Kinetic Properties of Saquinavir-Resistant Mutants of Human Immunodeficiency Virus Type 1 Protease and Their Implications in Drug Resistance *in Vivo*†

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ABSTRACT: In order to study the basis of resistance of human immunodeficiency virus, type 1 (HIV-1), to HIV-1 protease inhibitor saquinavir, the catalytic and inhibition properties of the wild-type HIV-1 protease and three saquinavir resistant mutants, G48V, L90M, and G48V/L90M, were compared. The kinetic parameter k_{cat}/K_m was determined for these proteases using eight peptide substrates whose sequences were derived from the natural processing site sequences of HIV-1. The k_{cat}/K_m values were determined using conventional steady-state kinetics as well as initial velocities of mixed substrate cleavages under the condition where the substrate concentrations $[S]_o << K_m$. The independently determined k_{cat} and K_m values for some of the substrates confirmed the accuracy of the mixed-substrate method and also permitted the calculation in all cases of true rather than relative k_{cat}/K_m values. The K_i values were also determined. Using a previously described kinetic model [Tang, J., & Hartsuck, J. A. (1995) *FEBS Lett. 367,* 112−116], the relative processing activities of HIV-1 protease variants were estimated in the saquinavir concentration range of $0-10^{-7}$ M. Although the protease activity of G48V, L90M, and G48V/L90M are only about 10, 7, and 3% of that of the wild-type HIV-1 protease in the absence of inhibitor, the resistance tendencies of the three mutants are clearly manifest by relatively less activity loss as inhibitor concentration becomes higher. Also, the ratios of the activities of the four protease species at certain saquinavir concentrations appear to correlate with the population ratios of the four protease species at different time points of clinical trials. This correlation suggests that the population ratio of the protease species is driven by *in vivo* saquinavir concentration, which appears to be in the range $10^{-10}-10^{-9}$ M during the clinical trials.

The activity of the protease (PR) of human immunodeficiency virus type 1 (HIV-1) is required for the processing of *gag* and *gag-pol* polyprotein precursors into viral structural proteins and enzymes. This processing is part of the HIV maturation, which is essential for the viral life cycle and infectivity (Kohl *et al.*, 1988; Peng *et al.*, 1989). An extraordinary world-wide effort to develop HIV-1 PR inhibitors has resulted in many potent synthetic compounds (Wlodawer & Erickson, 1993; Winslow & Otto, 1995) and four marketed inhibitor drugs: saquinavir (Ro 31-8959; Craig *et al.*, 1991), indinavir (L-735,524; Dorsey *et al.*, 1994), ritonavir (ABT-538; Kempf *et al.*, 1995), and nelfinevir (Patick *et al.*, 1996). Others are expected to reach clinics shortly. The ability of HIV-1 PR inhibitors to suppress HIV replication has been demonstrated in tissue culture and in clinical trials (Wei *et al.*, 1995; Ho *et al.*, 1995). The use of HIV-1 PR inhibitors along with other drugs in combination therapy has offered the best results so far in suppressing HIV propagation *in vivo* (Mellors, 1996).

A serious problem in the clinical use of PR inhibitor drugs is the development of drug resistance by HIV (Wei *et al.*, 1995; Ho *et al.*, 1995; Jacobsen *et al.*, 1996; Condra *et al.*, 1996; Molla *et al.*, 1996). The basis of HIV resistance is the mutation of its PR gene and the selection of PR mutants resistant to the inhibitors. This selection process can be simulated in *in vitro* experiments [for reviews, see Mellors *et al.* (1994) and Ridky and Leis (1995)]. Resistance in patients occurs within weeks owing to the fast replication of virus and fast turnover of the CD4+ T-cells (Coffin, 1995). *In vitro* and *in vivo* selection of HIV-1 in the presence of inhibitors produces, among many mutations, some prevailing PR mutants which have the characteristics of resistant mutations, *i.e.*, lower susceptibility to inhibitors in HIV-1 grown in cultured cells and increased IC_{50} or inhibition constants. Mutants selected with one inhibitor may cross-resist to other inhibitors (Gulnik *et al.*, 1995; Rose *et al.*, 1996; Condra *et al.*, 1996; Molla *et al.*, 1996). It is also interesting that *in vivo* inhibitor therapy produced a time-dependent increase of mutation sites per PR molecule. The mutant enzymes with more mutation sites are usually less sensitive to inhibitors (Condra *et al.*, 1995, 1996; Jacobsen *et al.*, 1996; Molla *et al.*, 1996).

