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# Genetic Correlates of In Vivo Viral Resistance to Indinavir, a Human Immunodeficiency Virus Type 1 Protease Inhibitor

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**Indinavir (IDV) (also called CRIXIVAN, MK-639, or L-735,524) is a potent and selective inhibitor of the human immunodeficiency virus type 1 (HIV-1) protease. During early clinical trials, in which patients initiated therapy with suboptimal dosages of IDV, we monitored the emergence of viral resistance to the inhibitor by genotypic and phenotypic characterization of primary HIV-1 isolates. Development of resistance coincided with variable patterns of multiple substitutions among at least 11 protease amino acid residues. No single substitution was present in all resistant isolates, indicating that resistance evolves through multiple genetic pathways. Despite this complexity, all of 29 resistant isolates tested exhibited alteration of residues M-46 (to I or L) and/or V-82 (to A, F, or T), suggesting that screening of these residues may be useful in predicting the emergence of resistance. We also extended our previous finding that IDV-resistant viral variants exhibit various patterns of cross-resistance to a diverse panel of HIV-1 protease inhibitors. Finally, we noted an association between the number of protease amino acid substitutions and the observed level of IDV resistance. No single substitution or pair of substitutions tested gave rise to measurable viral resistance to IDV. The evolution of this resistance was found to be cumulative, indicating the need for ongoing viral replication in this process. These observations strongly suggest that therapy should be initiated with the most efficacious regimen available, both to suppress viral spread and to inhibit the replication that is required for the evolution of resistance.**

The human immunodeficiency virus type 1 (HIV-1) protease is a virally encoded aspartyl protease that serves to cleave the Gag-Pol polyprotein precursor into mature proteins. This specific proteolysis occurs late in the viral life cycle and is essential for viral infectivity (24). Several peptidomimetic, competitive inhibitors of this enzyme are being developed as potential antiviral agents for the control of HIV-1 infection. Among these, indinavir (IDV) (also called CRIXIVAN, MK-639, or L-735, 524) (11, 40), is a potent and selective inhibitor of the enzyme that has recently received accelerated U.S. regulatory approval for HIV therapy.

One of the most serious impediments to the successful clinical use of antimicrobial drugs is the emergence of drug-resistant mutants. This has been especially evident for inhibitors of HIV-1 replication. The clinical antiviral effects of the many nucleoside and nonnucleoside reverse transcriptase inhibitors have been limited by selection of resistant viral variants. Viruses expressing reduced inhibitor susceptibility have also emerged during cell culture selection with different HIV-1 protease inhibitors, including A-77003, A-80987, ritonavir (ABT-538), BMS 186,318, RPI 312, IDV, saquinavir (Ro 31-8959), VX-478, XM323, and many others (5, 9, 10, 13, 14, 18–20, 23, 25, 32, 38, 39).

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Predictably, as some of these compounds (IDV, saquinavir, and ritonavir) entered prolonged clinical trials, resistance also developed in vivo (6, 7, 17, 28). The resistance exhibited by one viral isolate selected by IDV was traced to a combination of three amino acid substitutions in the protease (6). However, three other viral isolates evaluated in the same study exhibited such divergent patterns of substitutions in the protease that a simple basis for the resistance could not be defined. In this report, we examine the complex relationship between the genotypic and phenotypic changes occurring in the proteases of IDV-resistant viral variants selected in a subset of HIV-1-infected patients treated with the inhibitor.

## MATERIALS AND METHODS

**Primary viral isolates.** Isolation and phenotypic characterization of primary viral isolates were performed as previously described (33). Phenotypic testing entailed measuring the viral spread in cell culture over serial twofold dilutions of the test drug. Because of the inherent variability of the biological assay, increases in the 95% inhibitory concentration for viral spread in cell culture ($CIC_{95}$) of less than fourfold (two dilutions) were not considered indicative of changes in inhibitor susceptibility. Thus, resistance to IDV was defined as a $CIC_{95}$ of ≥400 nM, fourfold over the typical $CIC_{95}$ exhibited by wild-type virus.

**Molecular analyses.** HIV-1 protease genes were amplified and isolated from total RNA of viral cell culture supernatants. RNA isolations and amplifications and cloning and sequencing of protease genes were performed as previously described (6), except that the 5′ primer used for the first PCR (gag-RT) was d(CAGAGCCAACAGCCCCACCAG). Briefly, total RNA was reverse transcribed and then amplified in multiple (typically 8 to 12) independent nested PCRs. The product of each PCR was gel purified and separately cloned into plasmid pAMP19 (Gibco-BRL). Only one bacterial colony derived from each

PCR was picked for sequencing of the protease gene insert. All protease genes were completely sequenced on both strands. In all, the complete sequences of 421 independent protease genes from 57 primary viral isolates from 21 patients were determined.

