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Identification of a clinical isolate of HIV-1 with an isoleucine at position 82 of the protease which retains susceptibility to protease inhibitors

Robert W. King [a,*], Dean L. Winslow [a], Sena Garber [a], Helen T. Scarnati [a], Lee Bachelor [b], Sylvia Stack [b], Michael J. Otto [a,*]

[a] *The DuPont Merck Pharmaceutical Company, Virology Research, 500 S. Ridgeway Ave, Glenolden, PA 19036, USA*

[b] *Biotechnology, Experimental Station, Wilmington, DE 19880, USA*

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Abstract

The HIV-1 protease (PR) is essential for the production of mature virions. As such, it has become a target for the development of anti-HIV chemotherapeutics. Multiple passages of virus in cell culture in the presence of PR inhibitors have resulted in the selection of variants with decreased sensitivity to inhibitors of the PR. The most common alteration observed is a single amino acid change at position 82. This particular position has been well characterized by several laboratories as being important for the susceptibility of the virus to inhibitors of PR function. Mutations which result in the substitution of the wild-type valine with alanine, phenylalanine, threonine or isoleucine at position 82 of the PR have been associated with decreased sensitivity to several PR inhibitors. We describe here a clinical strain of HIV-1 that contains an isoleucine at position 82 of the PR instead of the usual valine. This strain is unique in that it was isolated from a patient that was anti-retroviral naive, and in the past, variants at position 82 of the PR have only been found after treatment of patients or cell culture with PR inhibitors. Moreover, this virus remains sensitive to PR inhibitors of the cyclic urea and C-2 symmetrical diol classes.

* Corresponding author. Present address: R.W. King, Avid Therapeutics, Inc., 3401 Market St., Suite 300, Philadelphia, PA 19104, USA. Fax: +1 (215) 386 5227.

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1. Introduction

The genome of the human immunodeficiency virus encodes an aspartyl protease which is essential for maturation, release and stability of the virus (Kohl et al., 1988; Seelmeier et al., 1988; McQuade et al., 1990; Park and Morrow, 1993). The mature, 198-amino acid homodimer is formed after an autocleavage event releases the monomeric subunits from the *gag/pol* polyproteins (Darke et al., 1989; Mergener et al., 1992). After dimerization, the mature PR cleaves the p160 *gag/pol* and p55 *gag* precursor polyproteins into the viral structural (p24, p17, p9 and p7) and metabolic proteins (protease, reverse transcriptase, and integrase) (Göttlinger et al., 1989; Page et al., 1990; Schneider and Kent, 1988). Mutations which encode a non-functional PR or exposure to compounds which inhibit the enzyme result in the inefficient processing of the viral polyproteins and the formation of immature, non-infectious particles (McQuade et al., 1990; Kaplan et al., 1993).

Conservation of sequence in 5 regions of the PR has been described previously (Fontenot et al., 1992; Winslow et al., 1995). Alterations in these regions have been observed only following growth of virus in the presence of PR inhibitors either in cell culture or patients. These HIV-1 variants have been less sensitive to inhibitors of the HIV-1 PR (Craig et al., 1993; Dianzani et al., 1993; Otto et al., 1993b; El-Farrash et al., 1994; Ho et al., 1994, King et al., 1995). Of particular interest is the amino acid at position 82 of the HIV-1 PR. While the susceptible wild type always contains valine at this position, many of the HIV-1 strains which exhibited reduced susceptibility to PR inhibitors had mutations in the codon for position 82 that resulted in an alanine, phenylalanine, threonine or isoleucine residue. Wild-type strains containing valine were sensitive to linear peptidyl and cyclic urea inhibitors, whereas variants that contained an alanine or phenylalanine were 4- to 8-fold less sensitive (Otto et al., 1993b; King et al., 1995). Variants containing threonine at this position were shown to be less sensitive to L735,524 (Emini et al., 1994).

