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Multiple mutations in the human immunodeficiency virus protease gene are responsible for decreased susceptibility to protease inhibitors

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Summary

The protease (PR) of the human immunodeficiency virus (HIV) is essential for replication of the virus, and accordingly has become an attractive target for the development of an antiretroviral drug. We have previously reported that passage of HIV-1 in the presence of increasing concentrations of the C-2 symmetrical, linear diol P9941 resulted in the isolation of virus with a valine-to-alanine change at position 82 (V82A) of the PR, and reduced sensitivity to certain PR inhibitors. In this study, we passaged four different variants of HIV-1 in increasing concentrations of XM323, and isolated variants with reduced sensitivity to inhibitors of PR. Twenty-three passages of HIV-1 (RF) in the presence of XM323 resulted in a variant that exhibited an approximately 100-fold reduction in susceptibility to XM323 and that contained V82F and I84V changes. When two other viruses, HIV-1(RF41D2) and HIV-1(RF41E4), previously derived from HIV-1 (RF) by passage in the presence of P9941, were passaged in the presence of XM323, variants with V82A/L97V and M46L/V82A/L97V changes, respectively, were obtained. The M46L/V82A/L97V variant showed a 6-fold reduction in sensitivity to XM323, whereas the susceptibility of the V82A/L97V mutant remained unchanged. Seventeen passages of a clinical isolate of HIV-1, HIV-1 (Pat.E), in the presence of XM323 produced a V82F/L97V mutant with an approximately 9-fold reduction in sensitivity to XM323.

Key-words: drug resistance; HIV-1 protease; mutations; XM323.

Introduction

Currently, the only antiviral agents available to those infected with the human immunodeficiency virus (HIV) are targeted to the viral reverse transcriptase. The efficacies of these drugs have been limited by the emergence of drug-resistant strains of HIV-1 in infected patients undergoing drug treatment. The development of this resistance has increased the importance of identifying potential inhibitors of other HIV targets.

The essential nature of the HIV-1 protease (PR) makes it a promising target for the development of anti-HIV compounds (Roberts *et al.*, 1990; Otto *et al.*, 1993b; Wlodawer and Erickson, 1993; Lam *et al.*, 1994; Vacca *et al.*, 1994). The mature PR consists of a 198-amino acid homodimer which is formed after an autocleavage event releases the monomeric subunits from the gag/pol polyproteins (Darke *et al.*, 1989; Loeb *et al.*, 1989). After dimerization, the PR sequentially processes the p160 gag/pol and p55 gag precursor polyproteins into the viral structural proteins (p24, p17, p9 and p7) and viral enzymes (protease, reverse transcriptase and integrase) (Gotlinger *et al.*, 1989; Richards *et al.*, 1989 Page *et al.*, 1990). Exposure of virus to compounds that inhibit PR function results in inefficient and incomplete processing of the viral polyproteins and the formation of immature, non-infectious particles (Lapatoo *et al.*, 1989; Kaplan *et al.*, 1993; Park and Morrow, 1993).

In the light of the resistance problems encountered with the nucleoside and non-nucleoside analogues, it has become important to try to identify, as early as possible during drug development, any potential HIV-1 strains that may exhibit altered susceptibility to the compound under investigation. One method for accomplishing this is to select mutants with decreased sensitivity to a potential inhibitor by passage of virus in cell culture in the presence of increasing concentrations of inhibitor. The particular mutations responsible for the resistant phenotype can then be determined by sequence analysis of the viral PR gene. Using this technique, we and others have isolated virus with decreased sensitivity to inhibitors of the HIV-1 PR (Otto *et al.*, 1993a; El-Farrash, 1994; Ho *et al.*, 1994; Kaplan *et al.*, 1994).

We report in this communication that continued passage of HIV-1 in the presence of increasing concentrations of XM323, a representative of the cyclic urea class of protease inhibitors, resulted in the isolation of strains of virus that contained multiple mutations in the PR gene, some of which had a large effect on the drug susceptibility

