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Binding of clinical inhibitors to a model precursor of a rationally selected multidrug resistant HIV-1 protease is significantly weaker than to the released mature enzyme

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Abstract

We have systematically validated the activity and inhibition of a HIV-1 protease (PR) variant bearing 17 mutations (PR^{S17}), selected to represent high resistance by machine learning on genotype-phenotype data. Three of five mutations in PR^{S17} correlating with major drug resistance, M46L, G48V and V82S, and five of eleven natural variations, differ from two clinically derived extreme mutants, PR20 and PR22 bearing 19 and 22 mutations, respectively. PR^{S17}, which forms a stable dimer (<10 nM), is ~10- and 2-fold less efficient in processing the Gag polyprotein relative to the wild-type and PR20, respectively, but maintains the same cleavage order. Isolation of a model precursor of PR^{S17} flanked by the 56 amino acid transframe region (TFP-p6pol) at its N-terminus, which is impossible when expressing an analogous PR20 precursor, allowed systematic comparison of inhibition of TFP-p6pol-PR^{S17} and mature PR^{S17}. Resistance of PR^{S17} to 8 protease inhibitors (PIs) relative to PR ranges from 1.5 to 5 orders of magnitude increase in K_i from 0.01 to 8.4 μ M. Amprenavir, darunavir, atazanavir and lopinavir, the most effective of the 8 PIs, inhibit precursor autoprocessing at the p6pol/PR site with IC₅₀ ranging from ~7.5 to 60 μ M. Thus this process, crucial for stable dimer formation, shows ~200 to 800-fold weaker inhibition than the mature PR^{S17}. TFP/p6pol cleavage, which occurs faster, is inhibited even more weakly by all PIs except darunavir (IC₅₀ of 15 μ M); amprenavir shows a 2-fold increase in IC₅₀ (~15 μ M), and atazanavir and lopinavir show increased IC₅₀ of >42 μ M and >70 μ M, respectively.

Graphical abstract

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#Equally contributed to the experimental work

SUPPORTING INFORMATION

One table comparing drug resistance mutations in PR^{S17} with PR, PR20 and PR22 and four figures pertaining to the estimation of kinetic parameters k_{cat} and K_m of PR^{S17}, K_i measurement by enzyme kinetics and ITC of PR^{S17}, and structure representations of mature PR^{S17}, PR20 and PR22 showing the positions of mutations matching with table details.

Keywords

HIV protease; drug resistance; autoprocessing; inhibitor binding; enzyme kinetics

INTRODUCTION

The human immunodeficiency virus type 1 protease (HIV-1 PR) plays a pivotal role by processing the viral polyproteins into mature proteins essential for the assembly and maturation of the virus in its replication cycle.¹ The mature PR is a homodimeric aspartic protease in which each 99 amino acid monomer contributes one of the two aspartic acid residues (Asp25) required for catalytic function. A single copy of PR is synthesized in the *pol* open-reading frame of the Gag-Pol precursor. The PR promotes its own release via transient dimerization and cleavages at its N- and C-termini, termed autoprocessing. The initial intramolecular cleavage at the N-terminus, between the transframe region (TFR, Fig. 1A) and PR, is essential for stable dimer formation and appearance of mature-like catalytic activity. Subsequent intermolecular cleavage at the C terminus of PR (PR/RT junction) yields the mature PR.² Since its initial discovery, PR has been a target for therapeutic intervention, and several drugs designed specifically to bind to the active site of PR are in current use.^{3,4} Protease inhibitors (PIs) are used in combination antiretroviral therapy (cART) with other antiretroviral agents that block reverse transcriptase and integrase activities, and viral entry⁵ (<https://aidsinfo.nih.gov/contentfiles/lvguidelines/adultandadolescentgl.pdf>). However, the short replication cycle of 1–2 days⁶ combined with error-prone nature of RT ($\sim 2 \times 10^{-5}$ errors/nucleotide per replication cycle)⁷ favor rapid evolution of mutations selected under drug pressure thus resulting in extreme drug resistance.^{8–11}

Several multi-drug resistant mutants of PR have been isolated and characterized structurally to explain weaker binding of clinical PIs and enable the design of future inhibitors targeting such mutants to be used in cART.^{4,8,9,12–14} One well characterized extremely drug resistant isolate of PR bearing 19 mutations (PR20)^{10,15–17} exhibits 3–4 orders of magnitude weaker binding to clinical PIs relative to the wild-type enzyme. However, it mediates Gag processing only about 4-fold more slowly and retains the same order of cleavage as the wild-type enzyme. Importantly, it undergoes efficient N-terminal autoprocessing, a requisite to release the fully active stable mature PR20. Consistent with the weak binding of SQV and DRV to mature PR20, autoprocessing of the PR20 model precursor (TFR-PR20) when expressed in *E. coli* is not inhibited up to a practical limit of 150 μ M by these PIs in contrast to the 1–2 μ M IC₅₀ observed for the wild type precursor.¹⁶

Exponentially growing databases in recent years have led to the application of machine learning and computational methods for the evaluation of inhibitors for use in drug therapy to optimize virological outcomes in HIV-1 infected patients.¹⁸ A new predictive method for drug resistance uses machine learning with a unified encoding of the sequence and 3-D structure of the target protein, such as HIV-1 PR.¹⁹ Superior results were obtained by including structural and sequence information as compared to other methods based only on sequence, with classification accuracies in the 93–99% range for PR instead of 60–87% accuracy.²⁰ The unified encoding allowed accurate prediction of drug resistance from genomic data with several different machine learning algorithms. It also gave excellent correlation between predicted and observed resistance in cross-validated regression to predict not just resistance but the degree of resistance.²⁰ The success of linear regression demonstrated that the unified encoding accurately encapsulated the structure and sequence information. This approach can be used to cluster genomic sequence data and select representative samples for further analysis.

