Articles

Relationships between Structure and Interaction Kinetics for HIV-1 Protease Inhibitors

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The interaction between HIV-1 protease and 58 structurally diverse transition-state analogue inhibitors has been analyzed by a surface plasmon resonance based biosensor. Association and dissociation rate constants and affinities were determined and displayed as $k_{on}-k_{off}-K_D$ maps. It was shown that different classes of inhibitors fall into distinct clusters in these maps. Significant changes in association and dissociation rates were found as a result of modifying the P1/P1′ or P2/P2′ side chains of a linear lead compound. Similarly, cyclic urea and cyclic sulfamide inhibitors displayed different kinetic features and the affinities of both classes of cyclic compounds were limited by fast dissociation rates. These results confirm that association and dissociation rates are important features of drug−target interactions and indicate that optimization of inhibitor efficacy may be guided by aiming for high association and low dissociation rates rather than high affinity alone. The present approach thus provides a new tool for structure−interaction kinetic analysis and drug discovery.

Introduction

Intermolecular interactions are essential for the function of all biomolecules, and studies of the interaction between drugs and their targets are important for the development of effective drugs. A variety of experimental and modeling techniques have been used to study different aspects of such interactions. Surface plasmon based biosensor technology has emerged as a new tool for kinetic analysis of biomolecular interactions.[1] Recent improvements in sensitivity and methodology now allows the technique to be used for interaction studies with low molecular weight compounds as analytes (for a review, see Rich and Myszka[2]). This has permitted the determination of association and dissociation rate constants (k_{on} and k_{off}, respectively) for the interaction between HIV-1 protease and various inhibitors.[3]

HIV-1 protease is an important target for anti-HIV chemotherapy, with six inhibitors currently being used in the clinic (see Tomasselli and Heinrikson[4] and Richman[5] for current reviews). Although various therapeutic regimes and drug combinations result in efficient reduction of viral replication, side effects and high cost limit the use of these compounds. Moreover, resistance development, due to selection of mutant viral strains that are not sensitive to drug treatment regimes, is a major concern in the long-term treatment of patients. Despite the structure-based drug design approach that has been adopted,[6,7] limited information concerning the interaction kinetics of the drugs with the enzyme is available.[8,9] In addition, the limitations of HIV-1 protease inhibition assays, for example, due to non-Michaelis−Menten conditions and enzyme instability, prevent determination of the inhibition constants of the most potent inhibitors.[10]

The possibility of determining the association and dissociation rates for the interaction between the enzyme and an inhibitor, thus resolving the affinity into the individual rate constants ($K_D = k_{off}/k_{on}$), provides an approach for optimization of inhibitors guided by the independent variables rather than by a compound variable. This is supported by our previous studies where we have found significant differences in k_{on} and k_{off} for inhibitors with similar inhibitory potencies, that the association and dissociation rate constants are not correlated to each other, and that they are related to the structural features of the inhibitors.[11−13] The aim of this study was to determine the structure−interaction kinetic properties of a set of HIV-1 protease inhibitors by identifying how different structural features influence association and dissociation rates. For this purpose, a structurally diverse set of inhibitors was selected from the original library[12] and the association and dissociation rates, and affinities, were determined. The structure−kinetic analysis was based on these parameters and on inhibition constants, determined by an enzyme inhibition assay,[14] as well as the chemical structures of the compounds. The kinetic consequences of the structural differences between these compounds reveal new

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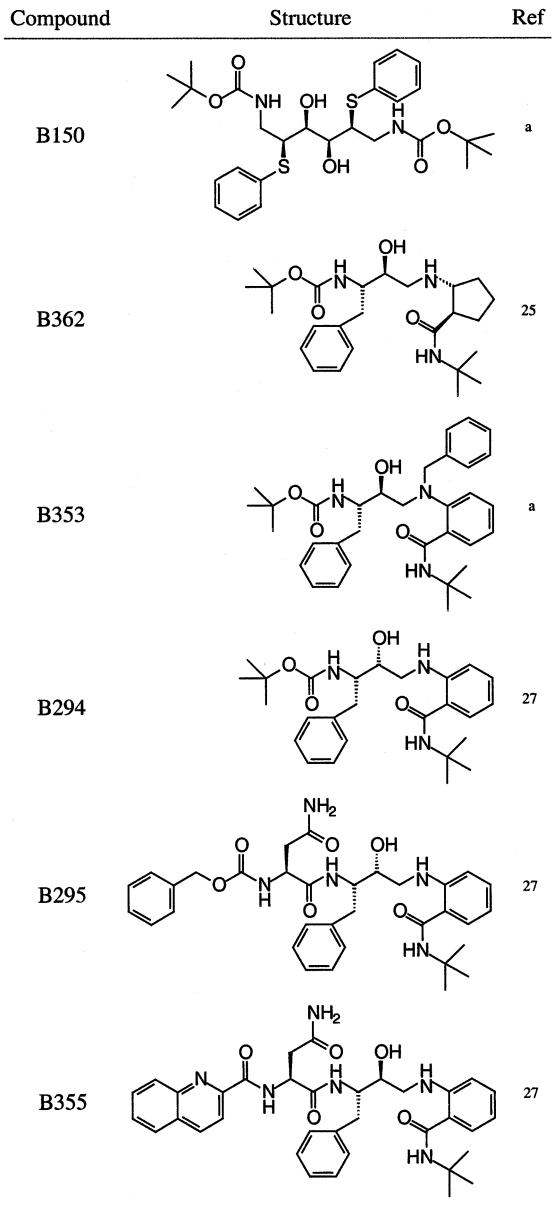


Figure 1. Selected compounds from HIV-1 protease inhibitor library: non-B268 analogues. [a] Unpublished data, Bertil Samuelsson.

features and aspects of inhibitor interactions with HIV-1 protease, expected to be of importance for designing more effective inhibitors.

Results

1. Selection of Inhibitors. A total of 58 inhibitors was selected for structure−kinetic analysis from a library of 290 HIV-1 protease inhibitors (Figures 1−5). The aim of the selection was to reduce the number of compounds while incorporating inhibitors that reflected the diversity of the larger dataset. To help accomplish this task, 10 chemical descriptors and 8 experimental variables were used to describe the compounds in the library. Principle component analysis (PCA) revealed seven components of statistical importance with a cumulative correlation coefficient (r^2) of 0.96. On the basis of this analysis, a diverse set of 50 compounds could initially be selected. To increase the number of interacting compounds, an additional 7 compounds were

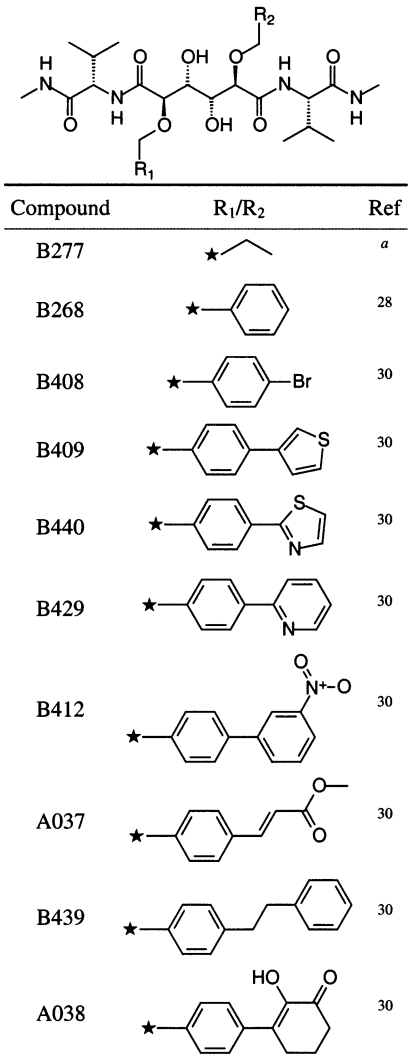
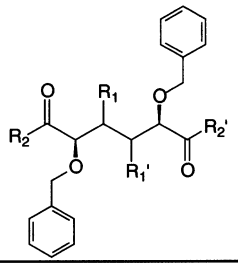


Figure 2. Selected compounds from HIV-1 protease inhibitor library: P1/P1′ analogues of B268. [a] Unpublished data, Bertil Samuelsson.

selected in a second complementary round, i.e., 7 compounds dissimilar to the previously chosen 50. Finally, amprenavir was added although it was not part of the initial library. Figure 6 shows the distribution of the chosen compounds in the first two dimensions of the PCA score plot. The 58 compounds finally selected from the library included five of the protease inhibitors in clinical use: U75875, linear monohydroxy isosteres, and dihydroxy isosteres (Figures 1−3) and cyclic urea and cyclic sulfamide inhibitors (Figures 4 and 5, respectively).

