Improved Structure−Activity Relationship Analysis of HIV-1 Protease Inhibitors Using Interaction Kinetic Data

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Despite the availability of large amounts of data for HIV-protease inhibitors and their effectiveness with wild type and resistant enzyme, there is limited knowledge about how this and other information can be systematically applied to the development of new antiviral compounds. To identify in vitro parameters that correlate with the efficacy of HIV inhibitors in cell culture, the relationships between inhibition, interaction kinetic, and cell culture parameters for HIV-1 protease inhibitors were analyzed. Correlation, cluster, and principal component analysis of data for 37 cyclic and linear compounds revealed that the affinities (K_D) determined from SPR-biosensor binding studies correlated better to cell culture efficacy (ED_{50}) than that of the inhibition constants (K_i), indicating that the conventional use of K_i values for structure−activity relationship analysis of HIV-1 inhibitors should be seriously reconsidered. The association and dissociation kinetic rate constants (k_{on} and k_{off}) alone showed weak correlations with ED_{50} values. However, ED_{50} values were most related to the free enzyme concentration in the viral particle ([E]), calculated from the rate constants and the total enzyme concentration in a viral particle. A structure−activity relationship analysis of the current data set was found to be valid for all classes of compounds analyzed. In summary, use of affinity, based on interaction kinetic rate constants, rather than inhibition constants, and theoretical consideration of the physiological conditions in the virus particle provide improved structure−activity relationship analysis of HIV-1 protease inhibitors.

Introduction

A functional HIV-1 protease is essential for the maturation and infectivity of HIV,[1] and HIV-protease inhibitors constitute some of the most effective drugs in the treatment of AIDS.[2] As not all potent inhibitors of the enzyme make good drugs, it is important to identify in vitro characteristics of HIV-1 protease inhibitors that may be used for structure−activity relationship (SAR) analyses that correlate with in vivo efficacy. Although SAR studies of HIV-1 protease inhibitors often contain cell culture data, at least for the more potent compounds (for example, refs 3−5), there is no critical evaluation of the correlation between the different types of data even when a discrepancy has been observed.[5] Though inhibition of HIV-protease is generally paralleled by a reduced rate of HIV replication in cell culture, the correlation between inhibition constants (K_i) and ED_{50} values is in fact rather poor. For example, data for C_2-symmetric compounds[5,6] reveal not only a poor correlation but also differences between structural classes of inhibitors (Figure 1). An analysis with a larger set of compounds of even greater diversity also showed a poor correlation between cell culture and inhibition data,[7] indicating that this is a general problem not limited to the C_2-symmetric compounds. The issue of correlation between enzyme inhibition and inhibition of antiviral replication has, however, been brought up in the context of resistance studies. For example, vitality values have been introduced in order to account for effects of mutations on k_{cat} and K_M values as well as on K_i values.[8] This has been extended to account for differences in kinetic constants for different substrates.[9,10] However, Klabe et al. have presented an elegant correlation analysis between the in vitro data for a number of mutants and corresponding cell culture data, showing that the correlation is low and that it is not influenced by adjusting K_i values with any combination of k_{cat} or K_M, for example as when using vitality values.[11] To our knowledge, there are no studies that

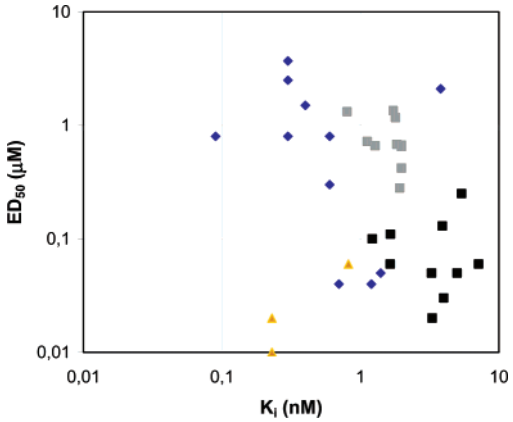

Figure 1. Relationship between ED_{50} and K_i values for three series of HIV-1 protease inhibitors. Fluoro-substituted P1/P1′ B268 analogues (■) and B369 analogues (■),[6] P1/P1′ analogues of B268 (◆),[5] and references (saquinavir, ritonavir, and nelfinavir) (▲).[6]

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address the weak correlation between inhibition of wild-type enzyme and viral replication.

Attaining a valid in vitro model system that correlates with the in vivo system it represents is not trivial, as the experimental conditions for enzymatic assays are typically quite different to those for viral replication. Cell culture assays obviously have higher complexity than enzymatic assays and will be influenced by secondary effects such as absorption and compound stability, making interpretations difficult. In addition, few models relate the degree of virus suppression to in vivo pharmacokinetic and pharmacodynamic parameters, such as binding constants to viral targets, although there are studies that attempt to resolve this deficiency.[12] Finally, to be of use for the design of effective antiviral drugs, the in vitro data obtained must be related to the in vivo efficacy in patients. Since medicinal chemists often rely on inhibition constants as a measure of inhibitory potency, especially in the early stages of a project, it is important that these values correlate with efficacy in cell culture and in vivo. Alternatively, complementary data must be incorporated into the drug discovery process at an early stage.

A biosensor-based analysis of the interactions between HIV-1 protease and a variety of inhibitors has successfully been adopted to obtain detailed interaction data,[13−16] but until now this type of data has not been compared to cell culture data. Data from these previous studies was therefore compiled and their correlation with ED_{50} values analyzed. A statistical analysis of the data set was also performed in order to identify structure−activity relationships of the inhibitors that correlated with their efficacy in cell culture.