Because each protease gene sequence was derived from a different PCR, independence of clones is assured, and the determined mutant frequencies should reflect the distributions of those mutants in the original sample. Moreover, molecular cloning prior to sequencing preserves the linkage relationships between different mutations in the protease gene and allows cocirculation of multiple variants to be detected.

Site-directed viral mutants were constructed as described previously (6). Transfection and growth of site-directed mutant viral clones were also performed as described previously (4, 6). After transfection of mutant proviral clones into HeLa cells and growth of viral stocks, the complete sequences of the viral protease genes from the mutant viral populations were verified as described above.

**Statistical methods.** Individual amino acid substitutions were assessed for correlation with phenotypic resistance one location at a time. At each time point (patient-by-week combination) assayed, the measure of amino acid substitutions at a particular location was the proportion of clones with an amino acid residue at that location that was different from the North American/European clade B consensus sequence (30). We term this proportion the mutation frequency. The natural logarithm of the $CIC_{95}$ was used as the measure of phenotypic resistance, with the values of ln (25 nM) and ln (3,000 nM) assigned when the $CIC_{95}$ was assessed to be less than or equal to 25 nM or greater than or equal to 3,000 nM, respectively.

With ln ($CIC_{95}$) as the response variable and the mutation frequency as the predictor variable, the correlation between phenotypic resistance and individual amino acid substitutions was measured by the slope of a weighted linear mixed-effects regression of ln ($CIC_{95}$) on the mutation frequency. Parameters of the model were estimated by using the restricted maximum-likelihood method (36). The random-effects components of the mixed-effects model allowed the data for each patient to have a different intercept. The total number of time points measured for each patient varied considerably. To prevent the patients with the most time points from unduly dominating the analysis, each time point from a particular patient was weighted inversely proportionally to the total number of time points measured for that patient. Thus, the total weight assigned to each patient was equal. The jackknife procedure (26, 29) was used to assess the variability of the estimated slope and to construct a statistical hypothesis test of whether the true slope is zero. All locations that had a minimum of four patients with amino acid substitutions at that location were analyzed to yield unadjusted *P* values. Since 30 locations met this criterion, the experiment-wide false-positive rate with the unadjusted *P* values is inflated. The step-down Bonferroni procedure (15, 16) was used to calculate adjusted *P* values, which yield the proper false-positive rate despite the multiplicity of tests performed.

The association between the number of amino acid substitutions for a given isolate and phenotypic resistance to IDV was assessed by using the jackknife and a linear mixed-effects model similar to the one described above. However, the predictor variable in this assessment was the sum of the mutant frequencies either over all 99 residues or over the 11 residues found to be correlated with IDV resistance.

**Nucleotide sequence accession numbers.** The nucleotide sequences described in this paper are available in GenBank under accession numbers U71606 to U72026.

## RESULTS

**Genotypic and phenotypic changes accompanying selection of IDV-resistant viral variants in vivo.** The studies described here were performed during phase I and early phase II clinical trials of IDV. During these trials (which will be described in detail elsewhere), patients were initially treated with suboptimal dosages of the inhibitor. At these dosages, the degree and duration of virus suppression were notably less than those observed in patients who initiated therapy at a higher (and subsequently recommended) dosage. For many patients in these earlier trials in whom some loss of viral suppression was observed, primary HIV-1 isolates were obtained by cocultivation of patient peripheral blood mononuclear cells with mitogen-stimulated normal human peripheral blood mononuclear cells (33). These were subjected both to inhibitor susceptibility analysis and to genotypic analysis by reverse transcription-PCR, molecular cloning, and DNA sequencing. As shown in Table 1, viral isolates from 17 of the 21 patients examined showed increases in the $CIC_{95}$ of IDV to 400 nM or greater, indicating the development of resistance.

Upon entry into the studies, viruses from these patients harbored between one and seven amino acid substitutions from the North American/European clade B consensus sequence (30). By the first appearance of phenotypic resistance ($CIC_{95}$, $\geq$400 nM), between 3 and 11 amino acid changes from these pretherapy sequences were observed among the viral isolates (Table 1). Over time, the resistance level of patient viruses increased, often to the highest measurable level ($CIC_{95}$, $\geq$3,000 nM). These increases in $CIC_{95}$ were accompanied by variable combinations of additional amino acid substitutions in the protease. Increased resistance was also accompanied by increasing numbers of these substitutions.