2. Identification of Binders. A primary screen at three different concentrations of each compound was used to identify those that interacted with the immobilized HIV-1 protease ("binders"). Out of the initially selected 50 inhibitors, 26 compounds were identified as binders at 1 μM or below and an additional 5 compounds when injected at 100 μM. The remaining 19 compounds could not be seen to interact with the target although several of the compounds have measurable K_i values (Table 1). The signal resulting from injection of these compounds was indistinguishable from that resulting from injection of buffer alone, a consequence of low affinity (k_{off}/k_{on} > 10 μM), slow association (k_{on} < 385 M^{-1} s^{-1}), or both. The complementary set of 7 com-



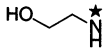
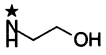
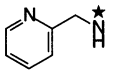
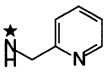
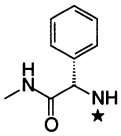
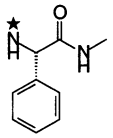
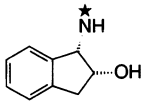
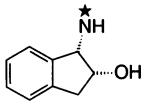
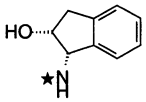
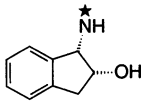
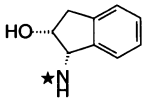
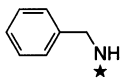
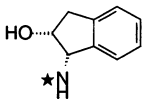
Compound	R_1	R_1'	R_2	R_2'	Ref
B366	(R)-OH	(R)-OH			28
A017	(R)-OH	(R)-OH	Thr-NMe	Thr-NMe	28
A016	(R)-OH	(R)-OH	Tyr-NMe	Tyr-NMe	28
A015	(R)-OH	(R)-OH	Phe-NMe	Phe-NMe	28
B316	(R)-OH	(R)-OH			28
B376	(R)-OH	(R)-OH			28
A018	(R)-OH	(R)-OH	Met-NMe	Met-NMe	28
B322	(R)-OH	(R)-OH	Ile-NMe	Ile-NMe	28
B365	(R)-OH	H	Val-NMe	Val-NMe	42
B385	(S)-OH	H	Val-NMe	Val-NMe	42
B347	(S)-OH	(S)-OH	Val-NMe	Val-NMe	42
B388	(R)-OH	(R)-OH		Val-NMe	44
B369	(R)-OH	(R)-OH			28
B425	(R)-OH	H			42
B435	(R)-OH	(R)-OH			44
B249	(R)-OH	(R)-OH	Val-OMe	Val-OMe	28
B248	(R)-OH	(R)-OH	Phe-OMe	Phe-OMe	28

Figure 3. Selected compounds from HIV-1 protease inhibitor library: P2/P2′ and central hydroxy analogues of B268.

pounds from the library and amprenavir all interacted with the enzyme, providing a final set of 39 binders.

3. Determination of Interaction Kinetic Constants. The association and dissociation rate constants (k_{on} and k_{off}, respectively) and the equilibrium dissociation constant ($K_D = k_{off}/k_{on}$) were determined for the interaction between HIV-1 protease and the 39 binders (Table 2). The graphical presentation of the data (Figure 7) shows that the compounds represent a wide range of association and dissociation rate constants and affinities. The combinations of k_{on} and k_{off} that result in the same K_D values are represented as diagonal lines. The compounds with the highest affinity are found in the upper left corner of the graph, representing high association and low dissociation rate constants.

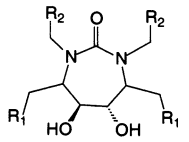
Very rapid association and dissociation rates may be difficult to measure accurately by the present method. When the association rate constant is very high, the observed association and dissociation rates may both be reduced by limited transport of analyte to and from the sensor surface. In the association phase, this is the result of a depletion of analyte close to the sensor surface whereas a corresponding enrichment of analyte may reduce the observed dissociation rate by causing rebinding in the dissociation phase. Furthermore, determination of fast dissociation rates is limited by the time needed for a complete exchange of solution in the measurement cell at the end of each sample injection. The upper limit for determination of k_{on} was estimated to be 3×10^6 M^{-1} s^{-1}, while the fastest k_{off} possible to determine accurately with the present experimental setup was judged to be approximately 0.1 s^{-1}. To some extent, analysis of the data with a model accounting for limited mass transport reduced this problem, permitting the estimation of association rates of up to at least 1×10^7 M^{-1} s^{-1}. Nevertheless, the affinity may be reliably determined even for interactions with associations and/or dissociations "out of range" because this parameter is determined independently.

The association rates for three compounds (DMP323, A008, and B376) were above the 1×10^7 M^{-1} s^{-1} limit. Although the sensorgrams appeared "normal" and it was possible to determine the association and dissociation rates with relatively low errors (Table 2), only the affinities were well defined for these compounds. By use of the affinities and the upper limit for association rate, the dissociation rates could be estimated to be higher than 0.01 s^{-1}, indicating that the affinities of these compounds are limited by high dissociation rates and are thus of little practical interest as drug leads.

4. Structure−Kinetic Relationships. Two major structural classes of transition-state analogue inhibitors were studied: linear and cyclic. Since many of the linear compounds are analogues of B268 (an early lead), the results are presented with a focus on B268.

4.1. Clinical Inhibitors. Although all the clinical inhibitors are linear, they are otherwise structurally unrelated. As expected from inhibition studies, these inhibitors all showed a high affinity interaction with the enzyme, with $K_D \leq 1$ nM (Table 2). However, each inhibitor exhibited unique kinetics, and association and dissociation rate constants varied 10-fold (Table 2 and Figure 7a). The compound with the highest affinity was saquinavir, which also had the lowest dissociation rate constant of all compounds analyzed.

4.2. Linear Non-B268 Analogues. Six of the selected compounds made up a distinct series of linear inhibitors (Figure 1) unrelated to the B268-analogues. Only two of these (B295 and B355) were seen to interact with the enzyme, having almost identical kinetic properties (Table 2 and Figure 7a). Interestingly, being somewhat structurally related to saquinavir, they had the same association rates as saquinavir but a 1000-fold faster dissociation rate. Three smaller analogues (B294, B353, B362) did not bind or show any or significant inhibition (Table 1), indicating the importance of the large hydrophobic N-terminal groups for



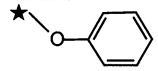
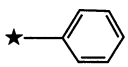
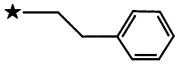
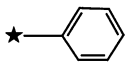
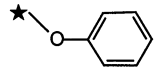
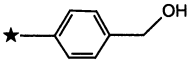
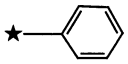
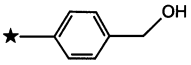
Compound	Chirality	R_1	R_2	Ref
A002	SRRS			26
A011	RSSR			26
A008	RSSR			26
DMP323	RSSR			46

Figure 4. Selected compounds from HIV-1 protease inhibitor library: cyclic urea compounds.

affinity. Similarly, B150, a symmetric compound with minor resemblance to the other compounds tested, was not seen to interact with the target and was not inhibitory (Table 1). Since no analogues of this compound were tested, it was not possible to identify the structural shortcomings of this compound for detectable interaction.

