The human immunodeficiency virus Gag- and Pol-encoded proteins are expressed as long polypeptide precursors that are proteolytically cleaved into mature proteins found in infectious virions. This cleavage is catalyzed by the virus-encoded aspartyl protease and is required for virus replication (1–3). As such, the viral protease has become a target for HIV1 therapeutics, resulting in many HIV protease inhibitors reaching clinical trials. Most of these drugs are substrate-based inhibitors, whose design has been facilitated by an abundance of crystal structure data for both the native enzyme and enzyme-inhibitor complexes (4). Additionally, there are now extensive biochemical data detailing both the catalytic mechanism and the molecular basis for substrate selection (5–7).

The primary difficulty encountered while administering HIV therapeutic agents to patients has been the rapid emergence of drug resistance by the virus. Inhibitors of retroviral enzymes have dramatic effects initially, lowering virus load to less than 1% of pretreatment levels and improving the clinical state of the patient (8–10). However, virus resistance to the inhibitory effects of the drugs develops rapidly, causing a continued progression of disease symptoms. This has been well documented in many clinical trials using reverse transcriptase inhibitors such as azidothymidine (AZT), (−)2′-deoxy-3′-thiacytidine (3TC), or nevirapine. Unfortunately, a similar progression is now being observed in patients treated with retroviral protease inhibitors (11).

The primary mechanism by which retroviruses develop this resistance has been thought to be a consequence of the relatively low fidelity of reverse transcriptase. Because the DNA polymerase does not have a proofreading function, base mismatch errors are incorporated into viral DNA before integration into the host cell genome. This proviral DNA then serves as a template for all new viral transcripts, passing along any mutations incorporated during the initial reverse transcription event. Although only one or two reverse transcription products are integrated successfully into each host cell genome, a large number of genetic mutations can accumulate in a very short time. This is made possible by the large pool of infected T-cells that turn over extremely rapidly. In a report earlier this year in Science, Coffin (12) calculates that at least 10^9 new cells are infected each day in a typical HIV-infected patient during the latent or steady state stages of infection (12) and that every point mutation that could possibly occur along the length of the viral genome does occur and at a staggering frequency of between 10^6 and 10^7 times daily! In addition to reverse transcriptase infidelity, mutations may be introduced into the viral genome through host cell RNA polymerase II transcription errors or through genetic recombination between homologous regions of the HIV genome and endogenous viral sequences. Thus, in the presence of a drug selecting for certain types of mutations in particular proteins, a passive and relatively random process leads rapidly to the emergence of drug resistance.

Several groups have sequenced protease genes from drug-resistant HIV phenotypes isolated from tissue culture systems (13–18). The protease mutants that have been analyzed contain a similar set of amino acid changes at very characteristic and predictable positions within the enzyme molecule. Although many different inhibitor compounds have been used in these studies, the same regions of the enzyme have mutated in the drug-resistant strains. Analysis of the data shows that mutations which produce inhibitor-resistant proteases are concentrated in regions of the enzyme that have been shown to form the subsites of the substrate binding pocket. This is best illustrated by the studies of Colonno and co-workers (18), who followed the appearance of drug-resistant viruses in HIV-1-infected cells grown in tissue culture. Multiple changes in protease amino acid sequence were detected with time. However, drug resistance emerged only after Val-82 changed to Ala (18). These mutations affect substrate recognition in the S1 and S1′ subsites (Fig. 1). Other key amino acids whose changes produce resistant phenotypes are at position 32 in the S2 and S2′ subsites, at positions 8, 46, 47, and 48 in the S4 and S4′ subsites, and at position 84 in the S1, S3, S3′, and S1′ subsites (Fig. 1). Positions 46, 47, and 48 are in highly flexible and exposed surface loops. These “flaps” cover the substrate binding cleft and form part of the S4 and S4′ subsites. The side chains of all of these residues, with the exception of Met-46, are orientated inward toward the inhibitor and contribute directly to its binding. The Met-46 → Leu mutation was shown by molecular dynamic simulations to decrease the flexibility of the flaps (19). This may prevent the flaps from opening as efficiently and thereby limit inhibitor access to the substrate binding pocket. Most of these residues that are mutating in these drug-resistant enzymes had been identified previously as being critical for determining retroviral protease specificity (7). Furthermore, it was predicted that these residues were those likely to play a significant role in developing drug resistance even before resistance data were available (7, 10).

Recent work by Condra et al. (11) describes four patients that developed resistance to the protease inhibitor MK-639. These drug-resistant HIV-1 protease mutants also have at least one mutation in a key amino acid residue positioned directly in one of the enzyme subsites forming the substrate binding pocket. For instance, patients A, B, and C had mutations at Val-82 or Ile-84 in the S1 and S1′ binding pockets. Patients A, C, and D had changes in Met-46 at the base of the flaps, and patients C and D had mutations at Val-32, which is a key residue in the S2 and S2′ binding pockets. Patient D had an additional flap mutation at Ile-47. Many of these positions were identified earlier by groups looking for drug resistance in tissue culture systems. In fact, many of the exact same mutations were seen in the human isolates. These include I84V, M46I, I47V, and V32I substitutions.