Curiously, studies with cloned enzymes have been less definitive. We have shown that either alanine, phenylalanine or isoleucine at position 82 in the purified enzyme reduced the sensitivity to C-2 symmetrical diols (Otto et al., 1993b; Ron Klabe, DuPont Merck Pharmaceutical Co., personal communication), whereas Emini et al. (1994) have shown that threonine or isoleucine at position 82 of the purified PR enzyme had little effect on the susceptibility of the enzyme to L735,524 (Emini et al., 1994). Kaplan et al. (1994) have reported that isoleucine alone had no effect on the sensitivity of the enzyme to the C-2 symmetrical diol, A77003, whereas a V32I/V82I double mutant was approximately 20-fold less sensitive.

As previously reported, all clinical strains of HIV-1 isolated from PR inhibitor naive patients contained the wild-type valine at amino acid position 82 of the PR (Winslow et al., 1995). However, here we report the identification of an HIV-1 isolate, HIV-1(Th16), which contained a naturally occurring isoleucine at position 82 of the PR and retained wild-type sensitivity to several classes of protease inhibitors, including the cyclic ureas and C-2 symmetrical diols. This virus was isolated from an asymptomatic, heterosexual male from Chiang Mai, Thailand, who previously had not been treated with any anti-retroviral therapies, and is, as far as we know, the only example of a clinical strain

of HIV-1 isolated from an anti-retroviral agent naive person that does not contain a valine at position 82. Since position 82 is located in the domain of the enzyme important for the binding of the substrate in the active site, it may be important for those involved in the development of PR inhibitors as an anti-HIV agent to be aware that isoleucine as well as valine may reside at this position in wild-type strains of HIV-1. Additionally, we present evidence that, unlike V82F and V82A changes, V82I does not affect the susceptibility of the virus to several different types of HIV-1 PR inhibitors.

2. Materials and methods

2.1. 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 (Th4), (Th5), (Th16), (Th18) and (Th26) were obtained from Jay Levy (UCSF, San Francisco, CA) as supernatants from infected human peripheral mononuclear cells (Ichimura et al., 1994). The virus was expanded by co-cultivation in non-infected PBMCs with IL-2 and PHA stimulation. HIV-1 virus stocks for all viruses were grown in either PBMCs or MT-2 cells and stored in liquid nitrogen. Virus titers were quantitated by plaque assay on MT-2 as previously described (Otto et al., 1993b; 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 μg/ml) at 37°C and 5% $C0_2$.

2.2. Protease inhibitor compounds

XM323 and P9941 are compounds in the cyclic urea and C-2 symmetrical linear diol classes, respectively, and were synthesized by chemists at DuPont Merck Pharmaceutical Co. (DMPC) (Otto et al. 1993a,b; Lam et al., 1994). A-80987 and Ro31-8959 have been described previously (Roberts et al., 1990; Kempf et al., 1991). Their equivalents were synthesized at DMPC.

2.3. Construction of recombinant HIV-1

A recombinant of HIV-1 containing only the I82 variation was constructed as described by Winslow et al. (1994). To create the V82I change, site directed mutagenesis of the HIV-1(HxB2) PR gene was performed using the Altered Sites in vitro Mutagenesis System (Promega Corp., Madison, WI) as follows. The HIV-1(RF) PR gene was subcloned into pAlter and the gene specifically mutated as described by the manufacturers. The HIV-1(HxB2) genome was digested with NcoI, and the 5′ and 3′ halves were subcloned into pGEM-3Z (Promega Corp., Madison, WI) creating p5′R and p3′R, respectively. The mutated PR gene was removed from pAlter and was subcloned

into p5′R such that it replaced the existing HIV-1(HxB2) PR gene located at that site. The new p5′R and p3′R plasmids were linearized with NcoI and ligated together to create infectious DNA. MT-2 cells were transfected with 1 μg of ligated DNA using the Transfectin System (Gibco/BRL, Gaithersburg, MD) as instructed by the manufacturers. The resulting virus was collected and its PR gene sequenced to determine if it contained the proper sequence.

2.4. Sequence analysis of the HIV-1 protease gene

In order to determine the nucleic acid sequence of the PR gene from the different HIV-1 strains, MT-2 cells were infected with virus at an m.o.i. 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 conc. 50 μg/ml) at 60°C for 1 h and the proteinase K 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 (Sequenase DNA Sequencing System, US Biochemical) and the sequence visualized by autoradiography.

