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In vitro isolation and identification of human immunodeficiency virus (HIV) variants with reduced sensitivity to C-2 symmetrical inhibitors of HIV type 1 protease

(AIDS/resistance)

M. J. OTTO*, S. GARBER, D. L. WINSLOW, C. D. REID, P. ALDRICH, P. K. JADHAV, C. E. PATTERSON, C. N. HODGE, AND Y.-S. E. CHENG

The DuPont Merck Pharmaceutical Company, Glenolden, PA 19036

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ABSTRACT **Protease inhibitors are another class of compounds for treatment of human immunodeficiency virus (HIV)-caused disease. The emergence of resistance to the current anti-HIV drugs makes the determination of potential resistance to protease inhibitors imperative. Here we describe the isolation of an HIV type 1 (HIV-1) resistant to an HIV-protease inhibitor. Serial passage of HIV-1 (strain RF) in the presence of the inhibitor, [2-pyridylacetylisoleucylphenylalanyl-ψ(CHOH)]$_2$ (P9941), failed to yield a stock of virus with a resistance phenotype. However, variants of the virus with 6- to 8-fold reduced sensitivity to P9941 were selected by using a combination of plaque assay and endpoint titration. Genetic analysis and computer modeling of the variant proteases revealed a single change in the codon for amino acid 82 (Val → Ala), which resulted in a protease with lower affinity and reduced sensitivity to this inhibitor and certain, but not all, related inhibitors.**

The aspartyl protease (PR) encoded by human immunodeficiency virus (HIV) is critical for replication of this virus (1). This enzyme is responsible for specific cleavages of *gag/pol* gene products that yield the viral structural proteins and essential enzymes, including reverse transcriptase, integrase, and the protease itself (2–4). Inhibition of this enzyme by synthetic inhibitors during infection *in vitro* leads to a reduction in the amount of infectious viral particles produced (5). This result is presumably due to insufficient processing of the p55 gag polyprotein to the essential structural proteins p24, p17, p9, and p7.

To date, most inhibitors of the HIV PR have been peptide analog transition-state mimetics. These have included reduced amides (6, 7), hydroxyethylene and hydroxyethylamine isosteres (6, 8–11), statine analogs (7), phosphinic acid derivatives (12), and difluoroketone derivatives (6, 13). Some of the more active inhibitors have incorporated the hydroxyethylene isostere into C-2 symmetrical peptide analogs (11, 14), which take advantage of the symmetrical nature of the HIV protease molecule. One such compound, synthesized at DuPont Merck, is [2-pyridylacetylisoleucylphenylalanyl-ψ(CHOH)]$_2$ (P9941) (see Structure I).

The development of viral resistance to both the nucleoside and nonnucleoside inhibitors of HIV reverse transcriptase has become an increasingly important concern in the treatment of HIV-infected patients and has highlighted the importance of understanding the potential for resistance to any new antiviral drug. We, therefore, attempted to isolate variants of HIV type 1 (strain RF) [HIV-1(RF)] *in vitro* with altered sensitivity to C-2 symmetrical diol protease inhibitors.

MATERIALS AND METHODS

Virus and Cells. HIV-1(RF) was obtained from Robert Gallo (National Institutes of Health, Bethesda, MD), as infected cultures of H9 cells. The cultures of persistently infected human lymphoblastoid cells were maintained in RPMI 1640 (GIBCO)/5% fetal calf serum/gentamycin at 5 μg/ml. Cell-free supernatants, containing infectious virus, were obtained by low-speed centrifugation of the infected cultures. Virus titers in these supernatants were determined by plaque assay, as described (15). MT-2 cells, human lymphoblastoid cells transformed by human T-lymphotrophic virus type 1, were obtained from David Montefiori (Vanderbilt University, Nashville, TN). The cells were grown in RPMI 1640/5% fetal calf serum/gentamycin at 5 μg/ml.