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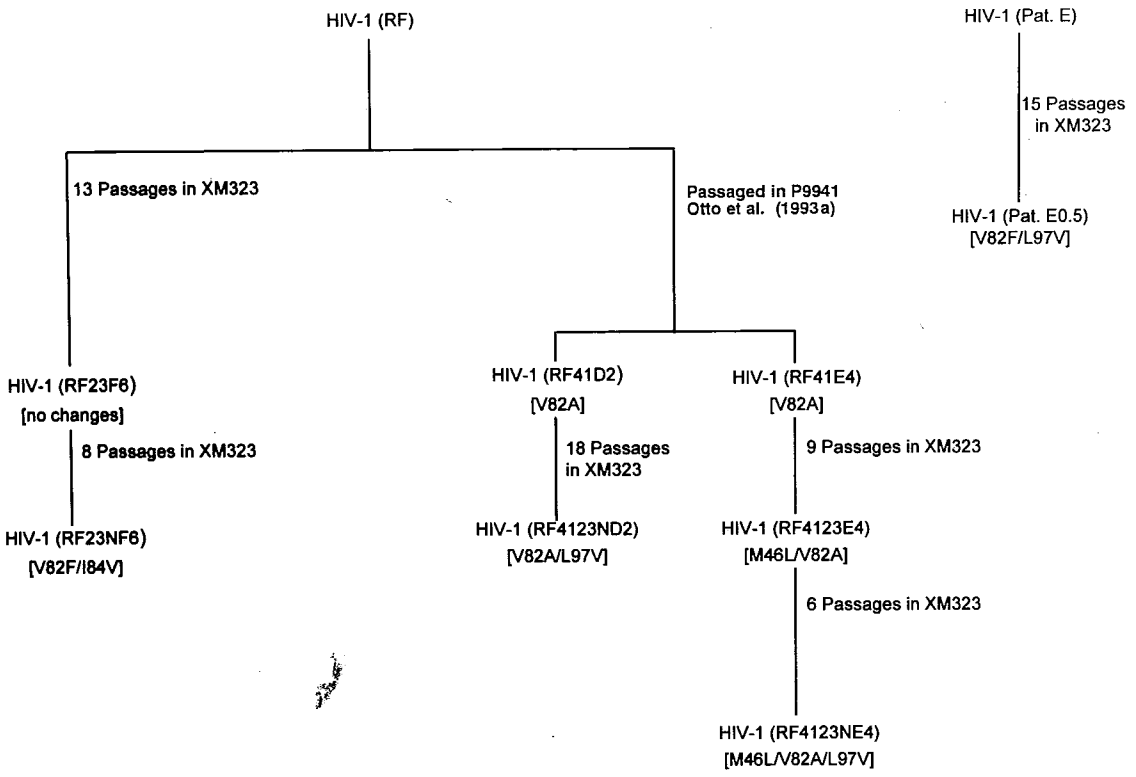

Fig. 1. The selection of (a) HIV-1(RF) and (b) HIV-1(Pat.E) PR variants with decreased sensitivity to inhibitors of PR. For the first passage, MT-2 cells were treated with XM323 at a concentration equal to approximately 75% of the IC_{90} for the particular virus and infected with virus at an MOI of 0.02. Subsequent passages consisted of 0.2–0.5 ml of the supernatant from the preceding passage added to fresh MT-2 cells in fresh medium and XM323.

phenotype and one of which had no or only a slight effect. In addition to the effects of these mutations on the susceptibility of the virus to different PR inhibitors, their effects on the replicative efficiency of the virus were also examined.

Results

Selection of HIV-1 mutants resistant to inhibitors of PR

In order to isolate variants of HIV-1 that were resistant to XM323, HIV-1 (RF), (RF41E4), (RF41D2) and (Pat.E) were passaged in the presence of the protease inhibitor as described in 'Materials and Experimental procedures', and outlined in Fig. 1. HIV-1 (RF), which has a wild-type phenotype with respect to protease inhibitor susceptibility, was passaged 13 times in increasing concentrations of XM323. The resulting virus, HIV-1 (RF23F6), was capable of replicating in a concentration of inhibitor equal to 5 times the original IC_{90}. This virus was plaque purified and assayed for sensitivity to a variety of different PR inhibitors, and its PR gene was sequenced. It then was passaged an additional 15 times in the presence of increasing concentrations of XM323, with the final concentration of XM323 being approximately 40 times higher than the initial level.

HIV-1(RF41D2) and HIV-1(RF41E4), previously isolated by passage of HIV-1(RF) in the presence of P9941 (Otto *et al.*, 1993a), contained V82A/R8A and V82A sequence changes, respectively. HIV-1(RF41D2) was passaged a total of 18 times in the presence of XM323 and the resultant virus, HIV-1(RF4123ND2), was able to tolerate only a slight increase in the concentration of inhibitor, whereas HIV-1(RF4123NE4), the surviving virus from the passage of HIV-1 (RF41E4) 17 times in the presence of XM323, replicated in the presence of XM323 at a concentration of approximately 5 times the original IC_{90}.

A clinical strain of HIV-1, HIV-1 (Pat.E), which was isolated from an HIV-positive male, was passaged 15 times in the presence of XM323. The virus selected, HIV-1 (Pat.E0.5), was capable of replicating in the presence of XM323 at a concentration of approximately 5 times the original IC_{90}.

Sequence analysis of the protease gene

In order to determine whether the changes in sensitivity to XM323 were the result of the accumulation of mutations in the PR gene of the respective virus, the PR gene for each isolate was sequenced as described in 'Materials and Experimental procedures'. Table 1 summarizes the

Table 1. Comparison of the amino acid sequences of proteases from strains and isolates of HIV-1

Virus	10	20	30	40	50	60	70	80	90
RF	PQITLWQRPIVTVKIGGQLKEALLDTGADDTVLEEMNLPGKWKPKMIGGIGGFIKVRQYDQILIECGHKAIGTVLVGPTPVNIIGRNLLTQIGCTLNF								
RF41D2	.....A.....							A.....	
RF4123ND2	.....A.....							A.....V..	
RF41E4	.....							A.....	
RF4123E4	.....				L.....			A.....	
RF4123NE4	.....				L.....			A.....V..	
RF23F6	.....								
RF23NF6	.....							F.V.....	
Pat. E	.....L..IR...I.....				R.....		P.....I.....		
Pat. E0.5	.....L..IR...I.....				R.....		P.....T.....F.....V..		

