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KINETIC AND BINDING STUDIES ON [^{125}I]SDZ-283471, A RADIOLABELED INHIBITOR OF HIV-1 PROTEINASE

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Kinetic and binding studies on a novel type of potent inhibitors of HIV-1 proteinase containing a 2-aminobenzyl substituted statine moiety as dipeptide mimetic are reported. The compounds were characterized as fast-binding competitive inhibitors of the enzyme. Using the radioiodinated derivative [^{125}I]SDZ-283471, monophasic association and dissociation curves were observed indicating a simple bimolecular reaction. While the association rate constant was similar to that of other inhibitors, the dissociation constant of SDZ-283471 was 20–500 times lower. Thus, the enzyme-inhibitor complex appears to be very stable in the case of the 2-aminobenzyl-statine compounds. Furthermore, we demonstrated that the inhibitors show appropriate specificity for HIV-1 and HIV-2 proteinases as compared to other aspartic proteinases. Using a competition assay, relative potencies of inhibitors modified in the P2 position were obtained and a preference for valine at this site was observed.

KEY WORDS: HIV proteinase, aspartic proteinases, radiolabeled inhibitor, transition state analogue.

INTRODUCTION

The aspartic proteinase of the human immunodeficiency virus, type 1, (HIV-1) has been recognized as an attractive target for the design of inhibitors which may be useful as anti-AIDS drugs[1–3]. The efforts of medicinal chemistry have aimed at the synthesis of potent, selective, antivirally active, and, finally, bioavailable inhibitors of this enzyme. In parallel, structure elucidation of proteinase-inhibitor complexes as well as studies on substrate specificity[3–9] and mechanism of peptide cleavage[10–11] have contributed to the understanding of enzyme-substrate and enzyme-inhibitor interactions. The latter enzymological studies have mainly used steady-state kinetic analysis; only recently, determination of association and dissociation rate constants of inhibitors using fluorimetric techniques have been reported[12, 13].

Here we present a novel type of active site-directed inhibitors and their characterization not only by using a standard kinetic approach, but also by binding studies with a radiolabeled inhibitor. Using a charcoal technique for separation of bound and unbound radioligand[14], we measured rate and dissociation constants, and determined enzyme specificity and relative potency of inhibitors.

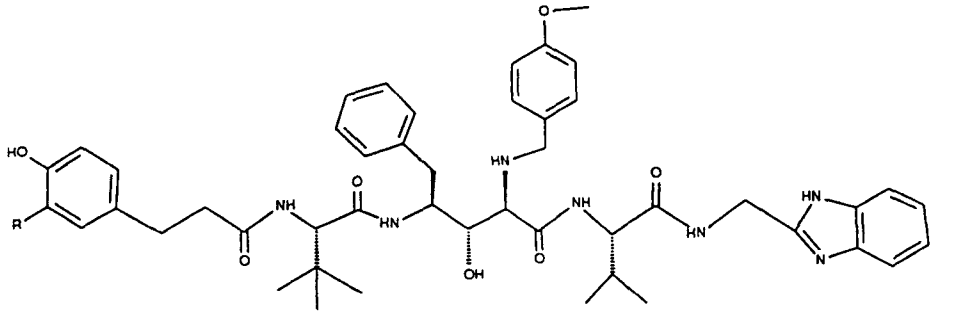

FIGURE 1 Chemical structure of compounds **1** (R = H) and **2** (R = I; = SDZ-283471)

MATERIALS AND METHODS

Materials.

Compounds **1–6** (see Figure 1 and Table 2) were prepared at Sandoz Research Institute, Vienna (D. Scholz, manuscript in preparation). Pepstatin A was bought from Fluka, Switzerland. HIV-1 proteinase was produced using the expressor plasmid[13] pTZprt$^+$ in *Escherichia coli* strain JM 105 and purified to homogeneity as described[16]. Protein concentration was estimated from the absorption at 280 nm using the molar extinction coefficient given by Nutt *et al.*[17]. HIV-2 proteinase was obtained from P. Strop, Institute of Organic Chemistry and Biochemistry, Prague, and human renin from J. Evenou, Sandoz Basle. Cathepsin D was purified from human spleen according to Ikeda *et al.*[18]. Porcine pepsin was bought from Boehringer-Mannheim, FRG.