Resistant mutants of HIV-1 PR should possess sufficient proteolytic activity (k_{cat}/K_m) to support HIV-1 life cycle but have reduced sensitivity (increased K_i) to inhibitors. These properties can be defined by kinetic parameters (Kaplan *et al.*, 1994; Ho *et al.*, 1994; Lin *et al.*, 1995; Gulnik *et al.*, 1995; Maschera *et al.*, 1996). Clinical trials of HIV-1 PR inhibitor drugs have now identified resistant mutation sites on the PR (Jacobsen *et al.*, 1996; Molla *et al.*, 1996; Condra *et al.*, 1996). Kinetic studies of these resistant mutant PR's

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provide insights into the *in vivo* development of resistance to HIV-1 PR inhibitors and understanding of the molecular basis of resistance. Saquinavir resistance *in vivo* is a particularly good model for such study since it gives rise to only three resistant mutants, G48V, L90M, and G48V/L90M (Jacobsen *et al.*, 1995), and the genotypes and phenotypes of resistant mutations in the course of saquinavir treatments are well documented (Jacobsen *et al.*, 1996).

A problem in the kinetic studies of HIV-1 PR resistant mutants is the choice of substrates which adequately represent HIV-1 intravirion processing. The use of chromogenic substrates (Kaplan *et al.*, 1994; Ho *et al.*, 1994; Lin *et al.*, 1995; Gulnik *et al.*, 1995; Wilson *et al.*, 1997) is convenient in assay procedures. But these peptide sequences are different from those of the native cleavage sites in the HIV-1 *gag-pol* polyprotein. Whether the kinetic data from the chromogenic substrates are representative of intravirion processing remains to be shown. The hydrolysis of peptide substrates derived from the HIV processing-site sequences seem to have the best chance of simulating the native processing event. However, many of these peptides have poor solubility leading to considerable difficulty in kinetic determinations (Ermolieff *et al.*, 1997; Maschera *et al.*, 1996).

In this paper, we report the kinetic constants of HIV-1 wild-type and three saquinavir-resistant mutant PR's using peptide substrates derived from eight natural HIV-1 processing sites. A mixed substrate assay method was used to overcome the low-solubility problem. Kinetic data from three *gag* cleavage site substrates correlate well with the pattern of *in vivo* resistance. Comparative data for a chromogenic substrate show it to be a poor choice for interpreting HIV-1 resistance.

EXPERIMENTAL PROCEDURES

Materials. Recombinant HIV-1 protease was prepared from *Escherichia coli* inclusion bodies and purified according to previously published procedures (Ido *et al.*, 1991; Lin *et al.*, 1994). Synthetic peptide substrates A, B, C, D, E, F, G, and H (see Table 1 for sequences) and the chromogenic substrate Lys-Ala-Arg-Val-Leu-Nph-Glu-Ala-Met (Nph = *p*-nitrophenylalanine) were synthesized at the Molecular Biology Resource Center, University of Oklahoma Health Sciences Center, using an Applied Biosystems peptide synthesizer 430A. Saquinavir and Ritonavir in commercially purchased capsules was extracted with dimethyl sulfoxide and purified on HPLC using a C-18 reversed-phase column. Indinavir was a gift from Merck. All other reagents were the highest grade available commercially and were used without further purification.

Determination of Kinetic Constants k_{cat}, K_m, and Relative k_{cat}/K_m. Peptide substrates were individually incubated with HIV-1 PR in 0.1 M sodium acetate with 0.1 M NaCl and 1% dimethyl sulfoxide, pH 5.0, at 37 °C. The reactions were stopped by the addition of guanidine-HCl stock solution to final concentration of 7 M. The peptide and hydrolytic products were quantified in a Beckman System Gold HPLC using a C-18 reversed-phase column, which was eluted with a gradient of acetonitrile in 0.06% trifluoroacetic acid. The substrate and product peaks were integrated using the software provided by the HPLC manufacturer. The initial velocities were fitted to the Michaelis−Menten equation using Enzfitter program (Leatherbarrow, 1987) to obtain k_{cat} and K_m values. The amount of PR was determined by active-site titration using saquinavir for each batch of PR. This result was used to calculate the specific activity of the PR, with the chromogenic substrate, which was used to determine active PR concentration for each kinetic experiment. The relative k_{cat}/K_m values were determined under the condition $[S]_o << K_m$ using a mixture of substrates. When the K_m was uncertain, the relative k_{cat}/K_m was determined for at least 2 [S] which agreed. The experimental conditions and the quantitation of hydrolysis were as described above. The relative k_{cat}/K_m is the ratio of the pseudo-first-order rates of cleavage of any two competing substrates (Fersht, 1985). In practice, the individual k_{cat} and K_m parameters were determined for nine substrate-PR pairs (Table 1, boldface) from which k_{cat}/K_m was calculated. Each PR species had at least one substrate whose k_{cat}/K_m was determined from individual constants. Other low-solubility substrates were paired with these substrates in the mixed-substrate assays to determine relative k_{cat}/K_m values and thus the actual k_{cat}/K_m values. The kinetic parameters were also obtained for the chromogenic substrate as previously described (Lin *et al.*, 1995).