An examination of the protease sequences in the viral isolates over time (Table 1) showed a high frequency of substitutions at several amino acid residues, especially at L-10 (to I, V, or R), K-20 (to M or R), L-24 (to I), M-46 (to I or L), I-54 (to V or A), L-63 (to P), A-71 (to V or T), V-82 (to A, F, or T), and L-90 (to M). However, these substitutions appeared in many different combinations and in different orders, and no invariant combination clearly coincided with the loss of inhibitor susceptibility.

**Correlations of genotype and phenotype.** Despite this genetic diversity, it was possible to correlate the appearance of genotypic changes, as reflected by specific amino acid substitutions in the protease, with phenotypic resistance of viral isolates. A regression analysis was performed in which substitution frequencies at specific residues were compared with ln ($CIC_{95}$) values for IDV. In all, both genotypic and phenotypic data were obtained for 57 primary viral isolates from 21 patients undergoing IDV therapy. The estimates of slope from the regression of ln ($CIC_{95}$) on the mutation frequencies are given in Table 2. The occurrence of changes at residues L-10, L-24, M-46, I-54, A-71, V-82, I-84, and L-90 was highly statistically significantly correlated with phenotypic resistance. Although the adjusted *P* value was not statistically significant at the 0.05 level, there is evidence that the mutation frequency at amino acid residue K-20 is also correlated with phenotypic resistance (Table 2). In addition, substitutions at residue L-63 also occurred frequently in resistant isolates. However, these were not found to be significantly correlated (adjusted $P = 0.328$) with resistance because the frequency of these mutations in untreated patients was also high (occurring in 8 of 13 pretherapy, IDV-susceptible isolates). Nevertheless, we have previously reported direct evidence that an L-63→P (L63P) substitution is required for IDV resistance in at least one genetic context (6). Therefore, this residue was also considered a contributor to IDV resistance.

Given the limited size of the data set, the residues identified as described above may be only a subset of those contributing to resistance. Inspection of the data revealed additional residues that may subtly influence viral susceptibility to IDV. One of these alterations, for which supplementary data from the study of site-directed viral mutants are available, is I64V. Coexpression of this amino acid alteration in the context of specific substitutions associated with IDV resistance consistently resulted in an approximately twofold enhancement of resistance (data not shown).

Overall, the data demonstrated that no single pattern of amino acid substitutions in the viral protease was required for the development of resistance to IDV. Rather, phenotypic resistance resulted from the combined effects of multiple, highly variable combinations of amino acid alterations. Substitutions of various combinations among at least 11 amino acid residues in the protease (L-10, K-20, L-24, M-46, I-54, L-63, I-64, A-71, V-82, I-84, and L-90) appeared to correlate with the loss of viral susceptibility to IDV as selected in vivo.

TABLE 1. Relationship of genotype to phenotypic resistance to IDV in primary viral isolates

