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# Cross-Resistance Analysis of Human Immunodeficiency Virus Type 1 Variants Individually Selected for Resistance to Five Different Protease Inhibitors

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**Human immunodeficiency virus type 1 (HIV-1) protease inhibitor-resistant variants, isolated on passage of HIV-$1_{HXB2}$ in MT-4 cells with five different protease inhibitors, have been examined for cross-resistance to five inhibitors. The protease inhibitors studied were Ro 31-8959, A-77003, XM323, L-735,524, and VX-478. Resistant variants with two to four mutations within their protease sequence and 9- to 40-fold-decreased susceptibility were selected for all five inhibitors within six to eight passes in cell culture. Passage of a zidovudine-resistant mutant in Ro 31-8959 generated a dual reverse transcriptase- and protease-resistant virus. Variants were cloned directly into a modified pHXB2-D infectious clone for cross-resistance analysis. Although the resistant variants selected possessed different combinations of protease mutations for each inhibitor, many showed cross-resistance to the other inhibitors, and one showed cross-resistance to all five inhibitors. Interestingly, some mutants showed increased susceptibility to some inhibitors. Further HIV passage studies in the combined presence of two protease inhibitors demonstrated that in vitro it was possible to delay significantly selection of mutations producing resistance to one or both inhibitors. These studies indicate that there may be some rationale for combining different protease inhibitors as well as protease and reverse transcriptase inhibitors in HIV combination therapy.**

Antiretroviral therapy for patients infected with human immunodeficiency virus (HIV) has involved predominantly drugs targeted to the reverse transcriptase (RT), an enzyme essential for virus replication (13, 23). Inhibitors targeted to a second essential virus enzyme, the HIV protease, are currently considered to be very promising anti-HIV therapies (49). The protease acts at a late stage in virus replication, cleaving the viral gag and gag-pol precursor polyproteins into mature structural and nonstructural proteins to produce infectious virus particles. Initially on the basis of noncleavable peptide mimetics and later on the basis of the three-dimensional structure of the HIV protease (32, 49, 50), numerous protease inhibitors which are potent inhibitors of HIV replication in cell culture have been designed. Several candidates are actively undergoing clinical development, including the Roche protease inhibitor (Ro 31-8959; Saquinavir) (5), the Merck protease inhibitor (L-735,524) (48), and the Abbott protease inhibitor (ABT-538) (14).

Considerable clinical experience with RT inhibitors has been gained in the treatment of AIDS (10). Resistant variants have arisen during prolonged therapy with many nucleosides (11, 24, 31, 45) and rapidly, i.e., within 1 to 6 weeks, with other nucleosides (43) and with nonnucleoside RT inhibitors (37, 41). Early in the development of HIV protease inhibitors it was suggested that resistance may not be as great a problem with protease inhibitors as it is with RT inhibitors, because the protease active site is highly conserved (4, 40). However, several studies have now shown that resistant variants may be selected in vitro by passage in increasing concentrations of protease inhibitors, including Ro 31-8959 (3, 6, 17), A-77003 (15, 18), RPI312 (7), and ABT-538 (29), although other researchers have failed to isolate resistant virus on in vitro passage with p9941 (34) and with L-735,524 (47). Ro 31-8959 was the first protease inhibitor to undergo clinical evaluation, demonstrating that protease was a valid target for the treatment of AIDS patients, with significant but modest reductions in p24 plasma levels and increases in CD4 counts (16). Clinical isolates have been monitored for resistance, and the same mutations have been observed both in the clinic and in vitro at residues 48 and 90. However, in the clinic, generally, these have been found to be single protease mutants, L90M and G48V, and not double mutants as seen in vitro (16, 17). More recent clinical studies with L-735,524 and with ABT-538 have produced impressive reductions in levels of viral RNA in plasma (14, 48). However, resistance to L-735,524 has been seen to develop in the clinic in some patients after about 3 months of therapy, with multiple mutations in protease producing cross-resistance to several protease inhibitors (2).

In view of the numerous candidate protease inhibitors in development and the conflicting information about protease resistance, we undertook this study to examine the abilities of a number of protease inhibitors to generate resistant variants in vitro by using methods previously developed for evaluation of RT inhibitors. Further, upon generation of variants resistant to each individual protease inhibitor, we examined cross-resistance patterns among the five different protease inhibitors. In addition, experiments involving the passage of virus in the combined presence of two protease inhibitors were included. Such studies may be valuable for determining beneficial combinations of protease inhibitors for subsequent use in the clinic in a manner analogous to that observed with certain RT inhibitors (28, 45).