Preparation of [^{125}I]SDZ-283471.

To a solution of 2 mg (2.4 μmol) compound **1** in 150 μl dimethylformamide, were added 15 μl 0.1 M sodium tetraborate, 100 μCi Na[^{125}I]iodide (9.7 mCi/μg iodine; Amersham), and 15 μl of a 2 mg/ml solution of chloramine *T*. After 30 min at room temperature, the reaction was stopped by addition of 50 μl 0.1 M sodium sulfite. The reaction mixture was separated by HPLC on a HP1050 chromatography system (Hewlett-Packard) with a Vydac RP-C18 column (4.6 × 25mm) which was eluted with 50% acetonitrile in 50 mM ammonium acetate; peaks were detected by UV-measurement (210 nm) and by liquid scintillation counting of eluate fractions. The reaction product was observed as a peak of radioactive substance which eluted at the position of the reference compound **2** and thus was identified as labeled SDZ-283471; its specific activity was $1.2 \cdot 10^6$ Ci/mmol. For binding experiments, the radiolabeled compound was diluted with a 10–1000-fold molar excess of unlabeled SDZ-283471.

Determination of Kinetic Constants K_i.

For measurements at pH 6.25, the substrate H-Lys-Ala-Arg-Val-Leu-(*p*-nitrophenylalanyl)-Glu-Ala-norleucyl-NH_2 described by Richards *et al.*[19] was used. HIV-1 proteinase (5.7 nM) was incubated at 37°C in 0.1 M MES, 1 M NaCl, 0.1% BSA, pH 6.25 (buffer A) with 280 μM of substrate in the presence of absence of compounds **2–6** or pepstatin A. From the decrease of absorbance at 298 nm initial rates were calculated.

For measurements at pH 4.7, the fluorogenic substrate H-Arg-Glu-(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Lys (DABCYL)-Arg-OH (bought from Molecular Probes, USA) described by Matayoshi *et al.*[20] was used. In detail, HIV-1 proteinase (1.5 nM) was incubated at 37°C with 2 μM of substrate in 0.1 M sodium acetate, pH 4.7, 1 M Nacl (buffer B) in the presence or absence of SDZ-283471. The increase in fluorescence intensity (λ_{ex} = 340 nm, λ_{em} = 490 nm) was recorded continuously to determine initial rates of substrate cleavage. To assess, if the proteinase would show decreased specific activity at low enzyme concentration, e.g., due to dissociation of the proteinase dimer, we performed measurements as described by Jordan *et al.*[21] using the fluorogenic assay and buffer B. In agreement with these authors, we found invariant specific activity over a concentration range of 0.4–8 nM.

The mode of inhibition of compound **2** was determined at pH 6.25 by plotting the data according to Lineweaver and Burk[22]. IC_{50}-values for compounds **2–6** were obtained by fitting the initial velocity data (V) from the inhibition of substrate hydrolysis to the equation

$$V = V_{max} \cdot IC_{50}/(I + IC_{50})$$

where I denotes the inhibitor concentration and V_{max} the velocity of the uninhibited reaction. From the IC_{50}-values kinetic constants K_i were calculated[23]:

$$IC_{50} = E_t/2 + K_i \cdot (1 + S/K_m) ,$$

where S is the substrate concentration, K_m the Michaelis constant (100 and 95 μM at pH 4.7 and 6.25 for the two substrates, respectively), and E_t the total concentration of enzyme. The latter was calculated by using B_{max} from a Scatchard plot[24] of the binding of SDZ-283471 (see below and Figure 5), assuming that one molecule of inhibitor binds per dimeric protease.

Binding assays.

HIV-proteinase binding assays at pH 4.7 and 6.25 were performed in buffers A or B (see above). For study of the pH-dependence of binding to HIV-1 proteinase, the buffer used was 20 mM acetic acid, 20 mM Mes, and 40 mM Tris at different pH[25], but ionic strength was kept constant (I = 1.0 M) by including sodium chloride. Binding to renin was assayed in 50 mM Tris-HCl, 0.1 M NaCl, pH 7.5, and in 0.1 M NaAc, 0.1 M NaCl, pH 5.6; binding to human cathepsin *D* was tested in 0.1 M citrate buffer, pH 3.5, and to porcine pepsin in 0.1 M glycine-HCl, pH 2.0. All assays were conducted at 20°C.