Determination of Inhibition Constant, K_i. The inhibition constant K_i, was determined as described by Bieth (1974). PR activity was determined, using chromogenic substrate, $[S]_o$, for a series of mixtures with constant PR, $[E]_o$, but increasing inhibitor concentration, $[I]_o$. The apparent inhibition constant, K_{iapp}, was determined from the plot of activity *vs* $[I]_o$ based on the equation

$$a = 1 - \frac{[I]_o + [E]_o + K_{iapp} - \sqrt{[([I]_o + [E]_o + K_{iapp})^2 - 4[I]_o[E]_o]}}{2[E]_o}$$

Inhibition constant, K_i, was determined from a series of K_{iapp} obtained from different substrate concentrations, ranging from 0.05 to 0.3 mM, where substrate concentration was extrapolated to zero, based on the equation $K_{iapp} = K_i (1 + [S]_o/K_m)$.

Preparation of HIV-1 PR Mutants. The mutants of HIV-1 PR were made in the designed HIV-1 gene (Ido *et al.*, 1991) using the PCR-based mutagenesis method as previously described (Lin *et al.*, 1995). The primer used for G48 mutants was 5′-GGAAGCCGAAAATGATCNNNGGCAT-CGGCGGTTTTATC-3′ (N = ATCG). The G48V mutant was selected from the sequenced individual mutants having changes at this position. The primer used for L90M was 5′-GCAACCGATCTGAGTCATCAGGTTACGGCCG-3′. Other primers were those described in Lin *et al.* (1995).

Calculation of Mutation Modulated Activity, MMA, of HIV-1 PR Mutants. The MMA values were calculated from the previously described kinetic model (Tang & Hartsuck, 1995), using the equation

$$\alpha = \sigma\{(k_{cat}/K_m)/[1 + ([I]_o/K_i)]\}$$

where α is the processing activity of an HIV-1 PR, σ is a constant, $[I]_o$ is inhibitor concentration, and other symbols are the conventional kinetic constants. MMA = (α of a mutant HIV-1 PR at any $[I]_o$/α of wild-type HIV-1 PR for $[I]_o = 0$) × 100.

RESULTS AND DISCUSSION

Kinetic Parameters of HIV-1 PR, Wild-Type, and Resistant Mutants G48V, L90M, and G48V/L90M. Individual k_{cat} and

Table 1: Catalytic Constants, k_{cat}/K_m, of HIV-1 PR, Wild-Type and Three Mutants Measured from Different Substrates and the Inhibition Constants, K_i, against Saquinavir

				G48V		L90M		G48V/L90M	
substrate	sequence[a]	processing site[b]	wild-type, k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	% of wild type	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	% of wild type	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	% of wild type
A	VSQNY/PIVQ	MA/CA	**34.5 ± 12.5**[c] (42 ± 6.8)	**3.20 ± 1.2**[h]	9.3	**2.72 ± 1.23**[i]	7.9	**0.58 ± 0.16**[k]	1.7
B	HKARVL/AEAMS	CA/P2	24.7 ± 6.5	4.4 ± 1.0	17.8	0.93 ± 0.23	3.8	0.36 ± 0.02	1.5
C	TATIM/MQRGN	P2/NC	**117.6 ± 38.8**[d] (123.5 ± 24.7)	11.4 ± 1.5	9.2	6.3 ± 0.42	5.1	2.36 ± 0.16	1.9
D	KLRPGNF/LQSRP	NC/P6	**1.98 ± 1.4**[e] (2.47 ± 0.6)	0.047 ± 0.024	2.4	0.68 ± 0.34	34.3	0.87 ± 0.24	43.9
E	RQGTVSFNF/PQITL	P6/PR	**116.1 ± 58.1**[f] (116.1 ± 22)	11.9 ± 1.1	10.2	8.5 ± 2.0	7.3	4.46 ± 0.4	3.8
F	CTLNF/PISPgr	PR/RT	7.41 ± 1.7	0.0003 ± 0.0001	0.004	0.88 ± 0.11	11.9	0.02 ± 0.004	0.3
G	GAETF/YVDGAA	RT	**51 ± 14**[g] (42 ± 9.2)	0.95 ± 0.1	1.9	**2.27 ± 1.3**[j] (2.89 ± 0.86)	4.5	0.27 ± 0.03	0.5
H	IRKIL/FLDG	RT/IN	8.64 ± 1.9	0.36 ± 0.004	4.2	0.94 ± 0.09	10.9	0.22 ± 0.003	2.5
chromogenic	KARVL/XEAM[l]		480 ± 101	600 ± 300	125	391 ± 151	81.5	*m*	
			Inhibition by Saquinavir						
			K_i (nM)	K_i (nM)	multiple of wild-type	K_i (nM)	multiple of wild-type	K_i (nM)	multiple of wild-type
			0.2 ± 0.06	2.7 ± 0.2	13.5	0.6 ± 0.04	3	83.7 ± 21.2	419