Isolation of HIV-1(RF) Variants. H9 cells were infected with HIV-1(RF) at a multiplicity of infection of 0.1 plaque-forming unit per cell in the presence of 0.1 μg of P9941 per ml. The virus was allowed to replicate for 3–4 days, which is equivalent to two to three rounds of replication; this was defined as one passage. Subsequent passages were initiated with uninfected H9 cells, fresh inhibitor, and cell-free supernatants from the previous passage. After five passages at 0.1 μg of inhibitor per ml, the virus was passaged three more times in the presence of 0.3 μg of inhibitor per ml, a concentration that had been shown previously to reduce the yield of HIV-1(RF) by 50%. Monolayers of MT-2 cells were infected with cell-free virus in the presence of 5 μg of P9941 per ml in an agar overlay. Plaques that appeared after 7 days were picked and pooled, and a virus stock was obtained that replicated in the presence of 5 μg of inhibitor per ml. Five separate isolates of this stock were subsequently obtained by endpoint dilution in 96-well microtiter plates in the presence of 5 μg of inhibitor per ml. These isolates were designated HIV(RF)41-D2, HIV(RF)41-D4, HIV(RF)41-E4, HIV(RF)-41-F4, and HIV(RF)41-G1.

Extraction of Viral DNA Encoding HIV-1 PR. Approximately 1 × 10^6 H-9 cells were pelleted by centrifugation at 13,000 rpm in a 1.5 ml Eppendorf tube, and the supernatant was discarded. The pellet was resuspended in 200 μl of solution A (100 mM KCl/10 mM Tris·HCl, pH 8.3/2.5 mM $MgCl_2$). Next, 200 μl of solution B (10 mM Tris·HCl, pH 8.3/2.5 mM $MgCl_2$/1% Tween 20/1% Nonidet P-40/proteinase K at 120 μg/ml) were added. After briefly mixing, the tubes were incubated at 60°C for 1 hr. The proteinase K was inactivated by heating to 95°C for 1–2 hr. The DNA encoding the HIV-1 PR was obtained by PCR amplification. Two PCR primers were synthesized corresponding to nt 1610–1636 on

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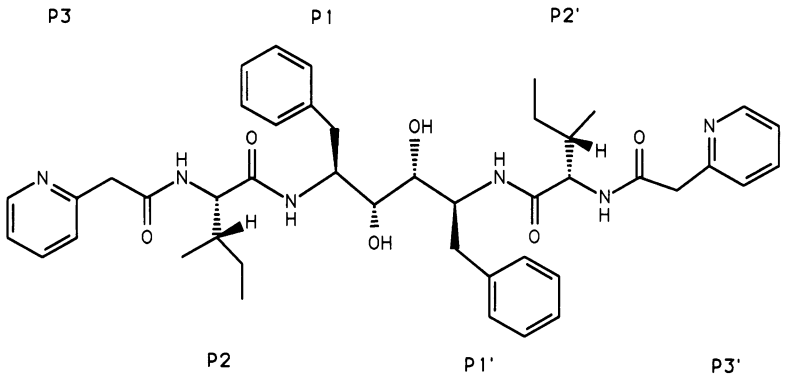
Abbreviations: HIV-1, human immunodeficiency virus type 1; PR, aspartyl protease; RMSD, rms derivative; Cbz, carbobenzyloxy.

*To whom reprint requests should be addressed.



P9941, (2-PyAc-Ile-Phe-ψ-[CHOH])2

Structure I

the sense strand (5'-TAGGGAAAATCTGGCCTTCCCA-CAAGG-3') and nt 2100–2119 on the antisense strand (5'-CCATCCATTCCTGGCTTTAA-3'). The 100 μl of reaction mix consisted of 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 mM each primer, *Taq* polymerase (Cetus) in Cetus PCR buffer with a 10 μl sample of DNA. Each tube was overlaid with 50 μl of mineral oil, and amplification was performed using a Perkin–Elmer thermal cycler for 35 cycles of 1 min at 94°C, 1 min at 60°C, and 1.5 min at 72°C. The products were visualized by ethidium bromide staining of samples separated on a 2% agarose gel.

