (Fig. 1). Following 6 passages (38 days in culture), isolate PD showed relatively unimpaired replication at 660 μM foscarnet. This is the maximum concentration of foscarnet in which cells remain viable. In contrast, the HX clone of HIV-1 required 16 passages (90 days) to replicate in the presence of 660 μM foscarnet.

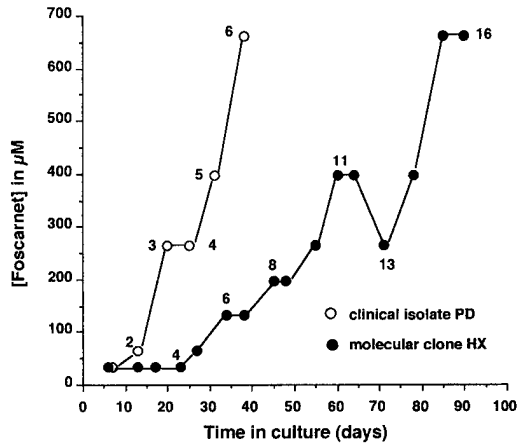

FIG. 1. *In vitro* generation in MT-2 cells of foscarnet-resistant HIV-1 from the clinical isolate PD and the molecular clone HX. Time in culture is plotted against the concentration of foscarnet at which the isolate was able to replicate at each passage level (as indicated by the circles).

To confirm that foscarnet-resistant virus was generated by the *in vitro* selection procedure, strain PD passaged six times in the presence of escalating concentrations of foscarnet and the same strain passaged six times without inhibitor (Fig. 1) were both passed once without drug to generate strains PD-R and PD-S, respectively. The foscarnet IC_{50} of PD-R was decreased 5.1-fold compared to PD-S (Table 1), whilst a 6-fold decrease was observed at the IC_{100} level (results not shown). One further passage of PD-R in the absence of inhibitor resulted in the reemergence of the foscarnet-susceptible phenotype (results not shown).

Foscarnet resistance observed at the level of HIV-1 replication was also evident in RT obtained from the lysed PD-R strain. The foscarnet IC_{50} of PD-R, 14 μM, was 40 times higher than that of PD-S (0.35 μM) (Fig. 2).

Relationship of phenotype to genotype for foscarnet-resistant strains of HIV-1 derived by *in vitro* passage

To encourage selection of virus with different genotypes and therefore varying degrees of foscarnet resistance PD-R was biologically cloned in the presence of foscarnet at concentrations of 165, 330, 495, and 660 μM. The resulting clones were designated PD165BC, PD330BC, PD495BC, and PD660BC, respectively. Isolate HX, passaged 16 times in the presence of inhibitor (Fig. 1) was biologically cloned in the presence of 330 and

TABLE 1

Amino Acid Sequences of RT Region and Foscarnet Susceptibilities of Foscarnet-Resistant Strains of HIV-1 Derived by *in vitro* Selection

	Amino acid at indicated RT codon number[a]																												
	pol domain												Connection domain								RNase H domain								
Isolate	89	92	103	123	156	172	211	214	245	272	275	277	326	329	350	357	358	376	400	403	447	460	468	469	483	512	519	Foscarnet IC_{50} ($\mu M \pm$ SD)[b]	Fold increase in foscarnet resistance[c]
HX	E	L	K	D	S	R	R	L	V	P	K	A	I	I	K	M	R	T	T	T	N	N	T	L	Y	Q	N	14.9 ± 9.2	1
HXBC[d]	–	–	R	–	–	–	–	–	–	–	R	–	–	–	R	–	–	–	–	–	–	–	–	–	–	–	–	5.61 ± 0.38	0.38
HX330BC[e]	–	–	–	–	A	K	–	F	–	–	–	–	–	–	–	–	–	–	–	–	S	–	–	–	H	K	S	88 ± 14	5.9
HX660BC[e]	–	–	–	–	A	K	–	F	–	–	–	–	–	–	–	–	–	–	–	–	S	–	–	–	H	K	S	90 ± 6.0	6.0
PD-BC[f]	–	–	–	S	–	–	K	F	M	A	–	K	V	L	–	–	K	A	A	M	–	D	S	I	H	K	S	23.9 ± 9.6	1
PD165BC[g]	–	I	–	S	–	–	K	F	M	A	–	K	V	L	–	–	K	A	A	M	–	D	S	I	H	K	S	179 ± 78	7.5
PD330BC[g]	K	–	–	S	–	–	K	F	M	A	–	K	V	L	–	–	K	A	A	M	–	D	S	I	H	K	S	255 ± 15	10.7
PD495BC[g]	K	–	–	S	–	–	K	F	M	A	–	K	V	L	–	–	K	A	A	M	–	D	S	I	H	K	S	183 ± 54	7.7
PD660BC[g]	K	–	–	S	–	–	K	F	M	A	–	K	V	L	–	–	K	A	A	M	–	D	S	I	H	K	S	231 ± 33	9.7
PD-S[h]																												36.3 ± 8.9	1
PD-R[i]																												185 ± 46	5.1

[a] RT amino acid residues shown are numbered as for HX (HXB-2 sequence) and are those that differ from isolate HX, as predicted from the observed nucleotide sequence. Underlined residues denote changes unique to foscarnet-resistant strains while others represent previously reported polymorphisms.

[b] IC_{50} determined in drug susceptibility assays performed in MT-2 cells and standard deviations (SD) calculated from at least two independent assays (see Materials and Methods).

[c] Compared with PD-S, PD-BC, or HX viruses, as appropriate.

[d] Biologically cloned from HX strain passaged 16 times in the absence of inhibitor.

[e] Isolates biologically cloned in the presence of 330 or 660 μM of foscarnet from passage 16 HX strain grown in the presence of foscarnet (Fig. 1).

[f] Biologically cloned from PD-S (see text).

[g] Strains biologically cloned in the presence of 165, 330, 495, or 660 μM of foscarnet from PD-R (see text).

[h] Uncloned HIV isolates derived from isolate PD that has had 7 passes in the absence of foscarnet. Nucleotide sequence not determined.

[i] Uncloned HIV isolate PD able to replicate in the presence of 660 μM foscarnet (passage 6 virus, see Fig. 1) which has undergone a further passage in the absence of foscarnet.

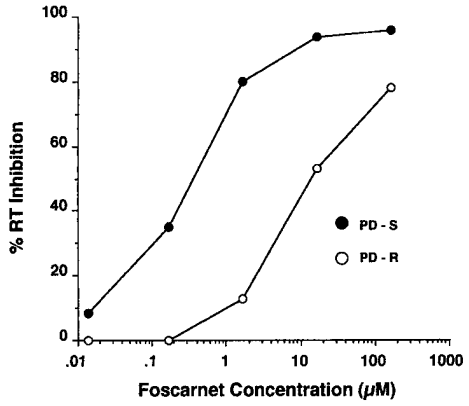

FIG. 2. Foscarnet susceptibility of RT extracted from wild-type isolate PD-S and foscarnet-resistant strain PD-R. The 100% cpm's incorporated for RT derived from strains PD-S and PD-R are 129,000 and 41,500, respectively.

660 μM foscarnet to generate HX330BC and HX660BC, respectively. Similarly, wild-type strain PD-S and HX, passaged 16 times and biologically cloned in the absence of foscarnet, were designated PD-BC and HX-BC, respectively (Table 1).

The *pol* gene from each of four foscarnet-resistant strains derived from the clinical isolate PD possessed a single amino acid substitution not present in wild-type PD-BC (Table 1). In PD165BC there was a Leu92-Ile substitution (TTA to ATA). In contrast, PD330BC, PD495BC, and PD660BC were wild-type at codon 92, but all possessed a single Glu89-Lys substitution associated with a GAA to AAA nucleotide change. No other genotypic differences in the RT gene between PD resistant strains and PD-BC were observed. Although there were differences between these strains and HX (Table 1), they occurred in previously described polymorphic regions (Myers *et al.,* 1993).

Nucleotide sequence analysis of the RT region of foscarnet-resistant strains HX330BC and HX660BC derived from the molecular clone HX showed changes different from those found in foscarnet-resistant PD strains. Both HX-derived clones had identical sequences with a total of seven amino acid changes compared to the wild-type HXB2 sequence (HX, Table 1). Only one of these, the Ser156-Ala (TCA–GCA) was not located in a polymorphic region. Codon 156 is present in a region of the HIV-1 RT highly conserved among all retroviruses (Larder *et al.,* 1987; Boyer *et al.,* 1992). In the context of foscarnet resistance, we considered this mutation to be significant.

Unexpectedly, examination of the nucleotide sequence of HXBC revealed three changes compared to the original molecular clone HX (Table 1). These may have resulted from repeated passage in MT-2 cells. None of the foscarnet-resistant clones had these mutations. Mutations at codons 275 and 350, both with the molecular change AAA to AGA, have not been reported in previous HIV-1 isolates (Myers *et al.,* 1993) and may represent unreported polymorphisms. While the Lys103-Arg observed in HXBC represents a polymorphic substitution (Myers *et al.,* 1993), another mutation, Lys103-Asn is associated with resistance to nonnucleoside RT inhibitors (Nunberg *et al.,* 1991; Richman, 1993). Given a previous report of allosteric interactions between the foscarnet and the L697-639 (nonnucleoside RT inhibitor) binding sites (Goldman *et al.,* 1991), we suspected that the Lys103-Arg change may have influenced foscarnet susceptibility, and in fact HXBC was 2.6 times more susceptible to foscarnet than HX itself ($P = 0.032$, Table 1). Consequently, isolate HX and not HXBC was used for the calculation of fold increase in foscarnet resistance for HX330BC, HX660BC, and HX92 strains. However the Lys103-Arg mutation in HXBC did not influence susceptibility to the nonnucleoside RT inhibitor nevirapine as there was no significant difference in the nevirapine susceptibility of HXBC and HX [IC_{50}s 0.09 $\pm$ 0.08 and 0.04 $\pm$ 0.03, respectively ($P = 0.17$)].

The foscarnet susceptibility of each of the biologically cloned strains is shown in Table 1. Since PD165BC possessed a different genotype to PD330BC, PD495BC, and PD660BC, a difference in foscarnet susceptibility was expected. However, while a trend towards increased resistance to foscarnet was observed with the Glu89-Lys containing genotypes compared with Leu92-Ile, the statistical significance of the trend was borderline ($P = 0.06$, $P = 0.13$, and $P = 0.09$, respectively). However, all had higher IC_{50} values than the wild-type virus.

As expected, the foscarnet susceptibilities of strains HX330BC and HX660BC were similar given their identical RT genotypes (Table 1). Of note, HX was more susceptible to foscarnet than PD-BC ($P = 0.055$), and the foscarnet IC_{50} values for the HX-derived foscarnet-resistant strains were substantially lower than those measured for resistant strains derived from PD. The former observation highlights the role of the genetic background of the RT region in influencing baseline drug susceptibility (Hooker *et al.,* submitted for publication).

Characterisation of foscarnet-resistant strains of HIV-1 derived by site-directed mutagenesis

Site-directed mutagenesis was performed to determine the role of the mutations Leu92-Ile, Glu89-Lys, and Ser156-Ala in conferring foscarnet resistance. HIV-1 with different genetic backbones but containing the Leu92-Ile mutation (HX92 and 92LAI-Ile) showed a 10-fold increase in the IC_{50} compared with the appropriate wild-type strain (Table 2), thus confirming the role of this substitution in foscarnet resistance. Similar analysis of the foscarnet susceptibility of strains 89LAI-Lys, 92LAI-Ile, and 156LAI-Ala in the HT4LacZ-1 system confirm the role of each mutation in conferring foscarnet resistance, with Glu89-Lys conferring the greatest decrease in susceptibility (>15.9-fold) and Ser156-Ala the lowest (4.5-fold, $P =$

TABLE 2

Foscarnet Susceptibilities of Recombinant HIV-1 Strains Generated by Site-Directed Mutagenesis

Isolate	Amino acid at RT codon introduced by mutagenesis			Foscarnet IC_{50} ($\mu M \pm$ SD)[a]	Fold increase in foscarnet resistance[b]
	89	92	156		
HX	E	L	S	14.9 ± 9.2	1
HX92[c]	–	I	–	129 ± 8.6	8.6
LAI[d]	–	–	–	39 ± 4	1
89LAI-Lys[d]	K	–	–	>600	>15.9
89LAI-Gly[d]	G	–	–	500 ± 13.3	13.3
92LAI-Ile[d]	–	I	–	367 ± 86	9.0
156LAI-Ala[d]	–	–	A	144 ± 4	4.5

[a] IC_{50} determined in drug susceptibility assays performed in MT-2 and HT4LacZ-1 cells for HX or LAI strains, respectively. Standard deviations (SD) calculated from at least two independent assays (see Materials and Methods).

[b] Compared with HX or LAI viruses, as appropriate.

[c] Recombinant strain of HX with change at position 92 introduced by site-directed mutagenesis (see Materials and Methods).

[d] Strains derived by mutagenesis of pXXHIV$_{LAI}$ and electroporation of clones into MT-2 cells (see Materials and Methods).

0.05). While strain 89LAI-Gly, which contains the previously reported substitution Glu89-Gly (Prasad *et al.*, 1991), was also foscarnet-resistant, its foscarnet IC_{50} was lower than the 89LAI-Lys strain ($P = 0.05$).

Drug susceptibility profiles of foscarnet-resistant strains

Foscarnet-resistant strains PD165BC, PD660BC, and HX660BC containing the Leu92-Ile, Glu89-Lys, and Ser156-Ala substitutions, respectively were tested for their susceptibility to several antiretroviral drugs (Tables 3a, 3b, 3c). Regardless of genotype, all foscarnet-resistant strains were 2.6–2.8 times more susceptible to zidovudine than the corresponding wild-type strain. These strains were also 4.1–36 times more susceptible to another azidonucleoside analogue, AZDU (Table 3). No difference in susceptibility to ddI and ddC was observed. In contrast, all foscarnet-resistant strains were also hypersensitive to the nonnucleoside RT inhibitors TIBO and nevirapine (Table 3).

DISCUSSION

Our data show that strains of HIV-1 with reduced susceptibility to foscarnet can be generated by *in vitro* selection, that resistance is due to a limited number of well-defined mutations in the reverse transcriptase enzyme, and that such resistant strains have a predictable pattern of altered susceptibility to other antiretrovirals. Phenotypic resistance to foscarnet was invariably manifested by reduced inhibition of both virus replication and reverse transcriptase. Strains generated with a foscarnet-resistant phenotype had mutations at codons 89, 92, or 156 of the RT, and site-directed mutagenesis confirmed the relevance of those mutations to the phenotypic changes observed at the level of virus replication.

The previously unreported Leu92-Ile change observed in Fos165BC was shown to mediate foscarnet resistance in both HXB2 and LAI genetic backbones. This was surprising given the conservative nature of this amino acid substitution, which represents a marginal increase in both accessible surface area and hydrophobicity (Ausu-

TABLE 3a

Drug Susceptibility Profile of Foscarnet-Resistant Isolate PD165BC

Isolate	IC_{50} (μM) for indicated drug[a]						
	Foscarnet	AZT	AZDU	ddI	ddC	TIBO	Nevirapine
PDBC	23.9 ± 9.6	0.024 ± 0.006	10 ± 0	5 ± 1.4	0.2 ± 0.11	0.26 ± 0.07	0.054 ± 0.006
PD165BC	179 ± 78	0.0086 ± 0.002	0.84 ± 0.51	4 ± 2.8	0.2 ± 0.12	0.079 ± 0.07	0.013 ± 0.01
Fold resistance[b]	7.5[c]	—	—	—	—	—	—
Fold hypersensitivity[b]	—	2.8[c]	12[c]	—	—	3.3[c]	4.2[c]

[a] Values are means ± standard deviations from at least two independent experiments.

[b] Increase (resistance) or decrease (hypersensitivity) in IC_{50} of foscarnet-resistant strains compared to wild-type virus.

[c] The fold increases in resistance and hypersensitivity were all statistically significant by the Wilcoxon rank-sum test ($P \leq 0.05$).

