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Evaluation of the Substrate Envelope Hypothesis for Inhibitors of HIV-1 Protease

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ABSTRACT **Crystallographic data show that various substrates of HIV protease occupy a remarkably uniform region within the binding site; this region has been termed the substrate envelope. It has been suggested that an inhibitor that fits within the substrate envelope should tend to evade viral resistance because a protease mutation that reduces the affinity of the inhibitor will also tend to reduce the affinity of substrate, and will hence decrease the activity of the enzyme. Accordingly, inhibitors that fit the substrate envelope better should be less susceptible to clinically observed resistant mutations, since these must also allow substrates to bind. The present study describes a quantitative measure of the volume of a bound inhibitor falling outside the substrate envelope, and observes that this quantity correlates with the inhibitor's losses in affinity to clinically relevant mutants. This measure may thus be useful as a penalty function in the design of robust HIV protease inhibitors. Proteins 2007;68:561–567.**

Key words: resistance; AIDS; drug design; mutant; docking

INTRODUCTION

Inhibitors of human immunodeficiency virus (HIV) protease revolutionized the treatment of patients infected with HIV in the mid 1990s, and remain a mainstay of therapy today.[1–3] However, recent years have seen the emergence of HIV strains that are resistant to protease inhibitors.[4,5] The appearance of resistance is traceable to the selective pressure of therapy, combined with the high replication rate of HIV and the low fidelity with which HIV replicates its genetic information. Overcoming the evolutionary power of this system and maintaining an active armamentarium against HIV may prove to be a substantial challenge. On the other hand, the challenge is circumscribed by the fact that a viable resistance mutant of HIV protease must still bind and hydrolyze the various cleavage sites of the virus's Gag-Pol gene product at an adequate rate to allow viral replication. Accordingly, an inhibitor which forms "substrate-like" interactions with the protease should tend to evade viral resistance, because a mutation that weakens inhibitor-binding should simultaneously weaken substrate-binding, and hence damage the activity of the enzyme.

Recent crystallographic studies of complexes of HIV protease with its substrates provide a basis for pursuing this design concept. The substrates adopt a rather uniform shape when bound, despite the differences among their amino acid sequences, and the border of the consensus volume they occupy has been termed the "substrate envelope."[6] Intriguingly, the consensus volume occupied by a number of bound inhibitors differs significantly from the consensus substrate volume, and key resistance mutations appear to cluster near locations where inhibitors protrude outside the substrate envelope.[7] These observations have led to the hypotheses that the protease recognizes its varied substrates largely on the basis of their shape, and that inhibitors that fit within the substrate envelope may be less susceptible to mutational resistance.[7] Indeed, it has been argued that the fit of inhibitor Darunavir (TMC114) to the substrate envelope helps explain its ability to retain affinity for clinically relevant protease mutants.[8,9]

These considerations suggest that the substrate envelope hypothesis may be useful as a basis for the design of new inhibitors that will tend to counteract the emergence of resistance mutants. The present study addresses this issue by devising a quantitative indicator of the degree to which a candidate ligand falls outside the substrate envelope, and then determining whether this indicator correlates with the inhibitor's sensitivity to clinically relevant resistant mutations. The resistance analysis is based upon new calorimetric data for the association of various inhibitors with wild-type and mu-