Cloning and Expression of HIV-1 PRs in *Escherichia coli*. The cloning of HIV-1 PRs was done essentially as described (16, 17). The DNAs coding for proteases were modified at the ends by PCR with two oligonucleotide primers, TCGGAAT-TCATGGCCTTCCCACAAGGGAAGGCCAG and TCGA-AGCTTACTAAAAATTTAAAGTGCAACCAATCTGA, and later inserted into an *E. coli* expression vector pET11 at the *Nde* I and *Bam*HI sites. The DNA constructs were transformed into *E. coli* BL21[DE3] cells. Proteases were produced by an induction with 0.1 mM isopropyl β-D-thiogalactoside and isolated as soluble and insoluble fractions. Expression was monitored by SDS/PAGE and immunoblot analysis.

Assay for Proteolytic Activity. The proteases were isolated from the soluble extracts of *E. coli* cells and were purified by pepstatin affinity columns as described (18, 19). Incubations were done in 20 mM Pipes buffer/100 mM NaCl, pH 6.0, with or without inhibitor. Radiolabeled gag protein fragment (20), containing a single cleavage site between the p17 and p24 gag proteins, was added to start the reaction. At the end of a 30-min incubation at 30°C, the radiolabeled gag substrate and products were separated by SDS/PAGE and detected by autoradiography. The amount of cleavage of gag protein was determined by densitometric measurement of the radiolabeled gag protein bands.

Molecular Modeling. The coordinates of HIV protease complexed with symmetrical mono-ol inhibitor A77404 were obtained from the Protein Data Bank (21). All calculations were done in INSIGHT/DISCOVER (Biosym Technologies, San Diego, CA) on Silicon Graphics workstations or a Cray-YMP computer; the system was visualized on a Silicon Graphics Indigo Elan workstation.

The diol was constructed by excision of the hydroxyl-bearing carbon and replacement with (*S,S*)-(CHOH-CHOH), while allowing the residues to move outward into the active-site pockets but maintaining initial torsion angles. Methyl groups were added to convert the valines at $P_2P_{2'}$ to isoleucines, and the carbobenzyloxy (Cbz) groups were modified to 2-pyridylacetyl by deleting the carbamate oxygens and replacing the *o*-phenyl carbon with nitrogen. Hydrogen atom positions were calculated (pH 7), and a 5-Å sphere of water was added to the complex. The following routine was executed to allow the model to find a local minimum: (*i*) All atoms except hydrogen were constrained, followed by steepest-descent minimization at 300 K to an overall rms derivative (RMSD) of <10 kcal per mol-Å. Side chains were freed, and the procedure was repeated to a RMSD of <1 kcal/mol-Å. All atoms were then allowed to move and the procedure was repeated to RMSD of <0.1 kcal/mol-Å. Overall RMSDs of <0.5 Å of backbone atoms from the original crystal structure were observed following this protocol. (*ii*) Molecular dynamics (step size = 1 fs) were run for 50 ps at 500 K with only inhibitor atoms allowed to move; structures were collected every ps and minimized by using steepest descents (to RMSD <1) followed by conjugate gradients (to RMSD <0.1) with all atoms allowed to move. This step allowed inhibitor to explore the active site in a computationally accessible manner. (*iii*) The lowest energy structure from *ii* was used as the starting point for molecular dynamics on the entire system at 300 K for 50 ps. The final structure was minimized as in step *ii*, and the interaction of Val^{82} and Val^{182} with phenyl rings P_1 and $P_{1'}$ was examined. The same protocol was followed for Ala^{82} and Ala^{182}.

The methyl groups of residues Val^{82} and Val^{182} of the wild-type model showed significant stabilizing van der Waals contacts with the phenyl rings of P_1 and $P_{1'}$. Under these conditions the inhibitor was unable to rearrange its conformation to permit effective van der Waals overlap with the alanine methyl group in the mutant enzyme model. Overall intermolecular-interaction energy between inhibitor and enzyme increased (was less negative) as a result of the mutation, and this trend is consistent with experimental observation. It should be noted that this method will not give accurate results, due to the short simulation times used, if significant side-chain repacking is occurring. Also, only interaction enthalpies are measured, and any entropic contributions are ignored, which may lead to significant error. Further efforts should be directed toward more exhaustive conformational searching with statistical methods (22) and toward more accurate energetic analysis via inclusion of solvation terms (23).

