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Kinetics Analysis of Consecutive HIV Proteolytic Cleavages of the Gag-Pol Polyprotein*

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The ordered, sequential cleavages of the Gag-Pol polyprotein by human immunodeficiency virus (HIV) protease present the virus with severe limitations on viable mutations of the enzyme. An extension of the method of Kuchel *et al.* (Kuchel, P. W., Nichol, L. W., and Jeffrey, P. D. (1974) *J. Theor. Biol.* 48, 39–49) for the analysis of consecutive enzyme reactions leads to a simple description of the catalytic efficiency of mutant and wild type HIV protease in the presence or absence of inhibitors. The overall catalytic efficiency of a mutant HIV protease relative to the wild type enzyme is given by the product of the ratios of their respective efficiencies for the 8 obligatory cleavages. Under no conditions is HIV viable when the geometric mean efficiency of a mutant HIV protease is less than 61% of the wild type activity for each cleavage. The lower catalytic efficiencies of the mutant enzymes coupled with the exponential dependence on $1/(1 + [I]/K_i)$ more than offset the inhibitor resistance acquired by HIV protease. The conclusion of this analysis is that inhibitor-resistant mutant HIV proteases are very unlikely to contribute to viral viability *in vivo*. The results strongly suggest that future protease inhibitor clinical trials should measure the infectivity of the virions in blood plasma instead of relying on viral RNA levels.

It is hoped that disrupting the proteolytic processing of HIV[1] precursor proteins will be of therapeutic benefit. Numerous *in vitro* experiments have demonstrated that impaired proteolytic activity, due either to the presence of protease inhibitors (1–4) or deleterious mutations (5–10) of HIV protease, results in noninfectious HIV particles. As a consequence of these studies and several human clinical trials (11–14), a number of HIV protease inhibitors have recently been approved for clinical use. However, the disappointing clinical efficacy of these inhibitors during the early trials led to the widespread belief (11, 13–20) that the HIV protease develops resistance to the inhibitors by mutating to less susceptible forms of the enzyme.

A number of investigators have attempted to quantify the effects of mutations on the kinetics of HIV protease, both in the presence and absence of inhibitors (3, 6, 7, 21–23). The sensitivity of the various mutants of HIV protease to inhibitors was determined for a battery of compounds. With the exception of Lin *et al.* (23), the effects of the mutations on the catalytic efficiency of HIV protease were measured for only one synthetic substrate, although the choice of substrate varied among investigators. Relying on a single substrate to characterize the efficiency of HIV protease mutants has been questioned. Gulnik *et al.* (22) point out that, "while inhibition constants should not depend on the substrate, k_{cat}/K_m ratios do . . . Thus future studies should focus on a panel of substrates that represent all the natural HIV (protease) cleavage sites in the Gag-Pol polyproteins."

At least eight obligatory cleavages of the Gag-Pol polyprotein by HIV protease have been identified for viable maturation of viral particles (24), and there is considerable evidence that the order of the eight cleavages is not random. *In vitro* studies clearly show time-dependent, sequential formation and disappearance of the various intermediates during processing of the Gag-Pol polyprotein by HIV protease (25–28). It is likely that this sequential processing is more than just an *in vitro* phenomenon. Indeed, Stewart and Vogt (29) have proposed that "the order of cleavages is critically important in virus maturation." For the avian retrovirus the "proper proteolytic maturation of the ALV Gag-Pol polyprotein and the consequent activation of reverse transcriptase . . . requires a specific sequence of cleavages that is dictated by the microenvironment of the budding virus particle." Sequential processing is characteristic of the maturation of picornaviruses as well. Shih and Shih (30) report that the proteolytic cleavage of the encephalomyocarditis virus capsid protein precursor occurs in a defined, stepwise manner.

The timing of the processing events is also crucial. Kageyama *et al.* (2) showed that the temporary presence of an HIV protease inhibitor resulted in irreversible damage to the infectivity of HIV-1 *in vitro*. It is important to point out that in this study the number of virions produced in the presence of the inhibitor was the same as produced in its absence. Even the viral RNA content was the same as the control. In addition, the authors could reduce the number of infectious particles in cell culture 10-fold with an inhibitor concentration that had little or no effect on the production of p24 antigen relative to a drug-free control. Similarly, Kaplan *et al.* (3) showed that submicromolar concentrations of a protease inhibitor profoundly reduced the number of infectious particles without noticeably affecting the production of p24 antigen. A consideration of these results and others led to the following hypothesis.