Methods

Inhibition and biosensor data were primarily compiled from previous studies.[14,16,17] The inhibition constant (K_i) for atazanavir was determined as described previously,[18] and the K_i value for lopinavir[19] was incorporated to this data set after normalization using ritonavir as an internal reference from both studies. Inhibition of HIV-1 replication in cell culture was determined by measuring the cytopathogenicity of the virus at different concentrations of the inhibitor in MT4-cells.[20] The concentration of inhibitor resulting in 50% inhibition (ED_{50}) was determined. Correlation, cluster and principal component analysis (PCA) were performed with STATISTICA.[21] For cluster and PCA analyses, the data was standardized according to standard procedures.[21]

Estimation of Free Enzyme Concentration. To quantify the amount of active enzyme inside the virus in the presence of different inhibitors, the concentration of free enzyme ([E]) was calculated from the interaction kinetic constants (k_{on} and k_{off}) and the total enzyme and inhibitor concentrations ($[E]_{tot}$ and $[I]_{tot}$, respectively) using eq 1:

$$[E] = -\frac{[I]_{tot} - [E]_{tot} + \frac{k_{off}}{k_{on}}}{2} + \sqrt{\left(\frac{[I]_{tot} - [E]_{tot} + \frac{k_{off}}{k_{on}}}{2}\right)^2 + \frac{k_{off}}{k_{on}} \times [E]_{tot}} \quad (1)$$

The parameters included in the equation were defined as:

$$K_D = \frac{[E][I]}{[EI]} = \frac{k_{off}}{k_{on}} \quad (2)$$

$$[E]_{tot} = [E] + [EI] \quad (3)$$

$$[I]_{tot} = [I] + [EI] \quad (4)$$

The total protease concentration ($[E]_{tot}$) in an HIV particle was estimated by assuming n protease molecules in a spherical viral particle with radius r. By including Avogadro's constant (N_A) the concentration was converted to molar units.

$$[E]_{tot} = \frac{3n}{4\pi r^3 N_A} \quad (5)$$

The volume was estimated by assuming a radius of 80 nm[22] and did not account for the space taken up by membranes and other molecules. The lowest theoretical enzyme concentration, representing the minimum concentration required for an infective virus particle, was determined by setting the number of protease molecules in a viral particle to one. The lowest theoretical inhibitor concentration was estimated in the same way.

Results

To elucidate which features of inhibitors and their interactions with HIV-1 protease correlate with their cell culture potencies, experimental data for a set of 58 inhibitors against HIV-1 protease was compiled. Compounds whose interaction with the target was undetectable ($k_{on} < 100\ M^{-1}\ s^{-1}$) or that associated faster than could be reliably measured with the current experimental setup ($k_{on} > 1 \times 10^8\ M^{-1}\ s^{-1}$) were excluded from the analysis. The total data set analyzed thus comprised 37 compounds (Table 1). The data set included a structural classification, the molecular weight, inhibition (K_i),[17] interaction kinetic (k_{on}, k_{off}, and K_D),[14,16] and previously unpublished cell culture data (ED_{50}). The logarithm of the parameters was used throughout the analysis.

A theoretical minimal concentration of 8.0×10^{-7} M enzyme ($[E]_{tot}$) was obtained using eq 5 and assuming one enzyme molecule per viral particle. By using this value, the corresponding value for the inhibitor concentration, and the interaction kinetic constants for different inhibitors, an estimate of the free enzyme concentration in the viral particle ([E]) was calculated. This allows estimation of the effective concentration of active enzyme.

Correlation between Kinetic and Cell Culture Data. The pairwise (linear) correlation (Pearson, r) between the eight parameters in Table 1 was analyzed. The molecular weight of the compounds did not correlate significantly with any of these parameters ($r < 0.5$, not shown), nor did the type of inhibitor, as classified by Hämäläinen et al.[7] The correlations between the other parameters are shown in Figure 2. The ED_{50} values showed the highest correlation ($r = 0.93$) with the free enzyme concentration in the virus. The correlation of affinity (K_D) to ED_{50} values was similar (0.90), while the individual rate constants, k_{on} and k_{off}, showed weak correlations (−0.66 and 0.71, respectively). The inhibition constants (K_i) showed a weaker correlation ($r = 0.79$) with the ED_{50} values than that of the K_D values, despite their theoretical equivalence. The correlation between K_D and K_i values was 0.9, indicating that these parameters were not identical.

Cluster analysis was also performed with the data in order to identify additional interrelationships between