| Patient | Wk[a] | IDV $CIC_{95}$[b] (nM) | Amino acid difference(s) from the consensus protease sequence[c] |
|---|---|---|---|
| A | 0[d] | 100 | L10V, T12I, G16E, R57K, I64V |
| | 12 | 100 | L10R, M46I, R57K, I64V |
| | 24[d] | 400 | L10R/V, T12A, M46I, R57K, L63P, I64V, V82T, I84V |
| | 37 | ≥3,000 | L10V, T12I, L24I, N37D, M46I, L63P, A71V, V82T, I84V |
| | 40[d] | 1,500 | L10R/V, T12I, L24I, N37D, M46I, L63P, A71V, V82T, I84V |
| | 44 | ≥3,000 | L10V, K20I/M, L24I, N37D, M46I, I54V, L63P, I66F, A71V, I72V, V82T |
| | 52[d] | ≥3,000 | L10R, K20I, L24I, M46I, I54V, R57K, L63P, A71V, G73S, V82T |
| B | 0[d] | 50 | E35D, M36I, R41K, I64V |
| | 24 | 100 | L10I, K20M, E35D, M36I, R41K, I64V, V82A |
| | 32 | 800 | L10I, K20M, E35D, M36I, R41K, I54V, L63P, A71T, V82A |
| | 36 | 200 | L10I, K20M, E35D, M36I, R41K, I54V, L63P, A71T, V82A |
| | 40 | 800 | L10I, K20M, E35D, M36I, R41K, I54V, L63P, A71T, V82A |
| | 48 | 1,500 | L10I, K20M, L24I, E35D, M36I, R41K, I54V, L63P, A71T, V82A |
| | 52[d] | 1,500 | L10I, K20M, L24I, E35D, M36I, R41K, I54V, L63P, A71T, V82A |
| C | 44[d] | 1,500 | V32I, M46I/L, L63A/P, I64M, A71V, I72V, V82A, Q92R |
| D | 24 | 50 | I13V, L63P, I64V, Q92K, I93L |
| | 36 | 1,500 | I13V, V32I, M46I, I47V, L63P, I64V, Q92L, I93L |
| | 44[d] | 800 | I13V, V32I, M46I, I47V, L63P, I64V, L90M, Q92L, I93L |
| E | 0 | 100 | I15V, P39S, I62V, L63P |
| | 16 | 100 | I15V, P39S, I62V, L63P |
| | 24 | 100 | P39S, L63P, I93L |
| | 32 | 800 | L10I, I15V, L24I, M36I, P39S, I54V, I62V, L63P, A71V, V82A |
| F | 44 | 25 | L19I, N37S, R41K, L63P |
| G | 16 | 400 | I15V, V32I, E35D, M36I, L63P, A71V, V82A |
| H | 0 | ≤25 | L33V, N37C, R41K, L63P |
| | 12 | 200 | I13V, L33V, N37C, I62V, L63P, A71V, I72M, G73S, V82T, L90M |
| I | 0 | 50 | E35D, N37D, L63P, I64L |
| | 12 | 400 | E35D, N37D, I54V, L63P, V82A |
| | 16 | 800 | L10H/I, L24I, E35D, N37D, M46I, L63P, V82A, Q92K |
| J | 0 | ≤25 | Q18E, L33V, E35D, N37S, L63P, L89M, I93L |
| | 24 | 200 | L10I, Q18E, K20R, L33I, E35D, M36I, N37S, I54V, L63P, V82F, L89M, I93L |
| | 32 | 800 | L10I, Q18E, K20R, L33I, E35D, M36I, N37S, I54V, D60E, L63P, V82F, L89M, I93L |
| | 36 | ≥3,000 | L10I, Q18E, K20R, L33I, E35D, M36I, N37S, I54V, L63P, V82F, L89M, L90M, I93L |
| K | 0 | ≤25 | N37S, I64V, V77I |
| | 18 | 400 | L24I, N37S, M46I, I64V, V77I, V82F, I93L |
| | 60 | ≥3,000 | L10I, M46I, I54V, Q58E, L63P, I64V, V77I, V82F, L90M, I93L |
| L | 0 | ≤25 | I13V, I62V, L63H, I64V |
| | 24 | 50 | I13V, I62V, L63H, I64V |
| M | 60 | ≥3,000 | L10I, L24I, M46L, I54V, L63P, A71V, V82A |
| N | 0 | 50 | L10I, N37C, I62V, L63S/T, I64M, I93L |
| | 24 | 200 | L10I, N37C, M46I, I62V, L63S, V77I, I85V, I93L |
| O | 0 | 100 | I93L |
| | 48 | ≥3,000 | L10I, M46I, L63P, V77I, I84V, N88T, I93L |
| | 60 | ≥3,000 | L10I, M46I, L63P, I66V, V77I, I84V, N88T, I93L |
| P | 0 | 50 | L10I, T12S, K14R, L33V, I64V |
| | 12 | 400 | L10I, T12S, I13V, I64V, V82F |
| | 48 | ≥3,000 | L10I, T12S, I13V, L33I, M36I, M46I, I64V, V82F, I84V, L89M |
| Q | 0 | 50 | R41K, L63S |
| | 24 | 200 | R41K, I54V, I62V, L63P, V77I, V82A |
| | 60 | ≥3,000 | L10I, N37S, R41K, M46I, I54V, I62V, L63P, A71V, V77I, V82A, L90M, I93L |
| R | 0 | ≤25 | M36I, L63P |
| | 24 | ≤25 | I15V, L63P, I64L |
| | 60 | ≤25 | M36I, L63P |
| S | 12 | 100 | I15V, V32I, N37D, M46I, I47V, L63P, V82A, Q92R, I93L |
| | 60 | ≥3,000 | L10I, I15V, L24I, I54V, L63P, A71V, V82A |
| T | 24 | 400 | V32I, E35D, N37D, M46I, I47V, L63P, G73S, V77I, L90M, I93L |
| U | 52 | ≥3,000 | L10I, K14R, E35D, M36V, N37D, I54V, L63P, I64V, A71T, V82F, L90M, I93L |

[a] Week of therapy with IDV. Data for week 0 were derived from pretherapy samples.