results of the sequence analysis by showing the predicted amino acid sequence of the PR genes of the respective viruses. The PR gene for HIV-1 (RF23NF6) contained two mutations. One mutation was a transversion (G→T) at the first base in the codon for position 82, which resulted in a phenylalanine in place of the wild-type valine. An additional mutation was found at the first base in the codon for amino acid 84. This transition (A→G) resulted in a valine at this position instead of isoleucine. An intermediate of HIV-1 (RF23NF6) isolated at passage 13 showed a 3-fold reduction in sensitivity to XM323 but contained no changes in the nucleic acid sequence that would alter the amino acid sequence of the PR gene (Tables 1 and 2).

As previously described by Otto *et al.* (1993a), HIV-1(RF41E4) and HIV-1(RF41D2) both contained mutations in the second base in the codon for position 82 of the HIV-1 PR gene, resulting in a valine-to-alanine change (G**U**X→G**C**X). Additionally, HIV-1(RF41D2) contained a double mutation in the codon for position 8 which resulted in an arginine-to-alanine change (**AG**X→**GC**X). HIV-1 (RF4123ND2), which showed little change in sensitivity to any of the PR inhibitors beyond what was exhibited by HIV-1 (RF41D2), contained the two codon changes found in its parental strain plus an additional mutation at the first base in the codon for position 97. This transversion of cytosine to guanine would result in a leucine-to-valine substitution at this position. HIV-1(RF4123NE4), which was resistant to both XM323 and P9941, maintained the V82A and acquired two additional changes, a C-to-G transversion in the codon for position 97 resulting in a L97V change, and a C-to-A transversion in the first base in the codon for position 46 resulting in an M46L change. HIV-1(RF4123E4), an intermediate of HIV-1(RF4123NE4) isolated at passage 9, contained the V82A

Table 2. Relative susceptibility of HIV-1 variants to selected protease inhibitors

	Fold change in IC_{90}			
Virus	XM323	P9941	Ro31-8959	A-80897
RF	1 [0.07][a]	1 [0.9]	1 [0.2]	1 [0.004]
RF41D2 (V82A)[b]	2.9	7.8	1	1.9
RF4123ND2 (V82A, L97V)	3.3	9	1	N.D.[c]
RF41E4 (V82A)	3.1	7.6	1	2.1
RF4123E4 (V82A,M46L)	7	9.9	1	3.2
RF4123NE4 (V82A,M46L,L97V)	11	9.8	1	N.D.
RF23F6 (No change)	2.7	1	1.7	1.1
RF23NF6 (V82F,I84V)	97	2.3	2	N.D.
Pat. E	1 [0.07]	1 [0.95]	1 [0.22]	1 [0.008]
Pat. E0.5	3.5	2.5	2	2.8

[a] IC_{90} (ug ml^{-1})
[b] Genotype of the protease gene.
[c] Not determined.