Table 1. Classification and Characterization of HIV-1 Protease Inhibitors

name	class [a]	MW g/mol	k_{on},[b] $M^{-1}\,s^{-1}$	k_{off},[b] s^{-1}	K_D,[b] nM	K_i,[b] nM	[E],[c] M	ED_{50},[d] μM
A015	4	711	1.09×10^5	0.938	7.03×10^3	140	7.37×10^{-7}	>140
A016	4	743	172	0.0605	3.09×10^5	5000	7.98×10^{-7}	>67
A017	4	619	4.36×10^4	0.179	4.99×10^3	700	6.86×10^{-7}	>80
A018	4	679	3.48×10^5	0.474	1.30×10^3	660	5.65×10^{-7}	>58
A021	6	635	6.87×10^5	0.0273	39.8	3.1	1.6×10^{-7}	1.55
A023	6	663	2.00×10^5	0.139	1.14×10^3	43	4.75×10^{-7}	>75
A024	6	663	2.21×10^5	0.0685	343	19	3.67×10^{-7}	>75
A030	6	689	5.12×10^5	0.0420	169	5.6	2.18×10^{-7}	2.90
A037	3	783	2.04×10^5	3.65×10^{-4}	1.95	0.09	3.7×10^{-8}	2.58
A038	3	835	2.93×10^4	4.87×10^{-4}	17.5	0.3	1.07×10^{-7}	5.60
A045	6	689	4.99×10^5	0.263	675	6.2	4.37×10^{-7}	13.0
A047	6	632	1.88×10^5	0.0697	577	10.1	3.9×10^{-7}	>79
B249	4	617	4.10×10^4	0.273	7.91×10^3	1400	7.22×10^{-7}	>56
B268	3	615	3.55×10^5	3.67×10^{-3}	10.8	0.4	8.6×10^{-8}	1.32
B277	3	519	134	4.85×10^{-3}	3.84×10^4	2400	7.83×10^{-7}	>192
B295	2	604	9.02×10^5	0.436	420	37	4.26×10^{-7}	3.31
B322	4	643	1.85×10^6	0.0677	48.2	0.91	1.54×10^{-7}	1.17
B347	4	615	9.20×10^3	0.0270	2.99×10^3	40	6.54×10^{-7}	22.9
B355	2	625	1.08×10^6	0.373	366	55	3.81×10^{-7}	10.12
B365	4	599	3.04×10^5	0.0309	102	2.1	2.39×10^{-7}	1.08
B369	4	653	6.39×10^6	1.33×10^{-2}	13.9	0.6	3.98×10^{-8}	0.10
B388	4	634	5.97×10^6	2.27×10^{-2}	4.42	0.054	5.33×10^{-8}	0.12
B408	3	773	8.89×10^5	1.69×10^{-3}	21.1	0.3	3.8×10^{-8}	0.65
B409	3	779	3.48×10^5	4.32×10^{-4}	3.04	1.2	3.09×10^{-8}	0.04
B412	3	857	1.81×10^5	8.17×10^{-4}	18.2	1.4	5.79×10^{-8}	0.16
B425	4	637	6.66×10^5	2.34×10^{-2}	35.8	1.4	1.51×10^{-7}	1.44
B429	3	769	3.23×10^5	3.73×10^{-4}	1.18	0.61	2.98×10^{-8}	0.64
B435	4	611	1.01×10^5	6.53×10^{-3}	69.7	0.1	1.97×10^{-7}	2.59
B439	3	823	8.11×10^4	1.63×10^{-3}	34.2	0.8	1.17×10^{-7}	2.05
B440	3	781	4.77×10^5	3.03×10^{-4}	0.643	0.55	2.22×10^{-8}	0.24
Amp	1	506	4.43×10^6	4.88×10^{-3}	1.10	0.23	2.92×10^{-8}	0.08
Ind	1	614	1.53×10^6	1.58×10^{-3}	1.07	0.31	2.82×10^{-8}	0.05
Nelf	1	664	6.63×10^5	6.68×10^{-4}	1.64	0.54	2.79×10^{-8}	0.02
Rit	1	721	3.92×10^6	2.16×10^{-3}	0.608	0.59	2.07×10^{-8}	0.05
Saq	1	671	8.17×10^5	2.27×10^{-4}	0.315	0.23	1.48×10^{-8}	0.01
Lop	1	629	6.63×10^6	6.54×10^{-4}	0.101	0.08	8.83×10^{-9}	0.01
Ataz	1	705	1.67×10^6	6.90×10^{-4}	0.403	0.24	1.8×10^{-8}	0.006

[a] Classification:[7] (1) clinical, (2) linear non-B268 analogues, (3) P1/P1′ analogues of B268, (4) P2/P2′ analogues of B268, (5) cyclic urea compounds, (6) cyclic sulfamide compounds. [b] Values were taken from previous published work.[14,16] The K_i value of atazanavir was determined as described previously[18] and an estimated value for the K_i value of lopinavir was obtained by normalizing data from Sham et al.[19] to this dataset using ritonavir as an internal reference. [c] [E] was calculated from the values of k_{on} and k_{off} with eq 1, assuming $[E]_{tot}$ and $[I]_{tot} = 8.0 \times 10^{-7}$ M. [d] ED_{50} represents the concentration of inhibitor that results in 50% inhibition of the replication of HIV-1 virus in MT4 cells as described in Materials and Methods.

the kinetic parameters. The analysis was initially performed with the structural classification included as one of the parameters. The clustering of compounds adhered to the structural classification (data not shown). Thus, to ensure that the results were not due primarily to this arbitrary parameter, the structural classification was removed from analysis. The values for the logarithm of each parameter were standardized to ensure that variability within the data set did not distort or bias the analysis.

The analysis showed that ED_{50} was most closely related to [E] and K_D, followed by K_i (Figure 3). The dissociation rate constant (k_{off}) was also in the same cluster but less related to the ED_{50}, [E], and K_D. The log k_{on} and molecular weight group together, separately from the other parameters.