2.5. Susceptibility assays

2.5.1. HIV-1 yield reduction assay

The HIV-1 yield reduction assay was performed as previously described (Otto et al., 1993b). Briefly, MT-2 cells were infected with virus (m.o.i. = 0.02) and treated with various concentrations of PR inhibitor. Three 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 the plaque assay. For each compound, the IC_{90}, the amount of inhibitor necessary to inhibit 90% of virus growth, was calculated.

2.5.2. ELISA assay for p24 antigen.

The AIDS Clinical Trial Group/Department of Defense (ACTG/DoD) consensus assay for PR inhibitors was used to determine virus sensitivity to the different PR inhibitors as measured by changes in p24 antigen concentrations (Japour et al., 1993). PBMCs were infected with virus (m.o.i. = 0.001 $TCID_{50}$) and treated with various concentrations of PR inhibitor. Seven days after infection, the cultures were diluted 1 : 156 in medium and the virus inactivated with NP-40 (0.5%). The concentration of p24 antigen in each sample was determined by ELISA as specified by the manufacturer (DuPont/NEN, Wilmington, DE).

3. Results

3.1. Sequence of the PR gene of HIV-1 isolates

The PR gene of the different strains of HIV-1 was amplified by PCR and sequenced by the Sanger method. The nucleic acid sequences of the PR gene of HIV-1(Th16) and