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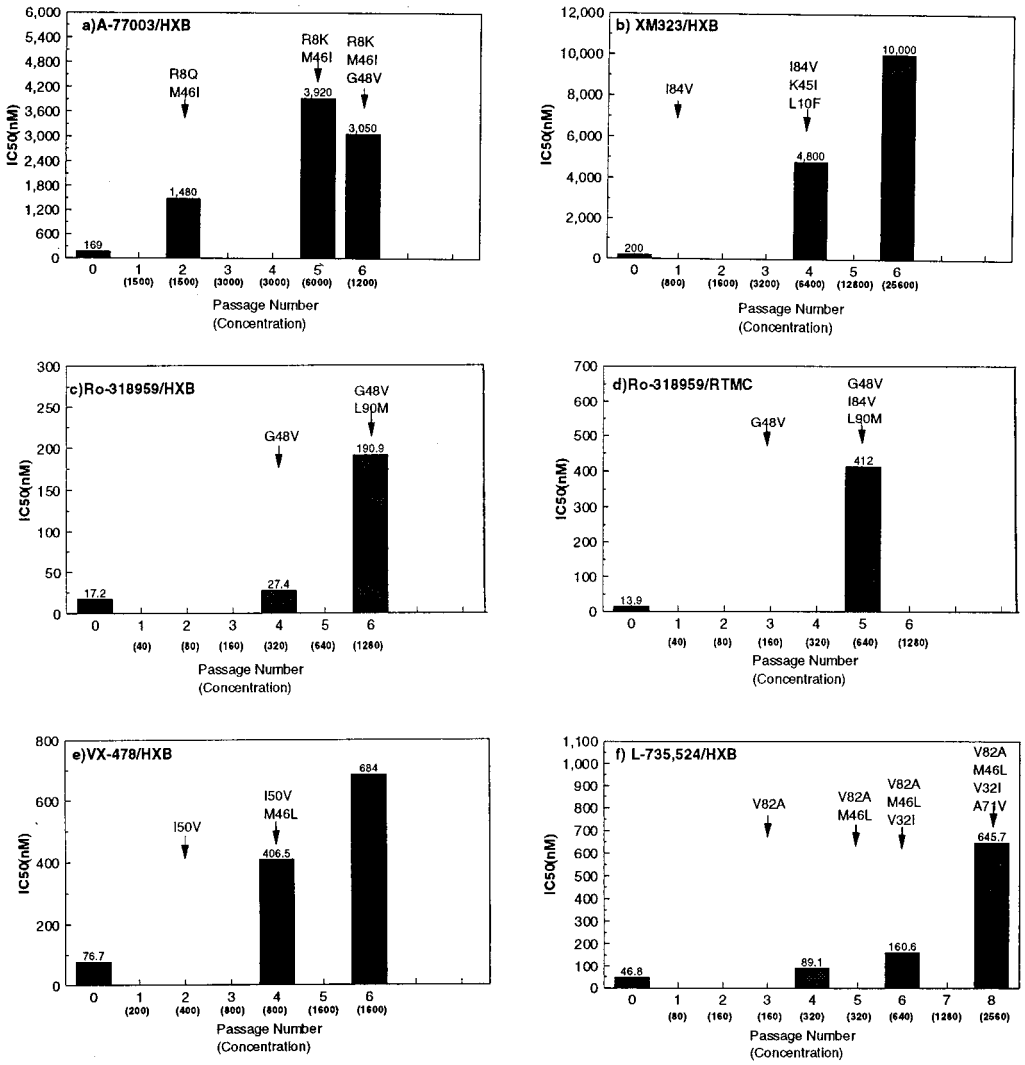


FIG. 1. Virus susceptibilities ($IC_{50}$s) and protease mutational analysis of HIV-$1_{HXB2}$ and the AZT-resistant variant HIV-$1_{RTMC}$ passaged in MT-4 cells in the presence of increasing concentrations (nanomolar) of five protease inhibitors. $IC_{50}$s determined at different passes in parallel with the $IC_{50}$s of the HXB2 control (0) are shown above the bars (generally averaged from results of two separate experiments). Arrows indicate passes at which mutations in amino acid residues within the protease region were first detected.

## MATERIALS AND METHODS

**Compounds.** Five protease inhibitors were used in this study. A-77003 (20) was provided by D. Kempf (Abbott Laboratories, Abbott Park, Ill.), Ro 31-8959 (Saquinavir) (39) was provided by I. B. Duncan (Roche Products Ltd., Welwyn Garden City, United Kingdom), and XM323 (8) was provided by M. J. Otto (Dupont Merck Pharmaceuticals, Wilmington, Del.). The Vertex inhibitors VB 11,328 and VX-478 (21) and the Merck inhibitor L-735,524 (47) were provided as part of an ongoing collaboration with Vertex Pharmaceuticals (Cambridge, Mass.). Compounds were dissolved and diluted in dimethyl sulfoxide and stored at 4°C. Immediately prior to use, compounds were diluted in culture medium and twofold dilutions were prepared directly in 96-well microtiter plates with a Biomek 1000.