TABLE 3b

Drug Susceptibility Profile of Foscarnet-Resistant Isolate PD660BC

Isolate	IC_{50} (μM) for indicated drug[a]						
	Foscarnet	AZT	AZDU	ddI	ddC	TIBO	Nevirapine
PDBC	23.9 ± 9.6	0.0077 ± 0.0028	1.8 ± 0.6	2.7 ± 1.1	0.23 ± 0.14	0.25 ± 0.16	0.15 ± 0.13
PD660BC	231 ± 33	0.003 ± 0.0012	0.44 ± 0.1	3.3 ± 0.7	0.27 ± 0.12	0.024 ± 0.006	0.02 ± 0.008
Fold resistance[b]	9.7[c]	—	—	—	—	—	—
Fold hypersensitivity[b]	—	2.6[c]	4.1[c]	—	—	10.4[c]	7.5[d]

[a] Values are means ± standard deviations from at least two independent experiments.
[b] Increase (resistance) or decrease (hypersensitivity) in IC_{50} of foscarnet-resistant strains compared to wild-type virus.
[c] The fold increases in resistance and hypersensitivity were all statistically significant by the Wilcoxon rank-sum test ($P \leq 0.05$).
[d] Fold increase in hypersensitivity was of borderline statistical significance ($P = 0.1$, Wilcoxon rank-sum test).

bel *et al.*, 1987). Nevertheless, Leu92 is highly conserved among HIV-1 and other lentiviruses (Boyer *et al.*, 1992), suggesting that changes to it will be significant at the phenotypic level. A Glu89-Lys substitution representing a change from an acidic to a basic amino acid, was also observed in three of four clones obtained from PD-R. The role of codon 89 in conferring foscarnet resistance has been previously documented by random mutagenesis and selection of drug-resistant RT using a novel screening assay (Prasad *et al.*, 1991). Subsequent mutagenesis studies have revealed that Glu89-Lys also results in a foscarnet-resistant RT enzyme (Song *et al.*, 1992). Elucidation of the foscarnet susceptibilities of RT enzymes with the Glu89-Gly or Glu89-Lys substitutions revealed 2000- and 8-fold increases in foscarnet IC_{50}s (Song *et al.*, 1992). This contrasts with our data showing that at the level of HIV-1 replication, HIV-1 with the Glu89-Lys substitution is more resistant than virus with the Glu89-Gly change. As the Glu89-Lys emerged in cell culture under selective pressure, it is likely that such a mutation will occur *in vivo*.

The polar to nonpolar substitution Ser156-Ala, which is located in conserved region C of all reverse transcriptases (Larder *et al.*, 1987), was found in biological clones HX330BC and HX660BC. HIV-1_{LAI} with the Ser156-Ala mutation was replication competent in contrast to a previous study with the BH-10 molecular clone showing that this mutation results in RT with intact polymerase activities but inactive RNaseH (Boyer *et al.*, 1992). Introduction of Ser156-Ala into the HXB2 backbone by site-directed mutagenesis also results in replication-competent HIV-1 (G. Tachedjian, unpublished results). The mechanism by which Ser156-Ala mutation abolishes RNaseH activity has been proposed to be due to repositioning of the template-primer to a position inconsistent with favourable catalysis at the RNase H active site (Boyer *et al.*, 1992; Tantillo *et al.*, 1994). One possible explanation for the difference in our results compared with those of Boyer *et al.* (1992) may be the emergence of compensatory mutations in either the polymerase or RNaseH domains following transfection of HIV-1 DNA with the Ser156-Ala mutation and recovery of infectious virus. Studies are underway to determine whether such compensatory changes are present in these HIV-1 strains. In relation to the viable strains HX330BC and HX660BC, the polymorphic substitutions either in the pol or RNaseH domains may also have a compensatory role.

In addition to Glu89-Gly and Glu89-Lys, a Val90-Ala change associated with foscarnet-resistant RT has also been reported (Im *et al.*, 1993). The mutations Trp88-Ser

TABLE 3c

Drug Susceptibility Profile of Foscarnet-Resistant Isolate HX660BC

Isolate	IC_{50} (μM) for indicated drug[a]						
	Foscarnet	AZT	AZDU	ddI	ddC	TIBO	Nevirapine
HX	14.9 ± 9.2	0.0128 ± 0.0011	27.6 ± 5.4	1.6 ± 1.1	0.22 ± 0.05	0.13 ± 0.03	0.18 ± 0.08
HX660BC	90 ± 71	0.0046 ± 0.0003	0.76 ± 0.64	1.8 ± 0.1	0.29 ± 0.01	0.09 ± 0.01	0.04 ± 0.02
Fold resistance[b]	6.0[c]	—	—	—	—	—	—
Fold hypersensitivity[b]	—	2.8[c]	36[c]	—	—	1.4[d]	4.5[c]

[a] Values are means ± standard deviations from at least two independent experiments.
[b] Increase (resistance) or decrease (hypersensitivity) in IC_{50} of foscarnet-resistant strains compared to wild-type virus.
[c] The fold increases in resistance and hypersensitivity were all statistically significant by the Wilcoxon rank-sum test ($P \leq 0.05$).
[d] Fold increase in hypersensitivity was of borderline statistical significance ($P = 0.1$, Wilcoxon rank-sum test).

and Gln161-Leu in association with His208-Tyr have also been reported in HIV-1 isolates from AIDS patients receiving foscarnet therapy and in strains generated *in vitro* (Mellors *et al.*, 1995). Codons 88, 89, 90, 92, and 156 all cluster in the same region on the three-dimensional structure of the HIV-1 RT (Jacobo-Molina *et al.*, 1993) and define a "hot spot" for foscarnet resistance-associated mutations where changes at one codon are involved.

Molecular modelling of a nucleoside triphosphate in the polymerase active site of the HIV-1 RT/DNA/Fab complex has revealed the likely deoxynucleoside triphosphate (dNTP) binding site, where pyrophosphate exchange and foscarnet binding is expected to occur (Nanni *et al.*, 1993; Tantillo *et al.*, 1994). The dNTP binding site comprises not only protein structural elements, but also nucleic acid (Tantillo *et al.*, 1994). Based on this model and calculated solvent accessible surface areas of amino acids in the vicinity of the dNTP binding site (Tantillo *et al.*, 1994), the protein secondary structural elements that appear to interact with the dNTP residue include β9, β10, the β9 and β10 hairpin, β6, the β6–αC loop and αC (see Fig. 3 in Tantillo *et al.*, 1994). Amino acids 89, 92, and 156, which are found to be implicated in foscarnet resistance, are located on β5a, the loop structure between β5a and β5b, and the N-terminus of α helix E, respectively (Jacobo-Molina *et al.*, 1993; Nanni *et al.*, 1993; Tantillo *et al.*, 1994). These residues are not located in structural elements composing the dNTP site and therefore cannot have a direct effect on foscarnet binding. However, all three residues cluster near the template strand of the template-primer within the palm subdomain (see Fig. 3B of Jacobo-Molina *et al.*, 1993). Therefore, we propose that the foscarnet resistance mediated by Glu89-Lys, Leu92-Ile, and Ser156-Ala is a result of altered template-primer positioning or conformation on the surface of the enzyme which causes a distortion of the geometry of the polymerase active site and leads to altered binding of foscarnet at the putative pyrophosphate site. A similar hypothesis has been proposed to explain the mechanism of resistance to nucleoside analogues (Nanni *et al.*, 1993; Tantillo *et al.*, 1994). Biochemical data to support the notion that template-primer movement can affect the active site has been previously shown by analysis of the susceptibility of wild-type and drug-resistant RT enzymes (with either Leu74-Val or Glu89-Gly mutations) to inhibitors in the presence of template-primers with template overhangs of varying lengths (Boyer *et al.*, 1994).

Three biologically cloned strains resistant to foscarnet, each with different genotypes, were hypersusceptible to the inhibitors AZT, AZDU, nevirapine, and TIBO, but had unchanged susceptibility to ddI and ddC. Similarly, foscarnet-resistant strains encoding Gln161-Leu and His208-Tyr or Gln161-Leu alone showed hypersusceptibility to AZT but no change in ddI, ddC, or D4T susceptibility at the level of HIV-1 replication (Mellors *et al.*, 1995).

It is noteworthy that the replication of our foscarnet-resistant strains was still inhibited by ddI and ddC, as previous studies with purified RT with previously reported mutations conferring foscarnet resistance (Glu89-Gly and Val90-Ala) are broadly cross-resistant to nucleoside triphosphate analogues including AZTTP, ddTTP, ddCTP, and ddATP (Prasad *et al.*, 1991; Im *et al.*, 1993). However, while RT with the Glu89-Gly mutation was also resistant to ddGTP in these assays, virus replication was still inhibited by conventional concentrations of ddG (Prasad *et al.*, 1991). The lack of correlation between HIV replication and enzyme assays, observed by Prasad *et al.* (1991), may be due to fundamental differences in the mechanism of inhibition by nucleoside analogues in these two systems.

The rapid emergence of foscarnet-resistant strains of HIV-1 after only 6–16 *in vitro* passages suggests that resistant virus may emerge in HIV-infected patients undergoing long-term treatment with foscarnet. We studied 12 HIV-1 isolates from seven AIDS patients on foscarnet therapy ranging from 2.5 to 16.5 months and found no evidence of HIV-1 significantly resistant to foscarnet (Tachedjian *et al.*, 1994; G. Tachedjian, unpublished data). However, others have reported the emergence of foscarnet-resistant HIV-1 strains in individuals on long-term foscarnet therapy (>3 months) for CMV retinitis (Mayers *et al.*, 1993). While our *in vitro* data support the latter study, other factors such as preexisting drug resistance and simultaneous therapy with other antiviral agents may have a role in determining whether foscarnet-resistant HIV-1 will emerge in an individual patient (Tachedjian *et al.*, 1994).

In conclusion we have shown that foscarnet-resistant HIV-1 emerges rapidly *in vitro*, and have identified several mutations in the HIV-1 RT which are able to confer foscarnet resistance. Foscarnet-resistant strains with different genotypes remain susceptible to several other classes of RT inhibitor antiretrovirals. In the context of an HIV-infected patient developing foscarnet-resistant HIV-1, these data indicate that antiretroviral drugs approved for clinical use (AZT, ddI, and ddC) or undergoing clinical trials (nevirapine and TIBO) are likely to retain their inhibitory activity against HIV-1.

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REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., Eds. (1987). "Current Protocols in Molecular Biology." Wiley, New York.

Bhattacharyya, G. K., and Johnson, R. A. (1977). "Statistical Concepts and Methods." Wiley, New York.

Birch, C. J., Tachedjian, G., Doherty, R. R., Hayes, K., and Gust, I. D. (1990). Altered sensitivity to antiviral drugs of herpes simplex virus isolates from a patient with the acquired immunodeficiency syndrome. *J. Infect. Dis.* **162,** 731–734.

Boyer, P. L., Ferris, A., and Hughes, S. H. (1992). Cassette mutagenesis of the reverse transcriptase of human immunodeficiency virus type 1. *J. Virol.* **66,** 1031–1039.

Boyer, P. L., Tantillo, C., Jacobo-Molina, A., Nanni, R. G., Ding, J., Arnold, E., and Hughes, S. H. (1994). Sensitivity of wild-type human immunodeficiency virus type 1 reverse transcriptase to dideoxynucleotides depends on template length; the sensitivity of drug-resistant mutants does not. *Proc. Natl. Acad. Sci. USA* **91,** 4882–4886.

Crumpacker, C. S. (1992). Mechanism of action of foscarnet against viral polymerases. *Am. J. Med.* **92,** 3–7S.

Eriksson, B. F., and Schinazi, R. F. (1989). Combinations of 3′-azido-3′-deoxythymidine (zidovudine) and phosphonoformate (foscarnet) against human immunodeficiency virus type 1 and cytomegalovirus replication *in vitro*. *Antimicrob. Agents Chemother.* **33,** 663–669.

Erlich, K. S., Jacobson, M. A., Koehler, J. E., Follansbee, S. E., Drennan, D. P., Gooze, L., Safrin, S., and Mills, J. (1989). Foscarnet therapy for severe acyclovir-resistant herpes simplex virus type-2 infections in patients with the acquired immunodeficiency syndrome (AIDS). An uncontrolled trial. *Ann. Intern. Med.* **110,** 710–713.

Fletcher, C., Collier, A., Rhame, F., Bennett, D., Para, M., Beatty, C., Jones, C., and Balfour, H. J. (1994). Foscarnet for suppression of human immunodeficiency virus replication. *Antimicrob. Agents Chemother.* **38,** 604–607.

Goldman, M. E., Nunberg, J. H., O'Brien, J. A., Quintero, J. C., Schleif, W. A., Freund, K. F., Gaul, S. L., Saari, W. S., Wai, J. S., Hoffman, J. M., Anderson, P. S., Hupe, D. J., Emini, E. A., and Stern, A. M. (1991). Pyridinone derivatives: Specific human immunodeficiency virus type 1 reverse transcriptase inhibitors with antiviral activity. *Proc. Natl. Acad. Sci. USA* **88,** 6863–6867.

Harada, S., Koyanagi, Y., and Yamamoto, N. (1985). Infection of HTLV-III/LAV in HTLV-I-carrying cells MT-2 and MT-4 and application in a plaque assay. *Science* **229,** 563–566.

Hawkes, R. A. (1979). General principles underlying laboratory diagnosis of viral infections. *In* "Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections." 5th ed., pp. 34–35. Am. Public Health Assoc., Washington, DC.

Hooker, D. J., Tachedjian, G., Solomon, A. E., Gurusinghe, A. D., Land, S., Birch, C., Roy, B. M., Arnold, E., and Deacon, N. J. (1995). An *in vivo* mutation from leucine to tryptophan at position 210 in human immunodeficiency virus type-1 reverse transcriptase contributes to high-level resistance to 3′-azido-3′-deoxythymidine (AZT). (Submitted for publication.)

Im, G.-J., Tramontano, E., Gonzalez, C. J., and Cheng, Y.-C. (1993). Identification of the amino acid in the human immunodeficiency virus type 1 reverse transcriptase involved in the pyrophosphate binding of antiviral nucleoside triphosphate analogs and phosphonoformate. *Biochem. Pharmacol.* **46,** 2307–2313.

Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D. J., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hizi, A., Hughes, S. H., and Arnold, E. (1993). Crystal structure of human immunodeficiency virus type I reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. *Proc. Natl. Acad. Sci. USA* **90,** 6320–6324.

Jacobson, M. A., Crowe, S., Levy, J., Aweeka, F., Gambertoglio, J., McManus, N., and Mills, J. (1988). Effect of foscarnet therapy on infection with human immunodeficiency virus in patients with AIDS. *J. Infect. Dis.* **158,** 862–865.

Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992). Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256,** 1783–1790.

Koshida, R., Vrang, L., Gilljam, G., Harmenberg, J., Oberg, B., and Wahren, B. (1989). Inhibition of human immunodeficiency virus in vitro by combinations of 3′-azido-3′-deoxythymidine and foscarnet. *Antimicrob. Agents Chemother.* **33,** 778–780.

Larder, B. A., Coates, K. E., and Kemp, S. D. (1991). Zidovudine-resistant human immunodeficiency virus selected by passage in cell culture. *J. Virol.* **65,** 5232–5236.

Larder, B. A., Kemp, S. D., and Purifoy, D. J. (1989). Infectious potential of human immunodeficiency virus type 1 reverse transcriptase mutants with altered inhibitor sensitivity. *Proc. Natl. Acad. Sci. USA* **86,** 4803–4807.

Larder, B. A., Purifoy, D. J. M., Powell, K. L., and Darby, G. (1987). Site-specific mutagenesis of AIDS virus reverse transcriptase. *Nature* **327,** 716–717.

Lowe, D. M., Parmar, V., Kemp, S. D., and Larder, B. A. (1991). Mutational analysis of two conserved sequence motifs in HIV-1 reverse transcriptase. *FEBS Lett.* **282,** 231–234.

Mayers, D., Erice, A., Lane, J., McCutchan, F., Jabs, D., Tudor-Williams, G., Sannerud, K., Weislow, O., Page, C., Felder, K., and Balfour, H. J. (1993). Foscarnet (FOS) resistant HIV isolates emerge during extended FOS therapy for CMV retinitis. The 33rd Interscience Conf. on Antimicrobial Agents and Chemotherapy, New Orleans. [Abstract 1068]

Mellors, J., Bazmi, H., Schinazi, R., Roy, B., Hsiou, Y., Arnold, E., Weir, J., and Mayers, D. (1995). Novel mutations in reverse transcriptase of human immunodeficiency virus type 1 reduce susceptibility to foscarnet in laboratory and clinical isolates. *Antimicrob. Agents Chemother.* **39,** 1087–1092.

Myers, G., Korber, B., Wain-Hobson, S., Smith, R. F., and Pavlakis, G. N. (1993). Human retroviruses and AIDS: A compilation and analysis of nucleic acid and amino acid sequences. Theoretical Biology and Biophysics Group T-10, Los Alamos National Laboratory, Los Alamos, New Mexico. pp. I.A. 76–98.

Nanni, R. G., Ding, J., Jacobo-Molina, A., Hughes, S. H., and Arnold, E. (1993). Review of HIV-1 reverse transcriptase three-dimensional structure: Implications for drug design. *Perspect. Drug Discovery Design* **1,** 129–150.

Neate, E. V., Pringle, R. C., Jowett, J. B. M., Healey, D. S., and Gust, I. D. (1987). Isolation of HIV from Australian patients with AIDS, AIDS related conditions and healthy antibody positive individuals. *Aust. N. Z. J. Med.* **17,** 461–466.

Nguyen, M. H., Schinazi, R. F., Shi, C., Goudgaon, N. M., McKenna, P. M., and Mellors, J. W. (1994). Resistance of human immunodeficiency virus type 1 to acyclic 6-phenylselenenyl- and 6-phenylthiopyrimidines. *Antimicrob. Agents Chemother.* **38,** 2409–2414.

Nunberg, J. H., Schleif, W. A., Boots, E. J., O'Brien, J. A., Quintero, J. C., Hoffman, J. M., Emini, E. A., and Goldman, M. E. (1991). Viral resistance to human immunodeficiency virus type 1-specific pyridinone reverse transcriptase inhibitors. *J. Virol.* **65,** 4887–4892.