[b] Viral susceptibility to IDV in cell culture was assessed as described in Materials and Methods.

[c] Amino acid differences relative to the North American/European clade B consensus sequence (30). Differences are reported only if present in ≥25% of sequenced clones (see Materials and Methods).

[d] Sequences were previously reported (6) and are included for comparison.

TABLE 2. Regression analysis of effects of protease amino acid substitutions on the viral $CIC_{95}$ of IDV

| Amino acid residue[a] | Estimate of slope | SE (slope) | P | |
|---|---|---|---|---|
| | | | Unadjusted | Adjusted |
| 54 | 2.53 | 0.30 | <0.001 | <0.001 |
| 82 | 2.82 | 0.35 | <0.001 | <0.001 |
| 24 | 2.57 | 0.34 | <0.001 | <0.001 |
| 10 | 2.62 | 0.36 | <0.001 | <0.001 |
| 84 | 2.81 | 0.42 | <0.001 | <0.001 |
| 71 | 2.46 | 0.39 | <0.001 | <0.001 |
| 46 | 2.54 | 0.41 | <0.001 | <0.001 |
| 90 | 2.51 | 0.55 | <0.001 | 0.005 |
| 20 | 1.67 | 0.49 | 0.003 | 0.060 |
| 63 | 2.22 | 0.84 | 0.016 | 0.328 |
| 77 | 1.53 | 0.78 | 0.065 | 1.000 |
| 9 | −6.26 | 4.16 | 0.149 | 1.000 |
| 93 | 1.02 | 0.71 | 0.164 | 1.000 |
| 36 | 1.02 | 0.72 | 0.174 | 1.000 |
| 35 | 0.78 | 0.59 | 0.200 | 1.000 |
| 32 | 1.15 | 0.88 | 0.206 | 1.000 |
| 96 | −9.70 | 7.44 | 0.208 | 1.000 |
| 41 | −1.09 | 1.08 | 0.326 | 1.000 |
| 47 | 5.08 | 5.24 | 0.345 | 1.000 |
| 64 | −0.70 | 0.74 | 0.358 | 1.000 |
| 16 | −9.38 | 9.96 | 0.358 | 1.000 |
| 87 | −8.46 | 9.00 | 0.359 | 1.000 |
| 37 | −0.70 | 0.79 | 0.384 | 1.000 |
| 15 | 0.45 | 0.61 | 0.469 | 1.000 |
| 62 | −0.54 | 1.03 | 0.610 | 1.000 |
| 89 | 0.88 | 1.76 | 0.621 | 1.000 |
| 33 | −0.53 | 1.11 | 0.641 | 1.000 |
| 72 | 0.45 | 1.30 | 0.733 | 1.000 |
| 92 | 0.22 | 1.06 | 0.841 | 1.000 |
| 13 | 0.08 | 1.36 | 0.955 | 1.000 |

[a] Listed in order of increasing adjusted *P* value, as described in Materials and Methods.

**Association between the number of protease amino acid substitutions and IDV resistance.** Increases in phenotypic resistance to IDV appeared to be accompanied by increasing numbers of amino acid substitutions in the protease. Table 3 cross-classifies each patient viral isolate by $CIC_{95}$ and the number of substitutions at the 11 protease residues associated with IDV resistance. There was a clear relationship between the total number of substitutions in the protease and the level of phenotypic resistance ($P < 0.001$). When substitutions at all 99 protease residues were considered, a strong association between the total number of substitutions and phenotypic resistance ($P < 0.001$) (data not shown) was also noted.

These observations were confirmed by phenotypic analysis of a number of site-directed viral mutants that were constructed in the NL4-3 viral background (1). We previously reported that expression of the single substitutions L10R, M46I, L63P, V82T, and I84V had no effect on viral susceptibility to IDV (6). We have now extended our observations to the single substitutions V32I, M36I, V82A, V82F, and L90M. The $CIC_{95}$s for each of these mutants were identical (50 to 100 nM) to that for wild-type virus. Attempts to reconstruct the viral resistance manifested by different patient isolates by using various combinations of double substitutions have also been unsuccessful (data not shown). The exhibition of measurable resistance to IDV has been reported to require minimally the coexpression of three protease amino acid alterations (6).