**Cell line and virus.** The human lymphoblastoid T-cell line MT-4 was obtained from R. Pauwels (Rega Institute for Medical Research, Leuven, Belgium). Cells were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum plus antibiotics. The HIV-$1_{HXB2}$ strain was derived from the molecular clone pHXB2-D (9), and the zidovudine (AZT)-resistant $HIV_{RTMC}$ strain was derived by site-directed mutagenesis of HIV-$1_{HXB2}$ with RT substitutions of N for D at position 67 (D67→N), K70→R, T215→F, and K219→Q (26). Virus grown in MT-4 cells was stored as cell-free virus supernatants at −70°C.

**Virus susceptibility assays in MT-4 cells.** Assays of virus infectivity (50% tissue culture infective dose) and susceptibility were performed by the dye uptake method with the vital dye 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) to quantify cell viability as described previously (36). For each assay 10 concentrations of each compound were used over a twofold dilution range with each dilution set up in quadruplicate. The concentration of each compound which protected 50% of the cells from virus killing ($IC_{50}$) was determined from regression analysis of the plot of percents cell death against drug concentrations.

**Selection of HIV-1-resistant variants by passage in MT-4 cells.** Cells ($2 \times 10^6$ per culture) were infected with $HIV_{HXB2}$ or $HIV_{RTMC}$ at a multiplicity of infection of <0.01 50% tissue culture infective doses per cell, and virus was passaged in increasing concentrations of inhibitor as described previously (46). Initially test compounds were added at 2, 4, and 10 times their predetermined $IC_{50}$s. Cultures were examined daily for viral cytopathic effect up to 21 days after infection, with fresh compound and medium added at 7 days after infection. Virus supernatants and infected cell pellets were stored at −70°C for later determinations of virus titers and $IC_{50}$s and for sequence analysis of viral DNA. Virus was passaged six to eight times in each inhibitor at the concentrations indicated (Fig. 1). Titers were determined for each pass, and $IC_{50}$s were determined generally at passes 4, 6, and 8. Combined protease inhibitor passage studies, using MT-4 cells with $HIV_{HXB2}$, were undertaken with equipotent concentration ratios of the two inhibitors based on their $IC_{50}$s, initially two and four times their combined $IC_{50}$s.

**Genetic analysis of protease by sequencing PCR products.** Initially, mutations were identified in passaged HIV-1 virus stocks by manual sequencing of the protease region, and they were subsequently confirmed by automated sequencing, as described below, by using the same PCR and sequencing primers. Briefly,

the HIV-1 protease region was PCR amplified from prokaryotic or eukaryotic DNA extracts by using the following PCR primers situated in the 5′ and 3′ flanking regions: 5′ PCR, 5′-GGGAAGATCTGGCCTTCCTACAAGG-3′, and 3′ PCR, 5′-GGGCCATCCATTCGTGGC-3′ (biotinylated at the 5′ end). The 500-bp amplified fragment was bound by the 3′ biotinylated end to small magnetic beads (Dynabeads M-280), and the bound DNA was denatured in 0.1 M NaOH. The positive-sense strand was removed from the negative-sense strand, which remained bound to the beads, and the bound or unbound strand was used for sequencing. Unbound DNA was precipitated in ethanol and resuspended in 5 μl of water. This DNA was sequenced by using an internal 3′ primer, 5′-CCG CTAGCCTGACACAGTCTCAATAGGGCTAA-3′. DNA bound to the beads was resuspended in 5 μl of water and sequenced from the 5′ end of the protease gene by using an internal 5′ primer, 5′-GGAGCCGATAGACAAGG-3′. DNA was sequenced with the Applied Biosystems Inc. PRISM sequence terminator single-stranded sequencing kit (catalog no. 401457), and samples were run and analyzed on an Applied Biosystems 373 automated sequencer.

**Cloning of HIV-1 protease variants into the infectious clone pHXB-NAPro.** The complete protease coding region was amplified from infected cellular DNA extracts from passages 6 and 8 by PCR using primers designed to produce *Not*I-*Asp*I DNA fragments for direct cloning into pHIV-NAPro as described previously (30). Proviral clones were sequenced as described above, and DNA was transfected into MT-4 cells by electroporation (19) for production of infectious virus clones. Virus supernatants and cell pellets were prepared when the cytopathic effect was complete.