Oberg, B. (1989). Antiviral effects of phosphonoformate (PFA, foscarnet sodium). *Pharmacol. Ther.* **40,** 213–285.

Peden, K. W. C. (1992). Instability of HIV sequences in high copy number plasmids. *J. Acquired Immune Defic. Syndr.* **5,** 313–315.

Prasad, V. R., Lowy, I., De Los Santos, T., Chiang, L., and Goff, S. P. (1991). Isolation and characterization of a dideoxyguanosine triphosphate-resistant mutant of human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **88,** 11363–11367.

Ratner, L., Fisher, A., Jagodzinski, L. L., Mitsuya, H., Liou, R.-S., Gallo, R. C., and Wong-Staal, F. (1987). Complete nucleotide sequences of functional clones of the AIDS virus. *AIDS Res. Hum. Retroviruses* **3,** 57–69.

Richman, D., Shih, C. K., Lowy, I., Rose, J., Prodanovich, P., Goff, S., and Griffin, J. (1991). Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture. *Proc. Natl. Acad. Sci. USA* **88,** 11241–11245.

Richman, D. D. (1993). Resistance of clinical isolates of human immunodeficiency virus to antiretroviral agents. *Antimicrob. Agents Chemother.* **37,** 1207–1213.

Rocancourt, D., Bonnerot, C., Jouin, H., Emerman, M., and Nicolas, J. F. (1990). Activation of a β-galactosidase recombinant provirus: Application to titration of human immunodeficiency virus (HIV) and HIV-infected cells. *J. Virol.* **64,** 2660–2668.

Safrin, S., Berger, T. G., Gilson, I., Wolfe, P. R., Wofsy, C. B., Mills, J., and Biron, K. K. (1991a). Foscarnet therapy in five patients with AIDS and acyclovir-resistant varicella-zoster virus infection. *Ann. Intern. Med.* **115,** 19–21.

Safrin, S., Crumpacker, C., Chatis, P., Davis, R., Hafner, R., Rush, J., Kessler, H. A., Landry, B., and Mills, J. (1991b). A controlled trial comparing foscarnet with vidarabine for acyclovir-resistant mucocutaneous herpes simplex in the acquired immunodeficiency syndrome. The AIDS Clinical Trials Group. *N. Engl. J. Med.* **325,** 551–555.

Sandstrom, E. G., Byington, R. E., Kaplan, J. C., and Hirsch, M. S. (1985). Inhibition of human T-cell lymphotropic virus type III *in vitro* by phosphonoformate. *Lancet* **i,** 1480–1482.

Sayers, J. R., Schmidt, W., and Eckstein, F. (1988). 5′-3′ exonucleases in phosphorothioate-based oligonucleotide-directed mutagenesis. *Nucleic Acids Res.* **16,** 791–802.

Song, Q., Yang, G., Goff, S. P., and Prasad, V. R. (1992). Mutagenesis of the Glu-89 residue in human immunodeficiency virus type 1 (HIV-1) and HIV-2 reverse transcriptases: Effects on nucleoside analog resistance. *J. Virol.* **66,** 7568–7571.

Studies of Ocular Complications of AIDS Research Group (SOCA), AIDS Clinical Trials Group. (1992). Mortality in patients with the acquired immunodeficiency syndrome treated with either foscarnet or ganciclovir for cytomegalovirus retinitis. *N. Engl. J. Med.* **326,** 213–220.

Tachedjian, G., Tyssen, D., Locarnini, S., Gust, I., and Birch, C. (1990). Investigation of topoisomerase inhibitors for activity against human immunodeficiency virus: Inhibition by coumermycin A1. *Antiviral Chem. Chemother.* **1,** 131–138.

Tachedjian, G., Hoy, J., McGavin, K., and Birch, C. (1994). Long-term foscarnet therapy not associated with the development of foscarnet-resistant human immunodeficiency virus type 1 in an acquired immunodeficiency syndrome patient. *J. Med. Virol.* **42,** 207–211.

Tantillo, C., Ding, J., Jacobo-Molina, A., Nanni, R. G., Boyer, P. L., Hughes, S. H., Pauwels, R., Andries, K., Janssen, P. A. J., and Arnold, E. (1994). Locations of anti-AIDS drug binding sites and resistance mutations in the three-dimensional structure of HIV-1 reverse transcriptase. Implications for mechanisms of drug inhibition and resistance. *J. Mol. Biol.* **243,** 369–387.

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

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Both foscarnet (PFA) and zidovudine (AZT) select for drug-resistant variants of human immunodeficiency virus type 1 (HIV-1), but the interactions between the mutations causing such resistance are unknown. The introduction of the previously identified PFA resistance mutation W to G at codon 88 (W88G), E89K, L92I, or Q161L into an HIV-1 strain having the four known AZT resistance mutations completely reversed high-level AZT resistance. Two additional PFA resistance mutations, W88S and S156A, partially suppressed AZT resistance. Phenotypic reversion of AZT resistance by W88S, W88G, E89K, L92I, and S156A was associated with a concomitant suppression of PFA resistance. The degree to which PFA resistance mutations reversed AZT resistance was directly correlated with each mutation's ability to confer high-level PFA resistance (≥5.0-fold) and AZT hypersusceptibility in a wild-type genetic background. Highly PFA-resistant HIV-1 strains were hypersusceptible to AZT; conversely, AZT-resistant strains with M41L and T215Y; M41L, L210W, and T215Y; or M41L, D67N, K70R, and T215Y mutations were 2.2- to 2.5-fold hypersusceptible to PFA. Prolonged in vitro selection of wild-type or AZT-resistant HIV-1 strains with the combination AZT and PFA failed to generate coresistant 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

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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 μg/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 μg/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 *Hin*dIII fragment of HXB2 encompassing the complete *pol* gene (coordinates 1258 to 5578) cloned into the *Hin*dIII 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 *pol* fragment from the M13mptac18.1 clones $HIVRT_{MQ}$ 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 $HIVRT_{MQ}$ encode the AZT resistance mutations M41L and T215Y and M41L, D67N, K70R, and T215Y, respectively (22). The derivation of pHIVΔRTBstEII (a gift from Brendan A. Larder) has been described previously (54). pKPHXB2ΔRT 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 pKPHXB2ΔRT 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 pHIVΔRTBstEII. 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 μg of *Msc*I-linearized pKPHXB2ΔRT or *Bst*EII-linearized pHIVΔRTBstEII with either *Xba*I- and *Eco*RI-linearized (pMQ- and pMN-derived constructs) or *Hin*dIII-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 pF4A4, respectively, were generated by cotransfection of *Bam*HI-*Eco*RI-digested phagemids with *Bst*EII-digested pHIVΔRTBstEII. 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 × *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 μM PFA and 0.2 μM 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, Kremsmünster, 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 (2.5×10^4 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 μl of Dulbecco's modified Eagle's medium–5% fetal calf serum containing 10 μg of DEAE-dextran (AMRAD Pharmacia Biotech) per ml at 37°C. After 1 to 1.5 h, the inoculum was removed and 1-ml 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 (IC_{50}) was derived from plots of the percentage inhibition versus the $\log_{10}$ concentration of inhibitor (45, 58). PFA resistance was defined as HIV-1 with a greater-than-twofold increase in IC_{50} compared with that of the corresponding wild-type strain. In HT4LacZ-1 assays, viral strains for which the IC_{50}s were ≤0.05 μM were considered AZT-sensitive; those for which the IC_{50}s were >0.05 and <1.0 μM were considered partially resistant; and those for which the IC_{50}s were ≥1.0 μM were considered highly resistant (21). The statistical significance of differences between IC_{50}s was determined by the Wilcoxon rank-sum test (2).

PCR amplification. HIV-1 strains were grown in phytohemagglutinin-stimulated PBMCs (2.5×10^6 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 μl 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			
						PFA		AZT	
	41	67	70	**88**	215	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] 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. The differences in IC_{50}s of AZT for HX and HX88S were not statistically significant ($P > 0.115$). In contrast, statistically significant differences in the IC_{50}s 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 IC_{50}s 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] IC_{50} for mutant strain divided by IC_{50} for wild-type HX. Values of >1 and <1 indicate resistance and hypersusceptibility, respectively.

[d] The IC_{50} 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 IC_{50} of PFA for this strain was determined in a different assay series in which the IC_{50} of PFA for HX was 22 μM. Accordingly, fold resistance has been calculated with this value.

[f] The IC_{50} 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 $MgCl_2$. 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_{LAI}161L and pHXXHIV-1_{LAI}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			
											PFA		AZT	
	41	67	70	88	89	92	156	161	215	219	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 $pXXHIV\text{-}1_{LAI}MC/Y$. 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 IC_{50}s 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 IC_{50} of PFA for HX and MQ156A was not statistically different ($P > 0.114$). In contrast, IC_{50}s 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 IC_{50} of the mutant strain by the IC_{50} 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 resistance in the LAIMC/Y background (Table 2). In contrast, introduction of S156A into the MQ background (strain MQ156A) resulted in only partial suppression of AZT resistance (Table 2). 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			
					PFA		AZT	
	89	92	156	161	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 $pXXHIV\text{-}1_{LAI}$ 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 IC_{50}s of AZT for HX89K and HX92I were significantly different from that for HX ($P = 0.05$), while IC_{50}s of AZT for LAI and LAI161L were of borderline significance ($P = 0.067$). IC_{50}s 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 IC_{50} 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] IC_{50}s and standard deviations were determined in drug susceptibility assays performed in HT4LacZ-1 cells and were calculated from at least two independent assays. The differences between IC_{50}s of PFA for HX/41+215 (MN), HX/3X, and MQ were significantly different compared with that for HX ($P \leq 0.05$). IC_{50}s of AZT for HX/41+215 (MN), HX/3X, and MQ were statistically different compared with that for HX ($P = 0.008$). IC_{50}s of AZT for LAI and LAIMC/Y were significantly different ($P = 0.014$).

[b] The fold increase in resistance was calculated by dividing the IC_{50} of the mutant strain by the IC_{50} of the corresponding wild-type strain.

[c] AZT-resistant strain with mutations M41L and T215Y.

[d] AZT-resistant strain with mutations M41L, 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 coresistant to both AZT and PFA could be produced by in vitro selection. The wild-type HIV-1 strain PD, previously used to select PFA-resistant strains (57), was passaged in the presence of increasing concentrations of PFA and AZT in MT-2 cells (results not shown). Following 25 passages (164 days in culture), we selected strain PFA330AZT0.2p25, which replicated in the presence of 330 μM PFA and 0.2 μM AZT. This strain was PFA resistant (7.8-fold increase in IC_{50}) but fully AZT susceptible compared with a wild-type PD that had undergone 23 passages in MT-2 cells in the absence of drug (results not shown).

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

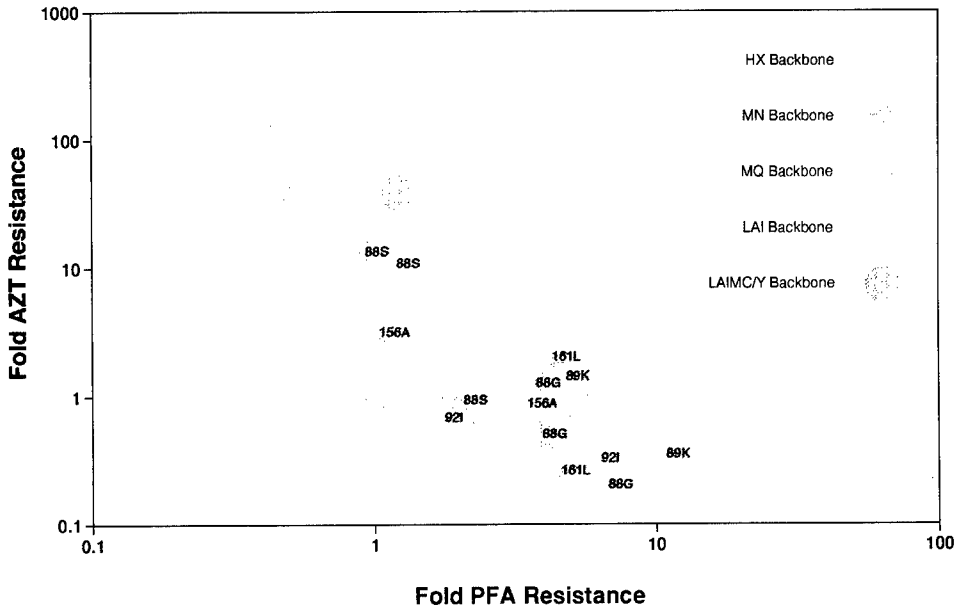

FIG. 1. Effect of HIV-1 RT mutations on relative susceptibility to AZT and PFA. Fold changes in PFA or AZT resistance were calculated relative to the corresponding wild-type strain (HX or LAI), from the data in Tables 1, 2, 3, and 4. Symbols depict different RT genetic backbones and are labelled with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

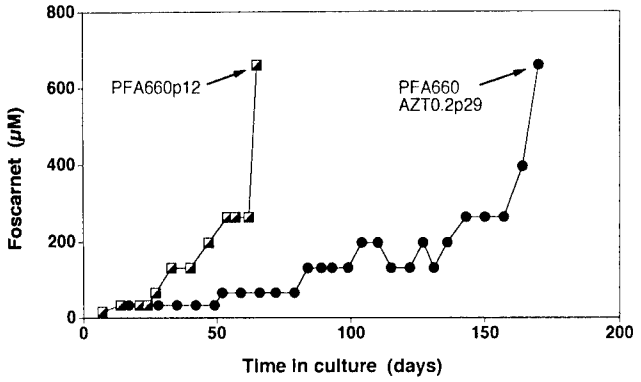

FIG. 2. Attempted selection of HIV-1 coresistant to AZT and PFA by in vitro selection in MT-2 cells starting with cloned AZT-resistant HIV-1 strains (MQ, HX/41+215, and HX/3X). See Materials and Methods for details. The time in culture is plotted against the concentration of PFA at which the isolate 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 μM 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 μM 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 μM 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 (pF1 to pF6) derived from this PFA-resistant strain showed four mutations common to all six clones (Table 5). Three of these were the original AZT resistance-associated mutations at codons 41, 70, and 215. The fourth was W88G, which has been previously reported for a PFA-resistant clinical isolate (35). Other changes were also observed in single clones at codons 91, 172, 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	
	41	67	70	88	91	172	177	211	215	217	326	386	Mean IC_{50} ± SD (μM)[b]	Resistance (fold)[c]	Mean IC_{50} ± SD (μM)	Resistance (fold)
Strains																
HX	M	D	K	W	Q	R	D	R	T	P	I	T	34 ± 19	1	0.02 ± 0.01	1
PFA660p12[d]													119 ± 20	3.5	0.026 ± 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 ± 0	4.8	0.04 ± 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 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 pF3 (F3) generated by cotransfection of MT-2 cells with pF3 and pHIVΔRTBstEII.

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	
	41	61	67	68	70	**88**	175	214	215	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 IC_{50}s of PFA for HX and PFA660AZT0.2p29 ($P = 0.05$) or FA4 ($P = 0.014$). The IC_{50}s 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* amplimers 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 ($IC_{50} \leq 0.05$ 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 ($IC_{50} < 0.2$ μM) as assayed in PBMCs (34), despite the presence of one or more AZT resistance mutations at codon(s) 41, 67, 70, 210, 215, and/or 219, including the atypical substitutions M41V, K70E/G, T215L, and K219R. These anecdotal data suggest that genotypic AZT resistance can be phenotypically reversed by PFA resistant mutations. However, since the timing and duration of AZT and PFA therapy in these patients varied, these data cannot be used to support our hypothesis that coresistance to AZT and PFA is difficult to achieve. Therefore, analysis of further clinical isolates to confirm the assertion that the combination of AZT and PFA imposes constraints on the mutability of HIV-1 RT will be required.

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

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

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

Elucidation of the crystal structure of the HIV-1 RT (17) and molecular modelling of a dNTP in the polymerase active site of the HIV-1 RT-DNA-Fab complex has revealed the likely dNTP binding site (61). PP_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_i exchange with improved pharmacological properties, might be able to take advantage of the favorable drug resistance interactions with AZT.