Analysis of Inhibitors. The distribution of the compounds in the ED_{50} vs [E] plot (Figure 2) was investigated more closely (Figure 4) so as to determine if any compounds deviated considerably from the correlation confidence interval or from other structurally similar compounds. The lead compound B268 was used as a reference throughout, and its structure is presented together with the structures of the discussed inhibitors in Figure 5. To ease the analysis, three different classes of compounds were highlighted in three different displays of the data set: P1/P1′ analogues of B268 (Figure 4a); P2/P2′ analogues of B268 with two reference compounds (Figure 4b); cyclic sulfamides (Figure 4c).

Of the P1/P1′ analogues of B268, only B277 was considerably different from the others (Figure 4a). It is the only analogue that has a smaller P1/P1′ residue than B268. The P2/P2′ analogues formed three groups, better, similar, and worse than B268 (Figure 4b), and the cyclic sulfonamides were all less effective than B268 (Figure 4c). Of the compounds outside the 95% confidence interval, P1/P1′ analogues had lower values of log [E] and higher values of log ED_{50} (below the line, Figure 4a). Other compounds generally had higher values of log [E] and lower values of log ED_{50} (above the line, Figure 4b,c).

To identify more complex relationships between inhibitors, cluster analysis was performed (Figure 6). Two main clusters defined the data set, visualized in the dendrogram in Figure 6 (groups 1 and 2). Group 1 contained the clinical inhibitors (amprenavir, indinavir, nelfinavir, ritonavir, saquinavir, lopinavir, and atazanavir) and other more effective compounds in two subgroups. The subgroup with all clinical inhibitors except amprenavir also contained the two most effective P2/P2′ analogues of B268 (B388 and B369) (group 1a). Note that B388 is the asymmetric combination of the

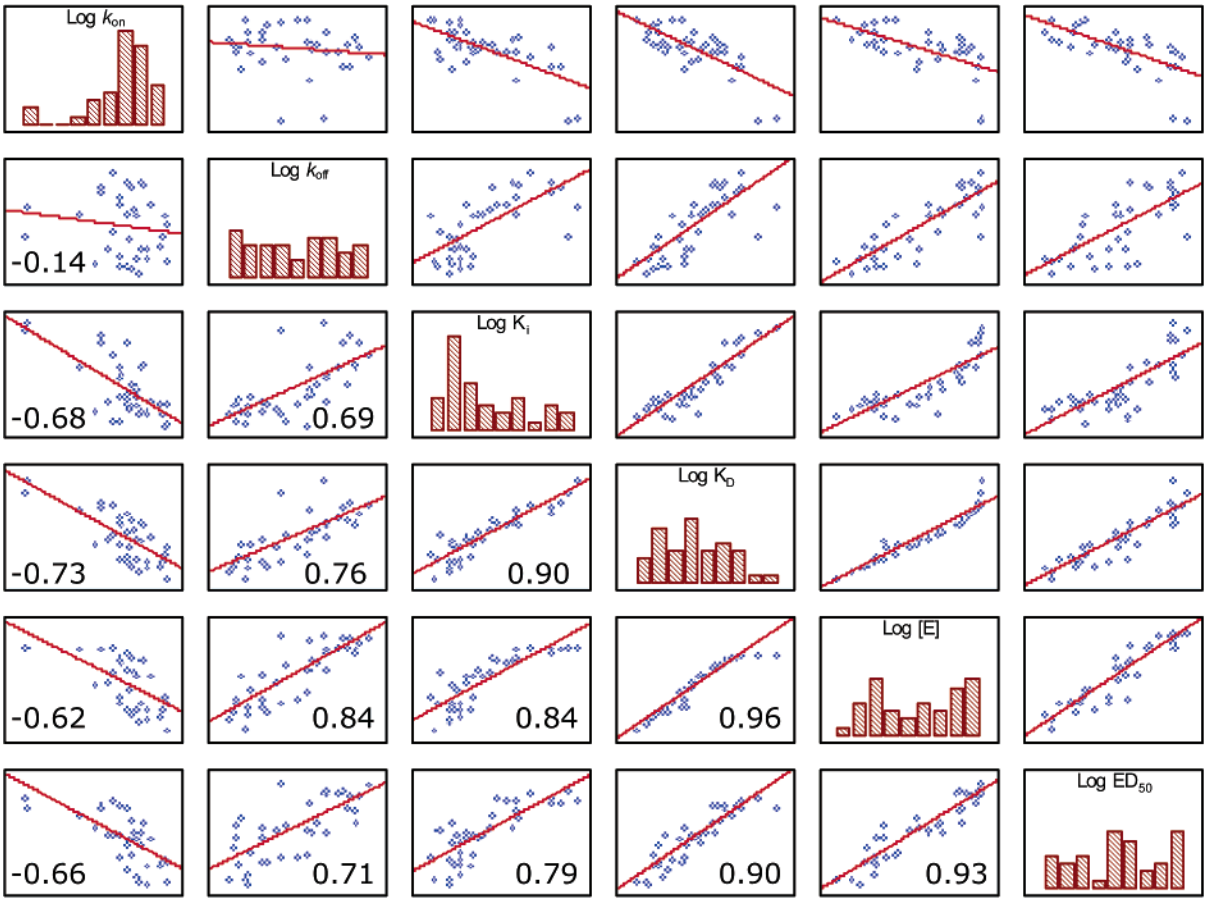


Figure 2. Correlation matrix of experimental variables from interaction (k_{on}, k_{off}, K_D), inhibition (K_i), and ED_{50} analysis for 37 compounds. The concentration of free enzyme ([E]) in a virus particle was calculated using eq 1, as discussed in the text. The numbers in the matrix represent the correlation coefficient (r).