[a] Sequences are in single-letter amino acid codes. Lower-case letters represent non-native residues. / represents cleavage position. [b] Abbreviations: MA, p17; CA, P24; P2, sequence between p24 and p7; NC, p7; PR, protease; RT, reverse transcriptase; IN, integrase (see Figure 1). [c] The k_{cat} and K_m values are 51.8 ± 4.9 s^{-1} and 1.5 ± 0.4, mM, respectively. [d] The k_{cat} and K_m values are 58.8 ± 7.6 s^{-1} and 0.5 ± 0.1 mM. [e] The k_{cat} and K_m values are 12.7 ± 4.2 s^{-1} and 6.4 ± 2.6 mM. [f] The k_{cat} and K_m values are 62.7 ± 14 s^{-1} and 0.54 ± 0.15 mM. [g] The k_{cat} and K_m values are 5.05 ± 0.4 s^{-1} and 0.1 ± 0.02 mM. [h] The k_{cat} and K_m values are 31.3 ± 5.2 s^{-1} and 9.8 ± 2.7 mM. [i] The k_{cat} and K_m values are 3.14 ± 0.46 s^{-1} and 1.15 ± 0.35 mM. [j] The k_{cat} and K_m values are 0.5 ± 0.07 s^{-1} and 0.22 ± 0.07 mM. [k] The k_{cat} and K_m values are 2.2 ± 0.2 s^{-1} and 3.8 ± 0.7 mM. Data in boldface are calculated from individually determined k_{cat} and K_m values. Other data are determined from relative k_{cat}/K_m from mixed-substrate assays. [l] X = *p*-nitrophenylalanine. [m] Activity too low for determination. The standard errors are calculated from average $n = 3$ (range 2−5) for relative k_{cat}/K_m and average $n = 6$ for individual kinetic constants.

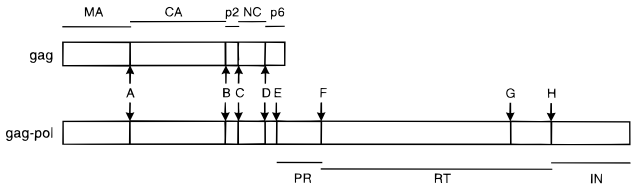

FIGURE 1: The relative positions of processing sites in HIV-1 *gag* and *gag-pol* polyprotein precursors. The arrows indicate the relative position of the natural cleavage sites by HIV-1 PR. The horizontal bars represent the final protein products. Abbreviations: MA, matrix protein or p17; CA, capsid protein or p24; NC, nuclear capsid protein or p7; RT, reverse transcriptase; IN, integrase.

K_m values (boldface, Table 1) were determined for HIV-1 PR's using synthetic peptides derived from the sequences flanking the cleavage sites in HIV-1 *gag* and *pol*. However, many peptides were poorly soluble and the kinetic parameters either could not be accurately determined using conventional methods or gave rise to data with large deviations (Maschera *et al.*, 1996; Ermolieff *et al.*, 1997). This problem is even more serious for PR mutants of reduced catalytic activity. The relative k_{cat}/K_m values of the wild-type and three saquinavir resistant mutants of HIV-1 PR were experimentally determined using the mixed substrate method (Table 1). The six sets of k_{cat}/K_m values determined by both methods show good agreement. The kinetic parameters from the chromogenic substrate were also determined for the wild-type and the two single mutants (Table 1). The activity of the double mutant was too low to be accurately determined with this substrate. The K_i values for saquinavir are also shown in Table 1. The mutant PR's have significantly increased K_i values compared to those of the wild-type PR: 3-fold for L90M, 13.5-fold for G48V, and 419-fold for G48V/L90M. Our K_i values for wild-type and three mutant HIV-1 PR's agree only moderately well with those reported by Maschera *et al.* (1996). It is our experience that the determination of apparent K_i at different inhibitor concentrations with extrapolation of the K_i value at zero substrate concentration is important for accurate values.

One of the main purposes of these kinetic experiments is to determine which substrates best represent HIV-1 intravirion processing. On the basis of the interior structure of immature HIV-1 virion, we had previously argued that the autoexcision of the HIV-1 dimer from the *gag-pol* polyprotein and the sequential cleavage of the sites in the tightly packed *gag* shell should be among the rate-limiting events (Tang & Hartsuck, 1995). This argument predicts that five sites, A, B, C, D, and E (Figure 1), are most representative of intravirion processing. This point will be further discussed below. We have examined the relationships among the k_{cat}/K_m values of the mutants and wild-type HIV-1 PR's determined using the eight natural-site substrates in order to identify substrates which respond consistently to these mutations. The comparison documented in Figure 2, which shows the k_{cat}/K_m of mutants as percent of that of the wild-type PR for each substrate, indicates that substrates A, C, and E respond most similarly to the mutations. Since these sites produced most consistent kinetic data with these mutations, we further examine the data from these three substrates in the next section. The catalytic activities of mutants and wild-type enzyme assayed by the chromogenic substrate did not agree with the data obtained from the peptide substrates (Table 1).