Representative sequences were extracted from the genotype sequence data in the Stanford HIV drug resistance database by mean shift clustering with regression analysis, in a procedure designed to identify mutants with the common features characterizing high level resistance.²¹ The clustering selected a small number of sequences that are able to quantitatively reproduce the resistance phenotype for the entire dataset with high accuracy in the regression. The method also selected sequences that are in the center of the distribution and therefore characteristic of a wide range of other mutations. Some sequences represented mutants resistant to more than one inhibitor. Analyzing such overlaps resulted in a single sequence predicted to have high level resistance to six of the inhibitors: atazanavir (ATV), amprenavir (APV), nelfinavir (NFV), indinavir (IDV), lopinavir (LPV), and SQV. This sequence, bearing 17 mutations, was termed PR^{S17} (S for selected) and chosen for experimental validation of the inhibition and further characterization.

Here we evaluate the dimer dissociation constant of PR^{S17}, and its catalytic efficiency by monitoring cleavage of a peptide substrate as well as the Gag polyprotein. The binding affinity of 8 clinical inhibitors to mature PR^{S17} was estimated by enzyme kinetics and isothermal titration calorimetry. Very little is known about the folding landscape, inhibition of autoprocessing and associated drug resistance for the PR precursor. The latter two are predicted to have distinct differences from the properties of the mature PR described in a majority of studies. Even though isolation of the wild-type model precursor is feasible, a systematic comparison of inhibition of autoprocessing (IC₅₀) *in vitro* with K_i values for inhibition of the mature PR was impractical because of the detection limit of the precursor below 1–2 μ M protein concentration by SDS-PAGE. On the other hand, it was also impossible to isolate and purify a model precursor (TFR-PR20) of the extremely drug resistant mutant PR20 because the precursor does not accumulate during expression due to efficient autoprocessing coupled with weak binding of PIs even at 150 μ M.¹⁶ Fortunately, the model precursor of PR^{S17}, TFR-PR^{S17}, provided an ideal window that is between the 2 extremes typified by PR and PR20. Thus, we were able to compare the K_i values for the inhibition of mature PR^{S17} by 8 PIs with the IC₅₀ values for inhibition of autoprocessing of TFR-PR^{S17} *in vitro* by the same PIs.

Our results indicate that PIs bind 1–5 orders of magnitude weaker to mature PR^{S17} relative to the wild type and PIs with higher affinity to the mature PR^{S17} show larger differences when compared with IC₅₀ values for inhibition of autoprocessing, providing the first such comparison. Hence, the PR^{S17} construct provided an important model for a comprehensive *in vitro* evaluation of drug resistance of the mature PR and its precursor.

MATERIALS AND METHODS

Cloning, protein expression and purification

The cloning, expression and isolation of TFR-PR, PR and PR20 have been described before.^{16,22} Inserts coding for TFR-PR^{S17} and PR^{S17} were custom synthesized (DNA 2.0, Menlo Park, CA), cloned in pJ414 flanked by NdeI and BamHI sites and transformed into *E. coli* BL-21 (DE3). Induction for protein expression, isolation of inclusion bodies and protein purification followed previously described protocols.^{23,24} Recombinant constructs were verified both by DNA sequencing and electrospray ionization mass spectrometry.

The clinical isolates, PR20 containing 14 PI resistance-associated mutations including those specific to DRV, and PR22 from a pediatric patient with high viral load despite HAART including PIs are described in references¹⁵ and¹¹, respectively.

Dimer dissociation and kinetic parameters

The dimer dissociation constant (K_{dimer}) for PR^{S17} was determined in 50 mM sodium acetate buffer, pH 5.0, containing 250 mM NaCl (buffer B) from the dependence of initial rate for hydrolysis of a chromogenic substrate (Lys-Ala-Arg-Val-Nle-[4-nitrophenylalanine]-Glu-Ala-Nle-NH₂, California Peptide Research, Napa, CA)²⁵ as a function of dimeric PR^{S17} concentration at 28 °C. Assaying at concentrations below 10 nM was impractical and only an upper limit for dimer dissociation could be estimated. K_m and k_{cat} for substrate hydrolysis by mature PR^{S17} were determined spectrophotometrically in the same buffer at an enzyme concentration of 0.5 μM and 20 - 400 μM of the chromogenic substrate (ε at 310 nm = 1797 Abs M⁻¹cm⁻¹) at 28 °C. Data were analyzed and plotted (Fig. S1) by use of the Enzyme Kinetics module of Sigmaplot 10.0 (Systat Software, Inc).

Inhibitor binding

Protein–inhibitor association constants (K_{assoc}) were measured by isothermal titration calorimetry (Microcal iTC200, Malvern, Westborough, MA). Freshly folded²⁶ PR^{S17} (~15 μM as dimer) in 50 mM sodium acetate buffer, pH 5.0 (buffer A), was titrated with 105 μM inhibitors in the same buffer at 28 °C. APV, IDV and DRV exhibited adequate thermal response upon titration and the data were processed with the instrument's Origin software (Fig. S2). For competitive inhibitors that bind at only one site, dissociation constants ($1/K_{\text{assoc}}$) are equivalent to the *inhibition* constants measured by enzyme kinetics (K_i).