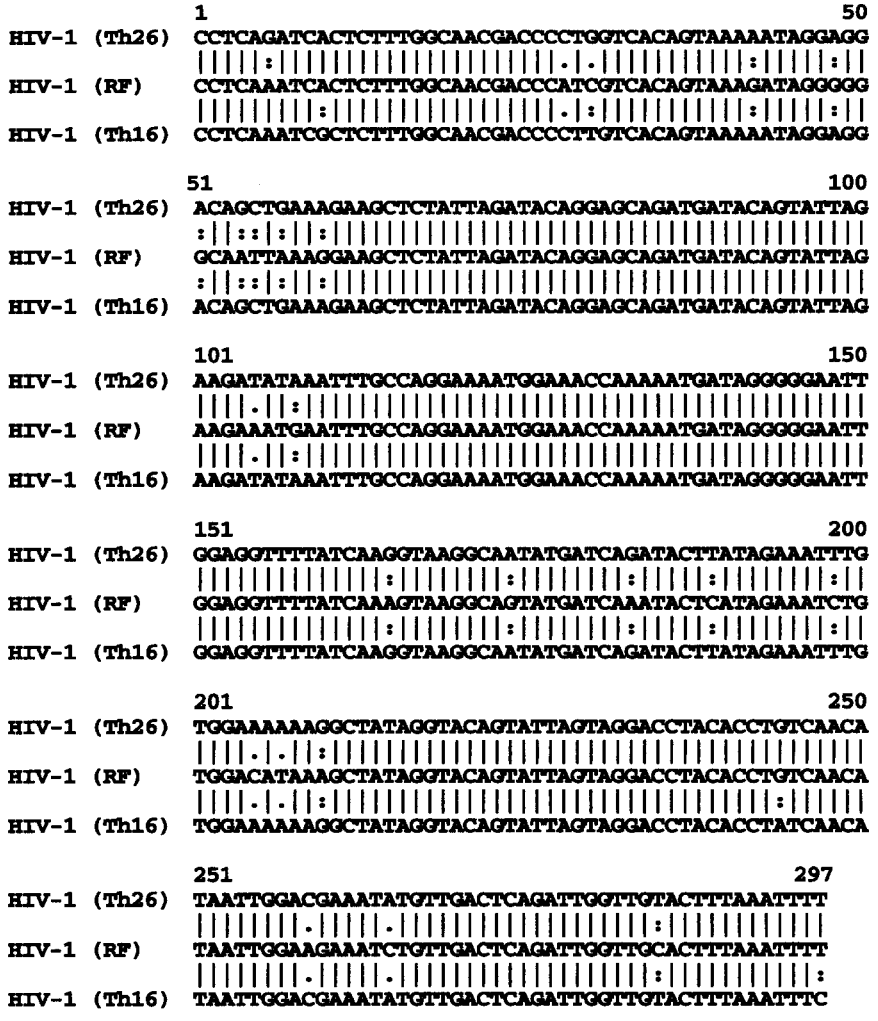

Fig. 1. Nucleic acid sequence comparison of the coding region for the protease genes of HIV-1(Th26), (RF) and (Th16). The protease genes of HIV-1(RF), (Th26) and (Th16) were amplified from the DNA of MT2 cells infected with HIV-1(RF), (Th26) or (Th16), respectively, by PCR amplification. The PCR products were sequenced by the Sanger method (US Biochemical, Cleveland OH) using primers for both the sense and antisense strands. Identical bases are denoted with (|), purine to purine or pyrimidine to pyrimidine variation with (:), and purine to pyrimidine or pyrimidine to purine variation with (.).

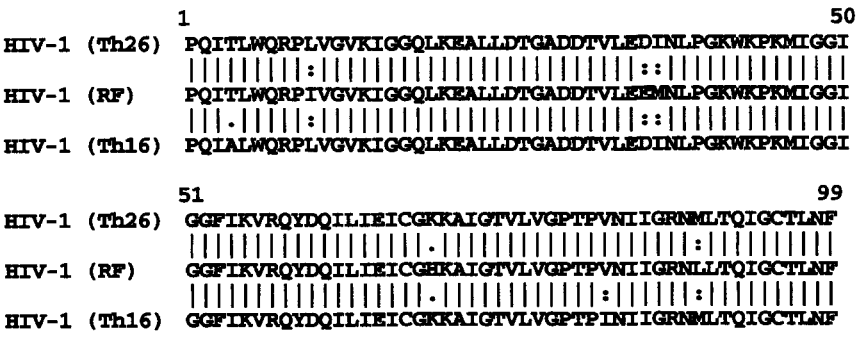

Fig. 2. Comparison of the predicted amino acid sequences of the protease genes of HIV-1(RF), (Th26) and (Th16). The nucleic acid sequence for each protease gene was translated using the Translate program and compared with the Bestfit program (Genetics Computer Group, Madison WI). Identical amino acids are denoted with (|), similar amino acids with (:), and dissimilar amino acids with (.).

(Th26) were compared to HIV-1(RF) (Fig. 1). The PR gene of HIV-1(RF) and (Th16) were 92% identical and 98% similar. Fifty-two percent of the variation between the PR gene of HIV-1(RF) and HIV-1(Th16) was due to purine to purine differences (13/25), whereas pyrimidine to pyrimidine differences accounted for 24% (6/25), and pyrimidine to purine and purine to pyrimidine differences accounted for 12% (3/25) each.

The sequence of the PR of HIV-1(RF) and (Th26) were 93% identical and 98% similar. Fifty-five percent of the variation between these two strains were due to purine to purine differences (12/22), pyrimidine to pyrimidine changes accounted for 18% (4/22), and pyrimidine to purine and purine to pyrimidine accounted for 14% (3/22) each. A comparison of the PR gene sequence of HIV-1(Th16) and (Th26) showed only 5 nucleotide differences. Additionally, the sequence of the PR gene of 3 other HIV-1 isolates from Northern Thailand, HIV-1(Th18), (Th4) and (Th5) were approximately 93% identical to HIV-1(RF) (data not shown).

The PR DNA sequences were translated into predicted amino acid sequences using the Translate program (Genetics Computer Group, Madison WI) and compared (Fig. 2). The amino acid sequences of HIV-1(RF) and (Th16) were 93% identical and 97% similar. The 25 nucleic acid differences resulted in a 7 amino acid difference, including T4A, I10L, E35D, M36I, H69K, V82I, and L89M. Therefore, most differences at the nucleic acid level occurred in the third or 'wobble' position of the codon and were silent. The amino acid sequence of HIV-1(Th26) was similar to that of HIV-1(Th16), except that it contained a threonine at position 4 and a valine at position 82 which are the same amino acids found at positions 4 and 82, respectively, in HIV-1(RF). Therefore, at the amino acid level, HIV-1(RF) and (Th26) were 95% identical and 99% similar (Fig. 2). Of the other 3 HIV-1 isolates from Northern Thailand, the PR from HIV-1(Th5) had an identical amino acid sequence as HIV-1(Th26) as did HIV-1(Th4) except that it contained a L63V change. HIV-1(Th18) had the same amino acid sequence as HIV-1(Th26) with additional changes at amino acids 5 (L5I), 13 (V13I), 20 (K20R), and 93 (I93V) (data not shown).