4.3. P1/P1′ Analogues of B268. All of the P1/P1′ analogues of B268 (Figure 2) showed detectable interaction with the enzyme (Table 2 and Figure 7b). The compound with the smallest P1/P1′ substituents (B277) showed a weak affinity (K_D > 10 μM), due to its very slow association rate. The association rate was increased 1000-fold from replacement of the P1/P1′ propyls with benzyl groups (B268) and an additional 2-fold from substitution of the benzyl ring with Br (B408). Other substitutions had little or even reduced effect on the association rate but decreased the dissociation rate. Of the compounds in this series, B440 showed the highest affinity and the slowest dissociation rate, second only to saquinavir.

4.4. P2/P2′ Analogues of B268. The structure–kinetic profile for the P2/P2′ analogues (Table 2 and Figure 7c) was quite different from that for the P1/P1′ analogues. Substitution of the valine side chain of B268 with a variety of groups resulted in a significant increase in k_{off} in all cases but had different effects on the association rate. Some of the compounds showed no affinity or weak affinities for the enzyme, while only two compounds (B369 and B388) had higher affinities than B268 did. The increased affinities were due to higher association rates compared with that of B268, thus compensating for the increased dissociation rates. In these compounds, one (B388) or both (B369) of the valine groups of B268 were substituted by a (2)-hydroxyindanyl, resulting in essentially the same overall effect. This indicates that the kinetics of asymmetric compounds (e.g., B388) is not simply an average of the symmetric parent compounds (B268 and B369).

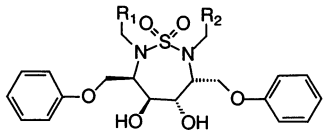
Substitutions in the termini of the inhibitor were also highly influential on the affinity. The affinity of B249, the terminal ester analogue of B268, was almost 1000-fold lower than that of B268. This was a consequence of reduced association and increased dissociation rates. Another ester terminal compound, B248, an analogue of the weak inhibitor A015, was not seen to interact with the target at all.

Furthermore, a significant effect on the interaction rates was observed for compounds that differed in the central mono- or dihydroxy group, directly involved in interactions with the catalytic aspartic acids. The dihydroxy compound B268 had a similar association rate but a 10-fold slower dissociation rate than the monohydroxy compound B365. Changing the stereochemistry of the central hydroxy groups from *R* to *S* reduced the affinity even more: (1) the affinity for B347, the *SS* analogue of B268 (*RR*), was 100-fold lower than for B268, an effect of changes in both association and dissociation rates; (2) the monohydroxy analogue B365 (*R*) had K_D =100 nM, while the *S* analogue B385, showed no interaction.

4.5. Cyclic Ureas. Only two of the four cyclic urea compounds interacted with the enzyme (DMP323 and A008). Both of these had a hydroxymethyl substituent on the P1/P1′ group, while the two noninteracting compounds lacked this group (Figure 4). Although the affinities (K_D) could be accurately determined for DMP323 and A008, it was not possible to resolve their association and dissociation rate constants because the association rates were above the maximum limit (see above). Nevertheless, the extremely high association rates for these compounds indicate that the affinities of these compounds were limited by fast dissociation rates (Figure 7d).

4.6. Cyclic Sulfamides. A total of 6 of the 15 cyclic sulfamide compounds interacted with the enzyme (A021, A024, A047, A045, A030, A023). As for cyclic ureas, the presence of hydoxyl groups or other polar P1/P1′ substituents was critical for measurable interaction (Figure 5). The cyclic sulfamide with the highest affinity for the enzyme was A021, although the other analogues had rather similar kinetics and are grouped in a small area in the graph (Figure 7d). The corresponding cyclic urea compound (A008) exhibited a 6-fold higher affinity, illustrating the importance of the identity of the scaffold in addition to the substituents.

5. Correlation between Inhibition (K_i) and Affinity (K_D). A comparison of the affinities determined with the biosensor technique (K_D) and the inhibition constants determined by the steady-state kinetic analy-



Compound	R_1	R_2	Ref
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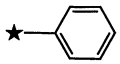
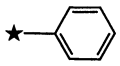
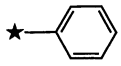
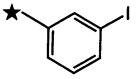
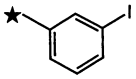
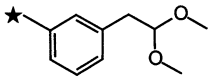
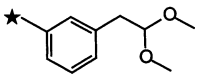
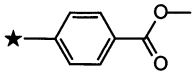
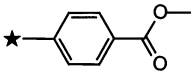
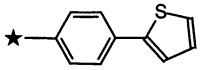
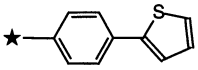
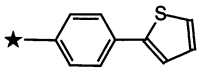
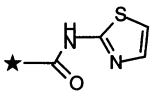
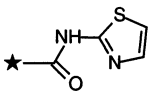
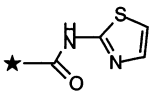
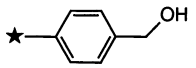
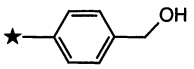
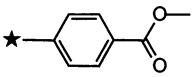
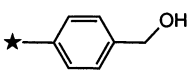
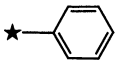
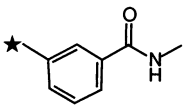
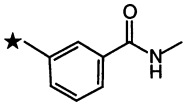
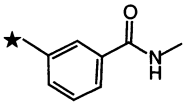
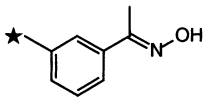
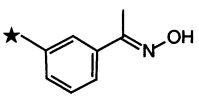
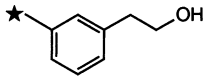
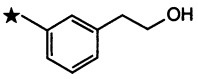
A044		Me	21
A006			26
A020			21
A019			41
A022			41
A031			21
A029			21
A026			21
A021			41
A024			21
A047			21
A045			21
A030			41
A023			41

Figure 5. Selected compounds from HIV-1 protease inhibitor library: cyclic sulfamide compounds.

sis (K_i) shows that they are linearly correlated, although the K_D value was approximately an order of magnitude higher than the K_i value (Figure 8). The correlation is quite high for compounds with K_D > 10 nM, while no correlation could be found below 10 nM. In fact, the 20 compounds with K_i below 2 nM showed K_D values between 0.3 and 100 nM. Moreover, in the biosensor assay the five drugs are among the 6 inhibitors with highest affinity, all having $K_D \leq 1$ nM, while in the steady-state inhibition assay they are accompanied by 12 other compounds having $K_i \leq 1$ nM. All compounds

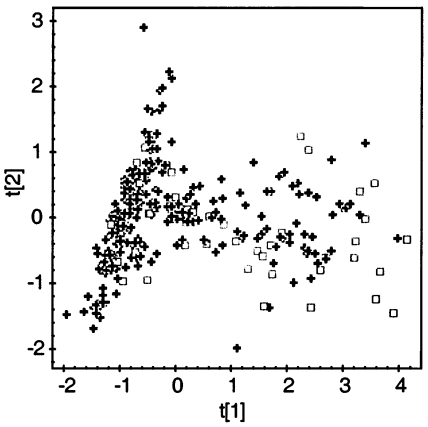


Figure 6. Score plot of the first two principle components (t[1] and t[2]) of the principle component analysis of the HIV-1 protease inhibitor library: selected compounds (□); unselected compounds (+).