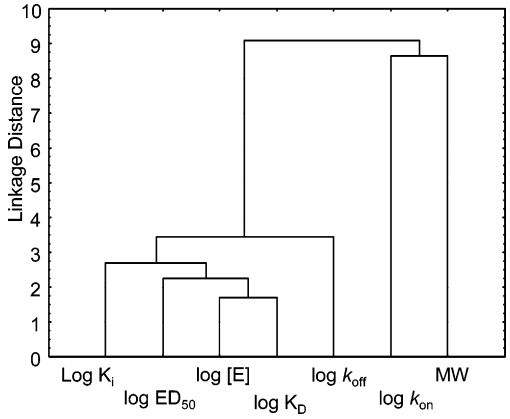


Figure 3. Dendrogram for cluster analysis of seven parameters (ED_{50}, [E], k_{on}, k_{off}, K_D, K_i, and molecular weight) for 37 compounds. Euclidean distances based on nearest neighbor (single linkage) are displayed.

two symmetric compounds B268 and B369. The other subgroup contained analogues of B268 with increased size of P1/P1′ (group 1b), and the lead compound B268 was located in the second cluster and grouped together with a number of P2/P2′ analogues (group 2a). Compounds in this cluster showed no improvement over B268 (Figure 4). The P2/P2′ analogues that were less effective than B268 were dispersed throughout the second cluster (Figure 6, group 2), except for A016 that was in a completely separate cluster. The linear reference compounds (B355 and B295) were structurally similar to each other and grouped together in the cluster analysis (Figure 6, group 2b). The cyclic sulfonamide compounds (scaffold no. 4 in Figure 5a) were all less effective binders and inhibitors than B268 (Table 1); however, the two most effective of these compounds, A030 and A021, appeared to be more closely related to a number of the P2/P2′ analogues, including B268 (group 2a). The remaining four cyclic compounds clustered together (group 2c). Two compounds were not included in either of the two clusters: B277, the only analogue with decreased size of P1/P1′, and A016, a P2/P2′ analogue of B268. Both of these were less effective compounds and had k_{on} values that were low as compared to the other 35 inhibitors included in the analysis (Table 1).

Principal Component Analysis. Principal component analysis (PCA) was performed to further characterize the dataset (Figure 7). Two factors were sufficient to describe 86.8% of the dataset (Figure 7a). All of the parameters contributed to factor 1 (69.26% of the dataset), although molecular weight contributed least. Primarily k_{on}, k_{off} and molecular weight contributed to factor 2 (17.6% of the dataset) (Figure 7b). The distribution of the compounds on the plane of factor 1 vs factor 2 is shown in Figure 7c. The distribution in the PCA analysis helped to explain the distribution of compounds in the cluster analysis. For example, the compounds B277 and A016 were clearly distinct from the other compounds in the PCA analysis, confirming their separate grouping in the cluster analysis. In addition,