**Sequence predictors of phenotypic resistance.** In an effort to identify a simple indicator of phenotypic resistance to IDV, we investigated the predictive value of assessing substitutions at the residue most frequently associated with resistance, V-82. Considering only this residue, 22 of 28 of the IDV-susceptible isolates ($CIC_{95} < 400$ nM) expressed the wild-type valine (in ≥50% of clones), while 23 of 29 IDV-resistant isolates ($CIC_{95} \geq 400$ nM) expressed prevailing substitutions of V-82 (to A, F, or T). Hence, these substitutions at residue 82 usually, but not always, accompany viral resistance to IDV.

If substitutions of either residue 46 or 82 are considered, a more sensitive predictor of resistance can be obtained. In this case, wild-type sequences at M-46 and V-82 were found in 20 of 28 IDV-susceptible isolates, while all 29 IDV-resistant isolates carried a mutation at one or both of these sites. Among the eight IDV-susceptible isolates carrying substitutions at either residue, five had IDV $CIC_{95}$s of 200 nM, below the 400 nM cutoff considered significant for measurable resistance but nonetheless suggestive of resistance. Moreover, four of the patients carrying these five isolates (patients A, B, J, and Q) eventually yielded more highly resistant viruses at later weeks of therapy.

It should be noted that these analyses were based on viral isolates from patients selected for potential development of resistance. The genotypic predictors of IDV resistance discussed above may perform differently in a random sampling of primary viral isolates. Nevertheless, an examination of amino acid changes at residue 82, with or without residue 46, may have predictive value in assessing the development of resistance.

**Cross-resistance among different protease inhibitors.** We previously reported that viral isolates from four patients treated with IDV also exhibited cross-resistance to a panel of five other structurally diverse protease inhibitors (6). We have since characterized the resistance patterns of isolates from 15 additional IDV-treated patients, as shown in Table 4. For comparison, some data from the original four patients (patients A to D) are also shown. All isolates in this analysis had been preselected for measurable resistance to IDV. Every viral isolate tested that exhibited resistance to IDV also expressed a loss of susceptibility to both XM412 and ritonavir. In contrast, only a subset of IDV-resistant viral isolates exhibited lessened susceptibility to saquinavir (63%), VX-478 (81%), or SC-52151 (74%). Although there was some correlation between the resistance patterns for these latter three compounds, within this data set, resistance to one inhibitor was not an absolute predictor of resistance to another. A comparison of these cross-resistance spectra with the sequence analyses depicted in Table

TABLE 3. Association between the number of protease amino acid substitutions and the level of resistance expression[a]

| No. of substitutions | No. of isolates with IDV $CIC_{95}$ (nM) of: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 25 | 50 | 100 | 200 | 400 | 800 | 1,500 | 3,000 |
| 0 | —[b] | — | 1 | — | — | — | — | — |
| 1 | 6 | 2 | 3 | — | — | — | — | — |
| 2 | 2 | 4 | 2 | — | 1 | — | — | — |
| 3 | — | 1 | 2 | 2 | 4 | — | 1 | — |
| 4 | — | — | — | 1 | 1 | 2 | 1 | 2 |
| 5 | — | — | — | 1 | — | 1 | — | 1 |
| 6 | — | — | — | 1 | — | 3 | 1 | 3 |
| 7 | — | — | — | — | — | — | 2 | 5 |
| 8 | — | — | — | — | — | — | — | 1 |

[a] All isolates ($n = 57$) analyzed in this study were classified by number of substitutions (sum of the mutation frequencies at the 11 residues associated with IDV resistance, rounded to the nearest integer).

[b] —, none.

TABLE 4. Susceptibility of IDV-resistant viral isolates to diverse HIV-1 protease inhibitors