K_i values for six inhibitors were determined kinetically in buffer B, 28 °C, at a concentration of 0.5 μM enzyme and 360 μM chromogenic substrate. Initial rates (v_i) of absorbance decrease at 310 nm were converted to μM/min as described for the determination of kinetic parameters. Values of $1/v_i$ plotted versus inhibitor concentration (Fig. S3) gave linear

regressions from which the slope and y-axis intercept were obtained; for competitive inhibitors, the negative x-axis intercept corresponds to the IC_{50} for inhibition and was determined from the relationship, $x\text{-intercept} = - (y\text{-intercept})/(\text{slope})$. Since the IC_{50} is substrate dependent, the K_i value was obtained from the relationship $K_i = IC_{50}/(1 + [\text{substrate}]/K_m)$ using the measured value of $K_m = 193 \mu\text{M}$ under the same conditions. Errors were estimated from the sum of the percent errors in the slope, y-intercept and independently measured K_m .

Autoprocessing

A stock solution of the TFR-PR^{S17} precursor (1.86 mg/ml, 54 μM in dimer concentration) maintained in 12 mM HCl was diluted with 2.3 volumes of 5 mM sodium acetate buffer at pH 6 in the presence or absence of inhibitor, followed immediately by the addition of 3.3 volumes of 100 mM acetate buffer at pH 5. In the absence of inhibitor, aliquots (10 μl) were drawn at the indicated times from 0.5–60 min, mixed with 4 μl sample loading buffer and subjected to SDS-PAGE on 20% homogeneous PhastGels (GE Healthcare). Similarly, autoprocessing reactions were carried out with varying concentrations of the inhibitor for 60 min, followed by SDS-PAGE.

Gag Processing

Assays (20 μl) to monitor the order and rate of cleavage of Gag, a truncated Gag construct spanning the matrix, capsid, SP1 and nucleocapsid domains, were carried out as described previously.^{10,27} Gag (50 μM) in 20 mM sodium phosphate, pH 6.5, 300 mM NaCl, 0.1 mM ZnCl_2 and 1 mM tris(2-carboxyethyl)phosphine was mixed with the appropriate protease to give a final concentration of 1 μM at room temperature. Aliquots (2 μl) were drawn at the indicated times and mixed with 3 μl of the above assay buffer and 2 μl of SDS-PAGE sample loading buffer, heated to 85 °C for 2 min and subjected to electrophoresis on 20% homogeneous PhastGels using 8 lane $\times$ 1 μl applicators (GE Healthcare). Gels were stained with PhastGel Blue R, destained and digitized.

RESULTS AND DISCUSSION

Description of mutations in PR^{S17}

Organization of the precursor Gag-Pol polypeptide and alignment of three mature protease sequences, PR, PR20 and PR^{S17} are shown in Figure 1A and 1B, respectively. The positions of all mutations in PR^{S17} are shown in comparison with the extremely drug resistant clinical isolate PR20 (3UCB¹⁷) in Figure 1C. In a recent study, we ascribed the autoprocessing, catalytic efficiency and extreme drug resistance of PR20 partly to cooperative effects of mutations in three clusters comprising residues 30–37, 60–67 and 87–94 (red stripes in Fig. 1B).¹⁰ These clusters in PR20 only partially overlap regions containing mutations in PR^{S17}, such that 3 out of 6 residues corresponding to cluster 1, 2 out of 3 in cluster 2, and 1 out of 3 in cluster 3 are represented in PR^{S17}. Eleven of all mutations either in PR20 or in PR^{S17} correspond to regions of natural variation (sequence not highlighted in Fig. 1B, see Fig. S1 in reference¹⁶, and reference²⁸ for details). Major mutations associated with drug resistance (DRMs; Fig. 1B) are localized between regions that are highly conserved, essential for dimerization and enzyme function, and regions showing natural variation. Highly conserved

and regions of natural variation account for about 28% and 46% of the total length of the PR monomer, respectively.

Mutations in PR20 and another highly drug resistant mutant, PR22,¹¹ compared with PR^{S17} and their associated resistance to specific clinical PIs are listed in Table S1 (<http://hivdb.stanford.edu/DR/PIResiNote.html> and reference²⁹). Mutations in PR^{S17} associated with resistance to multiple drugs are M46L, G48V, I54V, V82S and L90M. With the exception of L90M these mutations are not present in PR20. In contrast, mutations D30N, V32I, L33F, I47V, I54L, and I84V are present in PR20 but not in PR^{S17}. Thus, despite the large number of mutations in both PR20 and PR^{S17}, similarity between mutations at specific sites in the two constructs is limited to 2 conservative substitutions shown in blue and 6 exact matches (in red). Within this group of 8 mutations, the conservative substitutions I54L (in PR20) and I54V (in PR^{S17}) have similar but not identical drug-resistance profiles. In addition L90M, which confers major resistance to multiple drugs, is common to both PR20 and PR^{S17}. Unlike mutations V82S, proximal to the active site pocket, and G48V and I54V in the flap regions, non-active site mutations L90M and A71V may indirectly influence inhibitor or substrate binding. The latter mutations were proposed to distort the binding cavity due to increased volume of the side chains buried in the core region beneath the active site.³⁰

Dimer dissociation constant (K_{dimer}) and kinetic parameters of mature PR^{S17}

In earlier studies, we observed no strict correlation between K_{dimer} and stability of the tertiary fold, as measured by T_m . We were unable to make this comparison for PR^{S17} because no distinct peak by differential scanning calorimetry denoting the transition from a folded to an unfolded state was observed even in the presence of inhibitor DRV which is expected to enhance the overall stability of the dimer. Mature PR^{S17} exhibits a strictly linear relationship between protein concentration and catalytic activity, as measured by the initial rate of cleavage of a chromogenic substrate, from ~10 to >200 nM dimer concentration (Fig. S1A) consistent with a very low dissociation constant (K_{dimer}) that is comparable to the wild type PR ($K_{\text{dimer}} < 10$ nM^{2,3,4}). This observation strongly suggests that most of the substitutions selected in PR^{S17}, which map to the periphery of the dimer, with the exception of a few residues (see Fig. 1C), exert no long range effect on the dimer interface contacts to alter K_{dimer} .