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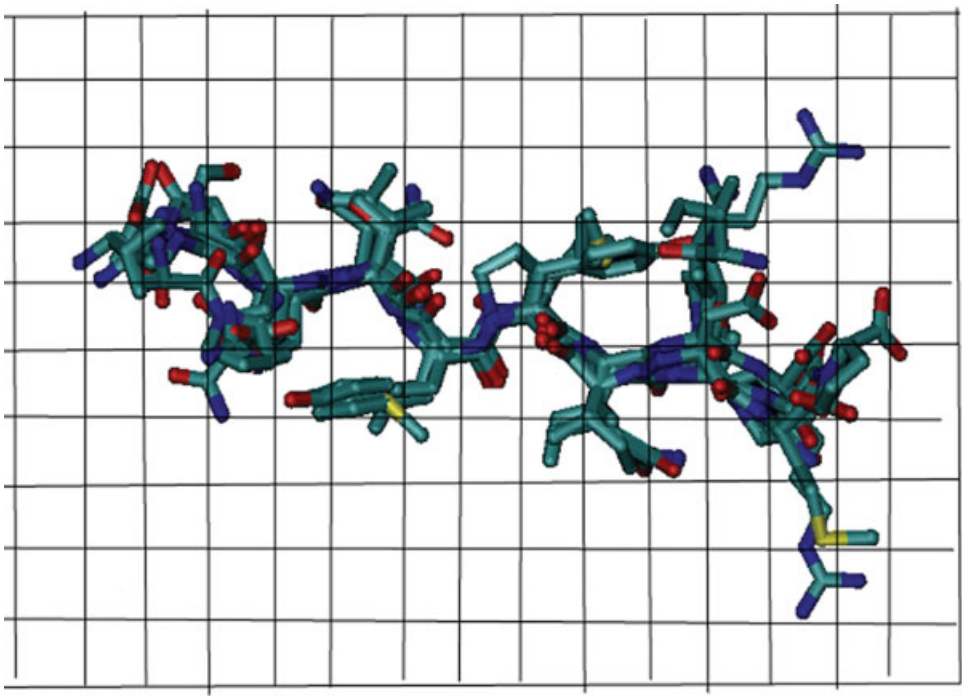

Fig. 1. Diagram of aligned substrates of HIV protease on a 2D representation of the 3D grid of substrate occupancy defined in Methods.

tant proteases, supplemented by additional calorimetric data from the literature.

MATERIALS AND METHODS

This section describes a method of quantifying the volume of a bound inhibitor falling outside the envelope, then details the measurement of affinities by isothermal titration calorimetry (ITC) for a group of inhibitors and proteases, and summarizes additional binding data drawn from prior publications. Finally, a novel measure of the "clinical relevance" of the mutations studied here is described.

Computational

Evaluation of the fit of an inhibitor to the substrate envelope

A 3D grid of substrate density in the binding site was generated as follows. The superimpose module of QUANTA (Accelrys Software Inc.) was used to superimpose six crystal structures of HIV protease having bound substrate peptides (1F7A, 1KJ4, 1KJ7, 1KJF, 1KJG, 1KJH[10]) on a crystal structure of HIV protease with indinavir (1HSG[11]), based upon the coordinates of backbone atoms (Fig. 1). The chemical C2 rotational symmetry of the receptor structure was accounted for by carrying out the symmetry operation and superimposing the six resulting structures on the original six by the same method, for a total of 12 overlaid substrates. Next, a cubic 3D grid with side-length 10 Å and grid spacing 0.2 Å was centered on the active site and an initial value of 0 was assigned to each grid point. Then a value g_{ijk} was incremented by 1 for every substrate structure that contains the grid point (i,j,k), where a grid point was considered to be contained by a substrate if it lay within the CHARMm[12] van der Waals radius of any nonhydrogen atom of the substrate. Because there are 12 overlaid substrates, the resulting grid values vary between 0 (outside all substrates) and 12 (inside all substrates).

The fit of an inhibitor to the substrate envelope is computed as follows. A crystal structure of HIV protease with the bound inhibitor is aligned with the substrate-bound structures, as described above. Then the effective volume of the inhibitor outside the substrate envelope, V_{out}, is computed by summing the values of the grid points g_{ijk} that lie within the van der Waals volume of the inhibitor, normalizing the sum by 12, and converting to a volume by multiplying by the 0.008 Å^3 volume of a grid box:

$$V_{\text{out}} \equiv \frac{0.008}{12} \sum_{i,j,k}^{\text{inside}} \left(12 - g_{ijk}\right) \tag{1}$$

Here "inside" implies that the sum runs only over grid points ijk that lie within the van der Waals volume of the inhibitor. As a control, the effective volume of the inhibitor that lies within the substrate envelope is computed as:

$$V_{\text{in}} \equiv \frac{0.008}{12} \sum_{i,j,k}^{\text{inside}} g_{ijk} \tag{2}$$

The total volume of an inhibitor, V_{tot}, is computed by adding these two quantities. The molecular weight and

the number of nonhydrogen atoms were also included as alternative measures of molecular size.