| Patient or range | Wk | $CIC_{95}$ (nM)[a] of: | | | | | |
|---|---|---|---|---|---|---|---|
| | | IDV | XM412 | Ritonavir | Saquinavir | VX-478 | SC-52151 |
| Wild-type range[b] | 0 | 25–100 | 100–400 | 100–400 | 25–100 | 50–200 | 50–200 |
| A | 24 | 400 | ≥3,000 | 1,500 | 50 | 200 | 200 |
| | 40 | 1,500 | ≥3,000 | ≥3,000 | 800 | 400 | ≥3,000 |
| | 52 | ≥3,000 | ≥3,000 | ≥3,000 | 800 | 800 | ≥3,000 |
| B | 32 | 800 | ≥3,000 | ≥3,000 | 200 | 200 | 400 |
| | 48 | 1,500 | ≥3,000 | ≥3,000 | 800 | 800 | 1,500 |
| C | 44 | 1,500 | ≥3,000 | ≥3,000 | 100 | 1,500 | 800 |
| D | 36 | 800 | ≥3,000 | ≥3,000 | 100 | ≥3,000 | 1,500 |
| | 44 | 800 | ≥3,000 | ≥3,000 | 100 | ≥3,000 | 1,500 |
| E | 32 | 800 | ≥3,000 | ≥3,000 | 200 | 400 | 800 |
| G | 16 | 400 | ≥3,000 | ≥3,000 | 50 | 400 | 100 |
| I | 12 | 400 | ≥3,000 | ≥3,000 | 100 | 400 | 400 |
| | 16 | 800 | ≥3,000 | ≥3,000 | 400 | 800 | 1,500 |
| J | 24 | 200 | ≥3,000 | ≥3,000 | 25 | 50 | 25 |
| | 36 | ≥3,000 | ≥3,000 | ≥3,000 | 200 | 1,500 | 200 |
| K | 18 | 400 | ≥3,000 | 800 | 50 | 200 | 200 |
| | 60 | ≥3,000 | ≥3,000 | ≥3,000 | 800 | ≥3,000 | ≥3,000 |
| L | 72 | 800 | ≥3,000 | 1,500 | 25 | ≥3,000 | 200 |
| M | 60 | ≥3,000 | 1,500 | ≥3,000 | 100 | 400 | 800 |
| O | 48 | ≥3,000 | ≥3,000 | 800 | 200 | 100 | 800 |
| | 60 | ≥3,000 | ≥3,000 | ≥3,000 | 800 | 50 | 800 |
| P | 12 | 400 | ≥3,000 | 1,500 | 25 | 800 | 100 |
| | 48 | ≥3,000 | ≥3,000 | ≥3,000 | 200 | ≥3,000 | 1,500 |
| Q | 24 | 200 | ≥3,000 | ≥3,000 | 100 | 400 | 400 |
| | 60 | ≥3,000 | ≥3,000 | ≥3,000 | 800 | 1,500 | 1,500 |
| S | 60 | ≥3,000 | ≥3,000 | ≥3,000 | 200 | 400 | 800 |
| T | 24 | 400 | 1,500 | 1,500 | 400 | 400 | 1,500 |
| U | 52 | ≥3,000 | ≥3,000 | ≥3,000 | 400 | 1,500 | 800 |
| V | 18 | 1,500 | ≥3,000 | ≥3,000 | 1,500 | 800 | ≥3,000 |
| W | 60 | 800 | ≥3,000 | ≥3,000 | 400 | 800 | 1,500 |

[a] $CIC_{95}$s were determined as described in Materials and Methods. References for inhibitors: IDV, 11 and 40; XM412, 42; ritonavir, 21; saquinavir, 8; VX-478, 22; SC-52151, 3.

[b] See Table 1, footnote *a*. The wild-type virus susceptibility range was determined by assay on multiple pretherapy virus isolates.

1 failed to reveal any obvious sequence determinants of cross-resistance.

## DISCUSSION

Treatment of HIV-1-infected individuals with IDV has resulted in the emergence, in some patients, of viral variants with reduced susceptibility to the inhibitor. The data reported here are derived from the earliest phase I and II clinical studies of the compound, in which patients were initially treated with either 200 or 400 mg every 6 h or 600 mg every 8 h, dosages which are now known to be suboptimal for long-term antiviral activity (unpublished data). Under these conditions, viral variants exhibiting various degrees of resistance began to appear 12 to 24 weeks after the initiation of therapy.

The evolution of resistance was characterized by gradual increases in the $CIC_{95}$ of IDV for viral isolates and was accompanied by the stepwise accumulation of amino acid substitutions in the viral protease. These substitutions involved multiple amino acid residues of the enzyme and appeared in highly variable combinations and in no consistent order. Molla et al. (28) have shown that the accumulation of amino acid substi-

tutions in patients treated with the protease inhibitor ritonavir usually began with mutations at V-82 and that this was followed by variable secondary substitutions at I-54, A-71, and M-36, M-46, or I-84 (listed in decreasing frequency of occurrence). This result suggests that among the many mutations contributing to ritonavir resistance, substitution at V-82 may represent the initial "path of least resistance" toward viral resistance to ritonavir but that the appearance of additional substitutions occurs with much less predictability. The data presented in Table 1 show that in IDV-treated patients, no preferred order of appearance of any substitutions was evident. This variability was also seen in a much larger database of serum virus sequences of multiple independent clones from 131 IDV-treated patients (5a).