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REFERENCES

1. **Barnes, W. M.** 1994. PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. Proc. Natl. Acad. Sci. USA **91:**2216–2220.
2. **Bhattacharyya, G. K., and R. A. Johnson.** 1977. Statistical concepts and methods. John Wiley and Sons, New York.
3. **Birch, C. J., G. Tachedjian, R. R. Doherty, K. Hayes, and I. D. Gust.** 1990. Altered sensitivity to antiviral drugs of herpes simplex virus isolates from a patient with the acquired immunodeficiency syndrome. J. Infect. Dis. **162:** 731–734.
4. **Cheeseman, S. H., D. Havlir, M. M. McLaughlin, T. C. Greenough, J. L. Sullivan, D. Hall, S. E. Hattox, S. A. Spector, D. S. Stein, M. Myers, and D. D. Richman.** 1995. Phase I/II evaluation of nevirapine alone and in combination with zidovudine for infection with human immunodeficiency virus. J. Acquired Immune Defic. Syndr. Hum. Retrovirol. **8:**141–151.
5. **Coffin, J. M.** 1995. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science **267:**483–489.
6. **Collier, A. C., R. W. Coombs, M. A. Fischl, P. R. Skolnik, D. Northfelt, P. Boutin, C. J. Hooper, L. D. Kaplan, P. A. Volberding, L. G. Davis, D. R. Henrard, S. Weller, and L. Corey.** 1993. Combination therapy with zidovudine and didanosine compared with zidovudine alone in HIV-1 infection. Ann. Intern. Med. **119:**786–793.
7. **Crumpacker, C. S.** 1992. Mechanism of action of foscarnet against viral polymerases. Am. J. Med. **92:**3S–7S.
8. **D'Aquila, R. T., V. A. Johnson, S. L. Welles, A. J. Japour, D. R. Kuritzkes, V. DeGruttola, P. S. Reichelderfer, R. W. Coombs, C. S. Crumpacker, J. O. Kahn, and D. D. Richman for the AIDS Clinical Trials Group Protocol 116B/117 Team and the Virology Committee Resistance Working Group.** 1995. Zidovudine resistance and HIV-1 disease progression during antiretroviral therapy. Ann. Intern. Med. **122:**401–408.
9. **Eriksson, B. F., and R. F. Schinazi.** 1989. Combinations of 3′-azido-3′-deoxythymidine (zidovudine) and phosphonoformate (foscarnet) against human immunodeficiency virus type 1 and cytomegalovirus replication in vitro. Antimicrob. Agents Chemother. **33:**663–669.
10. **Erlich, K. S., M. A. Jacobson, J. E. Koehler, S. E. Follansbee, D. P. Drennan, L. Goozé, S. Safrin, and J. Mills.** 1989. Foscarnet therapy for severe acyclovir-resistant herpes simplex virus type-2 infections in patients with the acquired immunodeficiency syndrome (AIDS). An uncontrolled trial. Ann. Intern. Med. **110:**710–713.
11. **Grob, P. M., J. C. Wu, K. A. Cohen, R. H. Ingraham, C.-K. Shih, K. D. Hargrave, T. L. McTague, and V. J. Merluzzi.** 1992. Nonnucleoside inhibitors of HIV-1 reverse transcriptase: nevirapine as a prototype drug. AIDS Res. Hum. Retroviruses **8:**145–152.
12. **Gurusinghe, A. D., S. A. Land, C. Birch, C. McGavin, D. J. Hooker, G. Tachedjian, R. Doherty, and N. J. Deacon.** 1995. Reverse transcriptase mutations in sequential HIV-1 isolates in a patient with AIDS. J. Med. Virol. **46:**238–243.
13. **Harada, S., Y. Koyanagi, and N. Yamamoto.** 1985. Infection of HTLV-III/LAV in HTLV-I-carrying cells MT-2 and MT-4 and application in a plaque assay. Science **229:**563–566.
14. **Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature (London) **373:**123–126.
15. **Hooker, D. J., G. Tachedjian, A. E. Solomon, A. D. Gurusinghe, S. Land, C. Birch, J. L. Anderson, B. M. Roy, E. Arnold, and N. J. Deacon.** An in vivo mutation from leucine to tryptophan at position 210 in human immunodeficiency virus type-1 reverse transcriptase contributes to high-level resistance to 3′-azido-3′-deoxythymidine (AZT). Submitted for publication.
16. **Hostetler, K. Y., G. D. Kini, T.-C. Chou, and D. D. Richman.** 1995. 1-0-Octadecylglycero-3-phosphonoformate exhibits enhanced activity and synergy with AZT against HIV, *in vitro*, abstr. I141, p. 230. *In* Abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
17. **Jacobo-Molina, A., J. Ding, R. G. Nanni, A. D. J. Clark, X. Lu, C. Tantillo, R. L. Williams, G. Kamer, A. L. Ferris, P. Clark, A. Hizi, S. H. Hughes, and E. Arnold.** 1993. Crystal structure of human immunodeficiency virus type I reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA. Proc. Natl. Acad. Sci. USA **90:**6320–6324.
18. **Jacobson, M. A., S. Crowe, J. Levy, F. Aweeka, J. Gambertoglio, N. McMa-**

nus, and J. Mills. 1988. Effect of foscarnet therapy on infection with human immunodeficiency virus in patients with AIDS. J. Infect. Dis. **158:**862–865.

19. **Japour, A. J., S. Welles, R. T. D'Aquila, V. A. Johnson, D. D. Richman, R. W. Coombs, P. S. Reichelderfer, J. O. Kahn, C. S. Crumpacker, and D. R. Kuritzkes for the AIDS Clinical Trials Group 116B/117 Study Team and the Virology Committee Resistance Working Group.** 1995. Prevalence and clinical significance of zidovudine resistance mutations in human immunodeficiency virus isolated from patients after long-term zidovudine treatment. J. Infect. Dis. **171:**1172–1179.
20. **Kaiser, L., L. Perrin, B. Hirschel, H. Furrer, J. Von Overbeck, M. Olmari, and S. Yerly.** 1995. Foscarnet decreases human immunodeficiency virus RNA. J. Infect. Dis. **172:**225–227.
21. **Kellam, P., C. A. B. Boucher, and B. A. Larder.** 1992. Fifth mutation in human immunodeficiency virus type 1 reverse transcriptase contributes to the development of high-level resistance to zidovudine. Proc. Natl. Acad. Sci. USA **89:**1934–1938.
22. **Kellam, P., C. A. B. Boucher, J. M. G. H. Tijnagel, and B. A. Larder.** 1994. Zidovudine treatment results in the selection of human immunodeficiency virus type 1 variants whose genotypes confer increasing levels of drug resistance. J. Gen. Virol. **75:**341–351.
23. **Kellam, P., and B. A. Larder.** 1995. Retroviral recombination can lead to linkage of reverse transcriptase mutations that confer increased zidovudine resistance. J. Virol. **69:**669–674.
24. **Koshida, R., L. Vrang, G. Gilljam, J. Harmenberg, B. Oberg, and B. Wahren.** 1989. Inhibition of human immunodeficiency virus in vitro by combinations of 3′-azido-3′-deoxythymidine and foscarnet. Antimicrob. Agents Chemother. **33:**778–780.
25. **Kozal, M. J., R. W. Shafer, M. A. Winters, D. A. Katzenstein, and T. C. Merigan.** 1993. A mutation in human immunodeficiency virus reverse transcriptase and decline in CD4 lymphocyte numbers in long-term zidovudine recipients. J. Infect. Dis. **167:**526–532.
26. **Kuritzkes, D. R., D. Shugarts, A. Griffin, M. Bakhtiari, J. Eron, J. Quinn, M. A. Fallon, M. Rubin, and S. Benoit.** 1995. Resistance to lamivudine (3TC) and zidovudine (ZDV) in a phase II comparative trial of 3TC vs ZDV vs 3TC plus ZDV. J. Acquired Immune Defic. Syndr. Hum. Retrovirol. **10**(Suppl. 3)**:**S26.
27. **Larder, B. A.** 1992. 3′-Azido-3′-deoxythymidine resistance suppressed by a mutation conferring human immunodeficiency virus type 1 resistance to nonnucleoside reverse transcriptase inhibitors. Antimicrob. Agents Chemother. **36:**2664–2669.
28. **Larder, B. A., G. Darby, and D. D. Richman.** 1989. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science **243:**1731–1734.
29. **Larder, B. A., P. Kellam, and S. D. Kemp.** 1991. Zidovudine resistance predicted by direct detection of mutations in DNA from HIV-infected lymphocytes. AIDS **5:**137–144.
30. **Larder, B. A., and S. D. Kemp.** 1989. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). Science **246:**1155–1158.
31. **Larder, B. A., S. D. Kemp, and P. R. Harrigan.** 1995. Potential mechanism of sustained antiretroviral efficacy of AZT-3TC combination therapy. Science **269:**696–699.
32. **Larder, B. A., S. D. Kemp, and D. J. Purifoy.** 1989. Infectious potential of human immunodeficiency virus type 1 reverse transcriptase mutants with altered inhibitor sensitivity. Proc. Natl. Acad. Sci. USA **86:**4803–4807.
33. **Larder, B. A., D. J. M. Purifoy, K. L. Powell, and G. Darby.** 1987. Site-specific mutagenesis of AIDS virus reverse transcriptase. Nature (London) **327:**716–717.
34. **Mayers, D. L., and J. Weir.** Personal communication.
35. **Mellors, J. W., H. Z. Bazmi, R. F. Schinazi, B. M. Roy, Y. Hsiou, E. Arnold, J. Weir, and D. L. Mayers.** 1995. Novel mutations in reverse transcriptase of human immunodeficiency virus type 1 reduce susceptibility to foscarnet in laboratory and clinical isolates. Antimicrob. Agents Chemother. **39:**1087–1092.
36. **Montaner, J. S., J. Singer, M. T. Schechter, J. M. Raboud, C. Tsoukas, M. O'Shaughnessy, J. Ruedy, K. Nagai, H. Salomon, B. Spira, and M. A. Wainberg.** 1993. Clinical correlates of *in vitro* HIV-1 resistance to zidovudine. Results of the Multicentre Canadian AZT Trial. AIDS **7:**189–196.
37. **Myers, G., B. Korber, S. Wain-Hobson, R. F. Smith, and G. N. Pavlakis.** 1993. Human retroviruses and AIDS: a compilation and analysis of nucleic acid and amino acid sequences. Theoretical Biology and Biophysics Group T-10, Los Alamos National Laboratory, Los Alamos, N.Mex.
38. **Nanni, R. G., J. Ding, A. Jacobo-Molina, S. H. Hughes, and E. Arnold.** 1993. Review of HIV-1 reverse transcriptase three-dimensional structure: implications for drug design. Perspect. Drug Discov. Des. **1:**129–150.
39. **Neate, E. V., R. C. Pringle, J. B. M. Jowett, D. S. Healey, and I. D. Gust.** 1987. Isolation of HIV from Australian patients with AIDS, AIDS related conditions and healthy antibody positive individuals. Aust. N. Z. J. Med. **17:**461–466.
40. **Nguyen, M. H., R. F. Schinazi, C. Shi, N. M. Goudgaon, P. M. McKenna, and J. W. Mellors.** 1994. Resistance of human immunodeficiency virus type 1 to acyclic 6-phenylselenenyl- and 6-phenylthiopyrimidines. Antimicrob. Agents Chemother. **38:**2409–2414.
41. **Nijhuis, M., D. de Jong, R. van Leeuwen, T. de Groot, W. Keulen, R. Shuurman, and C. Boucher.** 1995. The rise in HIV-1 RNA load during 3TC/AZT combination therapy is associated with the selection of viruses resistant to both 3TC and AZT. J. Acquired Immune Defic. Syndr. Hum. Retrovirol. **10**(Suppl. 3)**:**S25.
42. **Oberg, B.** 1989. Antiviral effects of phosphonoformate (PFA, foscarnet sodium). Pharmacol. Ther. **40:**213–285.
43. **Ragni, M. V., D. A. Amato, M. L. LoFaro, V. DeGruttola, C. Van Der Horst, M. E. Eyster, C. M. Kessler, G. F. Gjerset, M. Ho, D. M. Parenti, U. Dafni, S. Rasheed, J. A. Korvick, T. C. Merigan, and the AIDS Clinical Trials Group.** 1995. Randomized study of didanosine monotherapy and combination therapy with zidovudine in hemophilic and nonhemophilic subjects with asymptomatic human immunodeficiency virus-1 infection. Blood **85:**2337–2346.
44. **Richman, D. D., D. Havlir, J. Corbeil, D. Looney, C. Ignacio, S. A. Spector, J. Sullivan, S. Cheeseman, K. Barringer, D. Pauletti, C.-K. Shih, M. Myers, and J. Griffin.** 1994. Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. J. Virol. **68:**1660–1666.
45. **Rocancourt, D., C. Bonnerot, H. Jouin, M. Emerman, and J. F. Nicolas.** 1990. Activation of a β-galactosidase recombinant provirus: application to titration of human immunodeficiency virus (HIV) and HIV-infected cells. J. Virol. **64:**2660–2668.
46. **Safrin, S., T. G. Berger, I. Gilson, P. R. Wolfe, C. B. Wofsy, J. Mills, and K. K. Biron.** 1991. Foscarnet therapy in five patients with AIDS and acyclovir-resistant varicella-zoster virus infection. Ann. Intern. Med. **115:**19–21.
47. **Safrin, S., C. Crumpacker, P. Chatis, R. Davis, R. Hafner, J. Rush, H. A. Kessler, B. Landry, and J. Mills.** 1991. A controlled trial comparing foscarnet with vidarabine for acyclovir-resistant mucocutaneous herpes simplex in the acquired immunodeficiency syndrome. The AIDS Clinical Trials Group. N. Engl. J. Med. **325:**551–555.
48. **Sandstrom, E. G., R. E. Byington, J. C. Kaplan, and M. S. Hirsch.** 1985. Inhibition of human T-cell lymphotropic virus type III *in vitro* by phosphonoformate. Lancet **i:**1480–1482.
49. **Schuurman, R., M. Nijhuis, R. van Leeuwen, P. Schipper, D. de Jong, P. Collis, S. A. Danner, J. Mulder, C. Loveday, C. Christopherson, S. Kwok, J. Sninsky, and C. A. B. Boucher.** 1995. Rapid changes in human immunodeficiency virus type 1 RNA load and appearance of drug-resistant virus populations in persons treated with lamivudine (3TC). J. Infect. Dis. **171:**1411–1419.
50. **Shafer, R. W., A. K. N. Iversen, M. A. Winters, E. Aguiniga, D. A. Katzenstein, T. C. Merigan, and the AIDS Clinical Trials Group 143 Virology Team.** 1995. Drug resistance and heterogenous long-term virologic responses of human immunodeficiency virus type 1-infected subjects to zidovudine and didanosine combination therapy. J. Infect. Dis. **172:**70–78.
51. **Shafer, R. W., M. J. Kozal, M. A. Winters, A. K. N. Iversen, D. A. Katzenstein, M. V. Ragni, W. A. Meyer, P. Gupta, S. Rasheed, R. Coombs, M. Katzman, S. Fiscus, and T. C. Merigan.** 1994. Combination therapy with zidovudine and didanosine selects for drug resistant human immunodeficiency virus type 1 strains with unique patterns of *pol* gene mutations. J. Infect. Dis. **169:**722–729.
52. **Shirasaka, T., M. F. Kavlick, T. Ueno, W.-Y. Gao, E. Kojima, M. L. Alcaide, S. Chokekijchai, B. M. Roy, E. Arnold, R. Yarchoan, and H. Mitsuya.** 1995. Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides. Proc. Natl. Acad. Sci. USA **92:**2398–2402.
53. **Starnes, M. C., and Y. C. Cheng.** 1988. Inhibition of human immunodeficiency virus reverse transcriptase by 2′,3′-dideoxynucleoside triphosphates: template dependence, and combination with phosphonoformate. Virus Genes **2:**241–251.
54. **St. Clair, M. H., J. L. Martin, G. Tudor-Williams, M. C. Bach, C. L. Vavro, D. M. King, P. Kellam, S. D. Kemp, and B. A. Larder.** 1991. Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. Science **253:**1557–1559.
55. **Studies, of Ocular Complications of AIDS Research Group (SOCA), AIDS Clinical Trials Group.** 1992. Mortality in patients with the acquired immunodeficiency syndrome treated with either foscarnet or ganciclovir for cytomegalovirus retinitis. N. Engl. J. Med. **326:**213–220.
56. **Tachedjian, G.** Unpublished data.
57. **Tachedjian, G., D. J. Hooker, A. D. Gurusinghe, H. Bazmi, N. J. Deacon, J. Mellors, C. Birch, and J. Mills.** 1995. Characteristion of foscarnet-resistant strains of human immunodeficiency virus type 1. Virology **212:**58–68.
58. **Tachedjian, G., J. Hoy, K. McGavin, and C. Birch.** 1994. Long-term foscarnet therapy not associated with the development of foscarnet-resistant human immunodeficiency virus type 1 in an acquired immunodeficiency syndrome patient. J. Med. Virol. **42:**207–211.
59. **Tachedjian, G., D. Tyssen, S. Locarnini, I. Gust, and C. Birch.** 1990. Investigation of topoisomerase inhibitors for activity against human immunodeficiency virus: inhibition by coumermycin A1. Antiviral Chem. Chemother. **1:**131–138.
60. **Tachedjian, M., J. Krywult, R. J. Moore, and A. L. M. Hodgson.** 1995. Caseous lymphadenitis vaccine development: site-specific inactivation of the

Corynebacterium pseudotuberculosis phospholipase D gene. Vaccine **13:**1785–1792.