Table 1. Inhibitory Potency for Compounds without Detectable Binding in the Biosensor Assay

compound	K_i [a] (nM)	ref	compound	K_i [a] (nM)	ref
A002	>5000	26	A044	510	21
A006	19.1	26	B150	*b*	
A011	570	26	B248	*b*	28
A019	540	41	B294	550[c]	27
A020	93	21	B316	>5000	28
A022	84	41	B353	>5000[d]	
A026	2200	21	B362	>5000	25
A029	136	21	B366	>5000[c]	28
A031	920	21	B385	530	42
A042	710	21			

[a] Inhibition constants (K_i) were taken from previous publications as indicated. Values lacking references have not been published previously. [b] No inhibition at 10 μM. [c] Estimated from the I_{50} value. [d] Less than 20% inhibition at 2 μM.

except B409, B440, saquinavir, ritonavir, and U75875 had a lower K_D than K_i.

Although K_D values could thus be determined for compounds with K_i values up to 5 μM, a group of analogues did not show any interaction with the enzyme (Table 1). Considering the correlation between K_i and K_D (Figure 8), some of these compounds would be expected to have K_D values well within the range that could be determined. There was no obvious trend related to the different structural classes.

Discussion

By resolution of the affinity into the individual association and dissociation rate constants, it was possible to reveal kinetic characteristics for inhibitors of different structural classes and how minor structural modifications influenced the interaction kinetics. A graph of association versus dissociation rate constants clearly illustrated that many compounds with the same affinity in fact had large differences in association and dissociation rates and that the spread in rate constants was greater than that in affinities. Furthermore, since similar affinities may be a result of combinations of association and dissociation rates differing 2−3 orders of magnitude, changes in molecular structure may result in widely varying interaction kinetic constants without altering the affinities significantly. This can erroneously lead to the conclusion that the structural changes do not influence the ligand−target interaction.

Table 2. Association and Dissociation Rate Constants (k_{on}, k_{off}) and Equilibrium Constants (K_D) for the Interaction between Inhibitors and HIV-1 Protease[a]

	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (nM)	K_i [b] (nM)	ref
A008	*7.06 × 10⁹ ± 3.27 × 10⁹*	*43.8 ± 15.5*	6.95 ± 1.59	0.23	14
A015	1.09 × 10⁵ ± 8.43 × 10⁴	0.938 ± 0.794	7.03 × 10³ ± 1.06 × 10³	140	28
A016	1.72 × 10² ± 24.9	6.05 × 10⁻² ± 3.07 × 10⁻²	3.09 × 10⁵ ± 1.55 × 10⁵	5000	28
A017	4.36 × 10⁴ ± 1.95 × 10⁴	0.179 ± 3.50 × 10⁻²	4.99 × 10³ ± 1.01 × 10³	700	28
A018	3.48 × 10⁵ ± 1.18 × 10⁵	0.474 ± 0.213	1.30 × 10³ ± 209	660	28
A021	6.87 × 10⁵ ± 5.99 × 10⁴	2.73 × 10⁻² ± 2.41 × 10⁻³	39.8 ± 1.55	3.1	41
A023	2.00 × 10⁵ ± 8.58 × 10⁴	0.139 ± 3.56 × 10⁻²	1.14 × 10³ ± 560	43	41
A024	2.21 × 10⁵ ± 5.69 × 10⁴	6.85 × 10⁻² ± 1.53 × 10⁻²	343 ± 70	19[c] (7.3)	41
A030	5.12 × 10⁵ ± 2.74 × 10⁵	4.20 × 10⁻² ± 1.25 × 10⁻²	169 ± 55.4	5.6[c] (3.4)	41
A037	2.04 × 10⁵ ± 3.69 × 10⁴	3.65 × 10⁻⁴ ± 3.61 × 10⁻⁵	1.95 ± 0.443	0.09	30
A038	2.93 × 10⁴ ± 4.23 × 10³	4.87 × 10⁻⁴ ± 2.55 × 10⁻⁵	17.5 ± 2.99	0.3	30
A045	4.99 × 10⁵ ± 2.13 × 10⁵	0.263 ± 5.17 × 10⁻²	675 ± 244	6.2[c] (39)	21
A047	1.88 × 10⁵ ± 1.09 × 10⁵	6.97 × 10⁻² ± 1.52 × 10⁻²	577 ± 176	10.1	21
B249	4.10 × 10⁴ ± 1.94 × 10⁴	0.273 ± 0.140	7.91 × 10³ ± 2.73 × 10³	1400[e]	28
B268	3.55 × 10⁵ ± 5.77 × 10⁴	3.67 × 10⁻³ ± 4.07 × 10⁻⁴	10.8 ± 1.64	0.4	28
B277	1.34 × 10² ± 5.88 × 10¹	4.85 × 10⁻³ ± 1.88 × 10⁻³	3.84 × 10⁴ ± 1.12 × 10⁴	2400	11
B295	9.02 × 10⁵ ± 4.32 × 10⁵	0.436 ± 0.246	420 ± 69.9	37	27
B322	1.85 × 10⁶ ± 1.39 × 10⁶	6.77 × 10⁻² ± 4.52 × 10⁻²	48.2 ± 11.6	0.91	28
B347	9.20 × 10³ ± 1.64 × 10³	2.70 × 10⁻² ± 3.38 × 10⁻³	2.99 × 10³ ± 197	40	42
B355	1.08 × 10⁶ ± 2.10 × 10⁵	0.373 ± 7.92 × 10⁻²	366 ± 84.1	55[e]	27
B365	3.04 × 10⁵ ± 4.42 × 10⁴	3.09 × 10⁻² ± 4.26 × 10⁻³	102 ± 2.21	2.1	42
B369	6.39 × 10⁶ ± 3.59 × 10⁶	1.33 × 10⁻² ± 5.93 × 10⁻³	13.9 ± 11.8	0.6	43
B376	*2.05 × 10⁸ ± 1.87 × 10⁸*	*13.7 ± 13.6*	27.4 ± 21.6	2.3	28
B388	5.97 × 10⁶ ± 1.66 × 10⁶	2.27 × 10⁻² ± 7.62 × 10⁻³	4.42 ± 1.44	0.054[c] (0.2)	44
B408	8.89 × 10⁵ ± 5.35 × 10⁵	1.69 × 10⁻³ ± 7.75 × 10⁻⁵	21.1 ± 19.5	0.3	30
B409	3.48 × 10⁵ ± 1.53 × 10⁵	4.32 × 10⁻⁴ ± 1.29 × 10⁻⁴	3.04 ± 2.30	1.2	30
B412	1.81 × 10⁵ ± 9.89 × 10⁴	8.17 × 10⁻⁴ ± 3.62 × 10⁻⁴	18.2 ± 16.2	1.4	30
B425	6.66 × 10⁵ ± 7.82 × 10⁴	0.234 ± 1.83 × 10⁻³	35.8 ± 3.36	1.4	42
B429	3.23 × 10⁵ ± 4.11 × 10⁶	3.73 × 10⁻⁴ ± 8.07 × 10⁻⁵	1.18 ± 0.244	0.61[c] (0.3)	30
B435	1.01 × 10⁵ ± 3.08 × 10⁴	6.53 × 10⁻³ ± 1.39 × 10⁻³	69.7 ± 12.4	0.1	44
B439	8.11 × 10⁴ ± 2.52 × 10⁴	1.63 × 10⁻³ ± 2.34 × 10⁻⁴	34.2 ± 16.4	0.8	30
B440	4.77 × 10⁵ ± 3.20 × 10⁴	3.03 × 10⁻⁴ ± 1.10 × 10⁻⁵	0.643 ± 6.35 × 10⁻²	0.55	30
indinavir	1.53 × 10⁶ ± 2.42 × 10⁵	1.58 × 10⁻³ ± 1.29 × 10⁻⁴	1.07 ± 0.108	0.31	11
nelfinavir	6.63 × 10⁵ ± 3.04 × 10⁵	6.68 × 10⁻⁴ ± 5.88 × 10⁻⁵	1.64 ± 0.480	0.54	11
ritonavir	3.92 × 10⁶ ± 1.11 × 10⁶	2.16 × 10⁻³ ± 2.98 × 10⁻⁴	0.608 ± 7.50 × 10⁻²	0.59	11
saquinavir	8.17 × 10⁵ ± 1.61 × 10⁵	2.27 × 10⁻⁴ ± 3.04 × 10⁻⁵	0.315 ± 7.42 × 10⁻²	0.23	11
U75875	6.76 × 10⁶ ± 5.72 × 10⁶	5.44 × 10⁻³ ± 5.13 × 10⁻³	0.484 ± 0.190	2.8	
Amp	4.43 × 10⁶ ± 1.25 × 10⁶	4.88 × 10⁻³ ± 1.45 × 10⁻³	1.13 ± 0.111	0.23[d]	
DMP323	*2.52 × 10¹⁰ ± 9.99 × 10⁹*	*83.3 ± 22.1*	3.83 ± 1.25	0.27	14