The literature to date implies a finely tuned, ordered sequence of events leading to viable HIV replication and maturation. While HIV protease can cleave its viral proteins in a variety of ways, depending on the conditions, it is proposed that only a specific, ordered sequence of Gag-Pol polyprotein processing leads to infectious virions. Furthermore, the ordered processing sequence is kinetically equivalent to a series of

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[1] The abbreviations used are: HIV, human immunodeficiency virus; $[S_{p-1}]$, $p-1$ substrate concentration; V_{p-1}, V_{max} for the $p-1$ substrate cleavage; K_{p-1}, Michaelis-Menten constant for the $p-1$ substrate; $[S_p]_0^{(p-1)}$, the $p-1$ derivative of the product concentration S_p with respect to time; γ_j, ratio of jth mutant catalytic efficiency to that of the wild type catalytic efficiency; U_I, infectious units/ml in the presence of inhibitor; U_0, infectious units/ml in the absence of inhibitor.

consecutive enzyme catalyzed reactions depicted below, where PR is protease.

$$S_1 \xrightarrow[v_1]{\text{HIV PR}} S_2 \xrightarrow[v_2]{\text{HIV PR}} \cdots \xrightarrow[v_{p-1}]{\text{HIV PR}} S_p$$

REACTION 1

MATERIALS AND METHODS

Kuchel *et al.* (31) developed the theoretical basis for the analysis of the kinetics of consecutive enzyme catalyzed reactions involving single substrates, leading to the formation of the final product S_p. The procedure of Kuchel *et al.* assumes a steady state of enzyme-substrate complexes but not of intermediate reactants. The overall catalytic efficiency of the ordered sequence is represented by the rate of change in the molar concentration of the final cleavage product S_p (Equation 1).

$$\frac{d[S_p]}{dt} = [S_p]' = \frac{\frac{V_{p-1}}{K_{p-1}}[S_{p-1}]}{1 + \frac{[S_{p-1}]}{K_{p-1}}} \qquad \text{(Eq. 1)}$$

Kuchel *et al.* showed that the concentration of each species S_j is a continuous and differentiable function of t and may be expressed as a power series by means of a Maclaurin polynomial when expansion is made about the origin ($t = 0$, $[S_j]_t = [S_j]_0$). The production of S_p at any time t, then, is given by Equation 2.

$$[S_p]_t = [S_p]_0 + [S_p]_0' t + \frac{[S_p]_0'' t^2}{2!} + \ldots + \frac{[S_p]_0^{(n)} t^n}{n!} + \ldots \qquad \text{(Eq. 2)}$$

At $t = 0$, $[S_p]_0 = 0$. The expression for the first derivative of $[S_p]_0$ is directly obtained from Equation 1, while those for higher order derivatives are evaluated by successive differentiation with respect to time. The differentiation is facilitated by noting that $[S_j]_0 = 0$ for all $j \neq 1$, and at $t = 0$, $[S_1]_0$ is equal to the concentration of the first substrate at the beginning of the first proteolytic processing event. Obtaining the higher order coefficients in Equation 2 turns out to be a simple matter of successive substitution. The derivation of the next two higher order derivatives demonstrates the process.

$$[S_p]_0'' = \frac{V_{p-1}}{K_{p-1}}[S_{p-1}]_0' = \left(\frac{V_2}{K_2}\right)\left(\frac{V_1}{K_1}\right)\frac{[S_1]_0}{1 + \frac{[S_1]_0}{K_1}}, p = 3 \qquad \text{(Eq. 3)}$$

$$[S_p]_0''' = \frac{V_{p-1}}{K_{p-1}}[S_{p-1}]_0'' = \left(\frac{V_3}{K_3}\right)\left(\frac{V_2}{K_2}\right)\left(\frac{V_1}{K_1}\right)\frac{[S_1]_0}{1 + \frac{[S_1]_0}{K_1}}, p = 4 \qquad \text{(Eq. 4)}$$

Kuchel *et al.* showed that in general the first non-zero term for the expansion of $[S_p]_t$ is given by Equation 5.

$$\frac{[S_p]_0^{(p-1)} t^{(p-1)}}{(p-1)!} = \frac{\left(\prod_{j=2}^{p} \frac{V_{j-1}}{K_{j-1}}\right)[S_1]_0 t^{(p-1)}}{(p-1)!\left(1 + \frac{[S_1]_0}{K_1}\right)}, p \geq 2 \qquad \text{(Eq. 5)}$$

It is clear from the term $\prod_{j=2}^{p}(V_{j-1}/K_{j-1})$ of Equation 5 that $[S_p]_t$ depends on the outcome of all the processing steps of the ordered sequence.