3.2. Susceptibility to PR inhibitors

To determine how the above amino acid differences might affect the susceptibility of the virus to inhibitors of PR, we infected either PBMCs or MT-2 cells with HIV-1(RF) or one of the Thai isolates in the presence of various concentrations of each of 4 PR inhibitors: XM323, P9941, A-80987 and Ro31-8959. To compare the susceptibilities of HIV-1(Th16) and (Th26) to HIV-1(RF) for each of the 4 compounds, ratios of the IC_{90}s for the two isolates as compared to that of HIV-1(RF) were calculated. If the ratio was equal to or less than 3, the isolate was considered to have equivalent susceptibility as HIV-1(RF).

The IC_{90} values for each compound, whether calculated from the reduction in the supernatant levels of p24 antigen or from titers of infectious virus, illustrated that HIV-1(Th16) was as sensitive as HIV-1(RF) to each of these 4 compounds (Table 1). We have found that this is also true for several other cyclic urea compounds tested (data not shown).

Since the PR from HIV-1(Th16) differs from that of HIV-1(RF) in 6 additional amino acid positions other than V82I, it could be possible that the combination of changes in fact might be a combination of a super-sensitive and a resistant phenotype with the result being a sensitive phenotype. To examine this possibility, we tested another virus isolate from Northern Thailand, HIV-1(Th26) which, as stated above, was like HIV-1(Th16) except for a threonine at position 4 and a valine at position 82 (both differences made it more similar to HIV-1(RF)). This virus, when grown in the presence of the 4 different PR inhibitors, was not hypersensitive, but instead was equally susceptible as both HIV-1(RF) and (Th16) (Table 1).

3.3. Susceptibility of recombinant HIV-1

As stated above, the possibility existed that isoleucine at position 82 of the HIV-1 PR, like other changes at this position, might cause a phenotype of reduced susceptibil-

Table 1
Inhibition (IC_{90}) of HIV-1 (RF) and Thai isolates by PR inhibitors

Virus strain	XM323		P9941		Ro31-8959		A-80987	
	p24 [a]	Y.R. [b]	p24	Y.R.	p24	Y.R.	p24	Y.R.
RF	0.14 [c]	0.15	1.3	1.3	0.015	0.01	0.43	0.38
Th16	0.17	0.21	1.6	2.3	0.015	0.004	0.6	0.37
Th26	ND [d]	0.39	ND	3.0	ND	0.01	ND	0.37
Ratio of IC_{90}s (Th16/RF)	1.2	1.4	1.2	1.76	1	0.4	1.4	0.97
Ratio of IC_{90}s (Th26/RF)	ND	2.6	ND	2.3	ND	1	ND	0.97

[a] IC_{90}s were determined by ELISA for p24 antigen (DuPont, Wilmington DE).
[b] IC_{90}s were determined by yield reduction assay as described by Otto et al. (1993b).
[c] IC_{90}s are expressed in μM.
[d] Not determined.

Table 2
Inhibition (IC_{90}) of HIV-1 (HxB2) and recombinant HIV-1 by PR inhibitors

Virus strain	XM323	P9941	Ro31-8959	A-80987
HxB2	0.18[a]	1.2	0.013	0.37
V821	0.16	0.92	0.015	0.43
Ratio of IC_{90}s (V821/HxB2)	0.89	0.77	1.2	1.1

[a] IC_{90}s were determined by ELSIA for p24 antigen (DuPont, Wilmington DE) and are expressed in μM.

ity to PR inhibitors, but that other differences elsewhere in the virus compensated for I82. To eliminate this possibility, a recombinant virus containing the V82I change in the background of HIV-1(HxB2) was constructed.