The following crystal structures of HIV protease with bound inhibitors were drawn from the Protein Data Bank[13]: 1HPV[14] (amprenavir; APV), 1HXB[15] (saquinavir; SQV), 1HSG[11] (indinavir; IDV), 1OHR[16] (nelfinavir; NFV), and 1HXW[17] (ritonavir; RTV). These structures were used to compute the values of V_{out}, and V_{in} (see above) of the respective inhibitors.

Binding Data

The degree to which an inhibitor's affinity declines when a mutant protease is substituted for wild-type, is quantified as $\log(K_d^{mut}/K_d^{wild-type})$, where K_d^{mut} and $K_d^{wild-type}$ represent the inhibitor's dissociation constants for the mutant and wild-types, respectively. Dissociation constants from ITC were drawn from the literature. In each case, the ratio of mutant to wild-type is drawn from a single study to minimize noise due to experimental variations.

ITC data were utilized from a variety of studies, including results for protease variants with mutations only in the active site, only outside the active site, and both in and out of the site. One study examines a variant bearing two critical active site mutations (3X:V82T/I84V/L63P), and a prototypical multidrug-resistant variant (MDR5: L10I/G48V/I54V/L63P/V82A) (King et al., in preparation). Another study examines the consequences of mutants with a single mutation in the active site (I84V), multiple mutations outside the active site, (NAM10: L10I/M36I/S37D/M46I/R57K/L63P/A71V/G73S/L90M/I93L), and their combination (ANAM11: L10I/M36I/S37D/M46I/R57K/L63P/A71V/G73S/L90M/I93/I84V), upon resistance to a number of clinical inhibitors.[18] A third study examines cooperativity among mutations V82A/I84V in the active site, M46I/I54V in the active site flaps, and L10I/L90M in the dimerization region away from the active site, as well as the combinations, HM (L10I/M46I/I54V/V82A/I84V/L90M) and QM (V82A/I84V/M46I/I54V).[19] A fourth study examines the active site mutation V82F/I84V against the background of viral strains A, B, and C.[20]

Clinical Relevance of Mutations

Treatment of a patient with HIV protease inhibitors selects for mutations that disrupt inhibitor binding while preserving enzyme function. If the substrate envelope hypothesis is valid, then inhibitors that fit the substrate envelope well should tend to retain affinity in the face of such clinically relevant mutations, but not necessarily to artificial mutations that also disrupt the normal interactions of the enzyme with its substrates, since the latter should also disrupt interactions with the inhibitor. The clinical relevance of the mutations studied here is assessed based upon their tendency to occur in patients treated with protease inhibitors, and in the absence of concurrent mutations known to be major resistance mutations. Thus, a mutation is considered clinically relevant if clinical data suggest that it suffices to generate clinical resistance. Clinical data drawn from the HIV drug resistance database[5] are used to define the clinical relevance C_i of a mutation set i as

$$C_i = 100\ \frac{N_{i,\text{only}}}{N_{i,\text{all}}} \tag{3}$$

where $N_{i,\text{only}}$ is the number of isolates with mutation set i and no other major mutations, as defined at the drug resistance database (http://hivdb.stanford.edu/cgi-bin/PRMut.cgi); and $N_{i,\text{all}}$ is the total number of isolates with mutation set i. That is, $N_{i,\text{all}}$ includes isolates with other major mutations.

RESULTS

Fit of Inhibitors to Substrate Density

The fit of the inhibitors to the substrate envelope is based upon the computed density of the substrates within the binding site. Isodensity contours of the substrate density grid (Fig. 2) show a rather smooth gradation of density, rather than a sharp drop from the maximal value of 12 to the minimal value of 0. The absence of an unambiguous substrate envelope motivates the present use of a smoothly varying measure of the volume of an inhibitor lying outside the substrate region [Eq. (1)], rather than a sharp cutoff. The consensus volume—that covered by all 12 substrate poses—is rather constricted (red in Fig. 2). It seems unlikely that an inhibitor could achieve high affinity without reaching outside this region. In fact, the inhibitors studied here all extend to some degree outside the level 8 contour, APV the least, and RTV the most (see Fig. 3). This observation is quantified in the first row of Table I, which lists the computed values of V_{out} [Eq. (1)] which range over a factor of 2. The computed volumes within the substrate envelope are more uniform, varying by only about 20%. Table I also includes the number of nonhydrogen atoms and the molecular weight of each inhibitor as control measures of molecular size. Among all the measures of molecular size, V_{out} shows the largest variation in values across inhibitors.