## RESULTS

**Passage of HIV-1 in MT-4 cells in increasing concentrations of different protease inhibitors.** Before the initiation of passage studies with the five different protease inhibitors, the $IC_{50}$ for each inhibitor was determined in MT-4 cells. HIV-$1_{HXB2}$-infected MT-4 cells were then treated with three different concentrations of inhibitor, i.e., 2, 4, and 10 times the predetermined $IC_{50}$. The culture containing the highest concentration of inhibitor that failed to block virus replication, as assessed by the complete destruction of infected cells within a 21-day time limit (generally in <14 days), was used to infect the next culture passage. The concentration above the $IC_{50}$ at which virus was able to grow, in this first passage, varied slightly with the different inhibitors. With A-77003 and XM323, virus grew at approximately 10 times the $IC_{50}$s within 10 to 11 days. In the presence of the other three inhibitors, Ro 31-8959, VX-478, and L-735,524, virus grew slightly more slowly over 11, 14, and 16 days, respectively, with breakthrough observed only at two to four times the predetermined $IC_{50}$s.

Passage studies with HIV-$1_{RTMC}$ in increasing concentrations of Ro 31-8959 were also undertaken, and again breakthrough was observed at four times the $IC_{50}$. Initially, passage was limited to six passes for each inhibitor, and the results of susceptibility assays, compared with those for wild-type virus, and the consensus sequence changes in the protease gene, observed by automated DNA sequencing, are shown for each inhibitor in Fig. 1. For all the inhibitors except L-735,524, a significant increase in resistance (approximately 10-fold or greater) was observed by pass 6. For L-735,524, only an approximately threefold increase was observed, although three mutations were present within the protease substrate binding pocket. However, further passage resulted in the acquisition of a fourth mutation outside the binding pocket, with a concomitant increase in resistance of greater than 10-fold. Therefore, resistant variants were selected by passage in vitro with all the protease inhibitors examined. In addition, resistant virus was selected with Ro 31-8959 from both wild-type $HIV_{HXB2}$ and the AZT-resistant variant $HIV_{RTMC}$ at similar rates and with the same mutations. Sequencing of the RT region from pass-6 $HIV_{RTMC}$ confirmed that the AZT resistance mutations were still present, and susceptibility assays confirmed that this virus had reduced susceptibilities to both AZT ($IC_{50}$, 0.5 μM) and Ro-318959 ($IC_{50}$, 0.4 μM) compared with those of wild-type $HIV_{HXB2}$ ($IC_{50}$ of AZT, 5 to 20 nM; $IC_{50}$ of Ro 31-8959, 4 to 20 nM).

Sequencing data from all the passages revealed that, generally, the first mutation arose within one to four passes for each inhibitor, except A-77003, for which two mutations appeared within two passes. With each inhibitor besides L-735,524, up to three mutations were detected, and four mutations were detected with L-735,524, indicating that although the active site and substrate binding region are highly conserved, several mutations can be accommodated within this part of the molecule. Some mutations outside the substrate binding region, such as valine at residue 71 (A71V) and methionine at residue 90 (L90M), also appeared to contribute to reductions in susceptibility. By automated sequencing it was possible to determine the approximate percentage of mutant present at each residue (27). By pass 6, populations appeared 100% mutants for most of the substituted residues, suggesting that the mutations observed were linked. There were some exceptions, however; for example, the valine 84 mutation (I84V) appeared as a mixture at pass 6 with Ro 31-8959–$HIV_{RTMC}$, and the valine 48 mutation (G48V) appeared as a mixture with A-77003–$HIV_{HXB2}$. To study both the linkages present and the inhibitor susceptibilities of cloned isolates, the protease sequences from passage 6 and passage 8 viruses were cloned by direct ligation of PCR products into a modified pHXB-2D infectious clone.

**Determination of mutational linkage within the protease coding regions from clones isolated from passaged virus.** At least six clones from pass 6 or pass 8 virus for each inhibitor were obtained by PCR, and the protease region from each clone was sequenced (Fig. 2). As expected from the sequencing of passaged virus, the triple and quadruple mutations were linked. However, despite the confirmation that the mutations were linked, mixtures of double and triple mutants which were not apparent from the sequencing of uncloned DNA extracts were frequently present. For A-77003, mixtures of the double 8K/46L mutant and the triple 8K/46L/48V mutant were present, whereas with XM323 the double mutant 10F/84V predominated. Sequencing of VX-478-passaged virus revealed only the 46L/50V double mutant, but on cloning, the 46I/50V double mutant was also found to be present. At passage 6 with L-735,524, both double and triple mutants were present, whereas by passage 8 the quadruple mutant predominated. Additional mutations were observed in a few clones, with totals of 7, 13, and 15 mutations within the protease sequences of three clones. Not surprisingly, these multiple mutants were noninfectious when transfected into MT-4 cells, particularly since two clones had mutations at the critical residue 25 within the active site and one of these clones also had a stop codon at residue 42.