[a] Values were determined by global fitting of multiple sensorgrams, using a minimum of three sensorgrams for each inhibitor. Errors are given as standard errors. Since the kinetic constants were determined by global fitting and the values in the table are averages of each constant for all replicates for a certain inhibitor, the K_D is not identical to the ratio of the averages of the k_{off} to k_{on}. The variation in the errors for the different parameters for different compounds is a consequence of variations in the fitting in each case. Values in italics represent interactions outside the range of rate constants that can currently be measured (see text). [b] Inhibition constants (K_i) were taken from references as indicated. [c] Remeasured values, [d] Ahlsén et al. (submitted). [e] K_i values were converted from I_{50} values (taken from Alterman et al., 1998[28]) by assuming competitive inhibition and using the relationship $K_i = I_{50}/(1 + [S]/K_M)$, with [S] = 60 μM and K_M = 23 μM, from Nillroth et al., 1995.[45]

The association rate constants estimated for three of the inhibitors were apparently outside the limits of the method, considering limitations in mass transport and diffusion.[15] The values were apparently well defined and within the limits of the frequency of encounter of a small ligand with a protein, estimated to be within a range of 10^9−10^{11} s^{-1} M^{-1}.[16] However, simulation of sensorgrams with rate constants at these extremes illustrated the difficulties in resolving the individual rate constants although the affinities were well defined (not shown). The maximal dissociation rate observed was well below the limit (10^9−10^{12} s^{-1}) for maximal dissociation rates estimated for bimolecular complexes.[16]

The affinities obtained by steady-state inhibition measurements (K_i) and the current interaction analysis (K_D) correlated, except for the most potent compounds. There was a difference in the absolute values by a factor of 10 and a significant difference in the range and the magnitude of inhibition (0.05−5000) and dissociation constants (0.28−350000). However, these differences may be attributed to differences in the experimental design and the conditions under which these constant were determined. The present methods allows affinities to be determined under conditions expected to be more physiological than those used for inhibition studies, where the conditions have to be optimized for maximal activity, being pH 5−6 and high ionic strength. Therefore, the K_i values were determined at 30 °C in 0.1 M acetate, pH 5.0, 1 M NaCl, and 5% DMSO,[14] while the K_D values were determined at 25 °C in 9.7 mM Hepes, pH 7.4, 0.15 M NaCl, 2.9 mM EDTA, 0.0048% (v/v) Tween 20, and 3% DMSO (see Methods). High ionic strength is known to increase the catalytic activity of HIV-1 protease, thought to be a consequence of salting-out of the substrate[17,18] or of increased stability of the enzyme.[19] Moreover, it has recently been shown that, at least for the interaction between amprenavir and HIV-1 protease, an increased affinity at high ionic strength can primarily be attributed to a reduction in the dissociation rate constant.[9] Similarly, the differences in pH are also expected to influence the enzyme−inhibitor interactions. K_D values have been determined at different pH, including that for the inhibition assay, but the pH dependence of the interaction varies for

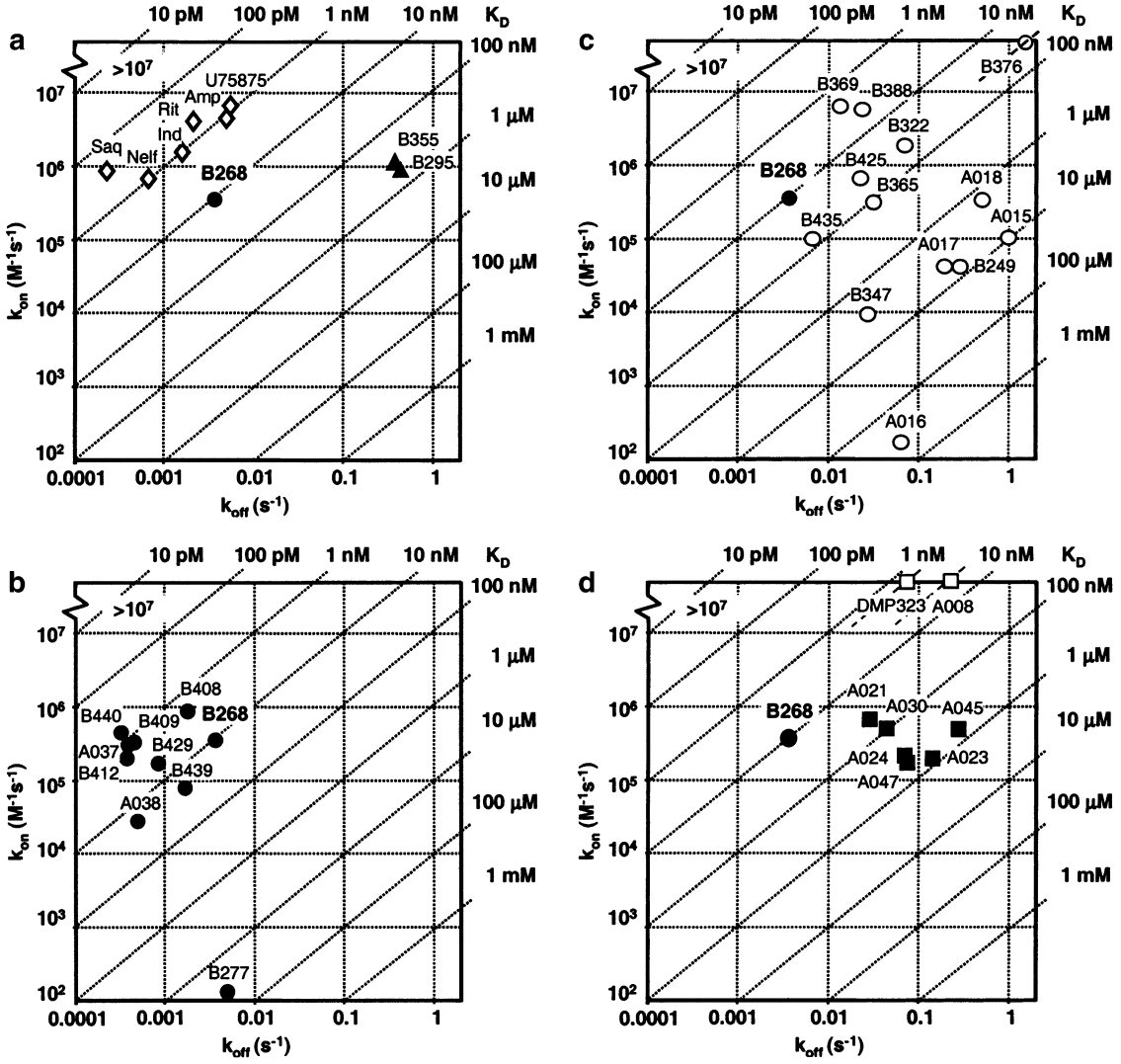


Figure 7. HIV-1 protease interaction kinetic maps for linear and cyclic inhibitors, shown as association versus dissociation rate constants (k_{on} and k_{off}), and the combinations of k_{on} and k_{off} that result in the same K_D values (diagonal lines): (a) drugs and related compounds (◇); (b) P1/P1′ analogues of B268 (●); (c) P2/P2′ analogues of B268 (○); (d) cyclic ureas (□) and cyclic sulfamides (■). B268 is included in all four graphs as a reference point. Since the K_D values in the graphs are obtained as the ratio of the rate constants, they may differ slightly from those in Table 1 determined by regression analysis.

different inhibitors (manuscript in preparation) and no general statement on how pH influences the correlation can be made at this stage. Since it remains to be established how changes in ionic strength or pH are related to the structural features of inhibitors, it is not possible at this stage to rationalize the differences in absolute K_i and K_D values or how well these should be correlated.