Binding Affinities to Wild-Type Protease and Mutants

Experimental binding affinities were taken from literature ITC data. Table II lists the sensitivities ($\log(K_d^{mut}/K_d^{wild-type})$) of 5 inhibitors to 13 protease variants. The affinity losses vary from 0.3 to nearly 4 logs, and the different inhibitors clearly show different sensitivities to mutation: APV tends to lose least affinity (mean sensitivity 0.87) while RTV tends to lose the most (mean sensitivity 2.3).

Clinical Relevance of Protease Mutants

The mutants analyzed here appear to span a range of clinical relevance, as computed with Eq. (3) as shown

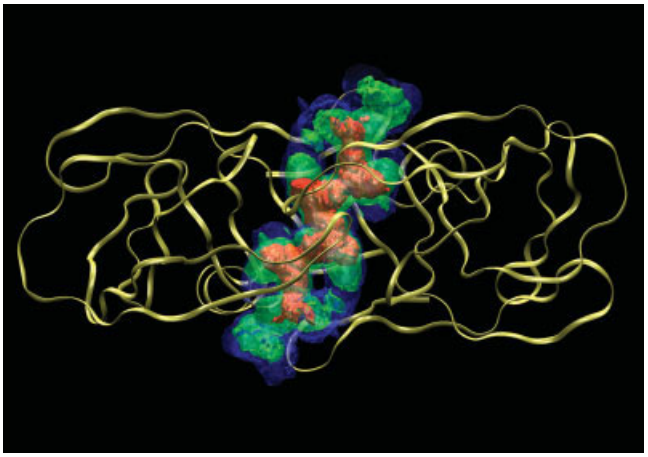

Fig. 2. Isosurface contours of the substrate density, whose values range from 0 to 12, in the context of a backbone trace of HIV-1 protease. Red: density 12. Green: density 8. Blue: density 4.

in Table III. The mutation sets of MDR5, QM, and HM are found in a significant fraction (19–44%) of clinical isolates having no other major mutations; mutation sets 3X, I84V, L10I/L90M, and V82A/I84V appear in 2–5% of isolates with no other major mutations; and V82F/I84V, M46I/I54V appear in <1% of isolates without other major mutations. Variants NAM10 and ANAM11 span a range of therapeutically relevant mutations, and ANAM11 was identified as a clinical isolate,[18] but these variants were not observed in any of the clinical isolates recorded in the Stanford database.

Correlation of V_{out} With Sensitivity to Clinically Relevant Mutations

The volume of each inhibitor outside the substrate envelope, and other properties listed in Table I, were compared with their affinities and relative clinical significance. Figure 4 examines the correlation of V_{out}, V_{in}, V_{tot}, number of nonhydrogen atoms, and molecular weight, with the loss of affinity of the various inhibitors on going from wild-type to the most clinically relevant protease mutants, MDR5, QM, and HM. The data are drawn from Tables I and II. The corresponding correlation coefficients for these mutants, and for the other, less clinically relevant mutants, are provided in Table IV. The volume of an inhibitor that lies outside the substrate envelope, V_{out}, correlates strongly with its susceptibility to the four most clinically relevant mutations, with correlation coefficients 0.94–0.97. Similar correlations are observed for many of the other mutants, but not all: the correlation coefficients range from 0.28 to 0.97. Interestingly, the other measures of molecular size show rather similar patterns. The correlations tend to be weakest for V_{in}, if only because this quantity has a rather small range of values.