RESULTS

Initially, our approach to isolating protease inhibitor resistant HIV was to pass the virus in the presence of P9941 at low concentrations followed by passage at higher concentrations. H-9 cells were infected with HIV-1(RF) in the presence of 0.1 μg of inhibitor per ml. The IC_{90} (concentration required to inhibit virus by 90%) of this compound in a yield reduction assay (15) is 0.9 μg/ml. The virus was allowed to replicate over five passages or ≈10–15 rounds of replication. The compound concentration was increased to 0.3 μg/ml for

three additional passages for a total of eight passages or >20 rounds of replication. At this point, the recovered virus stock showed no difference in IC_{90} from the initial virus stock, as determined by a yield reduction assay. Additional passages of this virus at concentrations of inhibitor $>IC_{90}$ resulted in undetectable virus. Because a very low concentration of resistant virus might escape detection by the yield reduction assay, the virus from passage at 0.3 μg/ml was allowed to form plaques in the presence of 5 μg of the inhibitor per ml. An average of five plaques appeared on plates that received between 100 and 200 plaque forming units, suggesting that these variants represented between 2 and 5% of the population. We note that no plaques formed on plates receiving parental virus at either 1000–2000 or 100–200 plaque forming units. Several plaques were picked and pooled, and a virus stock was grown in the presence of 5 μg of inhibitor per ml. Five isolates of this stock that grew in the presence of 5 μg of inhibitor per ml were obtained by endpoint dilution of the viral stock in 96-well plates. These isolates were designated HIV(RF)41-D2, HIV(RF)41-D4, HIV(RF)41-E4, HIV(RF)41-F4, and HIV(RF)41-G1. Fig. 1 shows the susceptibility of the parental HIV-1(RF) and the five isolates to P9941 in a yield reduction assay. The five isolates ranged from 6- to 8-fold less susceptible to inhibition by P9941 than the parental virus.

A likely explanation for viral resistance to this symmetrical diol is the selection of an HIV variant with an altered protease that can effectively process the gag and gag–pol polyproteins but has a reduced susceptibility to the inhibitor. To address this possibility, the proviral DNA segments coding for protease and its N-terminal flanking region were obtained from both the less sensitive viral isolates and the sensitive parental virus by PCR amplifications. The PCR fragments were purified and sequenced directly. In addition, the fragments were cloned into pTZ18R (Pharmacia) and the cloned DNAs were sequenced. A single point mutation of thymine to cytosine at nt 2014 of HIV-1(RF) was present in each of the DNAs derived from the resistant isolates, whereas a thymine was found in the sensitive isolate. This mutation results in a Val^{82} → Ala change (GTC → GCC).

Residue Val^{82} of HIV protease has been shown to be one of the 13 amino acid residues that contact the P_1 site of other inhibitors of HIV protease (24, 25). A molecular model of P9941 in the active site of HIV-1 protease was constructed from the published crystal structure of the enzyme with a similar inhibitor (21). Analysis of the interaction of residue 82 of the model supported the expectation that van der Waals contact at the $S_1(S_{1'})$ subsite is substantially decreased between $Ala^{82}(Ala^{182})$ and the $P_1(P_{1'})$ phenyl relative to wild type. Such a change in interaction would be consistent with reduced sensitivity to P9941. It should be pointed out, however, that the computational method used did not permit repacking of side chains of inhibitor or enzyme but would be indicative of the energetic trend if extensive conformational changes did not occur (see *Materials and Methods*).