The lack of correlation between these affinity constants for potent inhibitors may also be attributed to unreliable determination of subnanomolar inhibition constants by conventional initial rate kinetic inhibition measurements. Even when they are determined with an equation for tight-binding inhibitors, the stabilizing effect of substrate and inhibitors on dimer dissociation at low enzyme concentrations is significant and difficult to account for. In contrast, there is no such limitation in the estimation of dissociation constants (K_D) by the current method, apparently reliable for all compounds used in this study. The higher sensitivity and information content of the biosensor assay, as compared to the inhibition assay, is particularly relevant for high affinity inhibitors, since it is for these compounds that the possibility of resolving affinity into association and dissociation rate constants has the greatest impact. In addition, for several low affinity compounds, this method permitted the identification of the cause of the low affinity, i.e., which of the kinetic parameters (k_{on} or k_{off}) were primarily limiting the interaction.

This study reveals the kinetic importance of the choice of scaffold for inhibitors, most clearly seen for the cyclic compounds. Although some of the cyclic inhibitors had K_i values below 1 nM and all with association rates above 10^5 M^{-1} s^{-1}, none of the cyclic inhibitors had low dissociation rates. Clearly, the affinity of cyclic compounds was limited by fast dissociation rates. However, there is a significant difference in the binding geometry of cyclic sulfamides and cyclic urea compounds, with the sulfamides being twisted in the central core resulting in asymmetric binding where the apparent P2′ group binds to the S1′ pocket and the P1′ group binds to the S2′ pocket (by analogy to the cyclic urea compounds).[20] This is reflected in the different structure−activity relationships of these two classes of cyclic inhibitors.[21]

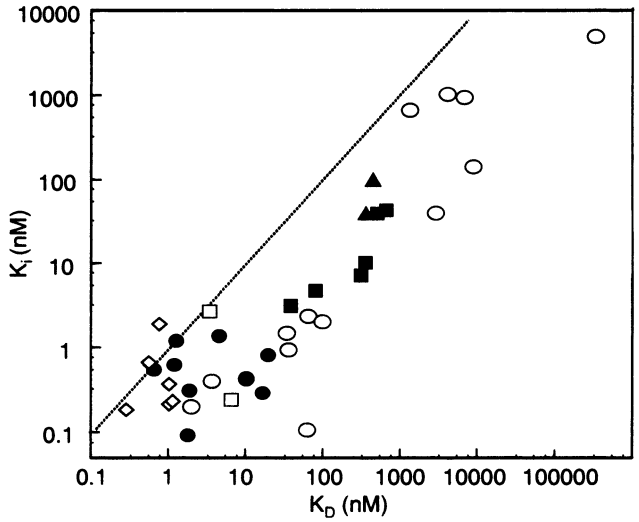


Figure 8. Correlation between inhibition (K_i) and dissociation constants (K_D) for HIV-1 protease inhibitors. K_i values were obtained with a steady-state inhibition assay,[14] and K_D was obtained from biosensor measurements (taken from Table 1). The symbols representing different structural classes are defined as in Figure 7.

The present data reveals differences also in the interaction kinetic profiles of these compounds. For example, the cyclic sulfamide analogue (A021) binds with lower affinity than the corresponding cyclic urea compound (A008), with at least a 10-fold reduced dissociation rate. However, the structural variations in this set of cyclic sulfamides had small effects on the association or dissociation rates, implying that more drastic structural changes are required to improve the kinetic properties of this class of compounds. So far, the most prominent difference in kinetic behavior for the cyclic compounds is observed between the nonbinding and the binding cyclic compounds, where the hydroxy group on the P2/P2′ side chain (P2/P1′ in cyclic sulfamides) is critical.

The analyzed set of C_2-symmetric linear inhibitors, with P1/P1′, P2/P2′, and other modifications, is presented with a focus on B268, a compound that can be seen as a parent compound to most of the others in the series. Reduction of the P1/P1′ group of B268 decreased the association rate, while all larger groups tested had minor effects on the association rate but decreased the dissociation rate significantly. On the basis of modification of the P1/P1′ position alone, a *p*-2-thiazolbenzyl group was most effective, resulting in an inhibitor with kinetics comparable to those for saquinavir and an affinity similar to all the clinical inhibitors. In contrast, modification of the P2/P2′ group of B268 increased the dissociation rate significantly, in some cases also coupled to large changes in the association rate. Evidently, substitution of the valine for a polar or larger group interferes with the stability of the enzyme−inhibitor complex. The termini of the inhibitor were also important for the interaction kinetics; when the terminal amide group was changed into an ester, the interaction was greatly reduced. Moreover, the identity of central hydroxy groups was critical; dihydroxy was preferred over monohydroxy and *R* was favored in both chiral centers. Symmetry in the rest of the molecule also had an influence. The kinetics for the asymmetric compound B388 illustrated that they are not simply the additive effects of the different structural entities in the molecule, although the asymmetric compound to a certain degree shares kinetic properties of both symmetric parent compounds.

Using building blocks from other inhibitors may provide a basis for designing new inhibitors with certain desirable features. For example, a non-B268-like series contains a building block also present in saquinavir. Only the largest of these inhibitors (B295 and B355) have detectable affinity, and their affinities are apparently limited by fast dissociation, since their association rates are similar to that for saquinavir. These data suggest that it is possible to identify structural features that are associated with certain kinetics. The limits for when this is possible are most likely dependent on the structural context, both regarding the inhibitors that are studied and the enzyme in question.

Since association and dissociation data for a large series of compounds have not been available previously, the correlation between these features and the pharmacodynamic properties of compounds is not known. While the association rate is concentration-dependent, slow association rates can be compensated by high intracellular concentrations; slow dissociation rates must be achieved by optimizing the interaction. When a complete block of an enzyme target is the goal, it would be beneficial to reduce the dissociation rate to a point where the compound acts like an irreversible binder. This would also be an advantage when rapid metabolism and other factors reduce the active concentration of the compound. One might argue that it is more important to optimize a compound with respect to slow dissociation than with respect to high affinity once it has a reasonably high on-rate with respect to the intracellular concentration. Recent data of exo-cyclic peptide mimetics of antibodies interacting with the HER2 tyrosine kinase receptor appear to support this hypothesis, since the efficacy of these peptides as inhibitors of cell proliferation was better correlated with k_{off} than with K_D.[22] Obviously the relative importance of the association and dissociation rates may be a unique property of a certain target and also dependent on the physiological conditions.

Conclusions

The present results shows that the resolution of affinity into interaction kinetic constants, displayed as $k_{on}-k_{off}-K_D$ maps, followed by a structure−kinetic analysis provides important information about enzyme−inhibitor interactions. It thus serves as a new important tool for identification, characterization, and optimization of drug leads. Further studies are required in order to discern the combinations of binding kinetics that are important for a certain target, especially concerning their effectiveness in vivo.