The highly variable nature of the observed amino acid substitutions has precluded the identification of simple, invariant rules diagnostic for IDV resistance. However, because all of our sequence data were derived from multiple independently amplified clones, mutation frequencies in these populations could be directly measured. This in turn permitted the statistical analysis of sequence data. The emergence of phenotypic resistance correlated with the appearance of substitutions at various numbers of amino acid residues among at least 11 sites in the protease: L-10, K-20, L-24, M-46, I-54, L-63, I-64, A-71, V-82, I-84, and L-90. Further, the level of resistance was correlated with the number of amino acid substitutions present. Thus, the evolution of resistance to IDV involves the stepwise accumulation of mutations during continued viral replication under the selective pressure of the inhibitor.

We have shown here and elsewhere (6) that the appearance of IDV resistance in clinical viral isolates has resulted in cross-resistance to other HIV-1 protease inhibitors. Every IDV-resistant viral isolate that we have identified to date (27 isolates from 19 patients) has also exhibited resistance to both ritonavir and XM412. Although not all genetic contributors to ritonavir and XM412 resistance have been defined, substitutions at either V-82 or I-84 appear to be major contributors to resistance to these agents (2, 25, 28). This might explain much, but not all, of the cross-resistance to these compounds that was observed in IDV-resistant isolates. The absence of these substitutions in the resistant isolates from patients D (week 36) and T (week 24), however, indicates that alterations other than the V-82 or I-84 substitutions can also confer viral resistance to ritonavir or XM412.

The pattern of cross-resistance between IDV, saquinavir, VX-478, and SC-52151 is more complex. Although most IDV-resistant viral isolates exhibited cross-resistance to these other inhibitors, the magnitude and spectrum of this cross-resistance varied widely among isolates, and no simple genetic determinants appeared to explain this phenomenon.

The emergence of cross-resistant variants has also been shown to occur during selection with other protease inhibitors. Although only limited virological data exist, the emergence of cross-resistant viruses has been reported to occur under selection by saquinavir (38), ritonavir (25, 35), VX-478 (31, 38), and other agents (31, 38). Consistent with our observations, the degree and spectrum of cross-resistance varied widely among isolates. It thus appears possible that the selection for viral variants that are cross-resistant to other protease inhibitors may be a general property of this class of compounds, all of which inhibit the same target and function by the same general mechanism.

A comparison of the mutations selected by the various protease inhibitors, both in vitro and in vivo, reveals a striking degree of overlap. Among the 11 mutations that we have identified as contributors to IDV resistance, all except I64V have so far been shown to be selected by ritonavir (25, 27, 28, 35, 37), and at least 8 of the 11, at L-10 (to I, V, or F), M-46 (to L), I-54 (to V), L-63 (to P or V), A-71 (to V), V-82 (to A, T, or I), I-84 (to V), and L-90 (to M) are selected by saquinavir (12, 17, 18, 34, 38, 41). Moreover, under conditions of initially equivalent selective pressure, all three possible pairwise combinations of IDV, saquinavir, and ritonavir have been shown to select in vitro for viruses carrying between 8 and 10 amino acid substitutions in the protease (37). Thus, when selective pressure was controlled, these three protease inhibitors selected for nearly identical collections of resistance mutations. In view of these overlapping patterns of resistance substitutions, the development of clinical cross-resistance among protease inhibitors is not surprising.

In conclusion, the development of IDV resistance has been shown to occur through multiple, overlapping genetic pathways, and this resistance results from the combined effects of several mutations that do not confer a measurable degree of resistance when occurring alone. In contrast, significant viral resistance to other protease inhibitors has been shown to result from the appearance of one or two amino acid substitutions in the protease. The finding that the evolution of IDV resistance is cumulative strongly suggests a requirement for ongoing viral replication in this process. Thus, potent inhibition of viral growth would be expected to delay the emergence of resistance to IDV. The results of ongoing clinical trials with high dosages of IDV, alone or in combination with other antiretroviral agents, have been consistent with this prediction. Patients who initiate therapy with these more potent therapeutic regimens exhibit more pronounced and durable antiviral effects than those who initiate at lower dosages and whose dosages are subsequently increased (data to be published elsewhere). This suggests that the initial and continued use of the most efficacious therapeutic regimens available may lead to maximal clinical benefit, both by preventing viral spread and by inhibiting the viral replication that drives the evolution of resistance.

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