Equation 6 can be used to compare the overall catalytic efficiency of the mutant proteases to the wild type enzyme. To do this it is necessary to compute the ratio, shown in Equation 6.

$$\frac{[S_p]_{t(\text{mutant})}}{[S_p]_{t(\text{wild type})}} = \frac{[S_p]_{0(m)}^{(p-1)} t^{(p-1)}/(p-1)!}{[S_p]_{0(w)}^{(p-1)} t^{(p-1)}/(p-1)!} = \frac{[S_p]_{0(m)}^{(p-1)}}{[S_p]_{0(w)}^{(p-1)}} \qquad \text{(Eq. 6)}$$

The higher order terms greater than $(p-1)$ were not included in Equation 5 to simplify the next steps. Substituting the appropriate versions of Equation 5 into Equation 6 results in Equation 7.

$$\frac{[S_p]_{t(m)}}{[S_p]_{t(w)}} = \frac{\left(\prod_{j=2}^{p} \frac{V_{j-1}}{K_{j-1}}\right)_m \left(\frac{[S_1]_0}{1 + \frac{[S_1]_0}{K_{1(m)}}}\right)}{\left(\prod_{j=2}^{p} \frac{V_{j-1}}{K_{j-1}}\right)_w \left(\frac{[S_1]_0}{1 + \frac{[S_1]_0}{K_{1(w)}}}\right)} \qquad \text{(Eq. 7)}$$

Let

$$\gamma_j = \frac{\left(\frac{V_j}{K_j}\right)_m}{\left(\frac{V_j}{K_j}\right)_w}. \text{ Then } \left(\frac{V_j}{K_j}\right)_m = \gamma_j \left(\frac{V_j}{K_j}\right)_w, \qquad \text{(Eq. 8)}$$

which gives Equation 9.

$$\frac{[S_p]_{t(m)}}{[S_p]_{t(w)}} = \frac{\left(\prod_{j=2}^{p} \gamma_{j-1} \frac{V_{j-1}}{K_{j-1}}\right)_w \left(\frac{1}{1 + \frac{[S_1]_0}{K_{1(m)}}}\right)}{\left(\prod_{j=2}^{p} \frac{V_{j-1}}{K_{j-1}}\right)_w \left(\frac{1}{1 + \frac{[S_1]_0}{K_{1(w)}}}\right)} = \left(\prod_{j=2}^{p} \gamma_{j-1}\right)\left(\frac{1 + \frac{[S_1]_0}{K_{1(w)}}}{1 + \frac{[S_1]_0}{K_{1(m)}}}\right) \qquad \text{(Eq. 9)}$$

Tang and Hartsuck (21) have argued that the effective concentrations of HIV substrates are below their K_m values. Whether or not this is true, the factor on the right can be dropped giving Equation 10 for HIV protease.

$$1 + \frac{[S_1]_0}{K_{1(w)}} \approx 1 + \frac{[S_1]_0}{K_{1(m)}}, \text{ resulting in } \frac{[S_p]_{t(m)}}{[S_p]_{t(w)}} \approx \prod_{j=2}^{p} \gamma_{(j-1)} \text{ or } \approx \prod_{j=1}^{8} \gamma_j \qquad \text{(Eq. 10)}$$

To a first approximation,[2] Equation 10 measures the overall efficiency of mutant HIV protease relative to the wild type enzyme. The overall efficiency is the product of the relative efficiencies of the mutant enzyme *versus* the wild type for all eight obligatory cleavages.

To assess the viability of HIV protease in the presence of an inhibitor it is necessary to compute

$$\frac{[S_p]_{t(\text{inhibitor})}}{[S_p]_t} = \frac{[S_p]_{0(I)}^{(p-1)} t^{(p-1)}/(p-1)!}{[S_p]_0^{(p-1)} t^{(p-1)}/(p-1)!} = \frac{[S_p]_{0(I)}^{(p-1)}}{[S_p]_0^{(p-1)}}. \qquad \text{(Eq. 11)}$$

Adjusting Equation 5 for the presence of competitive inhibitor yields Equation 12.

$$\frac{[S_p]_{0(I)}^{(p-1)} t^{(p-1)}}{(p-1)!} = \frac{\left(\prod_{j=2}^{p} \frac{V_{j-1}}{K_{j-1}}\right)[S_1]_0 t^{(p-1)}}{(p-1)!\left(1 + \frac{[S_1]_0}{K_1} + \frac{[I]}{K_i}\right)\left(1 + \frac{[I]}{K_i}\right)^{(p-2)}}, p \geq 2. \qquad \text{(Eq. 12)}$$

Substituting Equations 5 and 12 into Equation 11 gives Equation 13.