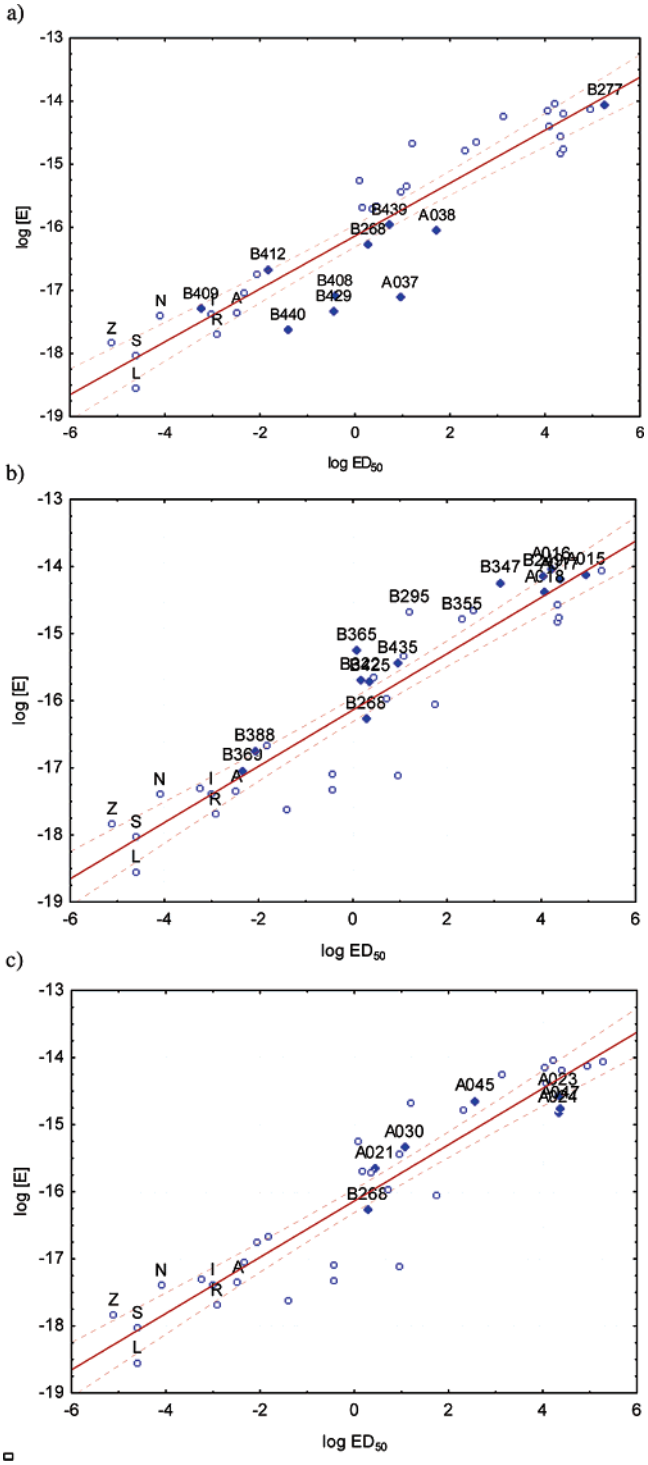


Figure 4. Correlation plot of log ED_{50} and log[E] for 37 compounds. The three panels include all compounds but highlight different sets of compounds: (a) P1/P1′ analogues of B268, (b) P2/P2′ analogues of B268, with two non-B268 analogues B295 and B355 labeled, and (c) cyclic sulfamides. All graphs show B268 and the seven clinical inhibitors: amprenavir = A, atazanavir = Z, indinavir = I, lopinavir = L, nelfinavir = N, ritonavir = R, and saquinavir = S.

amprenavir differed from the other clinical compounds primarily due to differences in factor 2. Finally, the most effective compounds all had a value of about 1 or more for factor 1 in the PCA analysis. There was no significant influence on the PCA when K_i values were excluded (data not shown).

Discussion

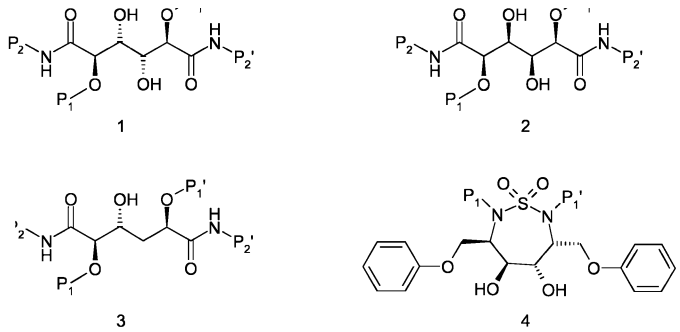
It is critical that an experimental system intended to model a more complex system, such as the interior of a viral particle, is well defined and that the parameters obtained are relevant. Inhibition constants are generally determined with enzyme concentrations in the nanomolar range, well below substrate or inhibitor concentrations. In a viral particle it is impossible to achieve such low concentrations of enzyme (or inhibitor), and enzyme concentrations are of the same order as substrate concentrations. Consequently, although the standard assumptions for enzyme inhibition measurements are useful for determining inhibition constants by steady-state analysis, this approach may not provide the most informative data.

Although the calculated theoretical minimum concentration of enzyme and inhibitor is much higher than the concentrations used in standard enzyme inhibition assays, the actual concentration of protease in the virus might be even higher. For example, it has been estimated that a virion contains about 2000 copies of the MA, CA, and NC proteins cleaved from the glycosaminoglycan (gag) precursor polyprotein (for review of the structural biology of HIV see ref 23). The polymerase (pol) precursor polypeptide is present in a 20-fold lower concentration and two copies are needed to produce the dimeric protease molecule. Consequently, the number of protease molecules could be up to 50 per virus (3.9×10^{-5} M) rather than one, as we assumed in the calculation of the theoretical minimum enzyme concentration. However, as long as the ratio of enzyme to inhibitor concentration was maintained, the correlation to ED_{50} values was unchanged (data not shown). Large differences between the concentration of enzyme and inhibitor would clearly alter the conditions in the virus particle considerably.

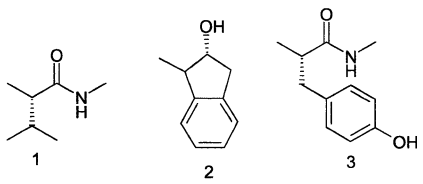
For a model system to be useful, the parameters that are determined must be relevant in the system to be described. Inhibition studies gave less reliable information about cell culture efficacy than biosensor analysis, since K_i values had a weaker relationship to ED_{50} values than that of K_D values. Although both parameters represent the dissociation constant for the inhibitor−enzyme complex, there is clearly a discrepancy between the two methods used to determine the values. This was in accordance with a previous study which showed that the parameters are not strictly correlated.[14] Significant differences in these two methods include pH and ionic strength, both of which can considerably influence the interactions of inhibitors to HIV-1 protease.[24,25] The conditions used in inhibition assays are selected to give high enzymatic activity; acidic pH and high ionic strength are therefore used. Biosensor analysis does not require a substrate as a reporter molecule to monitor the interaction with the inhibitor. The system is therefore less complex, i.e., contains fewer interactions. Furthermore, as biosensor analysis is not restricted to conditions of optimal enzyme activity; measurements can be performed at lower ionic strengths and neutral pH, closer to conditions in cell culture.