61. **Tantillo, C., J. Ding, A. Jacobo-Molina, R. G. Nanni, P. L. Boyer, S. H. Hughes, R. Pauwels, K. Andries, P. A. J. Janssen, and E. Arnold.** 1994. Locations of anti-AIDS drug binding sites and resistance mutations in the three-dimensional structure of HIV-1 reverse transcriptase. Implications for mechanisms of drug inhibition and resistance. J. Mol. Biol. **243:**369–387.
62. **Tisdale, M., S. D. Kemp, N. R. Parry, and B. A. Larder.** 1993. Rapid *in vitro* selection of human immunodeficiency virus type 1 resistant to 3′-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. Proc. Natl. Acad. Sci. USA **90:**5653–5656.
63. **Tudor-Williams, G., M. H. St. Clair, R. E. McKinney, M. Maha, E. Walter, S. Santacroce, M. Mintz, K. O'Donnell, T. Rudoll, C. L. Vavro, E. M. Connor, and C. M. Wilfert.** 1992. HIV-1 sensitivity to zidovudine and clinical outcome in children. Lancet **339:**15–19.
64. **Vrang, L., and B. Öberg.** 1986. PP_i analogs as inhibitors of human T-lymphocyte virus type III reverse transcriptase. Antimicrob. Agents Chemother. **29:**867–872.
65. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, M. S. Saag, and G. M. Shaw.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature (London) **373:**117–122.

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Impaired Fitness of Foscarnet Resistant Strains of Human Immunodeficiency Virus Type 1

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Running Title: Foscarnet-resistant strains of HIV-1 are less fit

Abstract

Foscarnet (PFA) is a pyrophosphate analogue antiviral active against human immunodeficiency virus (HIV-1) and herpesviruses. Strains of HIV-1 resistant to PFA have mutations in the HIV-1 reverse transcriptase (RT). We examined the influence of PFA resistance mutations, in different genetic backgrounds, on HIV-1 replication competency by both replication kinetics and growth competition assays. In replication kinetics assays the recombinant strains HX89K, HX92I and HX156A (with RT mutations, E89K, L92I and S156A, respectively, in the HXB2-D genetic background) replicated to lower titres than the wild-type parent in the absence of drug. Replication impairment increased as the degree of PFA resistance increased. PFA-resistant strains LAI 92I and LAI 156A (with RT mutations L92I and S156A, respectively) were replication impaired in comparison to the wild-type parent LAI to a similar degree as observed for strains in the HXB2-D background. In growth competition assays with wild-type LAI, strains LAI 92I and LAI 156A had relative fitness values of 0.5 and 0.8, respectively. These results show that the RT mutations E89K, L92I and S156A, observed in PFA-resistant strains selected in cell culture, reduce replication competence. Furthermore, these data show a correlation of increasing PFA resistance and decreasing replication competence mediated by single amino acid substitutions in the RT.

Foscarnet (PFA) is an inhibitor of viral DNA polymerases, including the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT). PFA inhibits HIV-1 replication *in vitro* and in patients.[1-3] PFA-resistant strains of HIV-1 emerge rapidly in cell culture in the presence of PFA[4-6] and, as predicted by these *in vitro* studies, PFA-resistant strains of HIV-1 have developed in patients with AIDS receiving long-term PFA therapy for the treatment of cytomegalovirus retinitis.[4]

PFA-resistant strains of HIV-1 selected by *in vitro* passage have single or double amino acid substitutions in the reverse transcriptase (RT) coding region which include, W88S, W88G, E89K, L92I, S156A, and Q161L/H208Y.[4-6] Except for Q161L, these mutations are located at a distance from the putative dNTP binding site in the three dimensional structure of HIV-1 RT. It has been hypothesized that most of these mutations mediate PFA resistance by altering binding of the template strand of the template-primer with RT leading to greater discrimination between the natural substrate, pyrophosphate (PPi) and PFA.[4, 5] These mutations impart only modest (2- to 14- fold) PFA resistance[4-6], which is in contrast to the >100-fold resistance achieved by mutations conferring decreased susceptibility to other HIV-1 RT inhibitors such as zidovudine and lamivudine.[7, 8]

Acquisition of mutations mediating drug resistance generally impairs viral replication kinetics. HIV-1 strains resistant to lamivudine or the nonnucleoside reverse transcriptase inhibitors (NNRTIs) S-2720 and (alkylamino)piperidine bis(heteroaryl)piperizine (AAP-BHAP) with RT mutations at codons 184 and 190, respectively, were replication impaired compared to wild-type strains.[9, 10] Mutations in the HIV-1 RT coding region that confer zidovudine resistance and which have also been shown to impart reduced fitness include T215Y and K219Q[11], T215Y[12], M41L[12] and M41L and T215Y[12]. Selection of HIV-1 strains

resistant to protease inhibitors requires multiple mutations for high-level resistance.[13] These mutations have been shown to cause impaired protease activity and decreased replication competence despite the appearance of compensatory mutations in the *gag* gene.[14]

PFA-resistant HIV-1 RT enzymes with different substitutions at codon 89 generally have impaired enzyme activity.[15] Mutagenesis of the HIV-1 RT to define important functional sites also generated PFA-resistant RT from mutations at codons 113, 114, 115, 151, 154 and 183; increasing PFA resistance was associated with decreasing RT activity.[16,17] Notably, infectious virus could not be recovered with any mutation which reduced RT activity by >80 % of wild-type.[18] Taken together, these studies suggest that mutations in RT that decrease susceptibility to PFA also impair viral growth kinetics.[9,10,19,20,21] To address this issue directly we examined the replication capacity of PFA-resistant strains of HIV-1, with RT mutations observed in *in vitro* selected strains, by both replication kinetic and growth competition assays.

We initially examined the replication kinetics of the recombinant PFA-resistant strains HX89K, HX92I and HX156A in parallel with the corresponding wild-type strain, HX (Fig. 1A). Strains HX89K, HX92I and HX156A have the PFA resistance mutations, E89K, L92I and S156A, respectively, which had been introduced by site-directed mutagenesis into the HXB2-D genetic background.[6] These strains exhibit a 12-, 7-, and 4- fold PFA resistance compared to strain HX as measured in the HT4LacZ-1 cell line.[6] Replication kinetics assays were performed in MT-2 cells[22] which were cultured in RPMI 1640 medium as previously described.[23] At least two independent assays were performed with each mutant. The titres of each HIV-1 inoculum were determined in every assay by end-point titration and $TCID_{50}$ were calculated using the Karber formula.[24] Culture supernates were removed daily from duplicate wells for each isolate, until viral CPE involved 100% of the

cells (6 - 8 days post-infection). Supernates collected on each day were titred for infectious virus in the HT4LacZ-1 cell line as previously described.[25] When MT-2 cells were infected with equivalent amounts of each virus [500 $TCID_{50}$ / 120 000 cells; multiplicity of infection (MOI) of 0.004] there was no difference in the time to peak infectious virus production (Fig. 1A), but there was a consistent reduction in peak infectious virus production by strains HX89K, HX92I and HX156A compared to HX (4.7 ± 1.8 - , 2.3 ± 0.5 - and 1.6 ± 0.5- fold reduction, respectively). Increasing PFA resistance was associated with increasing impairment of virus replication (r = 0.95; P = 0.05 by linear regression analysis). Serial passage of HX92I in the absence of drug resulted in a strain which had reverted to PFA susceptibility, confirming the reduced fitness of HX92I compared with wild-type (data not shown).

The HXB2-D infectious molecular clone of HIV-1 does not express the HIV-1 accessory proteins Vpu, Vpr and Nef.[26] Since defects in at least one of these proteins, Nef, effect proviral DNA synthesis[27], the impact of PFA resistance mutations in a genetic background containing a full complement of functional viral proteins needed to be assessed. In addition, since these PFA-resistant strains were recovered by homologous recombination of a 4.3-kb *Hin*dIII fragment of HXB2-D (used as the target for site-directed mutagenesis) with the molecular clone pKPHXB2ΔRT, mutations outside of the HIV-1 RT coding region, including *gag*, *pol*, *vif*, *vpr*, *rev*, *vpu*, and *tat* (present in the *Hin*dIII fragment), might contribute to PFA resistance. To exclude these possibilities, we investigated the effect of the L92I and S156A mutations on replication kinetics when inserted into a common LAI genetic background. This infectious molecular clone has a full complement of functional HIV-1 genes and was derived by mutagenesis of a 1.4-kb *Xma*I - *Xba* I *pol* fragment which was cloned, after mutagenesis, into pXXHIV-1_{LAI}.[6,28]

When MT-2 cells were infected with equivalent amounts of each virus (500 $TCID_{50}$ / 120 000 cells; MOI = 0.004) PFA-resistant strains clearly replicated less well than

wild-type, PFA-susceptible parental virus (Fig 1B). At day 5 postinfection, strains LAI 92I and LAI 156A produced 3.0 ± 0.4 - and 1.8 ± 0.3 - fold less infectious virus than the wild-type parent (Fig. 1B). These data were similar to that observed for the replication kinetics of the PFA-resistant clones HX92I and HX156A, relative to HX, thus confirming that the L92I and S156A substitutions are sufficient to impair virus fitness. However, when the experiment was repeated at a higher MOI of 0.017 (2000 $TCID_{50}$/120 000 cells), the PFA-resistant strains LAI 92I and LAI 156A appeared to replicate as well as the wild-type strain (Fig 1C); LAI 92I and LAI 156A produced only 1.2- and 1.3- fold, respectively, less infectious virus than wild-type parental virus after six days. These data show that replication kinetics assays are influenced by the MOI used, and that the presence of functional accessory proteins does not influence PFA resistance.

To assess the relative fitness of LAI 92I and LAI 156A compared to LAI, MT-2 cells were infected with a 1:1 mixture of wild-type and mutant strain (i.e. a mixture containing infectious titres of both viruses at an MOI of 0.01 PFU per cell). Titres were determined in HT4LacZ-1 cells. Infected cells were incubated without drug and progeny virus was serially passaged 5 and 8 times for the LAI:LAI 92I and LAI:LAI 156A mixtures, respectively. At each passage, chromosomal DNA from infected cells was purified as previously described[6] and the HIV-1 RT coding region (codons 1- 244) was PCR amplified from proviral DNA with M13-tailed primers using the Expand™ High Fidelity PCR system (Boehringer Mannheim, Germany). The sequences of the forward and reverse primer pairs, designated M13 5'v2 and M13Rcomb3, were as published.[6] Amplification of HIV-1 proviral DNA was performed in 100-µl volumes containing 10 µl of purified DNA in the presence of 2.6U of a mixture of *Taq* and *Pwo* DNA polymerases, 200 µM of each dNTP, 300 nM of each primer and 1.5 mM $MgCl_2$. Amplification conditions comprised one denaturation cycle (94°C for 2 min) followed by 10 cycles of denaturation (94°C for 15 sec), annealing (50°C for 30 sec) and extension (72°C

for 1 min), followed by a further 25 cycles as the previous 10 cycles, with the addition of 20 sec to each subsequent extension. PCR amplimers were purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol. The ABI PRISM™ Dye Primer Cycle Sequencing Ready Reaction kits with AmpliTaq DNA Polymerase FS (Perkin Elmer, Foster City, CA) with either M13 forward or reverse primers were used for sequencing the M13-tailed amplimers. The sequences of the sense and complementary strands of the amplimers were analysed to detect codons 92 and 156, respectively. The proportion of mutant and wild-type strains was determined by dividing the peak heights representing the mutant and wild-type nucleotides by the sum of these peak heights obtained from the same electropherogram.

The relative fitness of strains LAI 92 and LAI 156 compared to LAI was calculated using the formula: $p(t) = p(0)/q(0) \times (f)^N \times q(t)$: where p(t) and q(t) represent the proportions of the less fit and more fit population at time t, p(0) and q(0) are the proportions of these populations at time 0, N is the number of generations, and f is the relative fitness of the less fit population with respect to the more fit population as previously described.[12, 29] This equation describes the effects of selection at a single locus in an asexual haploid population, with the assumption of discrete generations of virus replication.[29] The number of generations, N, was assumed to be 2 for a 6 day passage.[12] The relative fitness of the less fit population was calculated from the slope of the plot ln(p/q) versus N [where the slope = ln(f)].

At passage one, the relative proportions of LAI to LAI 92I was 0.6 to 0.4 (Fig. 2). With further passage in the absence of drug the proportion of wild-type sequence at codon 92 (TTA) increased continuously and smoothly, and after 5 passages the wild-type virus had become the predominant species in the mixture (Fig. 2). From this experiment, the LAI 92I strain was calculated to have a relative fitness of

0.5 ± 0.14 compared to wild-type (set at 1.0). As a control to ensure that LAI 92I was genetically stable over the course of the experiment, a pure population of LAI 92I was passaged 7 times in MT-2 cells. No nucleotide substitutions other than L92I were detected in RT codons 1 through to 142.

When MT-2 cells were infected with 1:1 mixtures of LAI 156A and LAI, the relative proportions of LAI to LAI 156A after one passage in the absence of drug was 0.6 to 0.4 (Fig. 3). Continued passage of the mixture in the absence of drug resulted in outgrowth of wild-type sequence at codon 156 (TCA) over the mutant sequence (GCA), and after 8 passages wild-type virus predominated (Fig. 3). The relative fitness of LAI 156A compared to wild-type was 0.8 ± 0.13. A clonal population of LAI 156A, passaged 8 times in the absence of drug, was genetically stable at RT codons 100 through to 200.

This is the first report demonstrating that substitutions in RT (E89K, L92I and S156A) that impart resistance to the pyrophosphate analogue, PFA, reduce the replication fitness of HIV-1. Growth competition assays showed conclusively that PFA-resistant strains of HIV-1 with either the L92I or S156A have impaired replication fitness compared to the otherwise isogenic wild-type strain. PFA resistance substitutions impaired replication fitness to a similar degree in both LAI and HXB2-D genetic backgrounds, suggesting that defects in virion accessory proteins, including Nef, do not contribute to the effects of these RT mutations.

The degree to which replication of PFA-resistant HIV-1 strains were impaired increased in the order HX89K>HX92I>HX156A, exactly corresponding with increasing levels of PFA resistance ($r = 0.95$; $P = 0.05$). A similar pattern was observed for PFA-resistant strains in the LAI genetic background as LAI 92I was less fit than LAI 156A. In addition, the relative fitness for these strains was consistent with LAI 92I exhibiting a greater impairment of replication kinetics than

LAI 156A. Our calculations show a strong positive correlation ($r = 0.82$; $P = 0.004$, linear regression analysis) between the degree of PFA resistance and the relative reduction in *in vitro* RT activity for the 10 mutations at codons 113, 114, 115 and 151 previously reported by Larder and colleagues to reduce RT susceptibility to PFA.[16,18] A similar calculation by us yielded a positive though statistically insignificant correlation ($r = 0.45$; $P = 0.17$, linear regression analysis) between reduction in *in vitro* RT activity and PFA resistance for 10 mutations at codon 89.[15] Our data, together with those of Larder and colleagues[16,18] and Song and colleagues[15], suggest a relationship between the degree of PFA resistance conferred by single amino acid substitutions and impairment of RT activity. A recent study has shown that single amino acid substitutions in the domain II of CMV polymerase, V715M and T700A, were responsible for both PFA resistance and a slow growth phenotype[30] perhaps suggesting that the impairment of DNA polymerase activity by mutations conferring PFA resistance may not be confined to HIV-1 RT. A previous study[4] demonstrated that a PFA-resistant strain selected in cell culture with the Q161L/H208Y substitutions had similar replication kinetics to the wild-type strain HIV-1_{LAI}. It is possible that either subtle differences in replication kinetics was not observed by the assay system used or that no differences exist. The mutations conferring PFA resistance that were examined in this study were those observed in PFA-resistant strains of HIV-1 selected in cell culture.[5] Since the pool size and turnover rate of HIV-1 *in vivo* is much greater in cell culture, *in vitro* selection identifies only the most frequent mutants and not necessarily those with optimum fitness[31] which may also contain compensatory mutations.[32] Growth competition assays using molecular clones with PFA resistance mutations observed in patient isolates (W88S, W88G, Q161L, H208Y) will be required to establish if impaired fitness is a feature of all mutations conferring PFA resistance. Nevertheless, it is possible that second site mutations may be selected *in vivo*, that compensate for the impaired replication capacity imparted by single amino acid substitutions in the RT.

A previous study with the BH-10 HIV-1 molecular clone showed that insertion of S156A into the HIV-1 RT abolished RNase H activity while leaving the polymerase activity intact, suggesting that such a virus would not be viable.[33] It has been suggested that S156A abolishes RNase H activity by repositioning the template-primer on the HIV-1 RT to a position inconsistent with favourable catalysis of the RNA:DNA substrate at the RNase H active site.[33] However, we have shown in this study that both the HX and LAI strains of HIV-1 with the S156A mutation were viable, albeit with slightly impaired fitness; the replication competence of these mutants was not due to mutations in other regions of the HIV-1 RT coding region, although the involvement of compensatory mutations in other regions of the HIV-1 genome cannot be excluded.