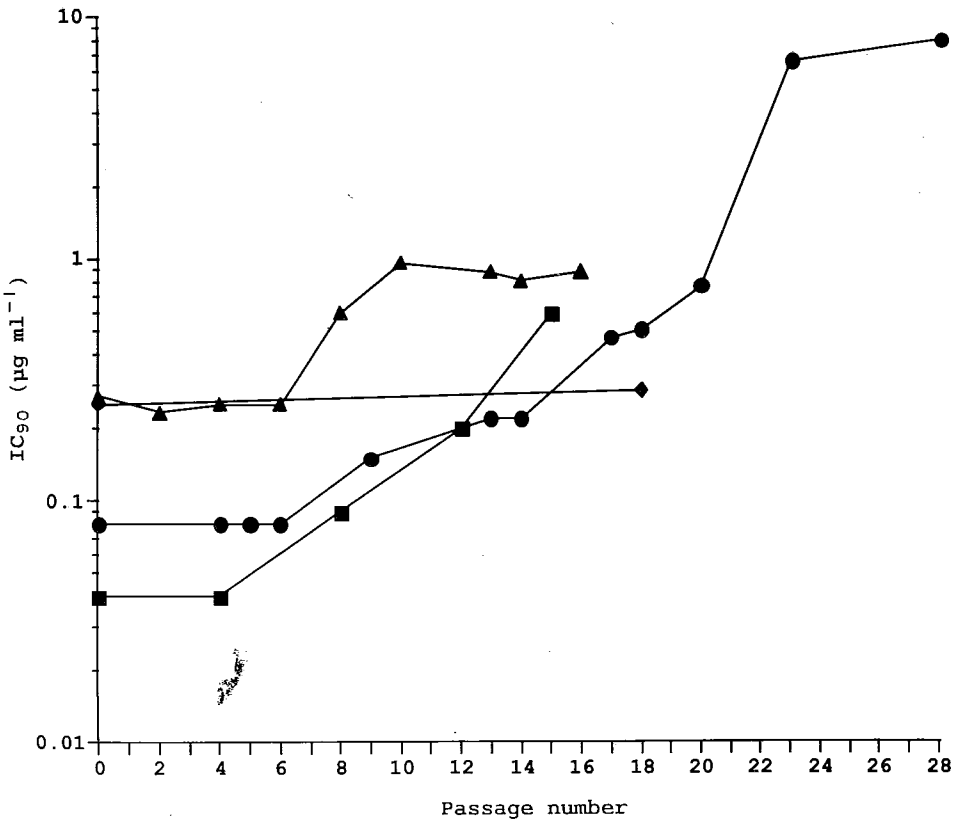

Fig. 2. Change in the IC_{90} of XM323 for the HIV-1 variants during the selection procedure. HIV-1 (RF), (RF41E4), (RF41D2) and (Pat.E) were passaged as shown in Fig. 1 and described in 'Materials and Experimental procedures'. The IC_{90} of XM323 for the resultant virus from each individual passage was determined by a yield reduction assay and compared to that of the parental strain. Symbols are as follows: –■– HIV-1 (Pat.E0.S); –●–, HIV-1 (RF23NF6); –▲–, HIV-1 (RF4123E4); –◆–, HIV-1 (RF4123ND2).

and M46L changes but not L97V. HIV-1(RF4123E4) and HIV-1 (RF4123NE4) exhibited nearly identical sensitivities to the PR inhibitors (Tables 1 and 2).

HIV-1 (Pat.E0.5) contained two mutations that resulted in amino acid changes when compared to its parental strain, HIV-1 (Pat.E). One change was the same V82F as seen in HIV-1(RF23NF6), and the other was the L97V change seen in HIV-1(RF4123ND2) and HIV-1(RF4123NE4).

Changes in the IC90 of XM323 during passage

After elucidating the exact mutations in the PR gene that were responsible for the reduction in susceptibility to PR inhibitor, we attempted to determine when during the selection procedure these changes occurred. The IC_{90} of XM323 for the virus from each individual passage was determined by yield reduction assay. These values were then compared to the original IC_{90} for the parental strains (Fig. 2).

For HIV-1 (Pat.E), the first change in IC_{90} was detected between passages 4 and 8. The IC_{90} increased until passage 15, when the selection procedure was terminated. The IC_{90} of XM323 for HIV-1 (RF41E4) also exhibited an initial change by passage 8, but quickly reached a plateau by passage 10 which was maintained for an additional seven passages. The IC_{90} of XM323 for HIV-1 (RF41D2), as noted above, did not change, although the virus was passaged 18 times.

The changes in the IC_{90} of XM323 for HIV-1 (RF) occurred in two stages. The first change took place between passages 6 and 9. The IC_{90} then stabilized until passage 13, at which time it increased until reaching a plateau at passage 22. Plaque purification and sequence analysis of the resultant virus from passage 12 showed that, while the virus demonstrated a slight but consistent measured reduction in susceptibility to XM323, it did not contain any mutations in the PR gene that would lead to amino acid changes. At passage 17, sequence analysis of 15 plaque isolates showed that 13 were wild-type and two had the mutations responsible for V82F/I84V. By passage 22, 10 out of 10 plaque isolates were the double mutant. The extended time between plateaus for this variant, as well as for the others, illustrated that the change