To separate enzyme-bound from free [^{125}I]SDZ-283471 we used a dextran-coated charcoal method. A suspension of 1.25% activated charcoal (Merck) in 0.25% dextran (Sigma), 0.5% bovine serum albumin was prepared; inclusion of albumin was found to be essential to prevent unspecific absorption. Assay samples (500 μl) were incubated for 1 min with the same volume of charcoal suspension and were centrifuged at $10,000 \times g$ for 1 min. Radioactivity in aliquots of the supernatants was measured by liquid scintillation counting; values were corrected by the radioactivity found in the supernatants in the absence of enzyme (less than 5% of total added radioactivity).

Determination of Association Rate Constant (k_{+1}).

The kinetic association constant was determined at ph 4.7 by incubating radioiodinated SDZ-283471 (1.5 nM) with HIV-1 proteinase (2.7 nM) in buffer A for various times and determination of bound inhibitor at each point of time.

Determination of Dissociation Rate Constant (k_{-1}).

The kinetic dissociation constant was determined at pH 4.7 by incubating labeled SDZ-283471 (63 nM) with HIV-1 proteinase (3 nM) in buffer A for 10 min to reach the equilibrium. Then, an excess of unlabeled SDZ-283471 (5 μM) was added, and bound radiolabeled SDZ-283471 was determined at various times.

Binding Data Analysis.

Binding data were analysed with the assumption that the binding of the inhibitor to the enzyme was a simple bimolecular reaction. The association rate constant was estimated from the equation[26]

$$\ln \frac{EI_e(I_t - EI \cdot EI_e/E_t)}{I_t(EI_e - EI)} = k_{+1} \cdot \left[\frac{I_t E_t}{EI_e} - EI_e\right] \cdot t$$

(where EI is the concentration of enzyme-inhibitor complex, EI_e is EI at equilibrium, and I_t and E_t are the total concentrations of inhibitor and enzyme, respectively) by regression analysis of a plot of the left side of the equation versus time (t) and calculation of k_{+1} from the slope. The dissociation rate constant was determined from regression analysis of the first-order plot of the data:

$$\ln (EI/EI_e) = -k_{-1} \cdot t \, .$$

The ratio of dissociation and association rate constants was used to give an estimate of the dissociation constant $K_D = k_{-1}/k_{+1}$ independent from the equilibrium saturation study (see below).

Equilibrium Saturation Studies.

HIV-1 proteinase was incubated for 10 min with increasing amounts of labeled SDZ-283471 in buffer A or B. Enzyme-bound radioactivity (B) was measured as described above. The concentration of free ligand (F) was calculated as the difference between