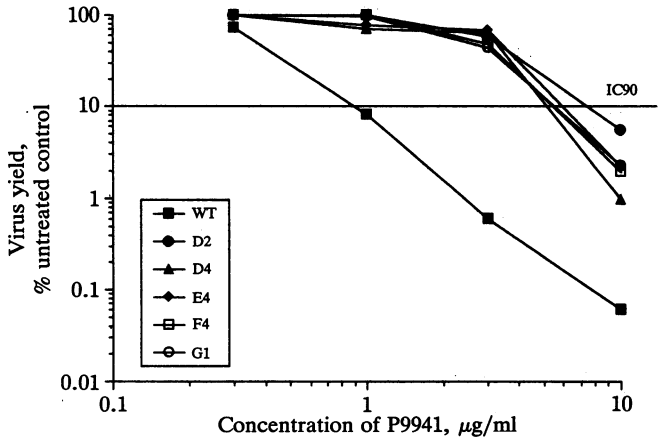


FIG. 1. Dose–response curves show reduced sensitivity of HIV-1(RF) variants to P9941. Sensitivity of HIV-1 (Val^{82}) and Ala^{82} variants (D2, D4, E4, F4, and G1) was determined in a yield reduction assay (15). The yield of virus is plotted as percentage of the untreated virus control versus the concentration of inhibitor.

To test whether the protease actually was less sensitive as predicted by the modeling, recombinant wild-type (Val^{82}) and resistant (Ala^{82}) proteases were produced by expression in *E. coli* of the cloned DNA from parental HIV-1(RF) and variant HIV(RF)41-D2. The expressed recombinant proteins consisted of the protease fused with its N-terminal 49 amino acid flanking peptide. The expression of this precursor protein has previously resulted in successful production of both HIV-1 and HIV-2 proteases in *E. coli* cells (16, 17). An additional advantage of this strategy is the ability to estimate the protease activity by the extent of autoproteolysis of the fusion proteins to the mature proteases and the N-terminal flanking peptides (16, 26). The protease constructs were successfully expressed in *E. coli*, resulting in the production of recombinant proteins in both soluble and insoluble forms (Fig. 2). The autoproteolysis of the precursor proteins indicated that the wild-type Val^{82}-protease was completely processed to the 11-kDa mature protease in the soluble extract (Fig. 2, lane 4). The autoproteolysis of the Ala^{82}-protease appeared less efficient with some of the protease antigen being found in the unprocessed form (Fig. 2, lane 2). Similarly, the ratio of processed to unprocessed protease in the insoluble fraction of the cell lysates was greater with the Val^{82}-protease than with the Ala^{82}-protease (Fig. 2, lanes 5 and 3, respectively).

The Val^{82}- and the Ala^{82}-proteases were isolated from the soluble extracts of *E. coli* cells and purified by pepstatin affinity chromatography (18). The ability of these enzymes to cleave radiolabeled HIV gag polyprotein (19, 20) in the presence or absence of P9941 was measured, and the 50% inhibitory concentrations (IC_{50} values) were determined. Fig. 3 shows the inhibitory dose responses for parental and variant proteases. The IC_{50} for the parental protease was 71 ng/ml, whereas the variant Ala^{82}-protease had an IC_{50} of 631

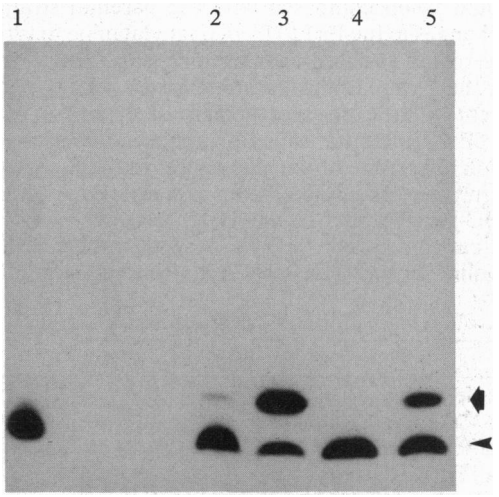


FIG. 2. Synthesis of HIV-1(RF) wild-type (Val^{82}) and its variant (Ala^{82}) proteases in *E. coli*. The DNAs coding for wild-type (Val^{82}) and mutant (Ala^{82}) proteases were transformed into *E. coli* BL21[DE3] cells. Proteases were produced and isolated as soluble (lanes 2 and 4) and insoluble (lanes 3 and 5) fractions. Purified HIV-1 (HXB2) protease (lane 1), Ala^{82}-(lanes 2 and 3), and Val^{82}-(lanes 4 and 5) proteases were separated in SDS/PAGE and detected by immunoblot analysis. Positions of the proteases and their precursors are indicated by an arrowhead and an arrow, respectively.