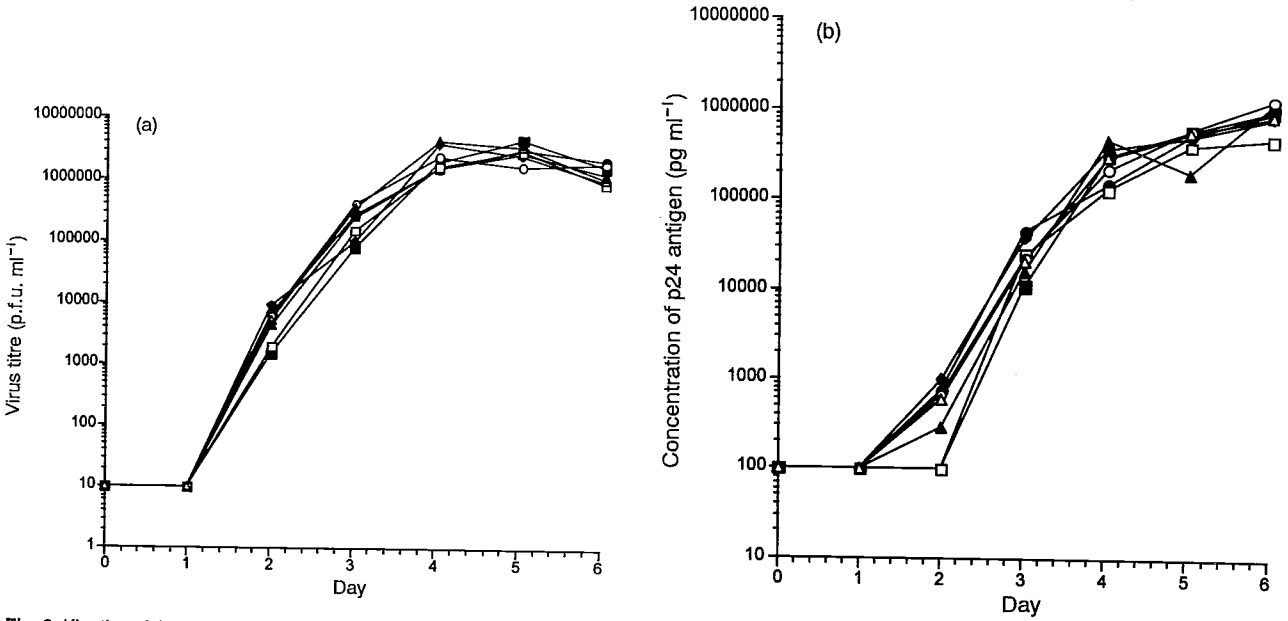

Fig. 3. Kinetics of the production of (a) infectious virus or (b) p24 antigen by the HIV-1 PR variants. MT-2 cells were infected with virus at an MOI of 0.01 and grown at 37 °C for 7 days. Every 24 h, the medium was sampled and assayed for the presence of infectious virus by plaque assay or p24 antigen by ELISA. Symbols are as follows: –■–, RF; –◆–, RF4123ND2; –●–, RF23NF6; –▲–, RF41D2; –□–, RF41E4; –○–, RF4123E4; –△–, RF4123NE4.

from one homogeneous population to the next did not occur in one or two passages but over several.

Cross-resistance of resistant variant viruses

Previous work in our laboratory had shown that variant virus with a V82A change had reduced susceptibility to both P9941 and XM323, but that their susceptibility to the PR inhibitor Ro31-8959 was not altered. The viruses recovered from multiple passages in the presence of XM323 were therefore tested for their sensitivity to all three of these PR inhibitors as well as to the linear diol A-80897 (Kempf *et al.*, 1993).

We classify changes in the sensitivity of the virus to PR inhibitors as follows: an increase in IC_{90} <3-fold is considered 'no change'; an increase ≥3-fold but <10-fold is classified as 'reduced sensitivity', and an increase ≥10-fold is 'resistant.' As shown in Table 1, HIV-1(RF23F6) exhibited a 3-fold reduction in susceptibility to XM323 and no change with respect to P9941, whereas HIV-1 (RF23NF6) became resistant to XM323 (97-fold increase in IC_{90}), but showed only reduced sensitivity to P9941 (3-fold change). HIV-1(RF4123NE4) became approximately 10-fold resistant to both XM323 and P9941, whereas HIV-1(RF4123ND2) was resistant to P9941 but only exhibited reduced sensitivity to XM323 (4-fold reduction). The sensitivity of these variants to Ro31-8959 did not change. The sensitivity of HIV-1(Pat.E0.5) to XM323 decreased 4.3-fold, but its sensitivity to P9941 and Ro31-8959 was not altered. Of all the variants tested, only HIV-1(RF4123E4) showed a greater than 3-fold reduction in susceptibility to A-80897.

Kinetics of p24 antigen and infectious virus production

An HIV variant with the I84V single mutation was reported to have lower infectivity than the wild-type virus (El-Farrash *et al.*, 1994). To determine whether the mutations found in our viruses had a similar effect, we infected MT-2 cells at MOIs of 1.0 and 0.01 and sampled the medium at 24-h intervals for 7 days and then tested the medium samples for the presence of infectious virus and p24 antigen. At either MOI, the above mutations in the PR gene and/or the predicted amino acid changes in the PR itself had no effect on the quantity of p24 or infectious virus released from the infected cells, or the rate at which either was produced (Figs 3 and 4).

Susceptibility of recombinant viruses

While the mutations found in the PR genes of the variant viruses were consistent with the changes in the susceptibility to the protease inhibitors, other mutations outside the PR gene might also play a role in the phenotype. In order to test this, recombinant viruses were constructed in which the genetic backgrounds were maintained as HIV-1(HxB2) and point mutations were made in the PR gene. In this manner the effect of the single amino acid changes