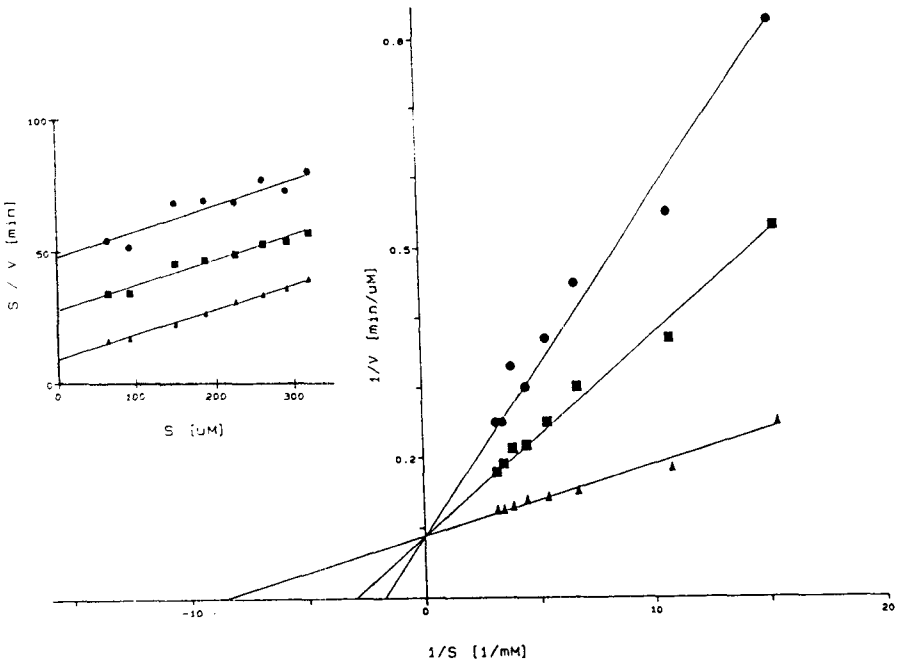

FIGURE 2 Lineweaver-Burk plot of the inhibition of HIV-1 proteinase by SDZ-283471 at pH 6.25. Inhibitor concentrations: (▲) 0.0, (■) 12.2, and (●) 24.4 nM. Inset: The same data shown in a S/V against S plot.

the total radioactivity and the bound fraction. Data were fitted to a binding curve assuming non-cooperative binding to a single site using the program EnzFitter (Elsevier); thus, dissociation constants (K_D) and concentration of binding sites (B_{max}) were obtained. Based on the known binding mode of statine-containing inhibitors, it is assumed that one molecule of SDZ-283471 binds per dimeric HIV-proteinase[27]; thus, B_{max} equals the concentration of enzyme dimers (E_t).

Competition Studies.

HIV-1 proteinase (3 nM) and [^{125}I]SDZ-283471 (5 nM) were incubated in buffer B in absence or presence of competitors. After 1 h, bound (B) and free radioligand were separated as described above. IC_{50}-values wree determined by regression analysis according to

$$B = B_0 \cdot IC_{50}/(I + IC_{50}) ,$$

where B_0 is the concentration of bound radioligand in the absence of competitor.

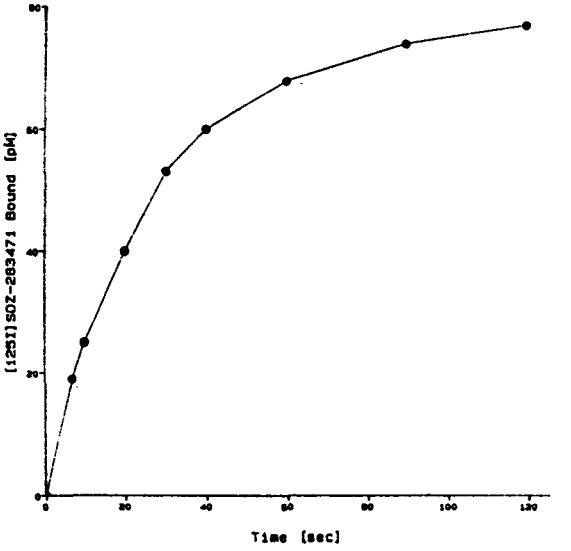

FIGURE 3 Association of [^{125}I]SDZ-283471 with HIV-1 proteinase. See Methods for experimental details. Data are the mean of three experiments.

RESULTS

Enzyme Inhibition Studies.

Inhibition of HIV-proteinase by SDZ-283471 was first studied using a kinetic approach. The same rate of peptide cleavage in the presence or absence of SDZ-283471 was observed whether the reaction was started by addition of enzyme or substrate; also, prolonged pre-incubation of enzyme with inhibitor (up to 60 min) did not increase the observed inhibition (data not shown).

The compound exhibited pure competitive inhibition of peptide cleavage as demonstrated using a Lineweaver-Burk plot (see Figure 2) and a plot of S/V against S (insert of Figure 2). Since the kinetic inhibition constant K_i was estimated from this experiment to be below 10 nM, K_i was calculated from the IC_{50}-value by using the equation of Cha *et al.*[23] which takes into account the mutual depletion of enzyme and inhibitor in the case of tight-binding inhibition (see Methods). Thus, $K_i = 4.3$ nM at pH 6.25 was determined.