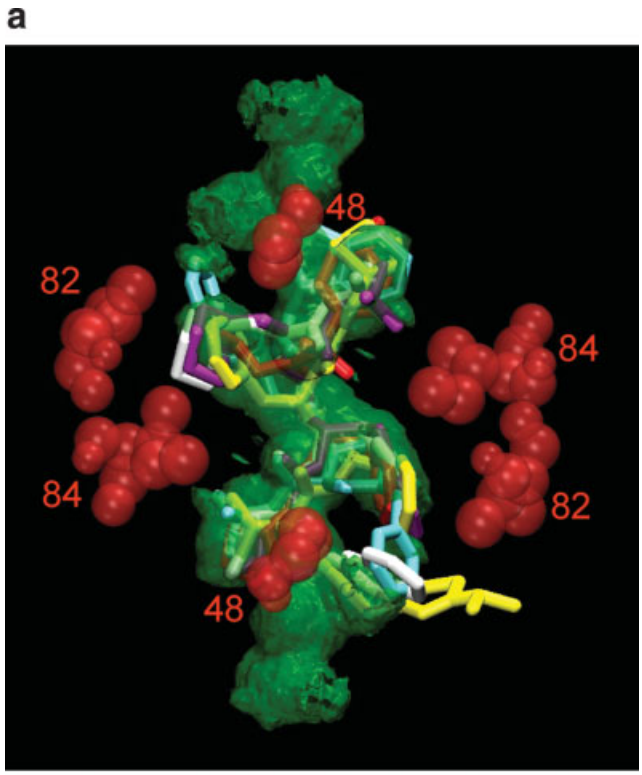

Fig. 3. Two views of level 8 isodensity contours of the substrate density (green) overlaid with crystal structures of bound APV (red), IDV (cyan), SQV (white), NFV (purple) and RTV (yellow). The active site residues I82, V84, and G48 are shown with red spheres. The hydroxyl groups common to all five inhibitors protrude downward in (**b**).

TABLE I. Computed Volumes (Å^3) of Inhibitors, With Other Measures of Molecular Size

	APV	IDV	SQV	NFV	RTV
V_{out}	128	180	213	166	256
V_{in}	267	315	319	288	308
V_{tot}	395	495	531	454	564
Nonhydrogen atoms	35	45	49	44	50
Molecular weight (Da)	506	614	671	664	721

V_{out}, volume outside the substrate envelope; V_{in}, volume within the substrate envelope; V_{tot}, total volume.

TABLE II. Values of Log($K_d^{mut}/K_d^{wild-type}$) for 5 Inhibitors and 13 HIV Protease Mutants

Mutation sets	APV	IDV	SQV	NFV	RTV
MDR5 (L10I/G48V/I54V/L63P/V82A)	0.52	1.88	2.55	1.94	
3X (L63P/V82T/I84V)	0.77	1.69	2.13	1.69	
NAM10 (L10I/M36I/S37D/M46I/R57K/L63P/A71V/G73S/L90M/I93L)		2.80	3.06	3.10	3.95
ANAM11 (L10I/M36I/S37D/M46I/R57K/L63P/A71V/G73S/L90M/I93/I84V)		2.98	3.29	3.13	4.56
I84V		0.57	0.60	0.54	1.73
V82F/I84V (Strain A)		1.78	1.34	1.34	2.58
V82F/I84V (Strain B)		1.83	1.32	1.30	2.57
V82F/I84V (Strain C)		1.85	1.32	1.30	2.58
L10I/L90M	0.60	0.48	0.78	0.47	0.58
M46I/I54V	0.31	0.16	0.85	0.28	0.65
V82A/I84V	0.74	1.20	0.90	0.28	1.31
QM (V82A/I84V/M46I/I54V)	1.19	1.46	2.19	1.27	2.15
HM (L10I/M46I/I54V/V82A/I84V/L90M)	1.93	2.30	3.29	2.33	3.18

TABLE III. Quantitation of Clinical Relevance [Eq. (3)] for HIV Protease Mutants

Mutation sets	$N_{i,\text{only}}$	$N_{i,\text{all}}$	Clinical relevance
MDR5 (L10I/G48V/I54V/L63P/V82A)	14	75	18.67
3X (L63P/V82T/I84V)	1	53	1.89
NAM10 (L10I/M36I/S37D/M46I/R57K/L63P/A71V/G73S/L90M/I93L)	0	0	N/A
ANAM11 (L10I/M36I/S37D/M46I/R57K/L63P/A71V/G73S/L90M/I93/I84V)	0	0	N/A
I84V	20	807	2.48
V82F/I84V (Strain A)	0	4	0.00
V82F/I84V (Strain B)	0	4	0.00
V82F/I84V (Strain C)	0	4	0.00
L10I/L90M	62	1264	4.91
M46I/I54V	1	390	0.26
V82A/I84V	4	166	2.41
QM (V82A/I84V/M46I/I54V)	7	28	25.00
HM (L10I/M46I/I54V/V82A/I84V/L90M)	4	9	44.44

$N_{i,\text{only}}$: number of clinical isolates having the listed mutations and no other major mutations.
$N_{i,\text{all}}$: total number of clinical isolates with the listed mutations.
Clinical relevance: $100\frac{N_{i,\text{only}}}{N_{i,\text{all}}}$.