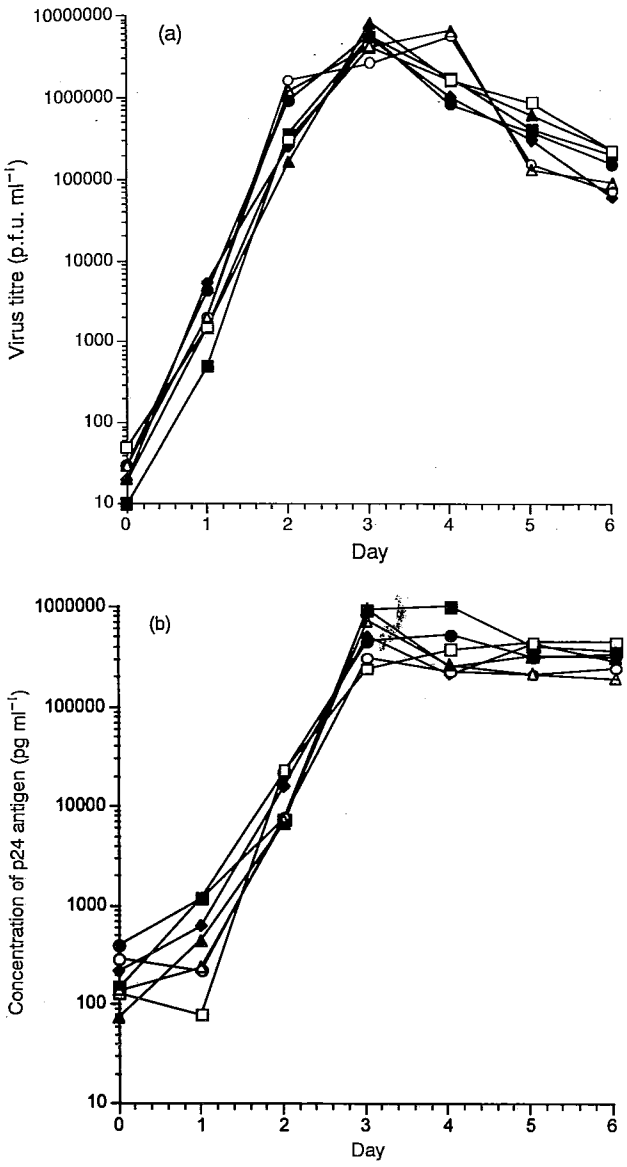

Fig. 4. Kinetics of the production of (a) infectious virus or (b) p24 antigen by the HIV-1 PR variants. Cells were infected at an MOI of 1.0 as described in the legend to Fig. 3. Medium samples were assayed for the presence of infectious virus by plaque assay or p24 antigen by ELISA. Symbols are as in Fig. 3.

in the PR on the susceptibility to XM323 or P9941 could be explored. As shown in Table 3, the changes observed with the single amino acid change V82A, V82F or I84V in the HIV-1 (HxB2) PR were similar to the changes observed for the selected viruses.

Discussion

The understanding of the development of drug-resistant strains of virus has become an important, if not essential, part of the drug discovery process. A significant contribution to this effort is the elucidation of the mutations that lead to reduced sensitivity to the compound under investigation. One method for accomplishing this is to select and characterize variants with reduced susceptibility to inhibitor which arise during multiple passages of virus in the presence of increasing concentrations of inhibitor.

Using this method, we originally selected strains of HIV-1 with reduced susceptibility to inhibitors of PR (Otto *et al.*, 1993a). Since then, other labs have isolated additional strains with altered susceptibility to various inhibitors (El-Farrash *et al.*, 1994; Ho *et al.*, 1994; Kaplan *et al.*, 1994). All the variant strains of HIV-1 that we have isolated using the C2 symmetrical diol P9941 or the cyclic urea XM323 have contained a change at position 82 from valine to either phenylalanine or alanine.

Passage of virus in the presence of other linear peptidyl mimetics has resulted in the isolation of PR-resistant variants that also contain changes at position 82, as well as variants that contain changes at positions 8 and 32. Passage of HIV-1(NL4-3) in MT-4 cells or HIV-1(HxB2) in CEM cells in the presence of the C2 symmetrical diol A-77003 has resulted in the isolation of a number of variants with reduced sensitivity (Ho *et al.*, 1994; Kaplan *et al.*, 1994). Kaplan *et al.* (1994) have isolated variants of HxB2 with either V82I (no reduction in sensitivity) or V32I (reduced susceptibility to A77003) or both V32I and V82I (even greater reduction in sensitivity). Ho *et al.* (1994) have used A-77003 to select a number of different PR mutants derived from HIV-1(NL4-3). All the mutants have changes at position 8 (R8Q or R8K), most also have a change at position 46 (M46I), and one has an additional V82A change. El-Farrash *et al.* (1994) have used an asymmetrical C-2 linear mono-ol, RPI-312, to select a resistant variant of HIV-1(III_B) from chronically infected MOLT-4 cells. This isolate contains a single amino acid change of isoleucine to valine at position 84.

It is interesting to note that, in the four published studies, different types of PR resistant virus were isolated. In two of the cases, passage of different strains of virus in different cell lines treated with the same inhibitor (A-77003) resulted in the selection of different genetic variants. This suggests that several factors may play a role in determining which variants arise. Not only is the specific inhibitor

Table 3. Relative susceptibility of recombinant HIV-1 virus

	Fold change in IC_{90}			
Compound	HxB2	V82A	V82F	I84V
XM323	1	2	8	50
P9941	1	6	6	8
Ro31-8959	1	1	1.5	2

important but the genotype of the parental virus and possibly the cell type may determine which amino acid positions are most likely to be altered.

To predict more accurately what types of mutation may lead to resistance to the cyclic urea class of PR inhibitors, we decided to passage four different strains of HIV-1 in the presence of XM323. HIV-1 (RF), our laboratory strain of wild-type virus, was used to determine the kinds of mutations that may arise in the wild-type sequence, and HIV-1 (Pat.E) was used to determine whether a fresh clinical strain would give results similar to or different from those obtained using the laboratory strain. Since the V82A change is the most commonly found variant, HIV-1 (RF41E4) and (RF41D2) were used to identify the types of mutation that may arise within strains that already contain a V82A substitution.