Association and Dissociation Rate Constants for [^{125}I]SDZ-283471

Binding of the radiolabeled inhibitor to HIV-1 proteinase was studied quantitatively by using an activated-charcoal assay (see Methods). Figure 3 shows the time course

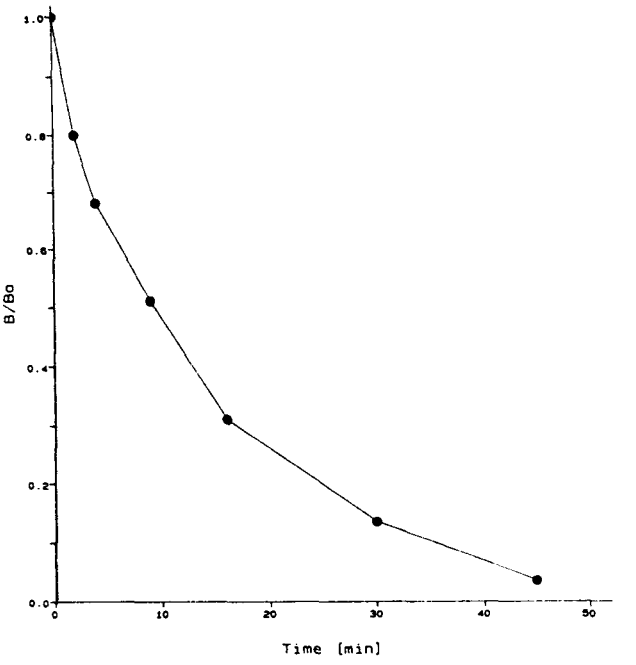

FIGURE 4 Dissociation of [^{125}I]SDZ-283471 from HIV-1 proteinase. See Methods for experimental details. Data are the mean of three experiments.

of enzyme/inhibitor association at nonsaturating concentration of [^{125}I]-283471 at pH 4.7; equilibrium was reached after about 2 min and did not vary after 16 h (not shown). The dissociation of radiolabeled inhibitor observed upon addition of an excess of unlabeled compound is shown in Figure 4. The curves are consistent with a one-step mechanism of inhibitor association and dissociation and allowed calculation of the rate constants k_{+1} and k_{-1}, respectively (see Methods for data analysis and Table I for results).

TABLE I
Kinetic and Equilibrium Constants for the Interaction between HIV-1 proteinase and SDZ-283471 at pH 4.7.

pH	K_i [nM]	k_{+1} [$10^7 \cdot M^{-1} \cdot min^{-1}$]	k_{-1} [min^{-1}]	k_{-1}/k_{+1} [nm]	K_D [nM]
4.7	3.0	4.1	0.07	1.7	1.5

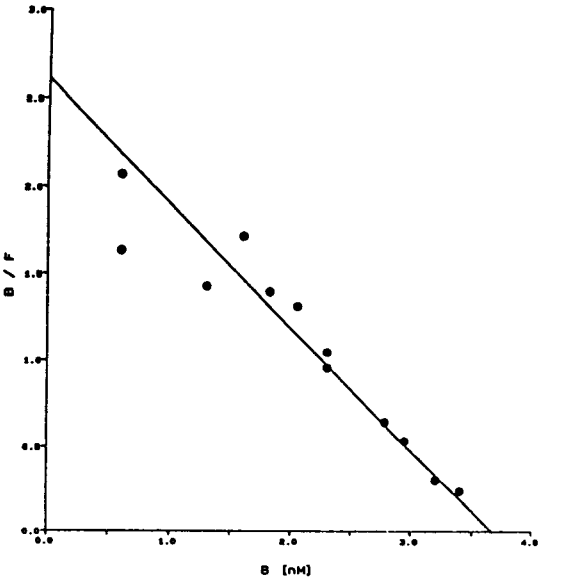

FIGURE 5 Scatchard plot of [^{125}I]SDZ-283471 binding to HIV-1 proteinase. See Methods for experimental details.