Mature PR^{S17} shows ~3-fold more favorable affinity ($K_m = 193 \pm 24$ μM , Fig. 1B) for the chromogenic substrate than PR20 ($K_m = 617 \pm 84$ μM).¹⁶ However, k_{cat} for PR^{S17} is about half the value estimated for PR20 [107 ± 6 (Fig. S1B) compared with 215 ± 19 min^{-1}].¹⁶ Because of compensation between k_{cat} and K_m , the overall catalytic efficiency of both proteases is comparable ($k_{\text{cat}}/K_m = 0.55 \pm 0.1$ and 0.35 ± 0.08 $\mu\text{M}^{-1}\text{min}^{-1}$ for PR^{S17} and PR20, respectively). Both these mutants, however, are ~10-fold less efficient catalytically than PR which exhibits a K_m and k_{cat} of 48 ± 3 μM and 173 ± 3 min^{-1} for the same substrate under identical conditions.³¹

Gag Processing mediated by mature PR^{S17}

PR mediated processing of the full length Gag polyprotein to release the structural proteins required for viral assembly occurs via a stepwise process with initial cleavage between SP1 and NC to give two fragments, MA-CA-SP1 and NC-SP2-P6¹. Thus, an assay utilizing Gag polyprotein substrate provides a realistic assessment of the catalytic efficiency of the proteases by monitoring the rate and order of multiple cleavages in a single reaction. We made use of a simplified recombinant Gag construct (termed Gag, Fig. 2) lacking the SP2 and P6 regions in which the SP1-NC cleavage (step 1) is followed by a cleavage between MA and CA (step 2) to give mature MA and CA-SP1 intermediate.^{10,27} The CA-SP1 is converted by a third, final cleavage (step 3) to CA and SP1. As indicated in Figure 2, this order of cleavage is essentially the same for processing by PR and the two drug resistant variants PR20 and PR^{S17}. SP1-NC cleavage monitored by the appearance of NC occurs most rapidly with PR. PR20 used for comparison with PR^{S17}, exhibits ~4-fold lower activity than PR consistent with a previous study,¹⁰ based on the initial SP1-NC cleavage. The corresponding cleavage catalyzed by PR^{S17} is ~10-fold slower than for PR. MA/CA cleavage catalyzed by PR20 to release CA (and CA-SP1) is essentially complete after 30–40 min (middle gel, lanes 5–6) whereas significant quantities of MA-CA are still clearly detectable with PR^{S17} even up to 3.5 h. The third cleavage (CA-SP1, step 3) is barely discernible as a doublet at 60 min only in the digest with PR^{S17} (bottom panel) under the conditions employed and thus no comparison is made with the other digests using PR and PR20. These observations suggest that catalytic efficiency for both SP1-NC and MA-CA cleavages is roughly 2-fold lower for PR^{S17} relative to PR20. The difference in the catalytic efficiency for hydrolysis of a chromogenic substrate (~1.5-fold higher for PR^{S17} than PR20) does not exactly match the rate estimate for Gag processing by these 2 enzymes, possibly due to differences in substrate recognition when using a synthetic substrate and a natural polyprotein substrate. In this context, some mutations in these two constructs could play a role in initiating transient encounter complexes between Gag and PR leading to substrate recognition and cleavage. Similarly, a 10–35 fold decrease in the cleavage efficiency of peptide substrates with PR20, relative to PR, is reflected by only ~4-fold decrease in the rate of Gag processing with no change in cleavage order.¹¹

Binding affinity of clinical inhibitors to mature PR^{S17}

Estimations of binding constants (K_{assoc}) of APV, IDV and DRV to PR^{S17} by isothermal titration calorimetry are shown in Figure S2. The dissociation constants ($1/K_{\text{assoc}}$) are equivalent to K_i for competitive inhibitors. Titration with SQV under the same experimental conditions did not give an interpretable sigmoidal isotherm because of a low c-value (product of PR concentration times K_{assoc}),³² due largely to its lower K_{assoc} of $\sim 10^5 \text{ M}^{-1}$ as compared to values ranging from 10^6 – 10^8 M^{-1} for the other three inhibitors. When satisfactory ITC data could not be obtained because of weak inhibitor binding or inadequate heat response, K_i values were determined kinetically (Fig. S3) from inhibition of hydrolysis of the chromogenic substrate in buffer B as described in Materials and Methods.

Experimental results for inhibition of PR^{S17} by PIs are summarized in Table 1 and Figure 3 and compared with previously published values for the wild type PR. As expected, there is no correlation between K_i values for inhibition of PR^{S17} and PR. The so-called first

generation inhibitors, IDV, NFV, SQV, and RTV, designed to fit sterically into the active site cavity of PR, are hydrophobic, and their affinity is largely determined by their shape and favorable binding entropy.³³ Newer, second-generation inhibitors such as DRV and LPV also take advantage of binding enthalpy due to specific interactions such as hydrogen bonds with the peptide backbone or residues in the binding cavity, and exhibit higher affinity to wild-type PR. The smallest difference (55-fold) in binding to PR^{S17} relative to PR is observed for APV, in part because it exhibits the tightest binding to PR^{S17} along with relatively weaker binding affinity (high pM) to PR. Although IDV, NFV and SQV show similar high pM binding to PR, these inhibitors exhibit a larger difference between PR and PR^{S17} because of their very poor binding (μ M) to PR^{S17}. Similarly ATV and LPV show K_i for PR in the mid-pM range, and 2000 to 4000-fold weaker inhibition of PR^{S17}. We note that PI resistance of PR^{S17} relative to PR (PR^{S17}/PR) increases with a similar trend to the K_i for binding of each inhibitor to PR^{S17} (see also Figure 3B and C), with the exceptions of DRV and RTV.