Processing Activity of Wild-Type and Mutant HIV-1 PR at Different Saquinavir Concentrations. Using a previously

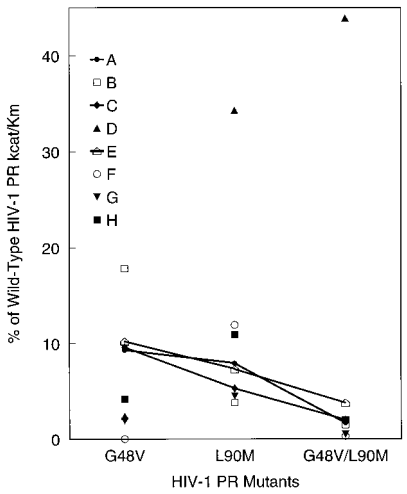

FIGURE 2: Relative k_{cat}/K_m values of HIV-1 PR mutants G48V, L90M and G48V/L90M as percentage of the wild-type enzyme. The data are the mean values from Table 1. Values were determined for peptide substrates A−H derived from 8 natural cleavage sites as shown in Figure 1.

proposed kinetic model (Tang & Hartsuck, 1995), the processing activities of the wild-type and mutant HIV-1 PR's inside HIV-1 virions were estimated as the mutation modulated activity, MMA, from their k_{cat}/K_m and K_i values. MMA is the activity of the mutant PR at a given inhibitor concentration expressed as the percentage of the activity of the wild-type HIV-1 PR in the absence of inhibitor. Figure 3 shows the plots of MMA values against saquinavir concentrations for substrates A, C, E, and the chromogenic substrate. The relationship of the activities of the wild-type and three mutant PR's are very similar when substrates A, C, or E were used (Figure 3). Examining the results from substrate A (Figure 3A), the wild-type HIV-1 PR activity declines rapidly from saquinavir concentrations 10^{-10} M and nearly disappears above inhibitor concentration of 10^{-8} M. In the absence of saquinavir, the mutants G48V, L90M, and G48V/L90M have about 9, 8, and 2% of the activity of the wild-type PR, respectively. However, the resistance tendency of the mutant enzymes can be clearly seen in their resistance to activity loss as inhibitor concentration is increased (Figure 3). The activities of the single mutants decline significantly only at and above saquinavir concentration of 10^{-9} M, while the activity of the double mutant persists to inhibitor concentration above 10^{-7} M. The MMA values estimated using the k_{cat}/K_m data of the chromogenic substrate produced MMA-inhibitor concentration relationships (Figure 3, Chr) very different from those of peptides A, C, and E. With chromogenic substrate, both single-site mutants appear to have about the same or higher activities at all inhibitor concentrations as compared to the wild-type PR. From the correlation of activities in PR mutant populations discussed below, the chromogenic substrate most likely overestimates the activities of the single mutants.

The inhibition constants were also determined for indinavir and ritonavir in order to examine cross resistance. For indinavir, the K_i values and standard errors are the following: wild-type, 0.1 ± 0.02 nM; G48V, 0.26 ± 0.02 nM; L90M, 0.48 ± 0.06 nM; and G48V/L90M, 0.83 ± 0.41 nM. For ritonavir, the K_i values and standard errors are the following: wild-type, 0.2 ± 0.1 nM; G48V, 0.10 ± 0.03 nM; L90M, 0.51 ± 0.06 nM; and G48V/L90M, 1.57 ± 0.55 nM. A plot of MMA values (from substrate A) against inhibitors concentrations (Figure 4) confirms the previous