**Characterization of infectious proviral clones obtained from virus passage.** A panel of 11 individual clones of interest comprising six double mutants, four triple mutants, and one quadruple mutant was identified from the sequencing data. DNAs from these clones were transfected into MT-4 cells for production of virus. All 11 clones produced virus in MT-4 cells, with titers ranging from $10^{2.5}$ to $10^{4.4}$ 50% tissue culture infective doses per ml. DNAs from infected cell pellets were extracted and sequenced to determine the viral protease genotype. From this analysis, it was determined that 10 of 11 clones had protease sequences consistent with that from the original transfected proviral vectors. However, one clone, the 48V/84V/90M mutant, selected during passage of $HIV_{RTMC}$ in the presence of Ro 31-8959, had an additional mutation at residue 71, an alanine-to-valine mutation. This mutation was also present in the L-735,524 quadruple mutant. Although growth from transfections may be variable, depending on the quality of the DNA and the electroporation efficiencies, it was apparent that some mutants had grown to higher titers than others. Notably, the

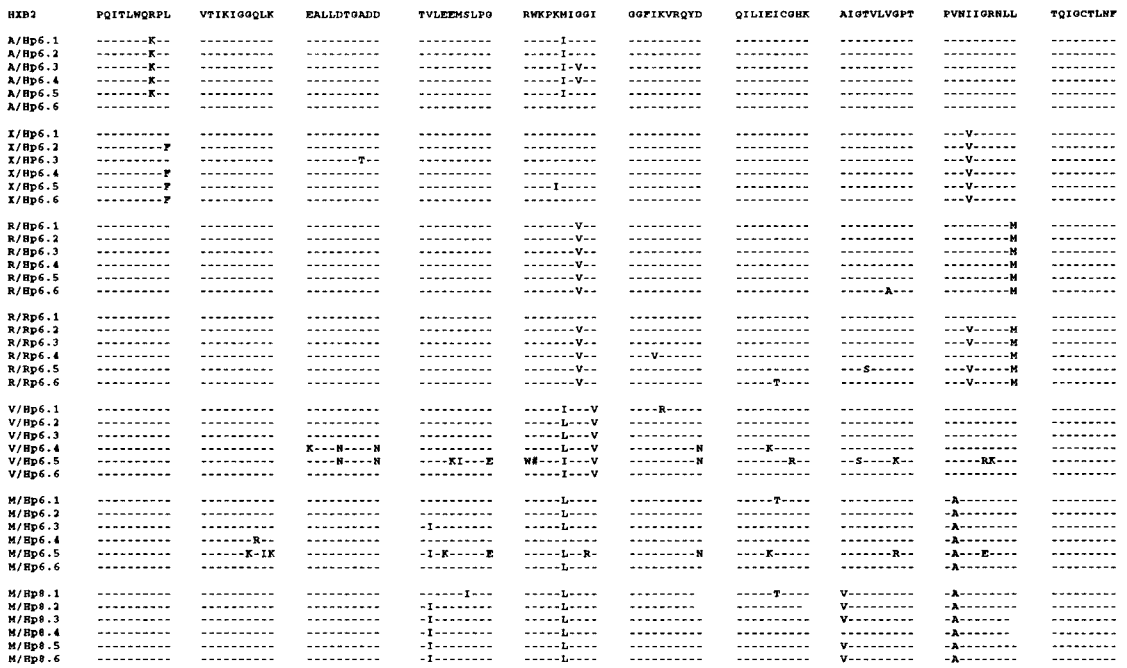


FIG. 2. Amino acid sequences of the protease regions of proviral clones obtained from passage 6 and passage 8 virus for five protease inhibitors. A/Hp6.1 to -6, A-77003/HXB/pass 6, clones 1 to 6; X/Hp6.1 to -6, XM323/HXB/pass 6, clones 1 to 6; R/Hp6.1 to -6, Ro 31-8959/HXB/pass 6, clones 1 to 6; R/Rp6.1 to -6, Ro 31-8959/RTMC/pass 6, clones 1 to 6; V/Hp6.1 to -6, VX-478/HXB/pass 6, clones 1 to 6; L/Hp6.1 to -6, L-735,524/HXB/pass 6, clones 1 to 6; M/Hp8.1 to -6, L-735,524/HXB/pass 8, clones 1 to 6.

L-735,524 quadruple mutant and the XM323 triple mutant produced titers comparable to that of the wild type.

Susceptibility assays were undertaken with all 11 variants, but growth of 3 variants was not sufficient in these assays to obtain accurate $IC_{50}$s. Neither of the mutants selected in the presence of A-77003 grew to a high titer, and it was therefore not possible to confirm in this study that these mutations were responsible for the high-level resistance that was observed against this inhibitor. Similarly, for the 32I/46L/82A triple mutant selected with L-735,524, no accurate susceptibility data could be obtained. In contrast, the double and quadruple mutants from the L-735,524 selection grew well.