Comparison of PR^{S17} with PR20

PR20 and PR^{S17} have six mutations in common including L90M in cluster 3 associated with resistance to all PIs used in this study except TPV and DRV. For comparisons between the two proteases, we chose second-generation inhibitors APV and DRV, and the first-generation inhibitor SQV, which differ in their binding affinity to both wild type and drug resistant proteases. Notably DRV, which exhibits pM affinity to wild type PR, binds to both PR20 and PR^{S17} with 4 orders of magnitude poorer affinity (40–50 nM), approximately the same for both drug resistant proteases. Although PR20 bears major DRV resistance mutations V32I, I47V, I54L, there are no corresponding mutations in PR^{S17} to explain the similar K_i values shown by these 2 enzymes. In contrast, APV binds to PR^{S17} with 16-fold higher affinity (K_i ~11 nM) relative to PR20 (K_i ~178 nM³⁴). This difference may relate to major APV drug resistance mutations V32I, I47V, I84V present in PR20 but not in PR^{S17} (see Table S1). Similarly SQV binds to PR^{S17} with ~10-fold poorer affinity relative to PR20, possibly also reflecting effects of SQV resistance-associated mutations G48V and I54V in PR^{S17} which are not present in PR20.

PR20 cluster 1 encompasses three mutations contiguous to the active site, D30N, V32I and L33F, that are not present in PR^{S17}. Other mutations in this region, E35D and M36I in both mutants, and S37D in PR^{S17}, are located in the flap hinge loop and likely contribute to altered flap conformation and dynamics.¹⁷ Mutations I62V and L63P in cluster 2 are common to PR20 and PR^{S17}, and interestingly, also occur naturally in PR group O.³⁵ L63P has been identified in drug resistant PR mutants^{30,36} and is classified as a minor drug resistance mutation, possibly associated with resistance to LPV.²⁹ However a basis for relating the structural effects of these two mutations to drug resistance is lacking. Outside of the clusters, mutations L10F in PR20^{10,15–17} and possibly L10I in PR^{S17} could disrupt the ion pair between R8 and D29' of the opposite subunit,³⁷ resulting in altered conformation of the binding cavity. I54L (in PR20) and I54V (in PR^{S17}) are major mutations associated with high-level resistance to most PIs, with the possible exception of DRV (for I54V).

Comparison of PR^{S17} with PR22

Another clinically derived, extremely drug resistant PR, bearing 22 mutations (termed PR22 for consistency with the present numbering system) has been described.¹¹ Interestingly, comparing the location of mutations in PR20, PR^{S17} and PR22 indicates that clinical isolates PR22 and PR20 are more alike than PR^{S17} (Fig. S4). Mutations corresponding to clusters 1 and 3 of PR20 are evident in PR22 whereas cluster 2 and a partial cluster 1 (residues 35–37) in PR^{S17} are in common with PR20. Only five of the mutations in PR22 are identical with those in PR^{S17}, namely, E35D, M36I, S37D, I54V, and L90M. Of these, I54V and L90M are major drug resistance mutations. Conservative substitutions L10V and L10I in PR22 and PR^{S17} respectively, are classified as minor resistance mutations. Interestingly, in spite of the differences between the two PR sequences, a similar trend of high resistance (large K_i) to SQV, RTV, IDV and NFV in comparison with second-generation inhibitors LPV, ATV, APV and DRV was observed for both PR^{S17} (Table 1 in this study) and PR22.¹¹ Whether the extremely high resistance of PR^{S17} is indeed a consequence of an open-closed flap equilibrium shifted towards an open conformation as in PR20^{17,38} and MDR 769³⁹, relative to the mainly closed conformation of wild type PR,^{17,38} is currently under investigation. Furthermore, multiple accessory mutations (13 out of 22 in PR22) that are not classified as DRMs may contribute to the viability of highly evolved proteases through indirect effects on the structure and dynamics of the mature protease and its precursor.

Autoprocessing of PR^{S17} precursor and inhibition by clinical inhibitors

The protease catalyzes its own release at its N terminus from its precursor Gag-Pol, which precedes the C-terminal cleavage, termed autoprocessing (Figure 1A and 4).⁴⁰ The protease is flanked at the N terminus by the 56 amino acid transframe region (TFR) comprising transframe peptide (TFP) and 48 amino acid p6pol, both separated by a cleavage site. N-terminal autoprocessing is characterized by two pH-dependent cleavages at the TFP/p6pol and p6pol/PR sites occurring independently. At pH 4, the ionization of a group, either in the active site or the EDL sequence within TFP, with a pK_a of 3.8 makes the cleavage at the TFP/p6pol site proceed faster than the N-terminal p6pol/PR cleavage.^{22,41,42} At pH 6, the reverse is evident. However, cleavage at the N-terminal p6pol/PR site is crucial for stable dimer formation ($K_{dimer} < 10$ nM) and ensuing catalytic activity.^{22,42} Thus, at pH 4, there is a lag in the appearance of catalytic activity coinciding with the accumulation of the intermediate p6pol/PR. Subsequent cleavage at the p6pol/PR site is concomitant with mature-like catalytic activity.^{22,42} At pH 5, both cleavages occur simultaneously, p6pol/PR cleavage being slightly faster. A similar pH dependent stepwise N-terminal autoprocessing from a model precursor was also described for the protease from HIV-1 group N.²³