Passage of HIV-1 (RF) in the presence of XM323 resulted in the identification of two isolates. The first variant, HIV-1 (RF23F6), which was isolated after 13 passages, had a reduced sensitivity to XM323 (approximately 3-fold) but did not contain any mutations in the coding region of the PR gene that affected the amino acid sequence. The 3-fold reduction in the susceptibility of this isolate to XM323 is unexplained, but may be attributed to changes in the upstream, non-coding sequences that play a role in the processing of the immature PR from the polyprotein or possibly affect dimerization. Replacement of the PR gene of HIV-1(HxB2) with the PR genes from HIV-1(RF) and (RF23F6), respectively, resulted in recombinant virus with similar susceptibilities to XM323, and so the change responsible for the 3-fold difference must map outside the PR gene (data not shown).

Additional passages in XM323 resulted in the second isolate, HIV-1 (RF23NF6), which contained V82F and I84V changes in the PR and was approximately 100-fold more resistant to XM323 than HIV-1 (RF). Even if the 3-fold increase in resistance seen in HIV-1 (RF23F6) was brought into the calculation for the reduction in susceptibility, these two mutations, at least, are responsible for a 33-fold reduction in the susceptibility of the virus to XM323. The hypothesis that the I84V change is responsible for a 33-fold reduction in sensitivity to XM323 was further supported by the construction of the HIV-1(HxB2) recombinant virus which contained this change and which was 50-fold less sensitive to XM323 than the parental virus. We are currently attempting to construct the V82F/I84V double mutant in the HIV-1(HxB2) background.

El-Farrash *et al.* (1994) reported that the I84V mutant had lower infectivity than the wild-type, HIV-1 (III_b). In order to determine whether any of the mutations found in the PR genes of HIV-1 (RF23NF6), (RF4123ND2), and (RF4123NE4) had a similar effect, we infected MT-2 cells at an MOI of 1.0 and 0.01, and determined the quantity of p24 antigen and infectious virus produced during a 24-h period (the approximate time of one round of replication), and the kinetics of production. The results of these experiments showed that the mutations found in these genes did not affect the ability of the virus to replicate in MT-2 cells. The discrepancy between the relative 'health' of our I84V mutant, HIV-1(RF23NF6), and that of the I84V mutant isolated by El-Farrash *et al.* can be reconciled by one of two explanations.

It is possible that a second mutation exists in the El-Farrash escape mutant that affects the infectivity of this virus. Given the high rate of genetic mutation of HIV-1, the occurrence of additional mutations at different sites in the HIV-1 genome that may affect other metabolic events would not be unexpected. Another explanation may be that the V82F change, which is the second mutation found in the HIV-1 (RF23NF6) mutant, may compensate for the I84V change, thus allowing normal growth. Positions 82 and 84 are both found in the S1/S1' substrate-binding pocket. The substitution of one of the amino acids found in this pocket for another with a larger or smaller side-chain may affect the interaction between substrate and enzyme. Molecular modelling has shown that substitution of a phenylalanine for valine at position 82 increases the distance between the P1/P1' side-chains of XM323 and the side-chains of the amino acid at position 82 (C.-H. Chang, The DuPont Merck Pharmaceutical Company, personal communication). The effect of this change may be tempered if a second amino acid substitution occurs in the S1/S1' pocket that restores the distance between enzyme and substrate/inhibitor. Similarly, any detrimental effect that may occur with an I84V variation may be rescued by a V82F substitution.

The passage of the two V82A variants in the presence of increasing concentrations of XM323 resulted in the accumulation of further mutations in the HIV-1 PR gene. An adenine-to-cytosine transversion at the first base of the codon for amino acid 46 resulted in a methionine-to-leucine substitution which was similar to the substitutions reported by Ho *et al.* (1994), except that Ho's variants contained an M46I change after serial passage of HIV-1 (NL4-3) in the presence of the linear diol A-77003.

The second mutation that occurred in three of the four XM323-resistant mutants was an adenine-to-guanine transition at the first base of the codon for amino acid 97 (L97V). It is interesting to note that, while the other amino acid substitutions occur in or around the catalytic site of the enzyme and may play a role in the interaction of enzyme with substrate, amino acid 97 is instead located in the domain responsible for dimerization. In at least two cases, this amino acid change had little or no effect on the susceptibility of the virus to inhibitors of PR. Any slight change in the IC_{90} of XM323 or P9941 to the virus with the L97V genotype was probably caused by further pas-

sage of virus in the presence of an increased concentration of XM323 to obtain a homogenous population of resistant virus rather than by any direct influence exerted by the individual amino acid located at position 97. Nevertheless, there seems to be some selective advantage in a valine at position 97 when virus is grown in the presence of XM323 which may not be reflected in a change of susceptibility to inhibitors of PR. For instance, the L97V variation may advantageously affect the formation or stability of the PR dimer when virus is exposed to inhibitor.

Finally, we once again showed that mutations that altered the particular amino acid at position 82 of the HIV-1 PR predominated in the escape mutants isolated from HIV-1-infected cells treated with XM323, and that these mutations, as well as those that caused amino acid changes at positions 46 and 84, must seriously be considered as the development of the cyclic urea class of PR inhibitors continues. However, we must remember that these mutations have been isolated from HIV-1-infected cell culture; the real predictive value of this methodology will be validated only when a member of this unique class of PR inhibitor reaches the clinic.