$$\frac{[S_p]_{t(I)}}{[S_p]_t} = \frac{\left(\prod_{j=2}^{p} \frac{V_{j-1}}{K_{j-1}}\right)\left(\frac{[S_1]_0}{\left(1 + \frac{[S_1]_0}{K_1} + \frac{[I]}{K_i}\right)\left(1 + \frac{[I]}{K_i}\right)^{(p-2)}}\right)}{\left(\prod_{j=2}^{p} \frac{V_{j-1}}{K_{j-1}}\right)\left(\frac{[S_1]_0}{1 + \frac{[S_1]_0}{K_1}}\right)} \qquad \text{(Eq. 13)}$$

[2] Equation 10a is the general expression of Equation 10 that includes the higher order terms. However, using the data of Lin *et al.* (23) as a test demonstrated that the higher order terms can be deleted, resulting in the simpler Equation 10 as a good approximation.

$$\frac{[S_p]_{t(m)}}{[S_p]_{t(w)}} = \left(\prod_{j=2}^{p} \gamma_{j-1}\right)\left(\frac{1 - \frac{\gamma_1}{p}\frac{V_{1(w)}t}{K_{1(w)}} + \frac{\gamma_1^2}{p(p+1)}\left(\frac{V_{1(w)}t}{K_{1(w)}}\right)^2 - \ldots}{1 - \frac{1}{p}\frac{V_{1(w)}t}{K_{1(w)}} + \frac{1}{p(p+1)}\left(\frac{V_{1(w)}t}{K_{1(w)}}\right)^2 - \ldots}\right) \qquad \text{(Eq. 10a)}$$

This simplifies to the equation below.

$$\frac{[S_p]_{t(I)}}{[S_p]_t} = \frac{1 + \frac{[S_1]_0}{K_1}}{\left(1 + \frac{[S_1]_0}{K_1} + \frac{[I]}{K_i}\right)\left(1 + \frac{[I]}{K_i}\right)^{(p-2)}} \qquad \text{(Eq. 14)}$$

If, $\frac{[S_1]_0}{K_1} < \frac{[I]}{K_i}$, as is likely, and Tang and Hartsuck (21) are right, Equation 14 can be approximated by Equation 15 for HIV protease.

$$\frac{[S_p]_{t(I)}}{[S_p]_t} \approx \frac{1}{\left(1 + \frac{[I]}{K_i}\right)^{(p-1)}} \text{ or } \approx \frac{1}{\left(1 + \frac{[I]}{K_i}\right)^{8}} \qquad \text{(Eq. 15)}$$

Equation 15 measures the overall reduction in catalytic efficiency of HIV protease in the presence of an inhibitor. It is important to note that the efficiency of overall proteolytic processing is inversely proportional to the eighth power of $(1 + [I]/K_i)$. For $[I] \neq 0$, overall catalytic efficiency falls off rapidly with increasing levels of inhibitor.

Finally, to compare the viability of the mutant HIV protease in the presence of inhibitor to the uninhibited wild type enzyme it is necessary to compute Equation 16.

$$\frac{[S_p]_{t(m,I)}}{[S_p]_{t(w)}} = \frac{[S_p]_{0(m,I)}^{(p-1)} t^{(p-1)}/(p-1)!}{[S_p]_{0(w)}^{(p-1)} t^{(p-1)}/(p-1)!} = \frac{[S_p]_{0(m,I)}^{(p-1)}}{[S_p]_{0(w)}^{(p-1)}} \qquad \text{(Eq. 16)}$$

The usual manipulations give Equation 17.

$$\frac{[S_p]_{t(m,I)}}{[S_p]_{t(w)}} = \left(\prod_{j=2}^{p} \gamma_{(j-1)}\right) \frac{1 + \frac{[S_1]_0}{K_{1(w)}}}{\left(1 + \frac{[S_1]_0}{K_{1(m)}} + \frac{[I]}{K_{i(m)}}\right)\left(1 + \frac{[I]}{K_{i(m)}}\right)^{(p-2)}}. \qquad \text{(Eq. 17)}$$

Employing the same rationale used to simplify Eqaution 14, Equation 17 reduces to Equation 18 for HIV protease.

$$\frac{[S_p]_{t(m,I)}}{[S_p]_{t(w)}} \approx \left(\prod_{j=2}^{p} \gamma_{(j-1)}\right) \frac{1}{\left(1 + \frac{[I]}{K_{i(m)}}\right)^{(p-1)}} \text{ or } \approx \frac{\prod_{j=1}^{8} \gamma_j}{\left(1 + \frac{[I]}{K_{i(m)}}\right)^{8}} \qquad \text{(Eq. 18)}$$

Equation 18 expresses the overall catalytic efficiency of mutant HIV protease in the presence of inhibitor as compared with the uninhibited wild type enzyme. In the absence of inhibitor, Equation 18 reduces to Equation 10. For any inhibited protease compared with itself, Equation 18 reduces to Equation 15. Therefore, Equation 18 is sufficient to characterize the overall catalytic efficiency of mutant or wild type HIV proteases in the presence or absence of inhibitors.