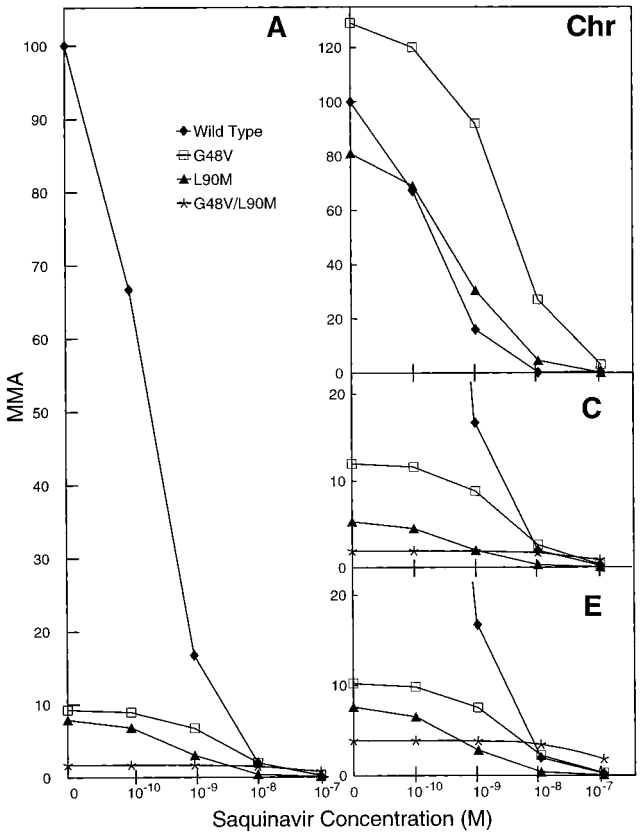

FIGURE 3: Simulated intravirion proteolytic activity, expressed as MMA values, at different saquinavir concentrations for the wild-type HIV-1 PR and saquinavir-resistant mutants G48V, L90M, and G48V/L90M. The MMA values are calculated from k_{cat}/K_m values of substrates A (left panel), C (right center panel), E (right lower panel), and chromogenic substrate (right upper panel) and K_i values using a previously described kinetic model (Tang & Hartsuck, 1995). MMA is the inhibited, mutant enzyme activity expressed as a percent of the wild-type PR activity in the absence of inhibitor.

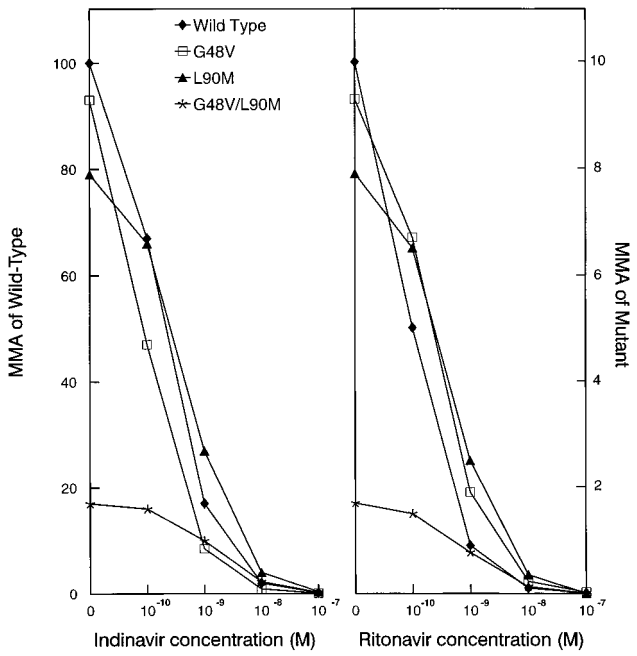

FIGURE 4: Proteolytic processing activity, MMA, of the wild-type and saquinavir-resistant mutants at different indinavir (left panel) and ritonavir (right panel) concentrations. The k_{cat}/K_m data used in MMA calculations are from peptide A.

conclusion (Jacobsen *et al.*, 1995) that the PR mutants are much less resistant to indinavir and ritonavir than to saquinavir (Figure 3).

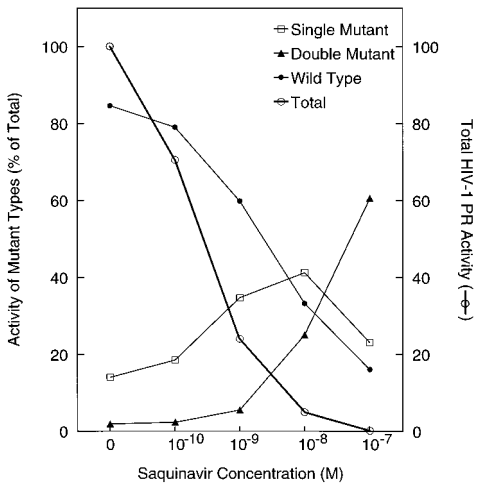

FIGURE 5: Individual MMA (light lines) as percent of total MMA (heavy line) at different saquinavir concentrations. HIV-1 PR's are considered as three groups: wild-type, single mutant, and double mutant. Total MMA is the sum of the MMA of three groups which is taken as 100% at $[I]_o = 0$.