Coffin[34] has postulated that drug resistance mutations that confer a selective disadvantage to the virus could result in the re-establishment of a lower steady state level of virus replication compared to that present before drug selection, resulting in a delay in disease progression in treated individuals. It has been hypothesized that the impaired fitness imparted by mutations conferring lamivudine resistance, M184V and M184I, is one possible explanation for the apparent long-term *in vivo* antiviral activity of lamivudine monotherapy despite drug-resistant mutants comprising 100% of the viral population within 12 weeks.[35] With the caveat that PFA-resistant strains *in vivo* are replication impaired, such strains could similarly result in lower levels of virus production in PFA-treated patients even after the emergence of strains with reduced susceptibility. Furthermore, mutations that confer PFA resistance have been shown to phenotypically reverse zidovudine resistance and result in delayed emergence of strains co-resistant to AZT and PFA *in vitro* .[6] Although PFA is far from an ideal antiretroviral[36] our studies suggest that it has desirable properties as an RT inhibitor. Development of PFA prodrugs or

other inhibitors of pyrophosphate exchange with improved pharmacological properties should be considered.

Acknowledgements

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References

1. Kaiser L, Perin L, Hirschel B, Furrer H, Von Overbeck J, Olmari M, and Yerly S: Foscarnet decreases human immunodeficiency virus RNA. J Infect Dis 1995;172:225-227.

2. Jacobson MA, Crowe S, Levy J, Aweeka F, Gambertoglio J, McManus N, and Mills J: Effect of foscarnet therapy on infection with human immunodeficiency virus in patients with AIDS. J Infect Dis 1988; 158:862-865.

3. Fletcher CV, Collier AC, Rhame FS, Bennett D, Para MF, Beatty CC, Jones CE, and Balfour HH Jr: Foscarnet for suppression of human immunodeficiency virus replication. Antimicrob Agents Chemother 1994; 38:604-607.

4. Mellors JW, Bazmi HZ, Schinazi RF, Roy BM, Hsiou Y, Arnold E, Weir J, and Mayers DL: Novel mutations in the reverse transcriptase of human immunodeficiency virus type 1 reduce susceptibility to foscarnet in laboratory and clinical isolates. Antimicrob Agents Chemother 1995; 39: 1087-1092.

5. Tachedjian G, Hooker DJ, Gurusinghe AD, Bazmi H, Deacon NJ, Mellors J, Birch C, and Mills J: Characterisation of foscarnet-resistant strains of human immunodeficiency virus type. Virology 1995; 212:58-68.

6. Tachedjian G, Mellors J, Bazmi H, Birch C, and Mills J: Zidovudine resistance is suppressed by mutations conferring resistance of human immunodeficiency virus type 1 to foscarnet. J Virol 1996; 70:7171-7181.

7. Larder BA and Kemp SD: Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). Science 1989; 246:1155-1158.

8. Boucher CA, Cammack N, Schipper P, Schuurman R, Rouse P, Wainberg MA, and Cameron JM: High-level resistance to (-) enantiomeric 2'-deoxy-3'-thiacytidine in vitro is due to one amino acid substitution in the catalytic site of human immunodeficiency virus type 1 reverse transcriptase. Antimicrob Agents Chemother 1993; 37:2231-2234.

9. Larder BA, Kemp SD, and Harrigan PR: Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. Science 1995; 269:696-699.

10. Olmsted RA, Slade DE, Kopta LA, Poppe SM, Poel TJ, Newport SW, Rank KB, Biles C, Morge RA, Dueweke TJ, Yagi Y, Romero DL, Thomas RC, Sharma SK, and Tarpley WG: (Alkylamino) piperidine bis(heteroaryl)piperizine analogs are potent, broad-spectrum nonnucleoside reverse transcriptase inhibitors of drug-resistant isolates of human immunodeficiency virus type 1 (HIV-1) and select for drug-resistant variants of HIV-1IIIB with reduced replication phenotypes. J Virol 1996; 70:3698-3705.

11. Chow YK, Hirsch MS, Merrill DP, Bechtel LJ, Eron JJ, Kaplan JC, and D'Aquila RT: Use of evolutionary limitations of HIV-1 multidrug resistance to optimize therapy. Nature 1993; 361:650-654.

12. Harrigan P, Bloor S, and Larder B: Quantitation of viral fitness and AZT selection pressure of AZT resistant HIV-1 variants. Antiviral Therapy 1996; 1 Suppl. 1:55.

13. Molla A, Korneyeva M, Gao Q, Vasavanonda S, Schipper PJ, Mo HM, Markowitz M, Chernyavskiy T, Niu P, Lyons N, Hsu A, Granneman GR, Ho DD, Boucher CA, Leonard JM, Norbeck DW, and Kempf DJ: Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. Nat Med 1996; 2:760-766.

14. Croteau G, Doyon L, Thibeault D, McKercher G, Pilote L, and Lamarre D: Impaired fitness of human immunodeficiency virus type 1 with high-level resistance to protease inhibitors. J Virol 1997; 71:1089-1096.

15. Song Q, Yang G, Goff SP, and Prasad VR: Mutagenesis of the Glu-89 residue in human immunodeficiency virus type 1 (HIV-1) and HIV-2 reverse transcriptases: effects on nucleoside analog resistance. J Virol 1992;66:7568-7571.

16. Larder B, Purifoy DJM, Powell KL, and Darby G. Site-specific mutagenesis of AIDS virus reverse transcriptase. Nature (London) 1987; 327:716-717.

17. Lowe DM, Parmar V, Kemp SD, and Larder BA: Mutational analysis of two conserved sequence motifs in HIV-1 reverse transcriptase. FEBS Lett 1991; 282: 231-234.

18. Larder BA, Kemp SD, and Purifoy DJ: Infectious potential of human immunodeficiency virus type 1 reverse transcriptase mutants with altered inhibitor sensitivity. Proc Natl Acad Sci USA 1989; 86:4803-4807.

19. Boyer PL and Hughes SH: Analysis of mutations at position 184 in reverse transcriptase of human immunodeficiency virus type 1. Antimicrob Agents Chemother 1995; 39:1624-1628.

20. Back NK, Nijhuis M, Keulen W, Boucher CA, Oude Essink BO, van Kuilenburg AB, van Gennip AH, and Berkhout B: Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme. EMBO J 1996;15:4040-4049.

21. Kleim JP, Bender R, Kirsch R, Meichsner C, Paessens A, and Riess G: Mutational analysis of residue 190 of human immunodefiency virus type 1 reverse transcriptase. Virology 1994; 200:696-701.

22. Harada S, Koyanagi Y, and Yamamoto N: Infection of HTLV-III/LAV in HTLV-1-carrying cells MT-2 and MT-4 and application in a plaque assay. Science 1985; 229:563-566.

23. Tachedjian G, Tyssen D, Locarnini S, Gust I, and Birch C: Investigation of topoisomerase inhibitors for activity against human immunodeficiency virus: inhibition by coumermycin A1. Antiviral Chem Chemother 1990;1:131-138.

24. Hawkes R. General principles underlying laboratory diagnosis of viral infections: In Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections. 5th ed. Am. Public Health Assoc. Washington DC, 1979, 34 - 35.

25. Rocancourt D, Bonnerot C, Jouin H, Emerman M, and Nicolas JF: Activation of a B-galactosidase recombinant provirus: application to titration of human immunodeficiency virus (HIV) and HIV-infected cells. J Virol 1990; 64:2660-2668.

26. Myers G, Korber B, Wain-Hobson S, Smith R, and Pavlakis G: Human retroviruses and AIDS. A compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory: Theoretical Biology and Biophysics, Los Alamos, N.M. 1993;76-98.

27. Aitken C and Trono D: Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. J Virol 1995;69:5048-5056.

28. Nguyen MH, Schinazi RF, Shi C, Goudgaon NM, McKenna PM, and Mellors JW: Resistance of human immunodeficiency virus type 1 to acyclic 6-phenylselenenyl- and 6-phenylthiopyrimidines. Antimicrob Agents Chemother 1994; 38:2409-2414.

29. Goudsmit J, De Ronde A, Ho DD, and Perelson AS: Human immunodeficiency virus fitness in vivo: calculations based on a single zidovudine resistance mutation at codon 215 of reverse transcriptase. J Virol 1996; 70: 5662-5664.

30. Baldanti F, Underwood MR, Stanat SC, Biron KK, Chou S, Sarasini A, Silini E, and Gerna G: Single amino acid changes in the DNA polymerase confer foscarnet resistance and slow-growth phenotype, while mutations in the UL97-encoded phosphotransferase confer ganciclovr resistance in three double-resistant human cytomegalovirus strains recovered from patients with AIDS. J Virol 1996;70:1390-1395.

31. Keulen W, Boucher C, and Berkhout B: Nucleotide substitution patterns can predict the requirements for drug-resistance of HIV-1 proteins. Antiviral Research 1996; 31:45-57.

32. Ho DD, Toyoshima T, Mo H, Kempf DJ, Norbeck D, Chen CM, Wideburg NE, Burt SK, Erickson JW, and Singh MK: Characterization of human immunodeficiency virus type 1 variants with increased resistance to a C2-symmetric protease inhibitor. J Virol 1994; 68:2016-2020.

33. Boyer PL, Ferris AL, and Hughes SH: Cassette mutagenesis of the reverse transcriptase of human immunodeficiency virus type 1. J Virol 1992; 66:1031-1039.

34. Coffin JM: HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science 1995; 267:483-489.

35. Schuurman R, Nijhuis M, van Leeuwen R, Schipper P, de Jong D, Collis P, Danner SA, Mulder J, Loveday C, Christopherson C, Kwok S, Sninsky J, and Boucher CAB: Rapid changes in human immunodeficiency virus type 1 RNA load and appearance of drug-resistant virus populations in persons treated with lamivudine (3TC). J Infect Dis 1995; 171:1411-1419.

36. Oberg B. Antiviral effects of phosphonoformate (PFA, foscarnet sodium). Pharmac Ther 1989; 40 :213-285.

Figure 1. Replication kinetics of PFA-resistant strains of HIV-1 in MT-2 cells infected at low MOI. Virus titres were measured by plaque assay of culture supernates from MT-2 cells infected at equivalent multiplicities with the wild-type strain HX or the PFA-resistant strains HX89K, HX92I and HX156A (A). Virus titres in culture supernates of MT-2 cells following infection with the wild-type strain, LAI or the PFA-resistant strains LAI 92I and LAI 156A at an MOI of 0.004 (B) or 0.017 (C), respectively. Data from representative assays of at least 2 performed are shown.

Figure 2. Change in the relative proportions of mutant (LAI 92I) and wild-type HIV-1 (LAI) sequences with sequential passage of a mixture in cell culture. Growth competition was performed in MT-2 cells starting with a mixture of equal amounts of LAI and LAI 92I which was then passaged 5 times in the absence of drug.

Figure 3. Change in the relative proportions of mutant (LAI 156A) and wild-type HIV-1 (LAI) sequences with sequential passage of a mixture in cell culture. Growth competition experiments were performed in MT-2 cells, starting with a mixture of equal titres of LAI and LAI 156A which was subsequently passaged 8 passages in the absence of drug.

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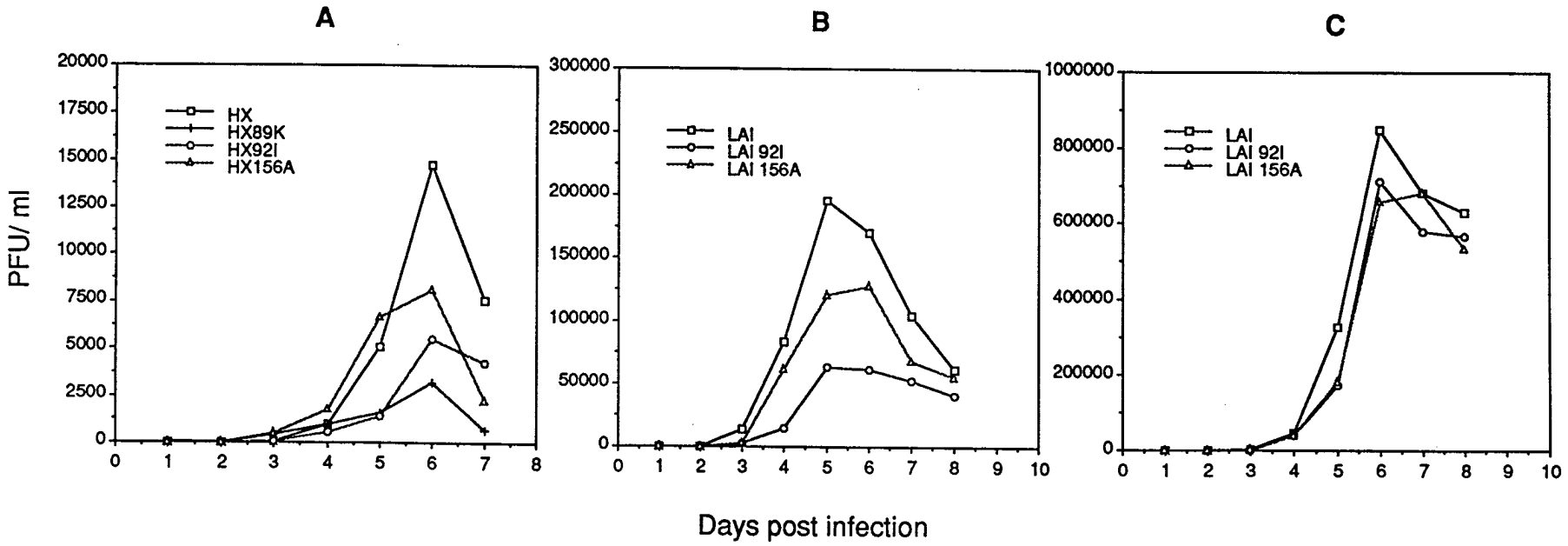
A
B
C
PFU/ ml
Days post infection
HX
HX89K
HX92I
HX156A
LAI
LAI 92I
LAI 156A
20000
17500
15000
12500
10000
7500
5000
2500
0
300000
250000
200000
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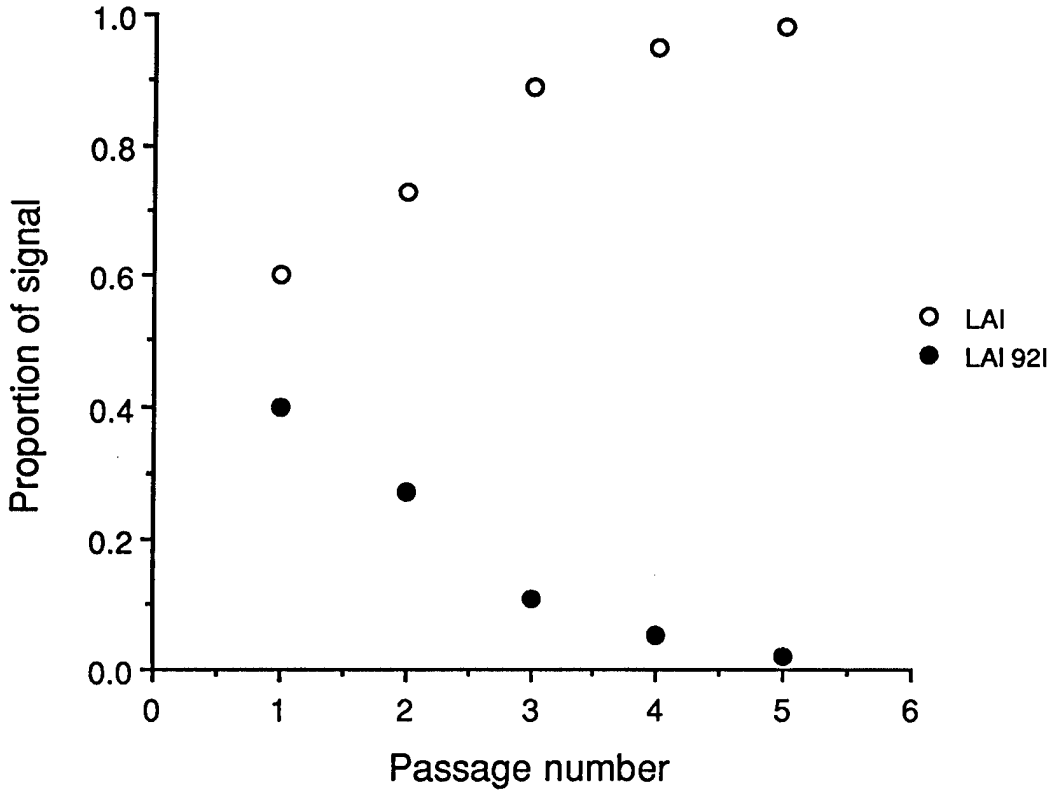
1.0
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Proportion of signal
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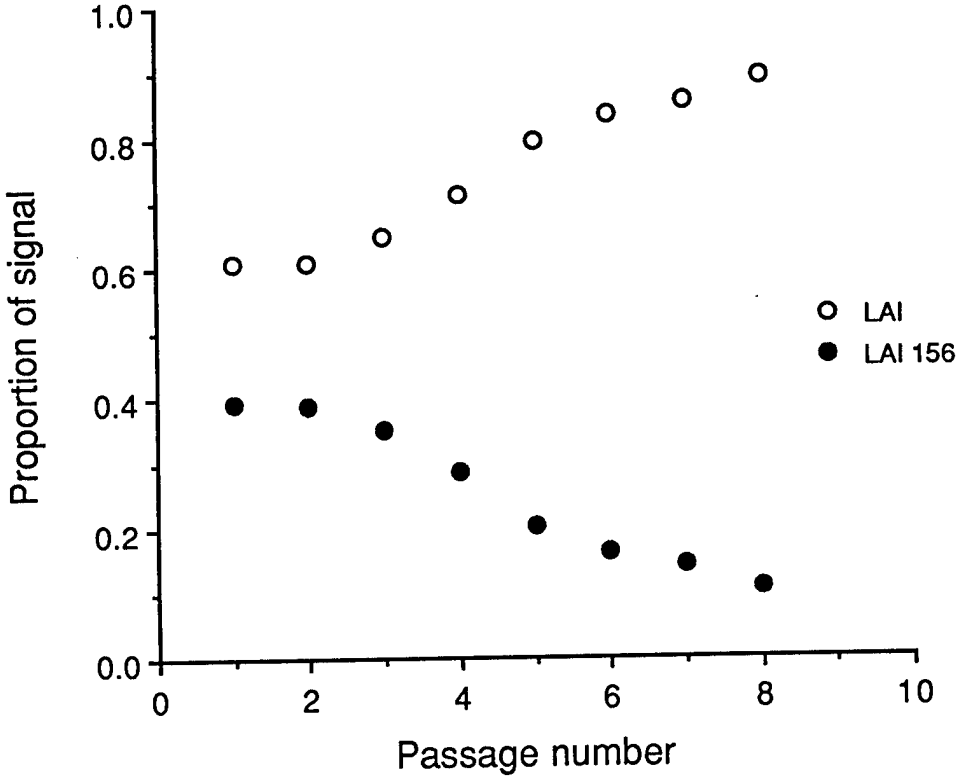
LAI 92I