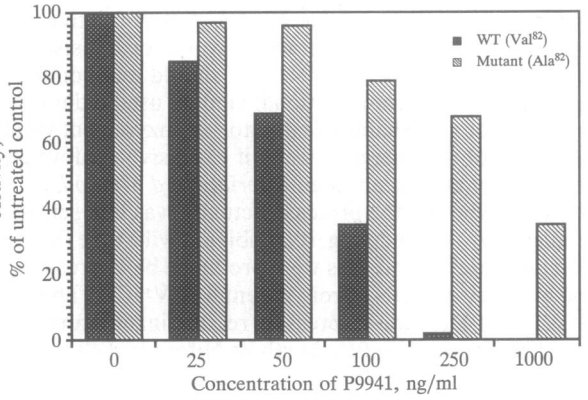


FIG. 3. Relative sensitivity of Val^{82}- and Ala^{82}-proteases to P9941. The Val^{82}- and the Ala^{82}-proteases were isolated from the soluble extracts of *E. coli* cells and purified by pepstatin affinity columns as described (18). Ala^{82}-protease and Val^{82}-protease were incubated with the indicated concentrations of P9941 and a radiolabeled gag protein fragment. Percentage of protease activity was calculated as the ratio of (amount of gag proteolyzed in the presence of inhibitor)/(amount of gag proteolyzed in the absence of inhibitor) × 100%.

ng/ml. The degree of change in enzyme sensitivity is essentially the same as the change for the inhibition of the respective viruses and strongly suggests that the Val → Ala mutation of the protease is the basis for the drug-resistant viral phenotype.

Having determined that the mutation affects the interactions between the inhibitor and the enzyme, we tested additional inhibitors with changes in P_3, P_2, and P_1. Table 1 shows the results of those tests. Changing from isoleucine to valine in the $P_2(P_{2'})$ positions had no significant effect on the reduction in efficacy for the variant viruses. However, a change in $P_3(P_{3'})$ from 2-pyridylacetyl to Cbz [Cbz-Val-Phe-ψ(CHOH)]$_2$ did affect the difference in susceptibility between the parental and the variant virus. The Cbz group apparently increases binding at $S_3(S_{3'})$, resulting in a more potent compound. This increase in potency was able to compensate for the decrease in interaction at $S_1(S_{1'})$ in the variants. The variants were only ≈4-fold less sensitive to this compound when compared with the parental strain. Additional changes in the $P_1(P_{1'})$ from phenylalanine to tyrosine or benzyltyrosine resulted in inhibitors with similar potencies against the parental and variant viruses. These results are consistent with the idea that an increase in the size of the side chains of the inhibitor at $P_1(P_{1'})$ might compensate for the reduction in the size of the side chains in the $S_1(S_{1'})$ position of the enzyme. An unrelated nonsymmetrical inhibitor, Ro31-8959 [QC-Asn-Phe-ψ(CHOHCH$_2$N)-DIQ, where QC is quinoline-2-carbonyl and DIQ is (4a*S*,8a*S*)-decahydro-3(*S*)-isoquinolinecarbonyl, described by Roberts *et al.* (10)], was also tested and found to be unaffected by this particular mutation. This result is consistent with crystallographic and modeling studies of Rich *et al.* (27) who showed that, while the P_1 phenylalanine can interact with the S_1 position, the (4a*S*,8a*S*)-decahydro-3(*S*)-isoquinolinecarbonyl group can occupy the $S_{1'}$ position and extend as far as the $S_{3'}$ position in the enzyme. The symmetrical change in the variant protease is apparently overcome by the asymmetric interactions of this inhibitor.