Methods

Enzyme and Inhibitors. HIV-1 protease and inhibitors were obtained and analyzed essentially as previously described.[3,23] The current set of inhibitors[24−30] (Figures 1−5) was selected from a library of 290 HIV-1 protease inhibitors, earlier used in a biosensor-based screen.[12] The selection was based on 10 chemical descriptors calculated using Sybyl 6.4:[31] polarizability,[32,33] Moriguchi LogP,[34,35] number of H-bond donors, number of H-bond acceptors, molecular weight, oval-

ity,[36] volume, and surface area (polar, apolar, and total).[37] Additionally, seven experimental variables, obtained from the previously published biosensor-based screen[12] (A1, A2, D1 and, D2 report points, the average of A1 + D1 + D2, pK_i, pEC_{50}), and pEC_{50} in the presence of 15% human serum (Lotta Vrang, personal communication) were used. Principle component analysis (PCA) of these 18 variables was performed in Simca[38] to normalize and extract the predominant trends in the data. Selection was performed using all seven components and a local implementation of the Kennard−Stone (maximum-dissimilarity) algorithm.[39]

Biosensor Analysis. The interaction between HIV-1 protease and inhibitors was determined at 20 °C with a Biacore 3000 surface plasmon resonance (SPR) based optical biosensor (Biacore AB, Uppsala, Sweden).[3] The Q7K mutant of HIV-1 protease was immobilized and stabilized on the sensor surface, and a total of 120 μL of the test compounds was injected for 3 min in a running buffer of 9.7 mM Hepes, pH 7.4, 0.15 M NaCl, 2.9 mM EDTA, 0.0048% (v/v) Tween 20, and 3% DMSO, using a flow rate of 40 μL/min. All compounds were initially screened at 10, 100, and 1000 nM in order to identify inhibitors that interacted with the enzyme and to estimate their affinity. Compounds that could not be detected to interact under these conditions were tested again at 100 μM. These initial estimates of affinity were used to determine a suitable concentration series for each individual compound. The association and dissociation phases of the entire set of sensorgrams for each concentration series were analyzed by simultaneous nonlinear regression analysis (global fitting) using BIAevaluation software (Biacore AB, Uppsala, Sweden). A kinetic model accounting for possible mass transport limitations was used.[3,40] The presented averages and standard errors are based on three or four independent experiments.

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References

(1) Jönsson, U.; Fägerstam, L.; Ivarsson, B.; Johnsson, B.; Karlsson, R.; Lundh, K.; Löfås, S.; Persson, B.; Roos, H.; Rönnberg, I.; et al. Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. *Biotechniques* **1991**, *11*, 620−627.

(2) Rich, R. L.; Myszka, D. G. Advances in surface plasmon resonance biosensor analysis. *Curr. Opin. Biotechnol.* **2000**, *11*, 54−61.

(3) Markgren, P.-O.; Lindgren, M. T.; Gertow, K.; Karlsson, R.; Hämäläinen, M.; Danielson, U. H. Determination of interaction kinetic constants for HIV-1 protease inhibitors using optical biosensor technology. *Anal. Biochem.* **2001**, *291*, 207−218.

(4) Tomasselli, A. G.; Heinrikson, R. L. Targeting the HIV-protease in AIDS therapy: a current clinical perspective. *Biochim. Biophys. Acta* **2000**, *1477*, 189−214.

(5) Richman, D. D. HIV chemotherapy. *Nature* **2001**, *410*, 995−1001.

(6) Wlodawer, A.; Vondrasek, J. Inhibitors of HIV-1 protease: a major success of structure assisted drug design. *Annu. Rev. Biophys. Biomol. Struct.* **1998**, *27*, 249−284.

(7) Thaisrivongs, S.; Strohbach, J. W. Structure-based discovery of Tipranavir disodium (PNU-140690E): a potent, orally bioavailable, nonpeptidic HIV protease inhibitor. *Biopolymers* **1999**, *51*, 51−58.

(8) Maschera, B. Human immunodeficiency virus. Mutations in the viral protease that confer resistance to saquinavir increase the dissociation rate constant of the protease−saquinavir complex. *J. Biol. Chem.* **1996**, *271*, 33231−33235.

(9) Porter, D. J.; Hanlon, M. H.; Carter, L. H., III; Danger, D. P.; Furfine, E. S. Effectors of HIV-1 protease peptidolytic activity. *Biochemistry* **2001**, *40*, 11131−11139.

(10) Xie, D.; Gulnik, S.; Erickson, J. W. Dissection of binding energy with native and ligand-bound protein stabilities: Determining the affinity of ultratight-binding inhibitors of HIV-1 protease and its drug resistant mutants. *J. Am. Chem. Soc.* **2000**, *122*, 11533−11534.

(11) Markgren, P.-O.; Hämäläinen, M.; Danielson, U. H. Kinetic analysis of the interaction between HIV-1 protease and inhibitors using optical biosensor technology. *Anal. Biochem.* **2000**, *279*, 71−78.

(12) Hämäläinen, M. D.; Markgren, P.-O.; Schaal, W.; Karlén, A.; Classon, B.; Vrang, L.; Samuelsson, B.; Hallberg, A.; Danielson, U. H. Characterization of a set of HIV-1 protease inhibitors using binding kinetic data from a biosensor-based screen. *J. Biomol. Screening* **2000**, *5*, 353−359.

(13) Markgren, P.-O.; Lindgren, M. T.; Gertow, K.; Karlsson, R.; Hämäläinen, M. D.; Danielson, U. H. Determination of interaction kinetic constants for HIV-1 protease inhibitors using optical biosensor technology. *Anal. Biochem.*, in press.

(14) Nillroth, U.; Vrang, L.; Markgren, P.-O.; Hultén, J.; Hallberg, A.; Danielson, U. H. Human immunodeficiency virus type 1 proteinase resistance to symmetric cyclic urea inhibitor analogs. *Antimicrob. Agents Chemother.* **1997**, *41*, 2383−2388.

(15) Karlsson, R. Affinity analysis of non-steady-state data obtained under mass transport limited conditions using BIAcore technology. *J. Mol. Recognit.* **1999**, *12*, 285−292.

(16) Fersht, A. *Structure and Mechanism in Protein Science. A Guide to Enzyme Catalysis and Protein Folding*; W. H. Freeman and Company: New York, 1999.

(17) Hyland, L. J.; Tomaszek, T. A., Jr.; Roberts, G. D.; Carr, S. A.; Magaard, V. W.; Bryan, H. L.; Fakhoury, S. A.; Moore, M. L.; Minnich, M. D.; Culp, J. S.; et al. Human immunodeficiency virus-1 protease. 1. Initial velocity studies and kinetic characterization of reaction intermediates by ^{18}O isotope exchange. *Biochemistry* **1991**, *30*, 8441−8453.

(18) Wondrak, E. M.; Louis, J. M.; Oroszlan, S. The effect of salt on the Michaelis Menten constant of the HIV-1 protease correlates with the Hofmeister series. *FEBS Lett.* **1991**, *280*, 344−346.

(19) Szeltner, Z.; Polgar, L. Conformational stability and catalytic activity of HIV-1 protease are both enhanced at high salt concentration. *J. Biol. Chem.* **1996**, *271*, 5458−5463.

(20) Bäckbro, K.; Löwgren, S.; Österlund, K.; Atepo, J.; Unge, T.; Hultén, J.; Bonham, N. M.; Schaal, W.; Karlén, A.; Hallberg A. Unexpected binding mode of a cyclic sulfamide HIV-1 protease inhibitor. *J. Med. Chem.* **1997**, *40*, 898−902.

(21) Schaal, W.; Karlsson, A.; Ahlsén, G.; Lindberg, J.; Andersson, H. O.; Danielson, U. H.; Classon, B.; Unge, T.; Samuelsson, B.; Hultén, J.; Hallberg, A.; Karlén, A. Synthesis and comparative molecular field analysis (CoMFA) of symmetric and nonsymmetric cyclic sulfamide HIV-1 protease inhibitors. *J. Med. Chem.* **2001**, *44*, 155−169.