Correlation of Processing Activity Ratios of Wild-Type and Mutant HIV-1 PR's to the Population Ratios of HIV-1 PR Genotypes during Saquinavir Treatment in Vivo. Figure 5 shows the percentage of proteolytic activity (MMA) of 3 HIV-1 PR species, wild-type, single mutant (G48V or L90M), and double mutant (G48V/L90M), in saquinavir concentration range $0-10^{-7}$ M. The wild-type HIV-1 PR accounts for 84% of the total MMA at 0 inhibitor concentration and decreases to 16% at 10^{-7} M saquinavir. The single mutants have a broad maximum of about 40% between 10^{-9} M and 10^{-8} M inhibitor. The double mutant percentage is small, between 0 and 10^{-9} M inhibitor, but rises sharply to 60% at 10^{-7} M saquinavir. A correlation exists between these activity percentages as a function of saquinavir concentration and the population percentage of the mutant species in clinical trials of saquinavir. Table 2 lists the latter (Jacobsen *et al.*, 1996) divided into three groups of similar population distributions. The first group consists mostly of the zero-time data where the HIV-1 genotype observed was virtually 100% wild-type. In the absence of inhibitor, the wild type HIV-1 PR, single mutant, and double mutant account for 84, 15, and 1% of total activity, respectively. These percentages correlate only moderately well with the HIV species at zero-time (first group in Table 2). The second and third groups of clinical HIV-1 population percentages are most consistent when G48V and L90M percentages are combined as a single mutant percentage. In these two groups, the average population percentages of wild-type, single mutants, and double mutant are about 84, 17, and 0% and 63, 30, and 6%, respectively. The corresponding values for MMA are 83, 16, and 1% and 62, 32, and 6%, respectively. These activity percentages are derived from saquinavir concentrations of 0.6×10^{-10} M (group 2) and 0.8×10^{-9} M (group 3). When the MMA value from substrates C and E were used, the correlation to the *in vivo* population ratio still shows a trend of similarity, but the actual values do not match as well as those from substrate A.

The remarkable correlation of the ratios of the second and third groups of Table 2 can be explained by the hypothesis that under the selection pressure of PR inhibitor, the population ratio of HIV-1 species is determined by their respective processing activity, which is represented by the estimated MMA values. Since the processing activities of

Table 2: Correlation between Percentage of Individual HIV-1 PR Species during Saquinavir Clinical Trails[a] and Percentage of Processing Activity (MMA) of the Corresponding HIV-1 PR Species (boldface)

	wild-type (%)	G48V (%)	L90M (%)	single mutant (%)	G48V/L90M (%)
week 0, trials V13329, V13330 (m)[b], V13330 (c)	100	0	0	0	0
trial ACTG229	98	0	2	2	0
% total MMA,[c] [I] = 0	**84**	**8**	**7**	**15**	**1**
week 46, trial V13330 (m)	82	4	15	19	0
week 43, trial V13330 (c)	84	2	14	16	0
week 45, trial ACTG229	85	0	15	15	0
% total MMA, [I] = 0.6×10^{-10} M	**83**	**9**	**7**	**16**	**1**
week 23, trial V13329	65	10	20	30	5
week 52, trial V13329	62	0	30	30	8
week 12, trial V13330 (m)	63	23	9	31	6
% total MMA, [I] = 0.8×10^{-9} M	**62**	**23**	**9**	**32**	**6**

[a] Data from Jacobsen *et al.* (1996). [b] m, monosaquinavir therapy; c, combination therapy. [c] % of total MMA = (individual MMA/sum of MMA from all PR's) × 100. MMA values were calculated from data using substrate A in Table 1.

all HIV-1 virions decline significantly in the presence of an inhibitor, the intravirion processing can be expected to be the rate-limiting step in the life cycle of HIV-1. We have observed in tissue culture experiments that the propagation of HIV-1 PR mutants is correlated to the MMA values of these mutants, indicating that the PR activity in the wild-type HIV-1 is close to limiting the rate of replication (unpublished results). In the presence of inhibitor, protease processing should decisively be the rate-limiting step of the processing. The reason that no single mutant genotype was observed at zero-time (Table 2) is possibly because the protease processing is not the rate-limiting step in the uninhibited HIV life cycle. In spite of this complication, these comparisons suggest the following points. (1) The population ratios of HIV-1 PR species studied here are driven by *in vivo* saquinavir concentration. The change of mutant ratios in trial V13330(mono) at 12 and 46 weeks (Jacobsen *et al.*, 1996) may result from the fall of *in vivo* saquinavir concentration (based on the data in Figure 5, from about 10^{-9}−10^{-10} M). This possibility suggests the importance of studying *in vivo* inhibitor concentrations in clinical trials in order to closely examine the validity of this point. The change of saquinavir metabolism in patients is a poorly documented area, and its relationship to current discussion may also require further studies. (2) Populations of single mutants G48V and L90M seem to be interchangeable, and the determining factor appears to be the sum of single mutants. This suggestion is inferred from both the current study and the data by Jacobsen *et al.* (1996). The reason the single mutants appear to function equivalently is obscure from the view point of enzyme kinetics and may lie with the propagation mechanism of the mutants. (3) Wild-type HIV-1 PR and the two single mutant species contribute significantly to resistance in the range of *in vivo* saquinavir concentration less than 10^{-9} M where total and individual HIV-1 PR activities are both significant (Figure 5). However, the direct contribution of double mutant G48V/L90M to HIV-1 pathogenesis seems much less significant in patients developing resistance against saquinavir. As shown in Figure 5, the activity percent of the double mutant is significant only above 10^{-8} M saquinavir where the total activity, and presumably the total HIV population, is less than 5% of the original. Figure 5 also predicts that when a higher *in vivo* saquinavir concentration is achieved, the double mutant may become prevalent. We recognize that correlating clinical data with a kinetic model risks oversimplification. The current discussion nevertheless provides new hypotheses for future clinical studies.