The susceptibility data for the eight cloned variants compared in Table 1 with those for the parent $HIV_{HXB2}$ confirmed that the mutations selected on passage with the different protease inhibitors were responsible for the resistance to each inhibitor. Generally, the greater the number of mutations the greater the level of resistance. For example, for XM323, the triple mutant was approximately twofold more resistant than the double mutant, and for L-735,524, the quadruple mutant was the only mutant selected which showed significant resistance. With Ro 31-8959, the quadruple mutant showed marginally more resistance than the double mutant. The two VX-478 double mutants showed different levels of resistance, i.e., the predominant 46L/50V mutant showed only low-level resistance, whereas the 46I/50V mutant was significantly more resistant.

TABLE 1. Susceptibilities of HIV-1 protease variants to five protease inhibitors

| Virus or virus clone[a] | Selecting inhibitor | $IC_{50}$ (nM)[b] of: | | | | |
|---|---|---|---|---|---|---|
| | | A-77003 | XM323 | Ro 31-8959 | L-735,524 | VX-478 |
| HXB2 | | 155.7 ± 13.7 (1) | 90.4 ± 17.2 (1) | 4.7 ± 1.2 (1) | 28 ± 3.2 (1) | 76 ± 25 (1) |
| 10F/84V | XM323 | 58 ± 8.3 (−2.7)[c] | 5,329 ± 2,299 (+58.9) | 18.3 ± 7.9 (+3.9) | 29 ± 1.3 (1.0) | 198 ± 34 (+2.6) |
| 10F/45I/84V | XM323 | 144.5 (−1.1) | >10,000 (+110.6) | 17.2 ± 2.7 (+3.7) | 86 ± 4.4 (+3.1) | 475 ± 67 (+6.3) |
| 48V/90M | Ro 31-8959 | 972 ± 179 (+6.2) | 31.6 (−2.9)[c] | 177 ± 61 (+37.6) | 50 ± 1.6 (+1.8) | 56 ± 1.9 (−1.4) |
| 48V/84V/71V/90M | Ro 31-8959 | 879.4 (+5.6) | 399 ± 65 (+4.4) | 192 (+40.9) | 106 (+3.8) | 137 ± 11 (+1.8) |
| 46L/50V | VX-478 | 92 ± 13 (−1.7) | 219 ± 7 (+2.4) | 1.0 ± 0.06 (−4.7)[c] | 7 ± 1.5 (−4)[c] | 229 ± 10 (+3.0) |
| 46I/50V | VX-478 | 255 ± 9 (+1.6) | 498 ± 44 (+5.5) | 1.7 (−2.8)[c] | 14 ± 0.6 (−2)[c] | 541 ± 76 (+7.1) |
| 46L/82A | L-735,524 | 256 ± 37 (+1.6) | 190 ± 44 (+2.1) | 1.3 ± 0.1 (−3.6)[c] | 45 ± 4.3 (+1.6) | 62 ± 25 (−1.2) |
| 32I/46L/71V/82A | L-735,524 | 1,530 ± 633 (+9.8) | 435 ± 65 (+4.8) | 0.8 ± 0.42 (−5.9)[c] | 172 ± 63 (+6.1) | 116 ± 43 (+1.5) |
| 8K/46I/48V[d] | A-77003 | 10,000 (+64) | 39.8 (−2.3) | 6.3 (+1.3) | 25.1 (−1.1) | 39.8 (−1.9) |

[a] Cloned protease variants obtained from passaged virus were transfected into MT-4 cells, virus stocks were prepared, and the protease genotypes were established (indicated by designations).

[b] $IC_{50}$s were determined in MT-4 cells (by using 10 concentrations of inhibitor diluted over a twofold range) at least twice, and the mean $IC_{50}$s ± standard deviations (SD) are recorded. Values for HXB2 controls are mean $IC_{50}$s ± SD for four separate assays. Relative $IC_{50}$s are shown in parentheses (+ and −, fold increase and fold decrease, respectively, in $IC_{50}$s compared with values for HXB2).

[c] Increased susceptibility compared with that of the wild type.

[d] A-77003 pass 6 virus; not cloned.

It was apparent from the data summarized in Table 1 that variants resistant to single inhibitors showed cross-resistance to several of the other protease inhibitors, particularly the triple and quadruple mutants. To examine cross-resistance patterns between the inhibitors, the $IC_{50}$ data were converted into shifts in susceptibility relative to that of the wild type; these values are shown in parentheses in Table 1. To include data for A-77003, we used the uncloned passage 6 virus for susceptibility assays.