(22) Berezov, A.; Zhang, H.-T.; Greene, M. I.; Murali, R. Biacore analysis of rationally designed anti-HER2 exocyclic mimetics of antibodies. *Biajournal* **2001**, *8*, 4−7.

(23) Markgren, P.-O.; Hämäläinen, M.; Danielson, U. H. Screening of compounds interacting with HIV-1 proteinase using optical biosensor technology. *Anal. Biochem.* **1998**, *265*, 340−350.

(24) Niklasson, G.; Kvarnström, I.; Classon, B.; Samuelsson, B.; Nillroth, U.; Danielson, H.; Karlén, A.; Hallberg, A. Synthesis of C_2 symmetric potential inhibitors of HIV-1 protease from D-mannitol. *J. Carbohydr. Chem.* **1996**, *15*, 555−569.

(25) Nöteberg, D.; Brånalt, J.; Kvarnström, I.; Classon, B.; Samuelsson, B.; Nillroth, U.; Danielson, U. H.; Karlén, A.; Hallberg, A. Synthesis of enantiomerically pure *cis* and *trans* 2-aminocyclopentanecarboxylic acids. Use of proline replacements in potential HIV-protease inhibitors. *Tetrahedron* **1997**, *53*, 7975−7984.

(26) Hultén, J.; Bonham, N. M.; Nillroth, U.; Hansson, T.; Zuccarello, G.; Bouzide, A.; Åqvist, J.; Classon, B.; Danielson, U. H.; Karlén, A.; Kvarnström, I.; Samuelsson, B.; Hallberg, A. Cyclic HIV-1 protease inhibitors derived from mannitol: Synthesis, inhibitory potencies, and computational predictions of binding afinities. *J. Med. Chem.* **1997**, *40*, 885−897.

(27) Brånalt, J.; Kvarnström, I.; Classon, B.; Samuelsson, B.; Nillroth, U.; Danielson, U. H.; Karlén, A.; Hallberg, A. A convenient synthesis of 1-(*S*)-[1′-(*S*)-(*tert*-butyloxycarbonylamino)-2′-phenethyl]oxirane. A useful building block in synthesis of HIV protease inhibitors. *Tetrahedron Lett.* **1997**, *38*, 3483−3486.

(28) Alterman, M.; Björsne, M.; Muhlman, A.; Classon, B.; Kvarnström, I.; Danielson, H.; Markgren, P.-O.; Nillroth, U.; Unge, T.; Hallberg, A.; Samuelsson, B. Design and synthesis of new potent C_2-symmetric HIV-1 protease inhibitors. Use of L-mannaric acid as a peptidomimetic scaffold. *J. Med. Chem.* **1998**, *41*, 3782−3792.

(29) Zuccarello, G.; Bouzide, A.; Kvarnström, I.; Niklasson, G.; Svensson, S. C. T.; Brisnader, M.; Danielson, H.; Nillroth, U.; Karlén, A.; Hallberg, A.; Classon, B.; Samuelson, B. HIV-1 protease inhibitors based on acyclic carbohydrates. *J. Org. Chem.* **1998**, *63*, 4898−4906.

(30) Alterman, M.; Andersson, H. O.; Garg, N.; Ahlsén, G.; Lövgren, S.; Classon, B.; Danielson, U. H.; Kvarnström, I.; Vrang, L.; Unge, T.; Samuelsson, B.; Hallberg, A. Design and fast synthesis of C-terminal duplicated potent C_2-symmetric P1/P1′-modified HIV-1 protease inhibitors. *J. Med. Chem.* **1999**, *42*, 3835−3844.

(31) *SYBYL*, version 6.5; Tripos Inc., 1699 South Hanley Road, St. Louis, MO 63144.
(32) MOPAC 6.0: Quantum Chemistry Program Exchange #455, Indiana University, 1990.
(33) Dewar, M. J. S.; Zoebisch, E. G.; Healy, E. F.; Stewart, J. J. P. AM1: A New General Purpose Quantum Mechanical Molecular Model. *J. Am. Chem. Soc.* **1985**, *107*, 3902−3909.
(34) Moriguchi, I.; Hirono, S.; Liu, Q.; Nakagome, I.; Matshushita, Y. Simple method of calculating octanol/water partition coefficient. *Chem. Pharm. Bull. (Tokyo)* **1992**, *40*, 1.
(35) Moriguchi, I.; Hirono, S.; Nakagome, I.; Hirano, H. Comparison of reliability of logP values for drugs calculated by several methods. *Chem. Pharm. Bull. (Tokyo)* **1994**, *42*, 4.
(36) Bodor, N.; Gabanyi, Z.; Wong, C. K. A New Method for the Estimation of Partition-Coefficient. *J. Am. Chem. Soc.* **1989**, *111*, 3783−3786.
(37) Pearlman, R. S.; Skell, J. M.; Deanda F. S., III. *Laboratory for Molecular Graphics and Theoretical Modeling*; College of Pharmacy, University of Texas: Austin, TX.
(38) *SIMCA*, version 6.0; Umetrics AB, Box 7960, SE-907 19 Umeå, Sweden.
(39) Kennard, R. W.; Stone, L. A. Computer Aided Design of Experiments. *Technometrics* **1969**, *11*, 137−148.
(40) Glaser, R. W. Antigen−antibody binding and mass transport by convection and diffusion to a surface: a two-dimensional computer model of binding and dissociation kinetics. *Anal. Biochem.* **1993**, *213*, 152−161.
(41) Hultén, J.; Andersson, H. O.; Schaal, W.; Danielson, U. H.; Classon, B.; Kvarnström, I.; Karlén, A.; Unge, T.; Samuelsson, B.; Hallberg, A. Inhibitors of the C_2-symmetric HIV-1 protease: Nonsymmetric binding of a symmetric cyclic sulfamide with ketoxime groups in the P2/P2′ side chains. *J. Med. Chem.* **1999**, *42*, 4054−4061.
(42) Wachtmeister, J.; Mühlman, A.; Classon, B.; Kvarnström, I.; Hallberg, A.; Samuelsson, B. Impact of the central hydroxyl groups on the activity of symmetrical HIV-1 protease inhibitors derived from L-mannaric acid. *Tetrahedron* **2000**, *56*, 3219−3225.
(43) Mühlman, A.; Lindberg, J.; Classon, B.; Unge, T.; Hallberg, A.; Samuelsson, B. Synthesis of novel, potent, diol-based HIV-1 protease inhibitors via intermolecular pinacol homocoupling of (2*S*)-2-benzyloxymethyl-4-phenylbutanal. *J. Med. Chem.* **2001**, *44*, 3407−3416.
(44) Oscarsson, K.; Classon, B.; Kvarnström, I.; Hallberg, A.; Samuelsson, B. Solid phase assisted synthesis of HIV-1 protease inhibitors. Expedient entry to unsymmetrical substitution of a C_2 symmetric template. *Can. J. Chem.* **2000**, *78*, 829−837.
(45) Nillroth, U.; Besidsky, Y.; Classon, B.; Chattopadhyaya, J.; Ugi, I.; Danielson U. H. Specific interaction between HIV-1 proteinase and 5′-phosphate peptidomimetic derivatives of nucleoside analogues. *Drug Des. Discovery* **1995**, *13*, 43−54.
(46) Lam, P. Y. S.; Jadhav, P. K.; Eyermann, C. J.; Hodge, N.; Ru, Y.; Bacheler, L. T.; Meek, J. L.; Otto, M. J.; Rayner, M. M.; Wong, N.; Chang, C.-H.; Weber, P. C.; Jackson, D. A.; Sharpe, T. R.; Erickson-Viitanen, S. Rational design of potent, bioavailable, nonpeptide cyclic ureas as HIV protease inhibitors. *Science* **1994**, *263*, 380−384.

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