Substrates Representative of Intravirion Processing and Methods for Determining Kinetic Parameters. On the basis of the relative activities of wild-type and mutant HIV-1 PR's, the best agreement of the results were produced from substrates A, C, and E (Table 1 and Figure 3). There are other reasons why these three sites are among the most important for the rate of HIV-1 virion maturation. The intravirion processing is thought to be initiated by the autocleavage of *gag-pol* precursor at site E (Figure 1), at the N-terminus of the PR (Tang & Hartsuck, 1995). The supporting evidence is the *in vitro* self-processing of HIV-1 PR model precursors (Co *et al.*, 1994; Louis *et al.*, 1994). The initial cleavage at site E results in a PR attached to *pol* enzymes but free to hydrolyze *gag* sites (Figure 1). We have argued that from structural considerations, the *gag* sites are hydrolyzed sequentially from C-terminus to N-terminus (Tang & Hartsuck, 1995). The relatively low k_{cat}/K_m value for site F suggests that the intermediate PR-RT-IN can adequately catalyze the processing of *gag* sites. The ability of C-terminal linked HIV-1 PR dimer to hydrolyze other substrates has been clearly demonstrated (Wondrak *et al.*, 1996). These observations suggest that the sites which control the speed of the processing maturation are A, B, C, and E. These cleavage steps are important because they are involved in the release of PR from the *gag-pol* polyprotein and release of structural proteins from the *gag* polyprotein, which is present in 20-fold excess to the *pol* polyprotein. Teleologically, the low k_{cat}/K_m value of site D probably occurs because its hydrolysis is not a prerequisite for the hydrolysis of sites A, B, and C. Site D is located between two small units, p7 and p6, on the surface of the *gag* shell structure (Nermut *et al.*, 1994). From the icosahedral packing of the rod-shaped *gag* polyproteins, site C may be directly accessible to HIV-1 PR. It should be noted also that once site C is cleaved, the hydrolysis rate for site D is increased due to the free diffusion of the substrate (Tang & Hartsuck, 1995). The above discussion on the representative sites is consistent with the agreement of activity-inhibition profiles in Figure 3. The exception is the substrate from site B which expresses much better activity for G48V and much worse activity for L90M than the corresponding data produced by peptides A, C, and E (Table 1). However, if the single mutants are considered equivalent as suggested above, then the consequence in mutant population and their contribution to resistance is about the same as concluded from other three substrates. Current results suggest that peptides derived from sites A, C, and E are most appropriate for kinetic simulation of intravirion activities.

The kinetic parameters for the hydrolysis of peptides derived from the natural cleavage sites have been reported by several groups of investigators. Some of these report the values of maximum velocity/K_m (Darke *et al.*, 1988; Krausslich *et al.*, 1989; Moore *et al.*, 1989; Tomasselli *et al.*, 1990), which cannot be directly compared to the current k_{cat}/K_m data. Of the reported k_{cat}/K_m values for the wild-type HIV-1 PR from four laboratories (Tözsér *et al.*, 1991; Maschera *et al.*, 1996; Schock *et al.*, 1996; this work), there is a general trend of relative values for the substrates, but considerable discrepancies are present. These disagreements do not appear to come from the different length of the peptides derived from the cleavage sites. Four processing site sequences used by Schock *et al.* (1996) are different from those used in other studies. This may contribute to some of the disagreement. However, most of the discrepancies cannot be traced to the substrates and are likely rooted in the methods of data collection. With low solubility of some of the substrates, the accurate determination of individual k_{cat} and K_m values is difficult even for the wild-type HIV-1 PR and not possible for mutant HIV-1 PR's with low activities (Maschera *et al.*, 1996; Ermolieff *et al.*, 1997). The use of initial velocity under the condition of $[S]_o << K_m$ to estimate k_{cat}/K_m values (Schock *et al.*, 1996) may also be subject to large deviations among substrates because of the low hydrolysis rates and differences in experimental conditions. We have found the combination of determinations of individual constants by conventional steady-state kinetics and the relative k_{cat}/K_m by the competitive hydrolysis method using mixed substrates to be very reliable. While the former is used to estimate the wild-type HIV-1 PR with substrates of adequate solubility, the latter is used to calculate the actual k_{cat}/K_m from the relative values.

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