DISCUSSION

The present results support the hypothesis that HIV protease inhibitors that conform better to the substrate envelope tend to be less susceptible to resistance mutations. The presumptive explanation is that a viable mutant must allow the protease to interact correctly with its substrates and so it will also tend to retain affinity for a substrate-like inhibitor. The present data do not definitively establish this mechanism, especially because nonspecific measures of inhibitor size, such as molecular weight, also are found to correlate with sensitivity to mutation. On the other hand, these additional correlations do not disprove the presumed mechanism; they may merely reflect the correlation of molecular weight, say, with V_{out}. Teasing apart the various correlations will require further studies. The ultimate aim of the present study, however, is to facilitate the design of new inhibitors that will resist mutation. It will therefore be of particular interest to observe the consequences of using the fit of candidate inhibitors to the substrate envelope as a figure of merit in computer-aided ligand-design. Forthcoming publications will describe such studies, and will report on the robustness of the resulting inhibitors to mutations.

The present analysis generalizes the notion of the substrate envelope to that of a substrate density, which falls rather gradually to zero from its maximum at the core of the substrate binding region. This approach provides more detailed information about the disposition of substrates in the binding site, as highlighted in Figure 2(a), and avoids the need to set an arbitrary level of substrate density at which to position a sharp substrate envelope. The substrate density is encoded on a 3D grid, allowing rapid calculation of the fit of a docked ligand to the substrate density. Analogous maps of ligand density, or of the density of a flexible receptor, could be useful in describing and modeling other molecular systems as well.

The present study also evaluates the clinical relevance of protease mutations based upon the sequences of clinical isolates. For example, L10I/L90M occurs rarely in the absence of other major mutations (clinical relevance 4.9, Table III), presumably because these two mutations alone confer less than one log of resistance to the clinical inhibitors studied here (Table II). An alternative approach to assessing clinical relevance might have been to