Although the free enzyme concentration was highly correlated to the cell culture efficacy of the studied inhibitors, the scatter plot of free enzyme concentration versus cell culture data revealed a nonuniform distribu-

a)



b)



c)

Compound	Scaffold	P_1 and P_1'	P_2	P_2'
B277	1		1	1
B268	1		1	1
A037	1		1	1
B429	1		1	1
B408	1		1	1
B409	1		1	1
B440	1		1	1
B439	1		1	1
A038	1		1	1
B412	1		1	1
B388	1		2	1
B369	1		2	2
B347	2		1	1
B425	3		2	2
A016	1		3	3
A021	4		-	-
A030	4		-	-

d)

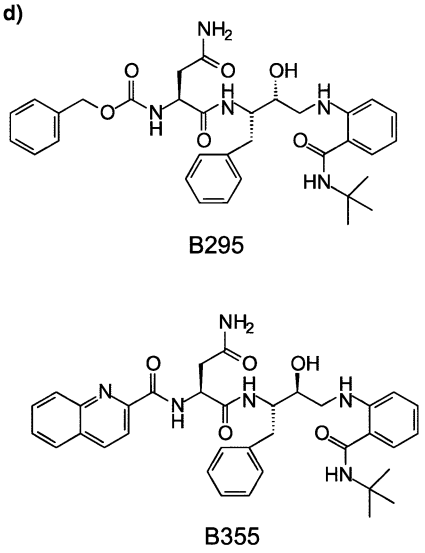


Figure 5. Structures of inhibitors: (a) scaffolds, (b) P2 and P2′ groups, (c) linear and cyclic compounds based on scaffolds defined in a, and P2/P2′ groups defined in b, and (d) linear compounds with unique scaffolds.

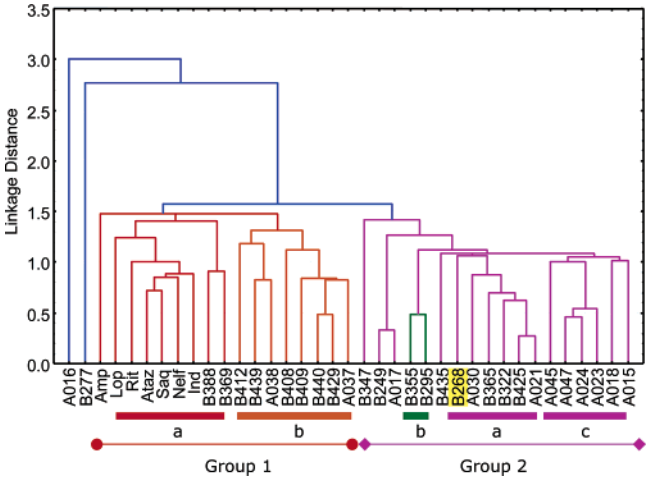


Figure 6. Dendrogram for cluster analysis of 37 inhibitors based on seven parameters (ED_{50}, [E], k_{on}, k_{off}, K_D, K_i, and molecular weight). Euclidean distances based on nearest neighbor (single linkage) are displayed. The main clusters (Group 1 and 2) and subgroups (a, b, and c) are labeled to clarify the presentation of the figure in the text.

tion of the compounds, with some compounds deviating significantly from the correlation. Since cell culture data actually describes inhibition of viral replication inside cells, there are obviously additional parameters that can be significant. Differences in membrane permeability, for example, could be responsible for this deviation. However, data for the interaction of the clinical inhibitors with liposomes (S. Cimitan et al., manuscript in preparation) did not explain the distribution of these compounds in the plot.

In addition to identifying kinetic parameters that correlate with ED_{50} values, this study also provided information about the characteristics of inhibitors and how compounds were related. The compounds in clinical use (amprenavir, indinavir, nelfinavir, ritonavir, saquinavir, lopinavir, and atazanavir) were generally the most effective in cell culture, and their potencies were well described by the free enzyme concentration in the viral particle. Amprenavir did not cluster closely together with the other clinical inhibitors. This may be accounted for by a lower molecular weight and faster association rate of amprenavir as compared to the other clinical inhibitors. However, amprenavir was not the most potent compound, indicating that reducing the molecular weight and increasing the association rate alone is not enough to achieve a potent inhibitor of viral replication.

Two linear compounds, B388 and B369, clustered together with the clinical inhibitors and were potent inhibitors in cell culture. B388 is the asymmetric combination of B369 and B268 (Figure 5), the latter compound clearly being less effective in cell culture. Other modifications of the P2/P2′ groups of the lead compound B268 generally had little impact or were unfavorable (a structural description of the current set of inhibitors has been published previously[14]). For B369 the changes in P2/P2′ were only beneficial when the central hydroxyls were maintained, as when one of these was removed (B425) the improved characteristics were lost. Similarly, when the stereochemistry of the central hydroxyls of B268 was inverted (B347), the inhibitory effect in cell culture was reduced. The different effects