Here we have assessed the approximate IC_{50} values for inhibition of N-terminal autoprocessing by the same 8 PIs used to monitor inhibition of mature PR^{S17}. Partial accumulation of the TFR-PR^{S17} occurs when expressed in the presence of ~25 μ M DRV. Thus, the TFR-PR^{S17} precursor was purified from inclusion bodies similar to the protocol described for mature PR^{S17}. Inhibition of autoprocessing was evaluated by folding the precursor to a final concentration of 8 μ M to initiate autoprocessing in the presence of various PIs. This methodology provided an ideal window to monitor the disappearance of the full length precursor and appearance of products by SDS-PAGE and staining as compiled

and shown in Figure 4. Our kinetics and NMR analysis indicate that only a small fraction of precursor exhibits a folded structure, consistent with a transient process of folding/dimerization concomitant with N-terminal autoprocessing in the early phase of the reaction.^{22,42,43} Thus the effective concentration of folded precursor at the onset of the assay is expected to be at least one order of magnitude lower than 8 μ M.

TFR-PR^{S17} undergoes efficient autoprocessing similar to the wild-type precursor construct (Fig. 4, compare wild type control and 4A).^{22,42} A majority of precursor is converted to products PR^{S17}, p6pol and TFP within 10 min. Figure 4B and C show the initial screening of inhibition of TFR-PR^{S17} by 8 PIs at a concentration of 50 μ M, except for the maximum permitted solubility of ATV and NFV at 42 μ M under these conditions. After 60 min, APV, ATV and DRV showed almost complete inhibition of autoprocessing. IDV showed partial inhibition whereas LPV, NFV, SQV and RTV showed almost no inhibition. RTV at 105 μ M, IDV at 50 μ M and LPV at 70 μ M (limit of solubility) showed ~50% inhibition of the p6pol/PR site and insignificant inhibition of the TFP/p6pol site while SQV even at 105 μ M showed almost no inhibition. Next, dose- response experiments were carried out for a total reaction time of 60 min in the presence of 2–40 μ M APV, ATV and DRV (Fig. 4E to 4G) and the approximate IC₅₀ values are summarized in Fig. 5A and Table 1. APV, DRV and ATV elicit inhibition with an IC₅₀ in the range of 7.5 μ M for APV and 15 μ M for DRV and ATV as judged by monitoring the appearance of product bands PR^{S17} and p6pol. Interestingly, however, cleavage of the TFP/p6pol site is less susceptible to inhibition by APV and ATV (higher IC₅₀ value) evident by the accumulation of the p6pol/PR intermediate (purple arrows, Fig. 4E and 4F). The IC₅₀ for the inhibition of TFP/p6pol cleavage by APV and ATV is estimated to be 15 and >42 μ M, respectively. The exclusive lack of accumulation of the p6pol/PR intermediate suggests that DRV inhibits both these cleavage steps equally efficiently with an IC₅₀ of ~15 μ M (Fig. 4G). A plot of the differential inhibition of autoprocessing at the TFP/p6pol and p6pol/PR sites by APV, ATV and DRV is shown in Fig. 4H. Thus, cumulative inhibition of autoprocessing steps of PR^{S17} precursor is most effective by APV and DRV followed by ATV. However, such differentiation of inhibition by PIs is not feasible to monitor for the wild-type precursor because of lower IC₅₀ in the range of 1–2 μ M.¹⁶ LPV ranks fourth with an IC₅₀ of ~60 μ M and >70 μ M for blocking autoprocessing at the p6pol/PR^{S17} and TFP/p6pol sites, respectively.

Overall, observed IC₅₀ values for inhibition of autoprocessing by PIs are significantly larger than the corresponding K_i values for inhibition of substrate hydrolysis by the mature enzymes. The tightest binders (APV, DRV, ATV and LPV) exhibit differences of ~200 to 800-fold and 300 to 1400-fold for the inhibition of autoprocessing at the TFP/p6pol and p6pol/PR^{S17} sites, respectively, relative to mature PR^{S17} (Fig 5 and Table 1). The inhibition of autoprocessing relative to mature activity by the weaker binders, as exemplified by SQV, differs by only ~10 to 100- fold. This suggests that different classes of inhibitors differ in their relative affinity for PR^{S17} and for its precursor depending on the effects of mutations on differences in the active site environment of the mature PR relative to the corresponding precursor. With the exception of DRV and NFV, the rest of the PIs exhibit differential inhibition of the 2 autoprocessing sites, with weaker inhibition of the TFP-p6pol site. It is worth noting that even though DRV is 2-fold less efficient than APV for blocking cleavage at the p6pol/PR^{S17} site, it is unique in blocking both sites with equal efficiency. It is unclear

if this phenomenon relates to the second binding site of DRV,⁴⁴ by targeting the flap of the precursor monomer, because APV, a structural analogue of DRV shows kinetics of inhibition of mature PR similar to DRV consistent with a second binding site.⁴⁴