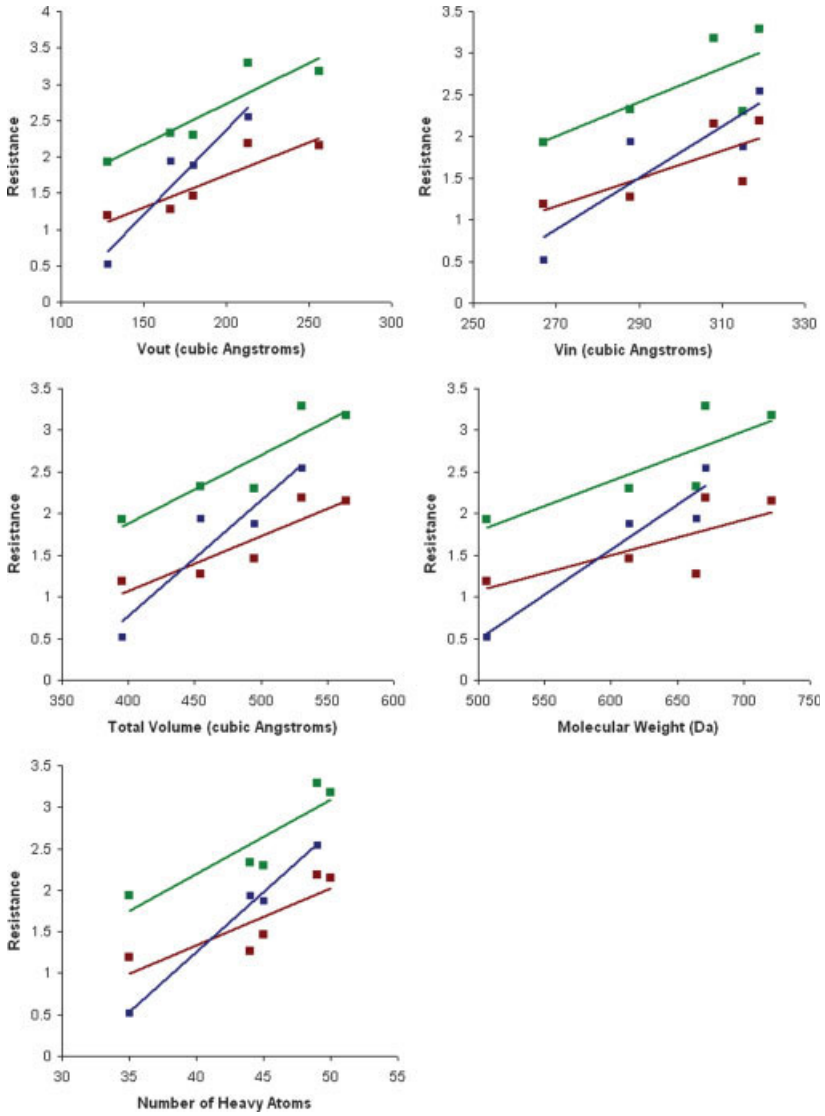

Fig. 4. Scatter plots of V_{out}, V_{in}, total volume, molecular weight, and number of nonhydrogen atoms of HIV protease inhibitors versus their log loss in affinity on going from wild-type to mutant, for the three most clinically relevant mutants. Blue, MDR5; green, HM; red, QM.

TABLE IV. Correlation Coefficients of $\log(K_d^{mut}/K_d^{wild-type})$ With Inhibitor Properties (Columns) for HIV Protease Mutants (Rows)

Mutation set	V_{out}	V_{in}	V_{tot}	Non-H atoms	Mol. weight
MDR5 (L10I/G48V/I54V/L63P/V82A)	0.96	0.88	0.94	0.99	0.96
3X (L63P/V82T/I84V)	0.97	0.89	0.95	0.99	0.95
NAM10 (L10I/M36I/S37D/M46I/R57K/L63P/A71V/G73S/L90M/I93L)	0.74	0.20	0.57	0.53	0.84
ANAM11 (L10I/M36I/S37D/M46I/R57K/L63P/A71V/G73S/L90M/I93/I84V)	0.94	0.17	0.84	0.79	0.83
I84V	0.89	0.10	0.77	0.71	0.81
V82F/I84V (Strain A)	0.77	0.17	0.71	0.55	0.56
V82F/I84V (Strain B)	0.75	0.20	0.69	0.52	0.50
V82F/I84V (Strain C)	0.75	0.20	0.69	0.52	0.50
L10I/L90M	0.28	0.28	0.30	0.04	0.10
M46I/I54V	0.68	0.46	0.65	0.60	0.53
V82A/I84V	0.62	0.57	0.63	0.44	0.22
QM (V82A/I84V/M46I/I54V)	0.91	0.75	0.91	0.84	0.73
HM (L10I/M46I/I54V/V82A/I84V/L90M)	0.91	0.75	0.91	0.89	0.82

rely on *in vitro* vitality scores of the mutant proteases,[21] which account for the enzymatic activity of the mutant against substrate. However, these data are unavailable for many mutants. In addition, the vitality may vary across substrates, whereas the clinical relevance score used here implicitly accounts for multiple substrates. This distinction may help explain why V82A/I84V (clinical relevance 2.4, Table III) appears more frequently than V82F/I84V (clinical relevance 0, Table III) in clinical isolates lacking other major mutations, despite the fact that the clinical inhibitors retain activity better against V82A/I84V than against V82F/I84V (Table II), and both mutants affect the catalysis of a model substrate similarly.[19,20] It is also worth noting that, although all the clinical inhibitors position the hydroxyethylene hydroxyl group outside the substrate envelope (see Fig. 3), this deviation should not provide a basis for resistance mutations because the hydroxyl contacts residues D25/D25′, which are essential for catalysis and therefore cannot mutate without eliminating the activity of the enzyme.

In summary, the failure of an HIV protease inhibitor to fit within the substrate envelope appears to correlate with its susceptibility to mutational resistance. Although this is a low resolution approach, the trend observed here suggests that designing ligands not only for tight-binding but also for fit to the substrate envelope could help accelerate the discovery of robust inhibitors of HIV protease.

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