Previous work had shown that HIV-1(HxB2) had similar susceptibility to the 4 PR inhibitors as that of HIV-1(RF), therefore, the susceptibility of the recombinant virus to these inhibitors was compared to that of the parental HIV-1(HxB2). Since recombinant virus does not plaque well on MT-2 cells, the susceptibility was determined only by the ACTG/DoD p24 consensus assay. Thus, PBMCs were infected with either HIV-1(HxB2) or (HxB2-V82I) (m.o.i. = 0.001 $TCID_{50}$) in the presence of varying concentrations of the 4 inhibitors and 7 days later the level of p24 in the supernatant was determined. The recombinant virus was as susceptible as HIV-1(HxB2) to the 4 PR inhibitors (Table 2).

4. Discussion

In this communication, we report the identification of an HIV-1 strain, HIV-1(Th16), from Northern Thailand that contains a naturally occurring isoleucine at position 82 of the PR. HIV-1(Th16) was isolated from an asymptomatic, heterosexual male who previously had not been treated with any anti-retroviral drugs. The I82 predicted in this virus is, to the best of our knowledge, the first example of a non-valine residue at position 82 of the HIV-1 PR isolated either in vivo or in vitro within a viral background without prior exposure to an inhibitor of the HIV-1 PR. The other reported isolation of an HIV-1 PR molecule with an I82 was encoded by a PR gene that was contained within a bacterial plasmid background (Loeb et al., 1989).

Along with the V82I variation, the PR of HIV-1(Th16) also differs from that of HIV-1(RF) at 6 other positions. These include T4A, I10L, E35D, M36I, H69K, and L89M. L10 occurs quite frequently in other PR inhibitor-sensitive strains of HIV-1 and is found in both HIV-1(HxB2) and (IIIb), whereas A4 has not been reported previously for HIV-1 (Fontenot et al., 1992; Ratner et al., 1985, Winslow et al., 1995). L10, D35, I36, K69 and M89 also occur in 4 other strains of HIV-1 isolated from patients in Northern Thailand. These strains also are sensitive to PR inhibitors in the C-2 symmetric diol and cyclic urea classes.

To determine if the isoleucine located at position 82 of the PR of HIV-1(Th16) affected the susceptibility of the virus to inhibitors of PR, we determined the sensitivity

of the isolate to 4 different PR inhibitors. We found that the IC_{90}s of the 4 different PR inhibitors for HIV-1(Th16) was equivalent to those for HIV-1(RF).

These results were unexpected, since studies with cloned enzymes containing isoleucine at position 82 in place of valine indicated that this specific variation was sufficient to reduce the potency of both C-2 symmetrical diols and cyclic ureas (Ron Klabe, personal communication). If an isoleucine at position 82 of the PR does indeed lead to a resistance phenotype to certain inhibitors of PR, our observations with HIV-1(Th16) could have been explained by one of the following. One possibility is that something inherent to HIV-1(Th16), for example, one of the additional amino acid differences found between the PRs of HIV-1(Th16) and (RF), may compensate for any affect exerted by the I82. In order to rule this out, we constructed a recombinant virus that contained only the V82I change in the PR in an HIV-1(HxB2) background, and tested its susceptibility to the 4 PR inhibitors. We found that this virus was as susceptible as the parental strain, HIV-1(HxB2), to the 4 inhibitors. Therefore, I82 alone did not affect the susceptibility of HIV-1(Th16) to inhibitors of PR.