1.0
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Proportion of signal
0
2
4
6
8
10
Passage number
LAI
LAI 156

K65R Mutation in HIV-1 Reverse Transcriptase Causes Resistance To (-)-β-D-Dioxolane-Guanosine and Reverses AZT Resistance

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ABSTRACT

HIV-1 variants resistant to DXG, a potent and selective nucleoside analog HIV-1 RT inhibitor, was selected twice by serially passaging HIV-1_{LAI} in increasing concentrations of drug (30 M maximum). After 13 and 14 passages, variants with a 7.6- and 12.2-fold respective increase in IC_{50} were isolated. Cloning of the RT region from resistant variants and subsequent DNA sequence analysis revealed the presence of a K65R substitution (AAA to AGA) in 10 of 10 resistant clones from the first selection procedure. Susceptibility testing of an HIV-1_{LAI} K65R recombinant confirmed the K65R mutation's role in conferring resistance to DXG. Cross resistance analysis of the the HIV-1_{LAI} K65R recombinant showed the K65R mutation confers cross resistance to ddC, ddI, D4T, 3TC, PMEA, D-CAPD, D-FDOC, D-DAPD ($P<0.05$). No change in susceptibility to AZT was observed in the K65R recombinant, however, when introduced into an AZT resistant genetic backgound, the K65R mutation significantly reversed AZT resistance ($P<0.05$). Cloning and DNA sequencing of resistant variants from a repeat selection revealed the presence of an L74V mutation, a mutation previously reported for ddI resistance (25). The L74V mutation was also found to increase AZT susceptibility when introduced into an AZT resistant genetic background, but to a more limited extent than the K65R mutation. From the results of this study it can be concluded that dioxolane and dideoxy compounds select for common resistance mutations, suggesting these compounds should not be used together in combination therapies. Additionally, results from this study indicate that dual AZT/DXG therapy should be explored due to the K65R and L74V mutation's suppressive effects on AZT resistance.

Introduction

The development of drug resistant viral variants is one of the greatest obstacles faced by researchers in the search for effective and useful treatments of human immunodeficiency virus type 1 (HIV-1) infection. Resistant viral variants have emerged in response to every class of antiviral compound, including nucleoside analogue reverse transcriptase (RT) inhibitors, nonnucleoside RT inhibitors and protease inhibitors. Additionally, variants resistant to combinations of antiviral compounds have been reported (4). Problems associated with toxicities, side effects and dosage requirements also hinder the development of effective and useful treatments of HIV-1 infection.

In response to the shortcomings and problems associated with many of the anti-HIV agents now in use, a vast number of antiviral agents have been developed. Testing each of these antiviral agents in clinical trials is neither economically feasible nor practical, making *in vitro* analysis a vital aspect of preclinial drug development. Previous studies have demonstrated that *in vitro* selection and characterization of resistant strains is a useful means of predicting both the mechanisms of drug resistance for specific compounds (22) as well as the time course for the development of resistance mutations (12). *In vitro* studies are also useful in predicting which resistance mutations will arise during clinical use of the antiviral (22). Knowledge of the mechanisms and time course of resistance can pinpoint the most promising antivirals, which can subsequently be taken to clinical trial. Additionally, *in vitro* studies can identify good candidates for combination therapy by identifying cross-resistance mutations as well as suppressor mutations that result in the phenotypic reversal of resistance (5, 12, 13). This type of information

is crucial for the development of effective drug combination protocols.

(-)-β-D-dioxolane guanosine (DXG), was recently synthesized by Schinzai and Chu and licensed to Triangle Pharmaceutical (7). DXG is a purine nucleoside analog with the natural D-configuration, and is both a potent and selective inhibitor of HIV-1 and HBV *in vitro* (3, 7, 20, 24). A unique feature of DXG is the substitution of a dioxolane ring system for the sugar moiety found in natural cellular nucleosides (7). The structure of DXG is illustrated in Figure 1 (7). The triphosphorylated form of DXG is recognized as substrate by viral reverse transcriptase (RT) and subsequently incorporated into DNA. This incorporation ultimately results in chain termination due to DXG's lack of the 3'C-OH moiety necessary for chain extension (Figure 1). The median effective concentration (EC_{50}) of DXG is 27 nM in activated periferal blood mononuclear cells (PBMC), making it the most potent of the purine analogs against HIV-1 in PBMCs. In addition, DXG has no apparent cytotoxity up to 100 μM. One potential drawback to DXG is its limited aqueous solubility (3), however, promising prodrugs of DXG are available. The compounds (-)-β-D-diaminopurine dioxolane (DAPD) and (-)-β-D-2-amino-6-chloropurine dioxolane (ACPD) undergo biotransformation *in vivo* to yield DXG and have favorable pharmakokinetic properties (3, 20). DXG is presently in the preclinical phase of development, and this study was undertaken to determine the potential for the development and mechanisms of HIV-1 resistance.

MATERIALS AND METHODS

Chemicals

Beta-D-guanosine dioxolane (DXG), 2',3'-dideoxycytidine (DDC), 3'-azido-3'-deoxythymidine (AZT), 3'-deoxy-2',3'-didehydrothymidine (D4T), 2',3'-dideoxy-3'-thiacytidine (3TC), β -D-2-amino-6-chloropurine dioxolane (ACPD), β -D-2, 6-diaminopurine dioxolane (DAPD), 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and D-dioxolanyl-5-fluorocytosine (D-FDOC) were provided by R. Schinzai (Emory University, Atlanta, GA). 2',3'-dideoxyinosine (DDI), and phophonoformate (PFA) were purchased from Sigma Chemical Company, St. Louis, Mo. The antiviral compounds were prepared as 10 or 30 μM stock solutions in sterile water or dimethyl sulfoxide as appropriate and stored at -20°C. Stock solutions were diluted to the appropriate concentrations with RPMI 1640 (Whittaker MA Bioproducts, Walkersville, MD) immediately before use.

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 medium supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS), 2 μM L-glutamine, 10mM HEPES buffer, and antibiotics (50 IU/ml penicillin, 50 υg/ml streptomycin). PBMCs were isolated from HIV-1 seronegative donors and PHA (10 μg/ml; Difco Labs, Detroit, MI) activated three days prior to infection with HIV-1. At the time of infection, PBMC were transferred to and maintained in RPMI 1640 medium supplemented with 10% FBS, 2 μM L-glutamine, 10% interleukin-2 (Cellular Products, Buffalo, NY) and antibiotics.

Viruses

Stock preparations of HIV-1_{LAI} were prepared as previously described (19). Briefly, MT-2 cells were electroporated with 10μg of plasmid DNA encoding the HIV-1_{LAI} proviral genome. Culture supernatants were harvested 5 to 7 days postinfection at peak viral cytopathic effect. Prior to selection for DXG resistant virus, the plasmid derived virus was passed 10 times as cell-free virus in MT-2 cells. The infectivity of the virus preparation was determined via threefold endpoint dilution in MT-2 cells (six cultures per dilution), and the 50% tissue culture infective dose ($TCID_{50}$) was calculated via the Reed and Muench equation (21).

Selection of Resistant Viruses

DXG resistant virus was selected twice by serially passaging HIV-1_{LAI} in MT-2 cells in increasing concentrations of DXG. MT-2 cells (1.0×10^6) were pretreated with DXG for 2 hours prior to inoculation with HIV-1_{LAI}. Virus replication was measured by daily monitoring of viral cytopathic effects (CPE). At +2 CPE, the culture fluids were clarified by centrifugation (200 x g for 10 minutes) and the supernatant was harvested. A 1.0 ml aliquot of the harvested supernatant was subsequently used to initiate a new passage in fresh MT-2 cells at a higher concentration of DXG. Virus was passaged two to three times at each concentration, depending on how successfully virus grew at the drug concentration. The selective pressure was increased from an initial DXG concentration of 2.5 μM to a final concentration of 30 μM during the course of 13 (selection #1) and 14 (selection #2) passages. Following every two passages the Log_{10} reduction in viral infectivity in 30μM DXG was measured to monitor the passaged virus for a reduction in susceptibility to DXG, as previously described (18).

Antiviral Susceptibility Determinations

Virus susceptibility to DXG was determined in MT-2 cells as previously described (18). Briefly, MT-2 cells were infected with a multiplicity of 0.01 $TCID_{50}$ per cell in the presence of

serial DXG dilutions. Each dilution was tested in triplicate. Culture supernatants were harvested day 7 post infection and assayed for p24 antigen production using a commercial assay (Dupont, NEN Products, Wilmington, Del.). Virus susceptibility to DXG was also determined in PBMCs as previously described (23).

Cloning and DNA sequencing of HIV-1 RT

HIV-1 RT was cloned from infected cell lysates as previously described (16, 17). The full-length RT coding region was PCR amplified and ligated into a PCRII TA cloning vector (Invitrogen, San Diego, CA). Escherichia coli INVαF' were subsequently transfected with the vector, and transformants were identified via EcoRI digestion. Plasmid DNA from transformants was purified (Quiagen Inc., Chatsworth, CA) and sequenced as previously described (18, 19).

Production of Mutant Recombinant HIV-1

Generation of mutant recombinant HIV-1 was performed as previously described (17). Mutant RT was generated via oligonuecleotide-directed mutagenesis and ligated into the pXXHIV-1_{LAI} cloning vector. Cloning was facilitated by the presence of two silent restriction sites at the 5' and 3' ends (*Xma*I and *Xba*I, respectively) of pXXHIV-1_{LAI} RT (16, 17). Infectious mutant recombinant HIV-1 were generated by electroporating MT-2 cells with the mutated pXXHIV-1_{LAI} clone. The RT region was PCR amplified from infected cell lysates and sequenced to verify the presence of the desired mutations.

Statistics

The Wilcoxon rank sum test for independent sample means was used to test the significance of the observed differences in mean IC_{50}s. One-tailed tests were used in comparing the IC_{50}s of wild-type and recombinant viruses as the hypothesis tested was the IC_{50} values for recombinant viruses were greater than the IC_{50} values for wild type viruses. Additionally, one-

tailed tests were used in comparing the IC_{50} cross-resistance values as the hypothesis tested was the HIV-1_{LAI} wild type IC_{50}s were less than those for the HIV-1_{LAI} K65R recombinant.

Molecular Modeling

Calculations were performed on a Silicon Graphics Indy workstation using SYBYL 6.1a software (Tripos Associates, St. Louis, MO). DDI, DAPD and DXG were constructed using routines available in SYBYL. The geometries of the molecules were optimized and charges were generated using PM3 hamiltonian in MOPAC interface. A grid search was performed on each molecule with 300 increment about the rotatable bonds O2-N1-C1'-O4' and C3'-C4'-C5'-O5'. The global minimum conformation was further relaxed. Energy minimizations were performed using Tripos force field with MOPAC charges, using conjugate gradient method. These molecules were superimposed for comparisons. The molecules were fit by "fit atoms" option in SYBYL, using N1, C2, N3, C4, N7, C8, N9 and C1' atoms in such a way that the RMS deviation is minimized.

RESULTS

Selection of DXG resistant HIV-1_{LAI} variants *in vitro* was accomplished by serial passage of virus in MT-2 cells in the presence of increasing concentrations of DXG. To increase the heterogeneity of the starting virus population, HIV-1_{LAI} was passaged 10 times prior to the start of selection. As shown in Figure 2, the IC_{50} of DXG increased only slightly during the first 6 passages. Variants with distinctly decreased susceptibility, however, emerged during passage 10, with a 5 fold increase in the IC_{50} of DXG. Subsequent passages resulted in the emergence of DXG resistant variants with even greater resistance capabilities. Susceptibility testing of variants from passage 13 found the IC_{50} of DXG had increased 7.3 fold when compared to the control HIV-1_{LAI} passaged in the absence of drug (from 1.6 to 11.6 μM).

The *in vitro* selection for DXG resistant virus was repeated a second time using the same methods. The second selection yielded a similar outcome (data not shown) with an IC_{50} of 15.9 M in variants from passage 14, a 12.2 fold increase from the baseline IC_{50}.

Genotypic Analysis. To determine the molecular basis for DXG resistance, the RT region of proviral DNA was PCR amplified from infected cells of passages 13 (selection #1)and 14 (selection #2), and analysed by DNA sequencing. Additionally, the proviral DNA amplified from control cells was sequenced. DNA sequencing revealed the prescence of a lysine to arginine mutation at position 65 (AAA → AGA) in 10 of 10 resistant clones from selection #1. The K65R mutation was not seen in any of the control clones (0 of 10). Sequence analysis of clones from selection #2 revealed the presence a leucine to valine mutation at position 74 (ATT→AGT) in 8 of 10 resistant and 0 of 10 control clones. The two single mutations described above were the only mutations identified in their respective clones. Additionally, no other mutations in the RT region were found in more than one clone from either selection procedure.

Susceptibility of recombinant viruses. To characterize the role of the K65R and L74V mutations in conferring resistance to DXG, each mutation was individually introduced into a wild-type background. Additionally, each mutation was individually introduced into an AZT resistant background (67N/70R/215Y/219Q). Introduction of these mutations into a wild-type background was necessary to determine if the K65R and/or L74V mutations were necessary and sufficient for the observed resistance to DXG as well as to determine if the K65R mutation confers cross-resistance to other antiviral agents. Introduction of these mutations into an AZT resistant background was necessary to ascertain the K65R and/or L74V mutations effects on AZT resistant variants. The susceptibilities of these mutant recombinant viruses to DXG and AZT were assayed in MT-2 cells via inhibition of P24 antigen production and are summarized in Table 1. The K65R mutation resulted in a level of resistance to DXG similar to that observed with the DXG-resistant virus selected during selection #1. The K65R mutation decreased HIV-1_{LAI}'s susceptibility to AZT in a wild type background ($P<0.05$), however, when introduced into an AZT resistant background, K65R actually increased the virus' susceptibility to AZT ($P<0.05$). The L74V mutation resulted in a level of resistance lower than that observed with the DXG-resistant virus selected during the second selection procedure. That is, the L74V mutant recombinant only displayed a 4.2 fold increase in IC_{50}, whereas resistant serially passaged virus displayed a 12.2 fold increase. Additionally, L74V did not significantly reduce HIV-1_{LAI}'s susceptibility to AZT in a wild type background ($P=0.19$). The L74V mutation did, however, increase HIV-1_{LAI}'s susceptibility to AZT when introduced into an AZT resistant background ($P<0.05$), but to a smaller extent than K65R (see Table 1).

Cross Resistance

The HIV-1_{LAI} K65R recombinant was tested for its susceptibility to a number of antiviral

agents in MT-2 cells and in PBMCs to determine the level of cross-resistance conferred by the K65R mutation. The susceptibility of the HIV-1_{LAI} K65R recombinant virus to the antiviral agents tested is summarized in Table 2. In MT-2 cells the K65R mutation significantly reduced HIV-1_{LAI}'s susceptibility to the other dioxolane derivatives tested (DCAPD and DDAPD) ($P<0.05$), as well as conferred cross-resistance to ddI, ddC, PMEA, 3TC, D4T, ($P<0.05$). The K65R mutation had no affect on HIV-1_{LAI} s susceptibility to AZT. The HIV-1_{LAI} (L74V) recombinant's susceptibility to other antiviral agents was not determined.