RESULTS

Kaplan *et al.* (3) report that partial inhibition of HIV proteolytic activity results in a profound reduction in the number of infectious particles, disproportionate to the effect of the inhibitor on the viral protease itself. At 100 nM, the Abbott HIV protease inhibitor A77003 reduced the viral titer 5-fold. However, a 3-fold increase in inhibitor concentration (330 nM) decreased the viral titer 80–100-fold. Surprisingly, at the higher inhibitor concentration there was only modest reduction of Gag precursor processing as determined by Western blot analysis of certain of the intermediates.

If we assume that the number of infectious units/ml (U_j) is proportional to the overall proteolytic processing of the Gag-Pol polyprotein, then we obtain Equation 19, which is just another form of Equation 15.

$$U_j \propto [S_p]_t \text{ and } \frac{U_I}{U_0} = \frac{[S_p]_{t(I)}}{[S_p]_t} \qquad \text{(Eq. 19)}$$

Equation 19 should describe the effects of HIV protease inhibitors on the production of infectious viral particles *in vitro*. Since the concentration of inhibitor that reaches the HIV protease in cell culture is unknown but likely to be substantially less than that in the culture media, K_i (K_i = 84 pM; Ref. 32) of Equation 15 should be replaced by the apparent inhibition constant $K_{i(app)}$, giving Equation 20.

$$\frac{U_I}{U_0} = \frac{1}{\left(1 + \frac{[I]}{K_{i(app)}}\right)^{8}} \qquad \text{(Eq. 20)}$$

Equation 20 describes the effect of A77003 on the production of infectious HIV particles (Table I).

TABLE I

Equation 20 explains the results of Kaplan et al.

The effect of HIV protease inhibitor A77003 on infectious virus production is shown.

[I][a]	U_i[a]	U_i from curve fit[b]
μM	*infectious units/ml*	*infectious units/ml*
0	7425	7425
0.1	1525	1525
0.33	95	95
1.0	0	0.7

[a] Data are taken from Fig. 1 of Kaplan *et al.* (3).

[b] Fitting $U_I = U_0/(1 + ([I]/K_{i(app)})^n$ to the data gives: U_0 = 7425, $K_{i(app)}$ = 466 nM, and n = 8.1.

Equation 20 can be used to calculate the EC_{50} value of the inhibitor for *in vitro* cell culture. For $K_{i(app)}$ = 466 nM (Table I), EC_{50} = 42 nM. This value compares with the EC_{50} = 30–150 nM reported by Kempf *et al.* (33) for the same inhibitor and cell line. The inverse eighth power dependence on $(1 + [I]/K_i)$ for the overall catalytic activity of HIV protease is responsible for the $EC_{50} < K_{i(app)}$ and the remarkably steep decline in the production of infectious virions in the presence of increasing levels of A77003. This result is general and has implications for extending the inhibitor approach to other viral targets.

The individual γ_j terms of Equation 18 are unknown for the mutant HIV proteases except for a few synthetic substrates (23). However, the geometric mean of the product of the eight γ_j terms can be used as a convenient substitute in the absence of the experimental values. The minimum overall catalytic efficiency of HIV protease for viable viral maturation has been estimated at between 0.25 and 0.02 times the wild type activity (6, 7, 21, 22). Substituting 0.25–0.02 and the geometric mean of the γ_j terms into Equation 18 gives the inequality, Equation 21, which determines the lower limit of HIV protease viability.

$$\left(\frac{\gamma}{1 + \frac{[I]}{K_i}}\right)^{8} < 0.25\text{–}0.02 \qquad \text{(Eq. 21)}$$

In the absence of inhibitor, Equation 21 gives $\gamma < 0.84$–0.61. If one is comparing the activity of the same enzyme in the presence and absence of inhibitor, then $\gamma = 1$ and the inequality in Equation 21 becomes $[I] > 0.19$–$0.63K_i$. Thus for the wild type HIV protease, inhibitor levels of only 20–60% of K_i (assuming 100% inhibitor bioavailability) are sufficient to render the virus nonviable.