An alternative explanation may be that an isoleucine at position 82 alone may not be sufficient to affect susceptibility. An additional change in the protease gene may be required to act in concert with the isoleucine to reduce sensitivity to inhibitors of the PR. This does not seem to be the case for V82A and V82F variants as these single amino acid variants are 4- to 8-fold less susceptible to PR inhibitors in the cyclic urea and C-2 symmetrical classes (Otto et al., 1993b). Although, Kaplan et al., have shown that cloned HIV-1 PR containing an isoleucine at position 82, which was produced in an *Escherichia coli* expression system, did not show reduced susceptibility to an inhibitor of PR when compared to similarly produced PR from HIV-1(HxB2); a PR molecule containing a V32I/V82I double mutation was 20-fold more resistant to the PR inhibitor than was the enzyme containing the V32I alone (Kaplan et al., 1994). Likewise, we have shown that M46L/V82A and V82F/I84V double mutants exhibited an increased resistance of approximately 3- and 10-fold, respectively, over the V82A or V82F single mutants to PR inhibitors in the cyclic urea class (King et al., 1995). Thus, additional changes in the amino acid sequence of the PR can act in concert with position 82 to affect the susceptibility of the virus to various inhibitors of the HIV-1 PR, and may be necessary for a change in sensitivity in association with I82 variant.

The three-dimensional crystal structure of the HIV-1 PR dimer has been elucidated, and residues 82 and 182 are located within the S1/S1′ subsites of the substrate binding pocket of the enzyme (Lapatoo et al., 1989; Navia et al., 1989; Wlodawer et al., 1989). These two subsites interact with the P1 and P1′ side chains of the substrate or inhibitor. Molecular modeling has shown that the V82A and V82F changes found in the PR of the HIV-1(RF) variants increase the distance between the amino acids that make up the S1/S1′ pockets and the P1/P1′ side chains of XM323 (data not shown). It is these differences in the distance between enzyme and inhibitor that may be responsible for the changes in the susceptibility of the virus to the inhibitor. Likewise, since the side chain of isoleucine is bulkier than that of valine, a V82I change also should alter the expected interaction between the P1/P1′ side chain and the S1/S1′ pockets. However, instead of increasing the distance between the S1/S1′ subsites and the P1/P1′ side chains of the inhibitor, I82 should reduce this distance.

The crystal structure of the HIV-1(HxB2) PR with a V82I and XM323 complex has been solved and the I82 does indeed project farther into the S1/S1′ pocket than the V82 thereby decreasing the distance between the P1/P1′ sidechains and the S1/S1′ subsites (C.-H. Chang, DuPont Merck Pharmaceutical Co., personal communication). With the HIV-1(HxB2) variants identified by Kaplan et al. (1994), the isoleucine at position 82 when paired with a V32I change appears to interfere with the usual interaction between the inhibitor and the enzyme, whereas the I82 alone does not. It is interesting that in our hands, the cloned enzyme containing the V82I does show a change in its interaction with the cyclic urea, XM323, but HIV-1(Th16), which contains an I82 in its PR, does not. We yet have not been able to explain this apparent contradiction between our observations with the cloned enzyme and the enzyme produced in the viral background.

It is interesting to note that the HIV-2 PR contains an isoleucine at residue 82 and, in general, is very similar biochemically to the HIV-1 PR (Richards et al., 1989; Mulichak et al., 1993; Tong et al., 1993). Moreover, HIV-2 is susceptible to XM323 (Otto et al., 1993a). It is thought that, in the HIV-2 PR, the bulkier side chain of isoleucine in the S1/S1′ pocket is accommodated by the proximal V47 (Tozer et al., 1992; Mulichak et al., 1993). The HIV-1 PR instead contains an isoleucine at position 47 necessitating that the surrounding amino acids have side chains that compensate for its size. It appears that at least for the PR of HIV-1(Th16) the size of the isoleucine side chain at position 82 phenotypically does not affect the interaction of the PR molecule with its substrate or those PR inhibitors tested in this study.

Finally, in the past we have been able to select mutants with increased resistance to PR inhibitors by passage of HIV-1(RF) and patient isolates in increasing concentrations of P9941 and XM323 (Otto et al. 1993b; King et al., 1995). All mutants contained at least a change in the amino acid at position 82. Numerous attempts to generate XM323-resistant mutants of HIV-1(Th16) under similar conditions failed to generate virus with altered sensitivity to PR inhibitors (data not shown). While this does not establish that I82 makes the emergence of resistance less likely, it certainly indicates that selection of XM323-resistant virus is more difficult with this virus.

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