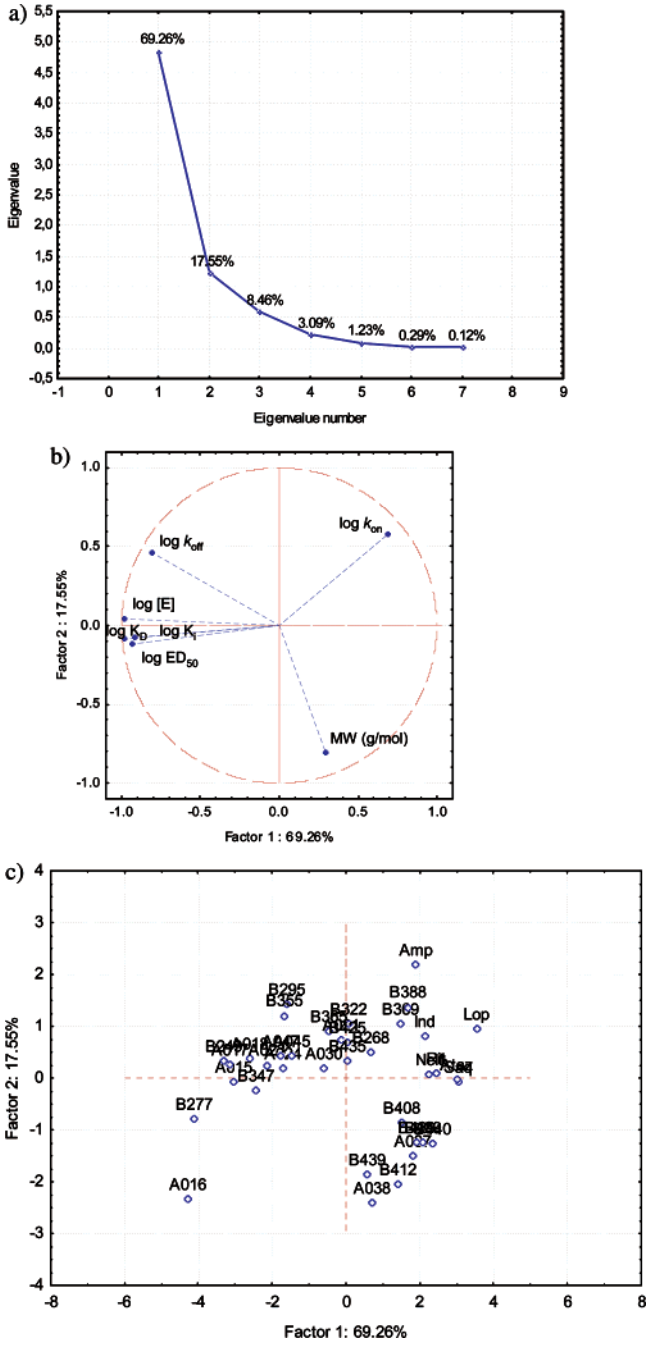


Figure 7. Principal component analysis (PCA) of the data set comprising 37 inhibitors and seven parameters (ED_{50}, [E], k_{on}, k_{off}, K_D, K_i, and molecular weight). (a) Eigen values for the factors describing the dataset, (b) contribution of the seven parameters to factors 1 and 2 of the PCA, and (c) distribution of the 37 inhibitors as described by factors 1 and 2 of the PCA.

of modifications depending on the lead compound (B268 or B369) highlights the importance of structurally optimizing the entire compound simultaneously, rather than region-by-region.

Interestingly, all P1/P1′ analogues of B268 did not cluster together with the lead compound B268 (Figure 6, group 2) but were more related to B369 and the clinical compounds (Figure 6, group 1). Increasing the size of the P1/P1′ side chain of B268 generally resulted in improved efficacy of the inhibitor. Decreasing the size of the P1/P1′ side chain resulted in an ineffective compound in cell culture, as illustrated by B277. Previ-

ous observations on the kinetic consequences of these structural changes[14] were now validated by cell culture data.

This study confirmed that compounds previously found to have low potential as drug leads[14] also had low efficacy in cell culture. These include two linear non-B268 analogues, B295 and B355, which have been found to have fast dissociation rates. Similarly, the cyclic sulfonamide compounds also have fast dissociation rates; this study confirmed that they also had low potency in cell culture. Moreover, their efficacy could be described by the same parameters as the linear compounds, although their interaction with the enzyme is slightly different and high cell culture efficacy has been difficult to achieve.

Cell culture studies provide an important bridge between cell-free in vitro assays and in vivo assays. However, it should be noted that correlating protease inhibition to viral inhibition in cell culture will by no means guarantee a sufficient description of a complex in vivo system. Additional considerations for describing in vivo efficacy include bioavailability (ADME), pharmacokinetics, resistance, and combinations with other drugs. Development of new drugs against HIV is based on addressing many of these questions; for a review see ref 2. In addition, recent biosensor-based studies of the ADME characteristics of HIV protease inhibitors have shown good correlation between the interaction parameters for liposomes, human serum albumin, and α_1-acid glycoprotein and bioavailability (Cimitan et al, manuscript in preparation). It is anticipated that the early stages of the drug discovery process can be improved considerably by extending the strategy presented here with this type of in vitro ADME data.

Conclusions

This study shows that interaction kinetic data are superior to inhibition data for describing the inhibitory potencies of HIV-1 protease inhibitors in a viral replication assay and that the theoretical in vivo conditions in the virus particle should be carefully accounted for. Thus, although inhibition measurements are still essential for the establishment of a novel compound as an inhibitor, interaction kinetic analysis should be used for a more detailed analysis of the interaction. The association and dissociation rates provide important information for understanding the interaction between HIV-1 protease and inhibitors; however, these individual parameters do not appear to be essential for description of viral inhibition in cell culture. The total protease concentration in HIV-1 virus particles ($[E]_{tot}$), calculated from k_{off}/k_{on} (K_D), can be used to model cell culture efficacy. It should therefore be possible to limit K_i measurements to cases when inhibition as such needs to be established. The approach employed here was found to be valid for all classes of compounds analyzed and can be used for structure−activity relationship analysis.

Appendix

Abbreviations: r, correlation coefficient; K_i, inhibition constant, K_D, dissociation constant; k_{on}, association rate constant; k_{off}, dissociation rate constant; ED_{50}, concentration of inhibitor inhibiting viral replication by 50%; SAR, structure−activity relationships; SPR, surface plasmon resonance; PCA, principal component analysis.

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