Materials and Experimental Procedures

Virus and cell lines

HIV-1 (RF) was obtained from Robert Gallo (National Institute of Health, Bethesda, MD), as infected H9 cell cultures. HIV-1 (RF41D2) and (RF41E4) were described previously by Otto *et al.* (1993a). HIV-1 (Pat.E) was isolated from an HIV-positive patient from Wilmington, DE (Winslow *et al.*, 1995). Virus titres were quantified by plaque assay on MT-2 as previously described (Otto *et al.*, 1993a, b; Smallheer *et al.*, 1993). 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 maintained in RPMI 1640 plus 5% (v./v.) fetal bovine serum and gentamycin (5 ug ml^{-1}) at 37 °C and 5% CO_2.

Protease inhibitor compounds

XM323 and P9941, compounds in the cyclic urea and C2 symmetrical linear diol classes, respectively, were synthesized by chemists at DuPont Merck Pharmaceutical Co. (DMPC) (Otto *et al.*, 1993a, b; Lam *et al.*, 1994). Ro31-8959 (Roberts *et al.*, 1990) and A-80897 (Kempf *et al.*, 1991) have been described previously. Their equivalents were synthesized at DMPC.

Selection of HIV-1 mutants

The procedure for the selection of HIV-1 mutants with decreased sensitivity to XM323 is outlined in Fig. 1. Briefly, MT-2 cells were infected with either HIV-1 (RF), (RF41D2), (RF41E4) or (Pat. E) at a multiplicity of infection (MOI) of 0.02 in the presence of XM323 (the concentration for the first passage was approximately 75–90% of the IC_{90}). Cells were checked daily for the presence of cytopathic effect (CPE). When CPE was greater than 75%, the cell suspension was centrifuged at low speed and the supernatant collected. Fresh cells were placed in RPMI medium containing XM323 and 0.2–0.5 ml of supernatant. The concentration of XM323 was kept constant until 75% CPE was achieved in ≤3 days for two consecutive passages, at which time the drug concentration was increased by 50–100%. Cells were passaged according to the above regimen until cells exhibited 75% or greater CPE in ≤3 days when grown in the presence of XM323 at a concentration ≥5 times the original IC_{90}. Virus was then expanded in MT-2 cells to create a working stock, the IC_{90} values of XM323, P9941 and Ro31-8959 were established, and the PR gene from the resulting virus was sequenced.

Plaque purification of HIV-1

Supernatant from HIV-1-infected cells was serially diluted using 10-fold dilutions and plated on MT-2 cells as previously described (Otto *et al.*, 1993b; Smallheer *et al.*, 1993). After 6 days the monolayer was examined for the presence of plaques. Wells that contained ≤20 plaques were chosen. Virus from isolated plaques was collected by removal of the agarose plug from above the monolayer with a sterile 5-ml pipette. The agarose plug was placed in 0.2 ml of medium, of which 0.1 ml was added to 5 ml of fresh medium containing MT-2 and incubated at 37 °C until >75% CPE was detected. At this time, cells were separated from the medium by centrifugation, the supernatant being saved as a viral stock solution and the cells used as a source of proviral DNA for sequencing the viral PR gene.

Sequence analysis of the HIV-1 protease gene

In order to determine the nucleic acid sequences of the PR genes from the different HIV-1 mutants, MT-2 cells were infected with virus at an MOI of 0.02 and incubated for 3 days at 37 °C. The cells were pelleted by low-speed centrifugation, resuspended in a Tris HCl buffer (0.1 M KCl, 10 mM Tris HCl and 2.5 mM $MgCl_2$), and then lysed in the presence of 0.5% NP-40 and 0.5% Tween-20. The proteins were digested with Proteinase K (final concentration 50 ug ml^{-1}) at 60 °C for 1 h, and the Proteinase K was then inactivated at 95 °C for 2 h. The PR gene was amplified from the DNA in these lysates by PCR (AmpliTaq DNA polymerase, Perkin Elmer) using primers Sn50 (TAGGGAAAATCTGGCCTTCCCACAAG) and Asn800 (CCATCCATTCCTGGCTTTATA). The amplified DNAs were purified (Magic PCR Preps Purification System, Promega Corp.), the sense and antisense strands were sequenced by the Sanger method (fmol Sequencing System, Promega Corp.), and the sequence was visualized by chemiluminescense (Images System, U.S. Biochemical). Amino acid sequences were predicted using the TRANSLATE program (Genetics Computer Group, Madison, WI).

HIV-1 yield reduction assay

The HIV-1 yield reduction assay was performed as previously described (Otto *et al.*, 1993a). Briefly, MT-2 cells were infected with virus (MOI = 0.02) and treated with various concentrations of PR inhibitor. 3 days after infection, the cells were examined

for CPE and the cell-free supernatants were collected. The concentration of virus in each supernatant sample was determined by plaque assay. For each compound, the IC_{90}, the amount of inhibitor necessary to inhibit 90% of virus growth, was calculated.

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