Lin *et al.* (23) are among only a few authors to report γ_j for $j > 1$, and these are all for synthetic substrates. An analysis of their data shows that most of the γ_j values < 0.61, the minimum mean value for the most stringent condition at the 0.02 level of wild type activity. Lin *et al.* report the V82D mutant of HIV protease was 5545 times less sensitive to a particular inhibitor than the wild type enzyme. However, γ_1 for this mutant was equal to 0.036, which is well below the 0.61 level. When the V82D mutant was compared with a battery of synthetic substrates, its overall catalytic efficiency was over a million-fold (*i.e.* $\Pi_{j=1}^{8} \gamma_j < 10^{-6}$) lower than the wild type enzyme for the same substrates. In fact, all the mutants, with

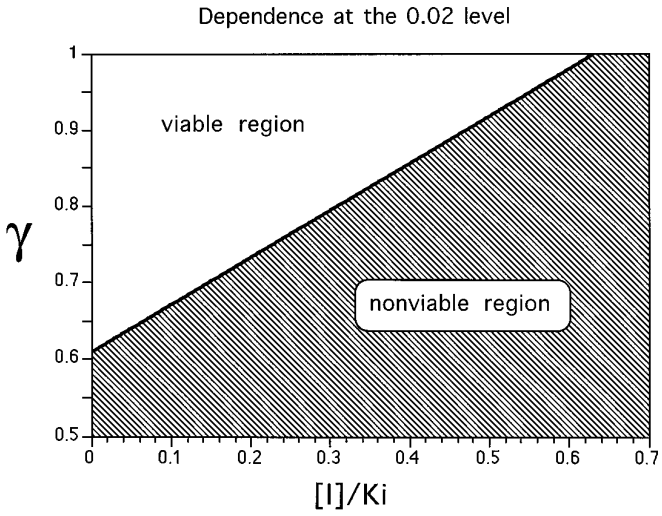

FIG. 1. **A plot of equation 21.** The minimum overall catalytic activity of HIV protease is set to 0.02 times that of the uninhibited wild type enzyme. Points above the line represent proteolytic activity leading to viable virus, whereas points below the line do not.

two exceptions, had $\Pi_{j=1}^{8}\ \gamma_j \ll 0.02$ and, consequently, would not be expected to lead to viable virus *in vivo*.

The greater activity of the G48Y mutant protease prompted Lin *et al.* to wonder "why the wild type HIV-1 does not select for a higher catalytic activity with a larger side chain at position 48." However, others have shown that elevations in the overall proteolytic activity of HIV protease above wild type levels are as deleterious to the virus as substantial reductions in activity (9, 34, 35). The apparent 10-fold higher activity toward one substrate referred to by the authors is a mere shadow of the overall gain in catalytic efficiency of the G48Y mutant, *i.e.* $\Pi_{j=1}^{8}\ \gamma_j > 10^5$, more than sufficient to explain the absence of this phenotype in nature.

Fig. 1 is a graphical representation of Equation 21, where the minimum overall catalytic activity of HIV protease is set to 0.02 times that of the uninhibited wild type enzyme. Points above the line represent viable protease, whereas points below the line do not. A striking feature of the graph is the very narrow region of viability. Under no conditions is HIV viable for $\gamma < 0.61$ or for $[I] > 0.63K_i$. Non-saturating levels of inhibitor are sufficient to render mutant as well as wild type HIV nonviable. Since virtually all mutant HIV proteases have $\gamma_j < 1$, then even lower levels of protease inhibitors are sufficient to prevent mutant viral maturation. A combination of $\gamma < 1$ and the inverse eighth power dependence on $(1 + [I]/K_{i(app)})$ offsets the inhibitor resistance the mutant enzymes may acquire.

It is instructive to examine the consequence to inhibition of mutations that result in overall catalytic efficiencies between 0.02 and 1.0 of the wild type HIV protease. If the overall catalytic efficiency of a mutant protease is twice the minimum level for viability, an inhibitor concentration greater than 9% of the $K_{i(m)}$ value (assuming 100% inhibitor bioavailability) is sufficient to render the mutant virus nonviable. If, for this mutant protease, $K_{i(m)} = 10K_{i(w)}$, the concentration of inhibitor needed for nonviability is only $0.9K_{i(w)}$, still well below the saturation level for even the wild type enzyme. If the mutant enzyme is lucky enough to achieve a 100-fold elevation in K_i over the wild type, then $[I] > 9K_{i(w)}$ is sufficient for nonviability. Even more dramatic, if the mutant protease has 40 times (*i.e.* $\gamma^8 = 0.80$, $\gamma = 0.97$) the minimum viable activity of the wild type at the 0.02 level, the amount of inhibitor needed for nonviability is still only 58% of $K_{i(m)}$. In this case, the mutant protease activity is virtually equivalent to the wild type and $[I] > 5.8K_{i(w)}$ results in nonviability for $K_{i(m)} = 10K_{i(w)}$.