Computer Modeling

Figure 3 illustrates the results of the computer modeling. The pictures of the fit of the atoms show a conformational difference among ddI, DAPD and DXG. DAPD and DXG seem to be closely related when the pseudorotation angle and other torsional angles are compared. However, ddI has a different sugar conformation than DAPD and DXG. The longer bond length of C2'-O3' in DAPD and DXG than C2'-C3' in ddI and the electronic effects of O3' play a key role in providing unique conformation to DAPD and DXG. Though this does not explain the *in vivo* behavior of these compounds, it does show a difference in the preferential conformations of these nucleosides and probably a different mode of interaction with the target site.

DISCUSSION

HIV-1 variants resistant to DXG, a nucleoside analog RT inhibitor, were selected twice by serially passaging HIV-1_{LAI} in increasing concentrations of drug. After 13 and 14 passages, variants with a 7.6- and 12.2-fold respective increase in IC_{50} were isolated. Sequence analysis of the RT region of resistant variants revealed the presence of a K65R mutation in resistant variants from the first selection procedure. Susceptibility testing of an HIV-1_{LAI} K65R recombinant confirmed the K65R mutation's role in conferring resistance to DXG. Cross resistance analysis of the HIV-1_{LAI} K65R recombinant showed the K65R mutation confers cross resistance to ddI, ddC, D4T, 3TC, PMEA, D-CAPD, D-FDOC, D-DAPD ($P<0.05$). Conversely, the K65R recombinant showed little change in susceptibility to AZT (Table 2). When introduced into an AZT resistant background, however, the K65R mutation significantly reduced AZT resistance ($P<0.05$). These results are similar to those of another study (27) which found that the K65R mutation results in resistance to ddC, cross resistance to ddI and no change in susceptibility to AZT. Sequence analysis of resistant variants from selection #2 revealed the presence of an L74V mutation. The IC_{50} of an HIV-1_{LAI} L74V recombinant was less than the IC_{50} of the DXG resistant virus selected in culture, indicating L74V was not the sole mutation responsible for the observed DXG resistance. While the L74V mutation was present in 8 of 10 resistant clones, no other mutations were found in more than one clone. The L74V mutation was also found to increase AZT susceptibility when introduced into an AZT resistant background, but to a more limited extent than the K65R mutation. A study by St. Clair, et al., identified the L74V mutation in ddI resistant variants isolated from patients who had changed from AZT to ddI therapy and determined that L74V confers cross resistance to ddC and increases susceptibility to AZT (25).

Differences in selection methodologies have been shown to yield different resistance

mutations (9). In this study, however, the exact same methods yielded two different resistance mutations during the course of the selection procedures. As previously mentioned, HIV-1_{LAI} was passaged 10 times in the absence of DXG prior to the start of the selection procedures, resulting in an increase in the heterogeneity of the starting virus pool. Recently, Kinjerski and Buckheit (8) reported that the genetic composition of the starting virus pool may result in genotypic differences of *in vitro* selected resistant viruses. In this study, the selection of two distinct resistant mutants was probably due to the heterogeneity of the starting virus pool. By chance two different resistant quasispecies present in the starting virus pool were selected for during the course of the selection procedures.

The existence of AZT suppressor mutations have been reported in other studies, but the K65R and L74V mutations are the first nucleoside analog RT inhibitor resistance mutations having suppressive effects on AZT resistance to be described. L100I (2), Y181C (11, 12) and the foscarnet resistance mutations (W88G, E89K, L92I, Q161L) (26), have also been shown to suppress AZT resistance when introduced into an AZT resistant background. The L100I and Y181C mutations confer resistance to nonnucleoside reverse transcriptase inhibitors while the foscarnet resistance mutations confer resistance to a PP_i analog. The K65R and L74V mutations confer resistance to dideoxy (6, 15, 25, 27) and dioxolane nucleoside RT inhibitors, while suppressing the mutations responsible for conferring resistance to AZT, another nucleoside analog RT inhibitor. This is a significant finding given that DXG and AZT target the same sight of HIV-1 RT, but appear to have counteractive resistance mutations. Additional studies on the interactions between the AZT resistance mutations and K65R and L74V would provide additional insight into this phenomenon.

The selection of the same resistance mutations by ddC and DXG as well as the extent of

the cross resistance conferred by these resistance mutations is a result of these nucleoside analogs interacting with the active site of RT in very similar fashions. Similarly, the lack of cross resistance conferred by K65R and L74V to AZT is a result of AZT's dissimilar interaction with the active site of RT compared to that of DXG and other dioxolane and dideoxy compounds. The azido group of AZT prevents the formation of 3',5' phosphodiester bonds and thus inhibits DNA synthesis, whereas the lack of the 3'C-OH moeity of other nucleoside analogs prevents chain elongation and thus terminates DNA synthesis. Different mutations of the RT active site would thus be required to counteract the inhibitory actions of each of these antivirals.

Gu, et al., reported the K65R mutation confers resistance to ddC, and noted that positions 65 to 71 of RT play important roles in RT binding of dNTP substrates (6, 10). Martin, et al., also reported finding the L74V mutation results in altered substrate recognition. Several AZT resistance mutations (67N and 70R) also lie in this region. Thus, the K65R and L74V mutations function to alter HIV-1 RT recognition of substrate, and the changes to the IKKK motif probably diminish the efficacy of the 67N and 70R mutations at reducing RT's susceptibility to AZT.

The results of this study yield several important conclusions. First, there are common patterns of resistance to dioxolane and dideoxy nucleotide analogs, which suggest that these compounds should not be used in combination with one another. Second, the lack of cross-resistance to AZT, indeed the reversal of AZT resistance by DXG resistance mutations, strongly supports the notion that therapies using biochemically unrelated compounds will be more efficacious than biochemically related compounds (4). Combination therapy of DXG and AZT should therefore be given further consideration.

REFERENCES

1. **Balzarini, J., A. Karlsson, M. J. Perez-Perez, M. J. Camarasa, W. G. Tarpley, E DeClerq**. 1993. Treatment of Human immunodeficiency virus type 1 (HIV-1)-infected cells with combinations of HIV-1-specific inhibitors results in a different resistance pattern than does treatment with single-drug therapy. J Virol 67:5353-5359.

2. **Buckheit, R. W., T. L. Kinjerski, V. Fliakas-Boltz, J. D. Russell, T. L. Stup, L. A. Pallansch, W. G. Brouwer, D. C. Dao, W. A. Harrison, R. J. Schultz, J. P. Bader, S. S. Yang**. 1995. Sturcture-activity and cross-resistance evaluations of a series of human immunodeficiency virus type 1-specific compounds related to oxathiin carboxanilide. Antimicrob Agents Chemother **39**: 2718-2727.

3. **Chen, H., F. D. Boudinot, C. K. Chu, H. M. McClure, R. F. Schinazi.** 1996. Parmacokinetics of (-)-β-D-2-aminopurine dioxolane and (-)-β-D-2-amino-6-chloropurine dioxolane, and their antiviral metabolite (-)-β-D-dioxolane guanine in rhesus monkeys. Antimicrob Agents Chemother **40**:2332-2336.

4. **Condra, J. H., W. A. Schleif, O. M. Blahy, L. Gabryelski, D. Graham, J. Quintero, A. Rhodes, H. Robbins, E. Roth, M. Shivaprakash, D. Titus, T. Yang, H. Teppler, K. Squires, P. Deutsch., E. Emini**. 1995. *In vivo* emergence of HIV-1 to variants resistant to multiple protease inhibitors. Nature **374**:569-571.

5. **DeClercq, E**. 1994. HIV resistance to reverse transcriptase inhibitors. Biochemical Pharmacology **47**:155-169.

6. **Gu, Z., Q. Gao, H. Fang, H. Salomon, M. A. Parniak, E. Goldberg, J. Cameron, M.A. Wainberg**. 1994. Identification of a mutation at codon 65 in the IKKK motif of reverse transcriptase that encodes human immunodeficiency virus resistance to 2'3'-dideoxy-3'-thiacytidine. Antimicrob Agents Chemother **38**:275-281.

7. **Kim, H. O., R. F. Schinazi, K. Shanmuganathan, D. L. Cannon, A. J. Alves, L. S. Jeong, J. W. Beach, C. K. Chu.** 1993. 1,3-Dioxolanylpurine nucleosides (2R, 4R) and (2R, 4S) with selective anti-HIV-1 activity in hyman lymphocytes. J Med Chem **36**:30-37.

8. **Kinjerski, T. L., R. W. Buckheit Jr.** 1997. The role of genotypic heterogeneity in wild type virus populations on the selection of non-nucleoside reverse transcriptase inhibitor resistant viruses. Antiviral Research **33**:109-115.

9. **Kleim, J. P., I. Winkler, M. Rosner, R. Kirsch, H. Rubsamen-Waigmann, A. Paessens, G. Riess**. 1997. *In vitro* selection for different mutational patterns in the HIV-1 reverse transcriptase using high and low selective pressure of the nonnucleoside reverse

transcriptase inhibitor HBY 097. Virology **231**:112-118.

10. **Kohlstaedt, L. A., J. Wang, J. M. Friedman, P. A. Rice, T. A. Steitz.** 1992. Crystal structure at 3.5Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science **256**:1783d-1790.

11. **Larder, B. A.** 1992. 3'-azido-3'deoxythymidine resistance suppressed by a mutation conferring human immunodeficiency virus type 1 resitance to nonnucleoside reverse transcriptase inhibitors. Antimicrob Agents Chemother **36**:2664-2669.

12. **Larder, B. A.** 1995. Viral resistance and the selection of antiretroviral combinations. J Acquired Immune Defic Syndr **10**:S28-33.

13. **Larder, B. A., S. D. Kemp, P. R. Harrigan**. 1995. Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. Science **269**:696-699.

14. **Markowitz, M., H. Mo, D. J. Kempf, D. W. Norbeck, T. N. Bhat, J. W. Erickson, D. D. Ho.** 1995. Selection and analysis of human immunodeficiency virus type 1 variants with inreased resistance to ABT-538, a novel protease inhibitor. J Virol **69**:701-706.

15. **Martin, J. L., J. E. Wilson, R. L. Haynes, P. A. Furman.** 1993. Mechanism of resistance of human immunodefiency virus type 1 to 2,'3'-dideoxyinosine. Proc Natl Acad Sci USA **90**:6135-6139.

16. **Mellors, J. W., G. J. Im, E. Tramontano, S. R. Winkler, D. J. Medina, G. E. Dutschman, H. Z. Bazmi, G. Piras, C. J. Gonzalez, Y. C. Cheng**. 1992. A single conservative amino acid substitution in the reverse transcriptase of human immunodeficiency virus-1 confers resistance to (+)-(5S)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)-thione (TIBO R82150). Molecular Pharm **43**:11-16.

17. **Mellors, J.W., G. E. Dutschman, B. J. Im, E. Tramontano, S. R. Winkler, Y. C. Cheng**. 1992. In vitro selection and molecular characterization of human immunodeficiency virus typel resistant to non-nucleoside inhibitors of reverse transcriptase. Molecular Pharm **41**:446-451.

18. **Mellors, J. W., H. Z. Bazmi, R. F. Schinazi, B. M. Roy, Y. Hsiou, E. Arnold, J. Wir, D. L. Mayers**. 1995. Novel mutations in reverse transcriptase of human immunodeficiency virus type 1 reduce susceptibility to foscarnet in laboratory and clinical isolates. Antimicrob Agents Chemother **39**:1087-1092.

19. **Nguyen, M. H., R. S. Schinzai, C. Shi, N. M. Goudgaon, P. M. NcKenna, J. W. Mellors.** 1994. Resistance of human immunodeficiency virus type 1 to acyclic 6-phenylseleneyl and 6-phenylthiopurimidines. Antimicrob Agents Chemother **38**:2409-2414.

20. **Rajagopalan, P., F. D. Boudinot, C. K. Chu, B. C. Tennant, B. H. Baldwin, R. F. Schinazi**. 1996. Pharmacokietics of (-)-β-D-2,6-diaminopurine dioxolane and its metabolite, dioxolane guanosine, in woodchucks (Marmota monax). Antiviral Chem and Chemother **7**:65-70.

21. **Reed, L. J., H. Muench**. 1938. A simple method for estimating fifty percent end points. Am J Hyg **27**:493-496.

22. **Richman, D. D**. 1993. Resistance of clinical isolates of human immunodeficiciency virus to antiretroviral agents. Antimicrob Agents Chemother **37**:1207-1213.

23. **Schinazi, R. F., J.-P. Sommadossi, V. Saalmann, D. L. Cannon, M.-Y. Xie, G. Hart, G. C. Smith, E. F. Hahn**. 1990. Activity of 3'azido-3'-deoxythymidine nucleotide dimers in primary lymphocytes infected with human immunodeficiency virus type 1. Antimicrob Agents Chemother **34**:1061-1067.

24. **Schinzi, R. F., H. M. McClure.** 1993. Development of (-)-β-D-2,6-diaminopurine dioxolane as a potential antiviral agent. Antiviral Res **23**:81-88.

25. **St. Clair, M. H., J. L. Martin, G. Tudor-Williams, M. C. Bach, C. L. Vavro, D. M. King, P. Kellam, S. D. Kemp, B. A. Larder**. 1991. Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. Science **253**:1557-1559.

26. **Tachedjian, G., J. Mellors, H. Bazmi, C. Birch, J. Mills**. 1996. Zidovudine resistance is suppressed by mutations conferring resistance of human immunodeficiency virus type 1 to foscarnet. J Virol **70**: 7171-7181.

27. **Zhang, D., A. M. Caliendo, J.J. Eron, E.M. DeVore, J.C. Kaplan, M.S. Hirsch, R.T. D Aquila**. 1994. Resistance to 2'3'-dideoxycytidine conferred by a mutation in codon 65 of the human immunodeficiency virus type 1 reverse transcriptase. Antimicrob Agents Chemother **38**:282-87.

Table 1 - DXG-AZT Mutational Interactions

Variant[b]	Susceptibility to: DXG[a] IC_{50} (μM)[c]	DXG[a] Fold Resistance	AZT[a] IC_{50} (μM)[c]	AZT[a] Fold Resistance
HIV-1_{LAI} (wild type)	0.278 ± 0.04	-	0.003 ± 0.000	-
HIV-1_{LAI} (K65R)	2.410 ± 0.346	8.67	0.010 ± 0.003	3.3
HIV-1_{LAI} (L74V)	1.168 ± 0.256	4.20	0.006 ± 0.002	1.93
HIV-1_{LAI} (4XAZT)[d]	0.575 ± 0.172	2.07	0.143 ± 0.043	47.7
HIV-1_{LAI} (4XAZT+K65R)	1.400 ± 0.16	5.04	0.004 ± 0.0004	1.33
HIV-1_{LAI} (4XAZT+L74V)	1.040 ± 0.144	3.74	0.019 ± 0.006	6.33

a Recombinant provirus genome
b Mean ± standard error (n = 3).
c AZT resistant strain with mutations 67N/70R/215Y/219Q

Table 2 - Resistance and Cross-Resistance of the HIV-1_{LAI} (K65R) Recombinant

	Result in:			
	MT-2 Cells[a]		PBMCs[b]	
Antiviral Agent	IC_{50} (μM)[c]	Fold Resistance[d]	Mean EC_{50} (μM)	Fold Resistance[d]
DXG	0.278 ± 0.04	8.67	5.50	8
AZT	0.040 ± 0.01	0.33	0.09	2
ddC	6.580 ± 0.96	3.13	0.30	45
ddI	14.18 ± 2.08	3.08	1.42	17
D4T	18.43 ± 3.64	3.04	0.52	6
3TC	>60 ± 0.00	>14.2	0.02	1
PFA	66.33 ± 1.83	2.99	29.00	40
DCAPD	12.90 ± 2.23	3.74	17.00	33
DDAPD	10.02 ± 1.397	4.54	8.05	10
DFDOC	2.63 ± 0.365	7.74	ND	ND
PMEA	>60 ± 0.00	>4.37	4.46	3

a IC50 determined via P24 antigen assay. See Materials and Methods.
b ????
c Mean ± standard error (n = 3 or 4).
d Fold resistance relative to IC_{50} of parental virus.

Figure Legends

Figure 1.

Figure 2.

Table 1.
a Recombinant provirus genome
b Mean ± standard error (n = 3).
c AZT resistant strain with mutations 67N/70R/215Y/219Q

Table 2.
a IC50 determined via P24 antigen assay. See Materials and Methods.
b ????
c Mean ± standard error (n = 3 or 4).
d Fold resistance relative to IC_{50} of parental virus.

Figure 3.

Figure 1 - Chemical Structure of DXG

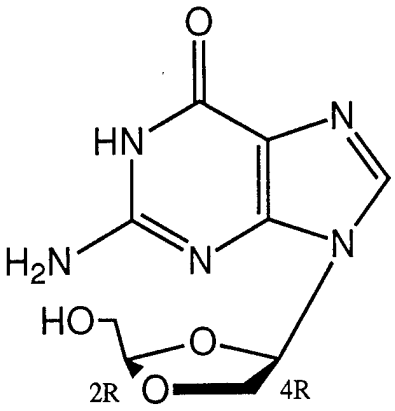

Figure 2 - In Vitro Resistance to Dioxolane G