The initial phase of the N-terminal autoprocessing reaction was shown to be first-order in protein concentration indicating an intramolecular process.^{40,42} As the reaction progresses, when sufficient active mature protease accumulates, an intermolecular process, involving the released mature protease cleaving the precursor, that becomes competitive with the intramolecular process could prevail.⁴⁰ Notably, complete autoprocessing (Figure 4E and F) occurs even in the presence of 5 μ M APV and 10 μ M ATV, ~500- and 100-fold higher in concentration than the estimated K_i for the binding of APV and ATV, respectively, to mature PR^{S17}, strongly suggesting that N-terminal autoprocessing proceeds even when the action of the released mature protease is inhibited. The TFP/p6pol site, 48 amino acids upstream to the protease, is inhibited even more weakly, relative to the p6pol/PR^{S17} site. In this context, it is worth noting that *in vitro* inhibition of the SP1/NC site of the wild type Gag-Pol precursor which was shown to precede the N-terminal autoprocessing sites, TFP/p6pol and p6pol/PR sites, is inhibited by RTV ~10,000-fold weaker than the corresponding mature protease,^{1,45} and similarly, significantly higher concentrations of PIs exert inhibition of a chimeric wild type TFR-PR precursor autoprocessing in cell culture.⁴⁶ These observations are consistent with the much weaker inhibition (IC₅₀ of 1–2 μ M) of autoprocessing of the wild type TFR-PR precursor by DRV and SQV during expression in *E.coli*,¹⁶ drastic increases in IC₅₀ of 8 PIs with the drug resistant TFR-PR^{S17} precursor described in this study, and no inhibition as in extreme instances of drug resistance such as the PR20 precursor, up to 150 μ M DRV or SQV.¹⁶

CONCLUDING REMARKS

This study systematically validates the functionality of a rationally selected HIV-1 protease PR^{S17} predicted to have high level drug resistance. In spite of a different mutation profile compared to the extremely drug resistant clinical isolates with multiple mutations, PR20 and PR22, PR^{S17} exhibits high resistance to 8 of the potent clinical inhibitors tested thus highlighting the malleability of the protease. Resistance of PR^{S17} ranges from 1.5 to 5 orders of magnitude increase in K_i relative to PR. A long term focus of our studies has been to compare the potency of PIs on the N-terminal autoprocessing reaction, a process crucial for shifting the equilibrium from a largely unfolded population of the precursor to a stably folded dimer, essential for mature-like catalytic activity. The accumulation of the TFP-p6pol-PR^{S17} precursor in the presence of PIs during expression permitted its isolation for *in vitro* comparison of inhibition of the autoprocessing reaction with the corresponding mature enzyme. PR^{S17} precursor presented a fortuitous window between the wild type precursor and the PR20 precursor ideal for estimation of the IC₅₀ values for inhibition of autoprocessing with inhibitor concentrations maintained well above the precursor concentration to exclude active site titration. The weaker binding of PIs to the precursor points to an active site environment mediating autoprocessing that is different from the mature PR, as well as an unfavorable entropy for PIs to compete with an intramolecular process.

Structural studies by crystallography and NMR would help to reveal if extreme resistance of mature PR^{S17} correlates with an open flap conformation as noted for PR20 and MDR 769, contrary to the mainly closed conformation of the wild type PR.^{17,38,39} Design of inhibitors with high affinity to extremely drug-resistant mutants such as the three described in this study presents a significant challenge because of the continuously evolving mutations under drug pressure. A database created by comparing the relative binding affinity of a panel of PIs to mutants such as PR20¹⁶, P51¹⁶ and PR22¹¹ may aid in selecting the drug regimen for treatment as exemplified by this study. Future attempts in defining inhibitors that block the N-terminal autoprocessing with IC₅₀ values in the low nM range, at least 1-order of magnitude better than those described here, will benefit by exploring structures of the protease bound to drugs prior to its cleavage at its N-terminus such as the model precursor TFR-PR and analogues thereof. Such studies will be challenging, however, because precursor maturation occurs via transient dimer formation from a mainly unfolded population.^{43,47}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HIV-1	human immunodeficiency virus type 1
PR	pseudo wild-type mature HIV-1 protease containing the mutations Q7K, L33I, L63I, C67A, C95A
PI(s)	protease inhibitor(s)
APV	zidovudine
DRV	darunavir
ATV	atazanavir
LPV	lopinavir
IDV	indinavir
NFV	nelfinavir
RTV	ritonavir

SQV	saquinavir
TPV	tipranavir

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Figure 1.

Organization of the Gag-Pol polyprotein and description of the drug resistant mutants. (A) Domain organization of HIV-1 Gag-Pol polyprotein. Abbreviations are MA, matrix; CA, capsid; SP1, spacer peptide 1; NC, nucleocapsid; TFR, transframe region; PR, protease; RT, reverse transcriptase; RN, ribonuclease; and IN, integrase. Transient dimerization leading to self-cleavage of PR at its N terminus (TFR/PR site) is concomitant with stable dimer formation and appearance of mature-like catalytic activity. At pH 5 and below, an additional cleavage at the N-terminus of TFR (Phe8/Leu9) precedes the TFR-PR cleavage. Our studies utilize the model precursor, TFR-PR, which upon folding at pH 5 from a denatured state, undergoes autoprocessing to release the products, transframe peptide (TFP) and the p6pol-PR intermediate in a first step followed by conversion of p6pol-PR to p6pol and the mature PR. Other cleavages (black arrows) in the Gag and Gag-Pol mediated by the protease are also indicated. (B) Sequence alignment of PR with drug resistant mutants PR20 (red) and PR^{S17} (black). PR, used in this study, denotes the pseudo wild-type protease with mutations Q7K, L33I and L63I to restrict autoproteolysis and C67A and C95A to avoid cysteine-thiol oxidation. Identical residues are denoted by dots in the alignment and red arrows indicate sites of autoproteolysis in the mature PR. Positions of major drug-resistance mutations (DRMs) are marked with black asterisks. Regions corresponding to the three clustered mutations in PR20¹⁰ are identified by red stripes. Highly conserved and regions of natural variability in PR are shown with (yellow) and without a highlight, respectively. Regions between these two where DRMs frequently occur are highlighted in gray. (C) Ribbon representation (3UCB¹⁷) showing location of 19 (left) and 17 mutations (right) in PR20 and PR^{S17}, respectively. Red and blue residue positions in PR^{S17} denote identical and non-identical residues, respectively.

conservative substitutions, respectively, matching those in PR20. Yellow (Q7K) substitution is introduced in both constructs to restrict autoproteolysis (self-degradation).