From Table 1 it is evident that the Ro 31-8959 quadruple mutant showed some level of resistance to all five inhibitors, although with VX-478 the shift was only twofold. Both the L-735,524 quadruple variant and the XM323 triple variants showed cross-resistance to four of the five inhibitors, the VX-478 mutants showed cross-resistance to just two inhibitors, and the A-77003 variants (pass 6 virus) did not show any cross-resistance. Interestingly, in some cases variants became more susceptible to certain inhibitors. For example, the VX-478 variants were three- to fourfold more susceptible than wild-type virus to Ro 31-8959 and two- to fourfold more sensitive to L-735,524. The L-735,524 quadruple variant had five- to six-fold-increased susceptibility to Ro 31-8959. The Ro 31-8959 double mutant 48V/90M was more susceptible than wild-type virus to XM323, but with the addition of the V-71 and V-84 mutations, to produce the quadruple mutant 48V/71V/84V/90M, resistance to XM323 appeared.

**Passage of HIV-1 in MT-4 cells in increasing concentrations of two combined protease inhibitors.** Further experiments were undertaken to evaluate resistance development with the previously evaluated inhibitor VB 11,328 (35) in combination with either Ro 31-8959 or L-735,524, both in clinical development. The passage number was limited to 6 for comparison with the single-protease passage experiments. In previous studies with MT-4 cells, passage of $HIV_{HXB2}$ with VB 11,328 produced a triple protease mutant (46I/47V/50V) with a 20- to 30-fold decrease in susceptibility (35). When VB 11,328 and Ro 31-8959 were combined in equimolar amounts, significantly lower concentrations (8- to 10-fold lower) of the two inhibitors were required to allow virus growth than were required with single-compound passage. Surprisingly, on analysis of the protease sequence, the double mutant 48V/90M was found to have arisen by pass 4, at a rate similar to that observed for Ro 31-8959 passage alone. Virus susceptibility analysis at pass 6 revealed about 10-fold resistance to Ro 31-8959 ($IC_{50}$s = 25 nM for HXB2 and 300 nM for pass 6 virus). In contrast, with VB 11,328 none of the mutations observed in single-passage studies were selected, with only a slight shift in virus susceptibility ($IC_{50}$s = 54 nM for HXB2 and 100 nM for pass 6 virus). With the L-735,524–VB 11,328 passage only one mutation, L-46, was selected by pass 3, and it remained the only mutation present at pass 6. The susceptibility assays, as expected from the mutational analysis, showed no major shift in susceptibility to either inhibitor ($IC_{50}$s of L-735,524 = 43 nM for HXB2 and 62 nM for pass 6 virus; $IC_{50}$s of VB 11,328 = 86.5 nM for HXB2 and 180 nM for pass 6 virus). This combination showed a marked reduction in the number of mutations selected compared with that in single-pass studies (one mutation compared with three mutations).

## DISCUSSION

Evaluation of in vitro development of resistance to five structurally distinct protease inhibitors has revealed that resistant variants may be selected at similar rates and with similar levels of resistance for all the inhibitors examined. This is in marked contrast to the case with RT inhibitors, for which there is considerable variation in both the level of resistance and the ease of resistance selection (12, 33, 44, 46). For all the protease inhibitors the level of resistance was below 100-fold and generally between 10- and 40-fold. In no case was a single mutation responsible for high-level (100- to 1,000-fold) resistance such as has been seen with the nonnucleoside RT inhibitors and 3′-thiacytidine (1, 12, 33, 38, 42, 46). In the majority of cases at least two and up to four mutations were required to produce a significant change in the phenotype, which is similar to the case with AZT (26). Interestingly, although all the inhibitors target the same active site, no two resistance mutation patterns were the same. A total of 11 different mutations were selected on passage in the presence of the different compounds, demonstrating that HIV-1 protease is able to accommodate a large number of mutations. However, certain mutations were observed more frequently than others; for example, the mutation of residue 46 to L or I was selected with three different inhibitors. This was one of the most frequently observed mutations in previous selection studies (15, 18, 29, 35), and it has been suggested to play a compensatory role in resistance development (15). Another mutation seen in two passage series was I84→V, and this mutation has also been reported previously, following selection with RPI312 (7), ABT-538 (29), and XM323 (22). From structural studies this appears to be an important residue within the substrate binding region, and the present study and previous studies imply that it is probably important in cross-resistance.