To put these numbers into perspective, a typical dosing level of HIV protease inhibitor ($M_r \approx 600$) for an AIDS patient is around 600 mg, three times a day (36). A low oral uptake of 6% translates roughly to 800 nM inhibitor in a 70-kg patient. The $K_{i(w)}$ values for the inhibitors range from nanomolar to picomolar. The upper limit of the daily dose then is 800–800,000 times the $K_{i(w)}$ values. The pharmacokinetic properties of the Roche inhibitor Ro 31-8959 have been reported. Patients receiving 1.8 g/day of the inhibitor had mean plasma concentrations around 44 nM (37). Recently, even higher doses of Ro 31-8959 have been administered (14). For patients receiving either 3.6 or 7.2 g of inhibitor per day, the mean plasma levels were 116 or 560 nM, respectively. As discussed earlier, the actual concentration of inhibitor that reaches the HIV protease is likely substantially less.

So far only one inhibitor-resistant mutant (K45E) of HIV protease (23) has been reported that comes anywhere near the minimum level of overall catalytic activity ($\Pi_{j=1}^{8}\ \gamma_j = 0.13$) necessary for viral viability. Nonetheless, a virus with this mutation is still sensitive to the HIV protease inhibitors because of the inverse eighth power dependence on $(1 + [I]/K_i)$. For example, The inhibitor U85548 has $K_i = 3$ nM for wild type and 31 nM for the K45E mutant (23), a 10-fold increase. Using the most optimistic conditions for inhibition, [I] = 800 nM and assuming that $K_{i(app)} = 100K_{i(m)}$, gives Equation 22, which is just at the limit of viability.

$$\frac{\prod_{j=1}^{8}\gamma_j}{\left(1+\frac{[I]}{K_{i(m)}}\right)^8} = \frac{0.13}{\left(1+\frac{800}{31\times 100}\right)^8} = \frac{0.13}{6.28} = 0.021 \qquad \text{(Eq. 22)}$$

DISCUSSION

It is important to point out that a number of approximations and assumptions were introduced into this analysis of consecutive cleavages in order to make the equations tractable. For instance, the Maclaurin polynomial (Equation 2) expanded about the origin ($t = 0$, $[S_p]_t = [S_p]_0$) is less predictive of the final substrate cleavage as time increases. While it is true that for an ordered, sequential process $[S_j]_0 = 0$ $(j \neq 1)$, the assumption that $[S_1]_0 < K_{1,w}, K_{1,m}$ (21) may turn out to be wrong. Even so, the effect on Equation 17 is not likely to be large.

For example, if

$$\frac{[S_1]_0}{K_{1(w\ or\ m)}} > \frac{[I]}{K_{i(m)}}, \qquad \text{(Eq. 23)}$$

then

$$\frac{[S_p]_{t(m,I)}}{[S_p]_{t(w)}} \approx \frac{\prod_{j=1}^{8}\gamma_j}{\left(1+\frac{[I]}{K_{i(m)}}\right)^7}. \qquad \text{(Eq. 24)}$$

It is also possible that future experiments will show that viable HIV actually requires more or fewer sequential proteolytic cleavages of the Gag-Pol polyprotein than the eight considered in this analysis. In spite of these shortcomings, Equation 18 is still a powerful tool for assessing the overall effects of inhibitors and mutations of HIV protease on the viability of the virus *in vitro*. Indeed, as shown above, Equation 18 (all the γ values = 1) explains the experimental data of Kaplan *et al.* remarkably well. However, to really test the results of Table I, the experiment of Kaplan *et al.* should be repeated comparing an inhibitor-resistant mutant protease to the wild type enzyme, and should include more than four data points within the

range of inhibitor concentrations that still allow for the production of infectious virus.

It is encouraging to note that Equation 18 is consistent with the growing number of observations that unexpectedly low inhibitor concentrations (well below those predicted by simple *in vitro* kinetic assays of the various HIV proteases) can interfere with Gag precursor processing and significantly reduce the production of infectious HIV in cell culture (1–3, 38). For example, Maschera *et al.* (38) found that, "[a]lthough the rank order of mutant virus resistance correlates with the increase in K_i for the mutant protease, the magnitude of the viral resistance increase is considerably smaller than the magnitude of the K_i increase." The authors' Table I shows that the disparity between simple K_i values and viral resistance grows as the K_i values go up. For the double mutant (G48V,L90M), the K_i increased 722-fold relative to the wild type, but the increase in viral resistance was only 9-fold: a discrepancy of over 80-fold between increase in K_i and viral resistance. Addressing this discrepancy, the authors pointed out that "[t]he enzyme assay measures inhibition of cleavage of a single peptide, whereas inhibition of HIV replication is dependent on cleavage at eight or more polypeptide sites." It is important to add that the authors observed that, "although the mutations may confer drug resistance, the enzymes [mutant HIV proteases] appear to autocatalytically be less active."