What is the molecular basis for these resistant phenotypes? The substrate binding pocket of protease is formed in a region along the central axis of the symmetric homodimer. Eight individual subsites are formed along the length of the enzyme surface and are designed to accommodate substrate amino acid
side chains. Substrate binding in the subsites is governed largely by an ability to form stabilizing van der Waal's interactions between substrate amino acid side chains and enzyme amino acid side chains lining the subsites (20, 21). The subsites have been shown to act largely independently in the selection of substrate amino acids (22, 23). For example, mutations can be made in amino acids forming the S2 subsite that change the preference for substrate amino acids at the corresponding P2 position. Protease amino acid residues in the substrate binding cleft can be mutated both singly and in combination to produce mutant proteases with altered substrate specificity (22, 23). Many of these mutants retain the ability to process the wild-type substrate sequences, albeit at lower rates. Recent work by Erickson and co-workers (24) has shown that the overall catalytic efficiency for each of 11 drug-resistant HIV protease mutants is between 1.2- and 14.8-fold lower than for the wild-type enzyme on a standard reference substrate. However, if drug treatment is continued, near wild-type titers of virus are restored over a period of a year without loss of a drug-resistant mutation (18). This occurs concomitantly with additional protease mutations, which presumably compensate for the decrease in activity toward the viral polyproteins due to the initial resistance-producing change.

The majority of the drug-resistant protease mutations that have been identified are relatively conservative changes involving the gain or loss of a methylene group (Table I). For example, I84V, V82I, V82A, I47V, and V32I all have significant effects on inhibitor binding. Other changes that are observed involve small changes in side chain length or character (Table I). The only exception is M46F. In the other positions (Table I), where changes in amino acids probably have little effect on specificity, more diversity in the substitutions is observed. It may seem surprising that such seemingly small changes in the critical regions can effect the IC50 concentrations by greater than 30-fold. However, in vitro biochemical studies using mutant retroviral proteases and altered peptide substrates have shown that rather modest changes in either enzyme or substrate can effect catalytic efficiency (kcat/Km) by greater than 10-fold (20, 22). A particularly vivid example demonstrating the extent to which retroviral protease specificity is easily altered by modest changes at the enzyme-substrate interface was seen in the Rous sarcoma virus protease. This enzyme is structurally very similar to HIV-1 protease. When the P2 position of an efficiently cleaved substrate was changed from valine to leucine, the activity was reduced to 10% of that seen with the unmodified peptide. However, changing Ile-44 to Val in the enzyme increased the ability to cleave the P2-modified peptide, restoring relative cleavage to levels approaching those seen with the unmodified peptide substrate. This result can be explained using crystallographic data with computer-modeled substrates. The addition of a methylene group from the substrate P2 posi-
tion can be compensated for by the removal of a methylene group from an amino acid side chain forming part of the S2 subsite. The simple gain or loss of a methylene group from an enzyme binding pocket can easily lead to 10-fold changes in $K_i$ for a given drug. This could render the drug ineffective by making it impossible to increase drug concentrations to therapeuatic levels. Recent analysis of crystal structure data for HIV-1 protease complexed with the symmetric inhibitor A-77003 confirms this conclusion and shows that favorable van der Waal's interactions in the S1' subsite are disrupted by a Val-82$\rightarrow$Ala substitution, which infers the drug-resistant phenotype (25). A similar conclusion with a different inhibitor complexed to a multidrug-resistant HIV-1 protease has recently been reported (28).

It has been realized that drug-resistant phenotypes selected in the presence of one drug often display resistance to many other protease inhibitors with different structures. This is not particularly surprising, for most protease inhibitors are substrate-based non-hydrolyzable peptide mimetics that are targeted initially against the wild-type enzyme. Mutating key substrate residues in the substrate binding pockets alters the ability to make favorable van der Waal's interactions in the S1' subsite are disrupted by a Val-82$\rightarrow$Ala substitution, which infers the drug-resistant phenotype (25). A similar conclusion with a different inhibitor complexed to a multidrug-resistant HIV-1 protease has recently been reported (28).

Typically, compounds in development that do not inhibit the wild-type enzyme are not examined further. However, it would seem that those in the business of designing HIV protease inhibitors could profit from examining compounds that may not bind especially tightly to the wild-type enzyme. Many of these compounds are likely to exist already and may bind tightly to the mutant proteases. Indeed, by the same arguments described above, an inhibitor designed against the mutant enzyme should not bind tightly to the wild-type protease. While the enzyme clearly demonstrates a remarkable ability to mutate and preserve function, there are almost certainly a limited number of mutations that will alter drug binding while preserving a sufficient level of specific activity on the normal substrates to allow for virus replication. A multidrug approach with protease inhibitors designed against wild-type and mutant form(s) most likely to develop may offer promise as a potentially effective therapy for HIV infection.

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Todd Ridky and Jonathan Leis

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