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Figure 2.

Gag processing mediated by mature PR, PR20 and PR^{S17}. (Left) Schematic representation of the sequential cleavages of Gag with the order of cleavage (blue), cleavage site positions and calculated molecular weight of the products indicated. (Right) Gel panels showing the time course of Gag processing by mature protease monitored by SDS-PAGE on 20% PhastGels. Reaction mixtures contained 50 μ M Gag and 1 μ M PR, PR20, and PR^{S17} as indicated. Cleavage of full length 48.4 kDa Gag between SP1 and NC (step 1) yields MA-CA-SP1 + NC, followed by cleavage between MA and CA (step 2) to generate the mature MA and the intermediate CA-SP1. The spacer polypeptide SP1 is cleaved subsequently (step 3) from the C-terminus of CA-SP1. No full-length Gag remains after 5 min of reaction with PR, and almost no MA-CA-SP1 is detectable after 10–15 min. SP1, because of its small size, is predicted to have run out of the gel. A faint band between the MA-CA and CA-SP1 bands corresponds to CA-SP1-NC formed by a minor MA-CA cleavage pathway.

Figure 3. Plots comparing the K_i values for the inhibition of PR and PR^{S17} and the relative resistance (K_i for PR^{S17}/K_i for PR) for each clinical inhibitor. Numerical values are listed in Table 1.

Figure 4.

Inhibition of N-terminal autoprocessing of TFP-p6pol-PR^{S17} precursor by clinical PIs. The precursor mimetic spanning the 2 cleavage sites at the N-terminus of PR and its cleavage products during autoprocessing is shown schematically on top. In wild type control and A through D, lanes C and M denote the full length precursor and molecular weight standards (in kDa), respectively. Purple arrows indicate precursor proteins TFP-p6pol-PR^{S17} (band a) and p6pol-PR^{S17} (band b). (A) Time course of autoprocessing of TFP-p6pol-PR^{S17} (band a) in the absence of clinical PI. Two cleavages occur at pH 5, one between Phe8 and Leu9 to release the transframe peptide (TFP) and p6pol-PR^{S17} (band b) which is faster than the cleavage at the N terminus of PR^{S17} [product bands c (PR^{S17}) and d (p6pol)]. At 10 min, majority of the precursor is converted to the end products, PR and p6pol. (B to D) Inhibition of autoprocessing of TFP-p6pol-PR^{S17} by clinical PIs. Autoprocessing was initiated in the presence of the protease inhibitor and samples were subjected to SDS-PAGE after 60 minutes of incubation at room temperature. (E to G) Dose-response profiles for inhibition of autoprocessing at two cleavage sites (TFP/p6pol and p6pol/PR^{S17}) by APV, ATV and DRV. Approximate IC₅₀ values are shown graphically in panel H for these inhibitors and all IC₅₀ values are summarized in Fig. 5 and Table 1.

Figure 5.

Plots comparing the inhibition of precursor autoprocessing at p6pol-PR^{S17} and TFP-p6pol sites and with mature PR^{S17}. Plots of approximate IC₅₀ values for inhibition of autoprocessing of TFP-p6pol-PR^{S17} at the p6pol/PR^{S17} (A) and TFP/p6pol (C) sites by clinical inhibitors based on results shown in Figure 4. Numerical IC₅₀ values are listed in Table 1 and above the bars in (C). (B and D) Difference in inhibition (ratio) between IC₅₀ for inhibition of autoprocessing [p6pol/PR^{S17} site (B) and TFP/p6pol site (D)] and K_i for inhibition of PR^{S17}.

Table 1

Comparison of the inhibition of mature PR^{S17} and PR, and autoprocessing of TFR-PR^{S17} precursor at the p6pol/PR^{S17} site, with a panel of clinical inhibitors. The inhibitors are listed in the order of increasing K_i (decreasing affinity) for PR^{S17}. Estimated K_i values for wild-type PR from published sources are listed here solely for comparison. Similar values from different sources were averaged. In the present work K_i values for inhibition of mature PR^{S17} were determined by ITC or by kinetic analysis as indicated by superscripts ITC and K, respectively.

Protease inhibitor (PI)	Inhibition of PR ^{S17} K_i (nM)	Inhibition of PR K_i (nM)	Relative resistance (PR ^{S17} /PR)	Inhibition of autoprocessing at p6pol-PR ^{S17} site IC ₅₀ (μM)	Difference in inhibition [autoprocessing, IC ₅₀ /mature PR ^{S17} , K_i]
APV	11 ± 3, ¹ ITC	0.20 ^a	55	7.5	682
DRV	50 ± 8.3 ¹ ITC	0.005 ^b	10000	15	300
ATV	70 ± 14 ^K	0.035 ^c	2000	15	214
LPV	73 ± 20 ^K	0.02 ^{a,c}	3650	~60	822
IDV	810 ± 75 ¹ ITC	0.25 ^c	3240	~50	62
NFV	5870 ± 1730 ^K	0.36 ^{a,b,d}	16300	>42	>7.2
RTV	6360 ± 1310 ^K	0.064 ^{a,d}	99380	~105	16.5
SQV	8390 ± 2640 ^K	0.39 ^{a,b,d}	21500	>105	>12.5

^{a,b,c,d} K_i values are cited from references^{48–50,30}, respectively, for comparison with PR^{S17}. K_i values cited in reference⁵⁰ were determined by kinetics and the rest by ITC.