The ability to explain and predict *in vitro* results does not mean that Equation 18 represents the detailed kinetic properties of HIV protease *in vivo*. However, since it predicts that small changes in γ, [I], or K_i can have effects that are magnified many orders of magnitude, Equation 18 does have considerable clinical relevance. For example, Equation 18 shows that the *in vitro* criteria currently being used to evaluate inhibitor-resistant HIV protease in clinical samples greatly overestimate the levels of viable, infectious virus.

Condra *et al.* (15) report the emergence in clinical samples of HIV-1 variants resistant to multiple protease inhibitors. The mutant HIV proteases themselves were not assayed for proteolytic activity or for sensitivity to the inhibitors. The authors measured the production of p24 antigen to determine the effects of a battery of protease inhibitors on HIV grown in cell culture. As Kaplan *et al.* (3) and Kageyama *et al.* (2) have demonstrated, simply measuring the amount of p24 antigen produced does not necessarily indicate the level of mature, infectious virus in cell culture, especially at low inhibitor concentrations. Since Condra *et al.* did not report the level of viability and infectivity of the mutant viruses relative to the wild type, it is difficult to assess the survival value to the virus of the mutations of HIV protease.

Jacobsen *et al.* (39) report the appearance of two inhibitor-resistant mutations in the proviral DNA taken from patients being treated with the Roche compound Ro 31-8959. These two mutants (L90M,G48V) were previously identified *in vitro* by repeated passage of wild type virus in infected CEM cells in the presence of increasing concentrations of the inhibitor (40). While the mutants with either single or double amino acid substitutions resulted in reduced sensitivity (3.4–20-fold) to the inhibitor, all the mutants showed significantly delayed processing of the *pol* gene products p66 and p51 as compared with the wild type (40). Using the p24 antigen assay, the authors concluded that the double mutant virus grew as well as wild type in CEM cells. However, to reiterate, the p24 antigen assay by itself is not a reliable indicator of the level of infectious virions. Therefore, it is difficult to assess the significance of the double-mutant inhibitor-resistant virus.

The meaning of inhibitor-resistant clinical isolates is further brought into question when looking at the sequence diversity of HIV protease in human samples. Lech *et al.* (41) obtained 246 protease coding domain sequences from 12 HIV-infected persons (median, 21/subject), none of whom had received protease inhibitors. The authors dropped two of the patients from their analysis since they could not find any DNA sequences that coded for active HIV protease in those individuals. The results from the remaining patients showed that some inhibitor-naive HIV positive subjects had proviral DNA that codes for amino acid substitutions (R8Q, V82I, M46F, M46I, and I50V) predicted to give rise to HIV proteases resistant to inhibitors. However, the "inhibitor-resistant" mutants V82I and M46I identified by Lech *et al.* were shown by others (42, 43) (who also used human sources of the proteases) to be no less sensitive to a battery of HIV protease inhibitors than the reference strains. Furthermore, Kozal *et al.* (44) showed that "[t]he DNA sequence of USA HIV-1 clade B proteases was found to be extremely variable and 47.5% of the 99 amino acid positions varied . . . Many of the amino acid changes that are known to contribute to drug resistance occurred as natural polymorphisms in isolates from patients who had never received protease inhibitors." Since up to 99.9% of the genetically distinct variants of the HIV genome are defective (45, 46), there is no reason to believe that all or most of the large number of variants of HIV protease that are coded for by proviral DNA will lead to infectious virus.

The amplification inherent in PCR and co-culture techniques makes it easy to overestimate the *in vivo* significance of the changes in the gene products being investigated. Logically, then, there is no reason to believe that the presence of p24 or viral RNA are any more indicative of infectious virus in the blood samples of AIDS patients than in cell culture. The results of Kageyama *et al.* (2) and Kaplan *et al.* (3), and the analysis of consecutive cleavages argue strongly that future HIV protease inhibitor clinical trials should determine the level of infectivity of the virions in blood plasma in addition to measuring the amounts of p24 antigen and viral RNA.

It is becoming clear that the degree to which the mutant proteases are resistant to inhibitors is meaningful only in the context where the viability and infectivity of the mutant viruses are also quantitated. As the data continue to accumulate, it seems increasingly unlikely that mutations of the HIV protease, substantial enough to protect the enzyme against inhibition, will at the same time leave virtually unimpaired its ordered, sequential processing of all eight cleavage sites of the Gag-Pol polyprotein. The conclusion of this analysis is that inhibitor-resistant mutant HIV proteases are very unlikely to contribute to viral viability *in vivo*.

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