Our results for A-77003 passage are comparable to those previously reported by Ho et al. (15), with selection of the 8K/46I double mutant, although the triple mutant with V-48 has not been observed previously. However, in contrast to the observations of Ho et al. (15), we found that the double mutant grew poorly. This difference may be due to variations between the parental $HIV_{NL-43}$ and $HIV_{HXB2}$ strains. The XM323 inhibitor has been observed to select resistant variants with the V-84/A-82 double mutation (22). This, together with our data, would indicate that valine 84, which confers about 10-fold resistance to XM323, is the mutation crucial for conferring resistance to this inhibitor. The pattern of resistance mutations with VX-478 was again similar, but not identical, to those observed previously for VB 11,328 and VX-478 selection in CEM cells (35). Only a double mutant, 46L/I/50V, was selected in the present study; the mutation at residue 47 within the active site was not observed. Passage in Ro 31-8959 consistently selected the 48V/90M double mutant, as reported in previous studies (17).

In contrast to earlier reports of a lack of in vitro resistance selection with L-735,524 (47), we were able to obtain resistant variants during selection passage in MT-4 cells, although up to three mutations produced only a marginal change in sensitivity and at least four mutations were required to produce relatively stable resistant variants. Interestingly, the fourth apparently critical mutation was V-71, which is not directly involved with substrate binding and has been described as another compensatory mutation (29). In our passage series the additional V-71 mutation improved virus growth and appeared to contribute to the resistance phenotype. However, the triple mutant grew only poorly, so it was not possible to determine accurately the specific contribution of V-71 to resistance. This mutation has also been observed in clinical isolates from patients receiving L-735,524 (2). Again, the 71V mutation was observed to arise spontaneously upon growth of the Ro 31-8959 triple mutant in the absence of drug pressure, suggesting a role in protease and/or virus stability.

On the basis of these and previous studies (15), there appear to be similar processes of resistance development with most

protease inhibitors. Many of the protease mutants with mutations within the substrate binding region that arise have reduced processing capabilities which significantly affect virus growth and which in turn drive selection of further compensatory growth mutations, either in the presence or in the absence of inhibitor, resulting in more stable resistant variant populations. Detailed studies of growth kinetics to investigate this further are under way. These stable resistant variants might be selected more rapidly in the clinic, where greater heterogeneity exists. However, the use of proviral clones in cell culture systems makes analysis of resistance selection simpler, since there is much less heterogeneity. Despite this fact, partially defective growth variants severely hinder the evaluation of the contributions of different mutations to the resistance phenotype. In contrast, with RT variants most show only a limited effect (28) or no detectable effect on in vitro virus growth kinetics (19).

Combination trials with RT and protease inhibitors are already planned and under way in the clinic. However, selection of a dual protease and RT resistant mutant had not been previously studied in vitro. We were therefore interested in examining this selection by using an AZT-resistant mutant. Not surprisingly, selection of protease inhibitor resistance with an AZT-resistant strain was comparable to that observed with the wild type. This suggests that dual target resistance may occur in the clinic despite the potential high-level potency of such combinations and highlights the importance of assessing such combinations in therapy-naive patients. With the number of mutations required to produce resistance to both a protease and an RT inhibitor such as AZT, selection of dual resistance in therapy-naive patients may be significantly delayed.

In this study we did not aim to determine the contributions of individual mutations to a particular phenotypic resistance profile, partly because much of this work has been undertaken previously (15, 18). We were interested in determining the cross-resistance patterns of the protease-resistant variants isolated in the passage experiments (eight in total). This analysis revealed that there was considerable cross-resistance between the different inhibitors, which is not surprising since they all target the same active site. These data would suggest that with the high-level accumulation of mutations within protease it should be possible to obtain multidrug resistance, as has been observed in vitro with RT inhibitors (25). This, in fact, is already proving to be the case in the clinic with L-735,524 monotherapy (2). However, the in vitro studies also show differences in cross-resistance patterns which may be exploitable in the clinic to delay resistance development. To examine this potential further, we passaged virus in the presence of two protease inhibitors and found that, at least in cell culture, it was possible to delay selection of variants resistant to one or both of the inhibitors. The VB 11,328 plus Ro 31-8959 passage unexpectedly produced resistance to Ro 31-8959 alone. The reason for this selection is difficult to interpret, since both inhibitors are equipotent, but the selection may be due to pressure by Ro 31-8959 against selection of VB 11,328 resistance, since mutations to VB 11,328 show increased susceptibility to Ro 31-8959. Although we have isolated variants with one protease inhibitor which show increased susceptibility to another protease inhibitor, we have not observed reversal of resistance by addition of mutations, as seen with some RT inhibitors. Interestingly, we have observed some synergy between certain protease combinations in cell culture (34a). At present, further studies to investigate in vitro protease resistance development by comparing combined drug selection pressure with sequential drug selection pressure are under way.

In summary, this study suggests that resistance will be a problem with all protease inhibitors in the clinic, as observed with the RT inhibitors. To attempt to overcome these problems, strategies for the use of protease inhibitors in combination therapy will need to be developed.

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