DISCUSSION

HIV protease inhibitors have the promise of providing the next generation of treatments for HIV disease. Therefore, understanding the potential for selecting resistant phenotypes is very important. The variant viruses described here have a modestly reduced sensitivity to P9941, which can be overcome by increasing the dose or by using related but structurally different inhibitors. The fact that a change at Val^{82} results in an enzyme with modified activity and reduced sensitivity may not be surprising because Grinde *et al.* (28) had shown that Val^{82} is specifically involved in substrate recognition. By replacing S_1 amino acids in Rous sarcoma virus PR with analogous HIV PR amino acids, including Val^{82}, they were able to produce a hybrid enzyme with the ability to recognize an HIV-specific substrate. In addition, the observations made here and in previous studies with recombinant HIV PRs (26, 29) indicate that even a conservative change at Val^{82} results in a less efficient protease. These facts may have some bearing on the difficulty in generating a resistant population by sequential passage. Under the selective conditions used in this study, only a small fraction of the population contained the Ala^{82} mutation, even after many cycles of replication in the presence of P9941. Under similar conditions high level resistance was obtained after only one or two passages with nonnucleoside HIV reverse transcriptase inhibitors (30, 31). Similarly, passage of HIV-1 in cell culture with 3-azido-3'-deoxythymidine (AZT) for five or six passages resulted in a population of virus with a 10-fold decrease in sensitivity (32). Our results suggest that single step mutation to high level resistance (>10-fold) of the type seen for nonnucleoside reverse transcriptase inhibitors does not readily occur for P9941. However, extrapolation from results obtained for P9941 to different classes of HIV protease inhibitors is uncertain at this time.

The clinical setting is significantly more complex than the *in vitro* model used in these studies. In particular, the genotypic and phenotypic diversity of HIV with respect to protease structure and activity is poorly understood. Analysis of additional clinical isolates with emphasis on amino acid sequences and protease inhibitor sensitivity should provide a picture of the diversity that already exists in the general population. Sequence data obtained thus far from our

Table 1. IC_{90} values for HIV-1(RF) subclones, μg/ml

Inhibitor	HIV-1(RF)	HIV(RF)41-D2	HIV(RF)41-D4	HIV(RF)41-E4	HIV(RF)41-F4	HIV(RF)41-G1
[2-PyAc-Ile-Phe-ψ(CHOH)]$_2$*	0.9	7.2	5.4	6.0	5.7	5.6
[2-PyAc-Val-Phe-ψ(CHOH)]$_2$	1.3	>5	>5	>5	>5	>5
[Cbz-Val-Phe-ψ(CHOH)]$_2$	0.09	0.35	0.3	0.35	0.4	0.3
[Cbz-Val-Tyr-ψ(CHOH)]$_2$	0.4	0.9	0.9	0.9	0.9	0.9
[Ac-Val-BzlTyr-ψ(CHOH)]$_2$	0.3	0.3	0.3	0.3	0.3	0.3
Cbz-Val-Phe-CHO	2.5	5.3	6.3	7	6.5	6.3
QC-Asn-Phe-ψ(CHOHCH$_2$N)DIQ†	0.004	0.004	0.004	0.004	0.004	0.004
AZT	0.02	0.02	0.02	0.02	0.02	0.02
ddC	0.26	0.25	0.26	0.25	0.26	0.25

IC_{90} values were determined by yield reduction, as described in ref. 15; values are the average of two separate assays done in duplicate. QC, quinoline-2-carbonyl; DIQ, (4a*S*,8a*S*)-decahydro-3(*S*)-isoquinolinecarbonyl; AZT, 3'-azido-3'-deoxythymidine; Bzl, benzyl.
*P9941.
†This inhibitor was described by Roberts *et al.* (10) as Ro31-8959.

studies (data not shown), from the Los Alamos data base (33), and recently from Fontenot *et al.* (34) indicate that the region of the protease from Gly^{78} to Asn^{88} is highly conserved with no changes in the amino acid sequence. Only after patients have been treated with the compounds will we know what variants will be generated by selective pressure during therapy.

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