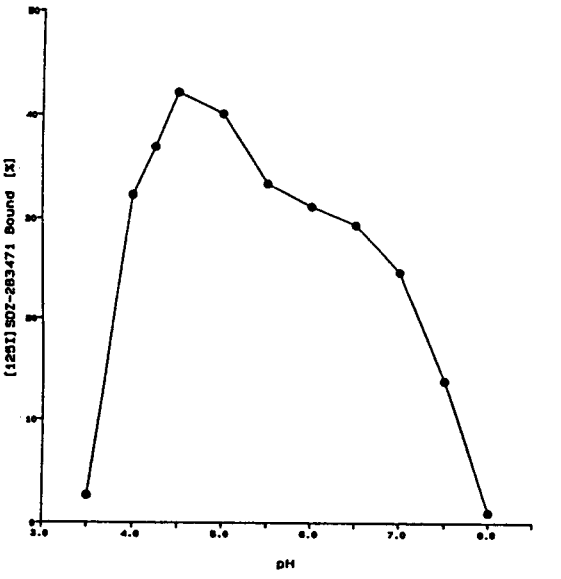

FIGURE 6 Effect of pH on the binding of [^{125}I]SDZ-283471 to HIV-1 proteinase.

Equilibrium Saturation Studies.

Figure 5 shows a Scatchard plot of [^{125}I]SDZ-283471 binding to HIV-1 proteinase at pH 4.7. The data are consistent with a single binding site model and yield a dissociation constant of 1.5 nM. The value is in good agreement with that deduced from the kinetic constants (see Table I). The K_D at pH 6.25 was 3.1 nM (data not shown).

Since it is known from the structure of HIV-1 proteinase/inhibitor complexes that there is one active site per proteinase dimer, the maximal concentration of binding sites (B_{max}) should be equal to the concentration of active enzyme. The value taken from the plot in Figure 5 (3.7 nM) is in good agreement with the protein concentration estimated from UV-absorption (3.5 nM).

Figure 6 shows the dependence of binding of [^{125}I]SDZ-283471 on the pH-value of the buffer.

Specificity of [^{125}I]SDZ-283471 Binding.

The labeled compound not only bound to the proteinase of HIV-1, but also to the HIV-2 enzyme. Affinity, however, is reduced: The K_D-value for binding to HIV-2 proteinase at pH 6.25 was 20.4 nM, to the HIV-1 enzyme 3.1 nM. The K_i-value for inhibition by SDZ-283471 at pH 6.25 also was about sevenfold higher (30 nM) for HIV-2 than for HIV-1 proteinase (4.3 nM).

[^{125}I]SDZ-283471 did not show any binding to human renin (tested at pH 4.7 and 7.4) and cathepsin *D* (pH 3.5), or to porcine pepsin (pH 2.0). Thus the compound shows selectivity for the retroviral enzymes.

Competition Assays.

Table 2 summarizes data on competitive binding of [^{125}I]SDZ-283471 and unlabeled inhibitors. Pepstatin A was used as reference compound and compared to compounds containing a central 2-aminobenzylstatine moiety with different amino acid

TABLE 2

Competitive Binding to HIV-1 Proteinase of [^{125}I]SDZ-283471 and Unlabeled Inhibitors at pH 6.25

The general structure of inhibitors **3**–**6** is *N*-benzyloxycarbonyl-*X*-BAHPP-*L*-valine-*N*-benzylamide, where BAHPP is 2(*R*)-(benzylamino)-4(*S*)-amino-3(*S*)-hydroxy-5-phenyl-pentanoic acid, and *X* is a *L*-amino acid.

Compd	*X*	[^{125}I]SDZ-283471 Binding IC_{50} [nM]	Inhibition of Peptide Hydrolysis K_i [nM]
3	valine	4.5	6.1
4	alanine	40	39
5	serine	10	11
6	histidine	29	23
Pepstatin A	–	520	450

substitutions. The IC_{50}-values for binding competition are comparable to the K_i-values yielded by initial rate studies (see Table 2).

DISCUSSION

In this study, a novel type of potent inhibitors of HIV-1 proteinase was used; they contain a 2-aminobenzyl substituted statine moiety as a dipeptide mimetic. While details of the synthesis and of structure-activity relationships in an extended series of these inhibitors will be described elsewhere (D. Scholz, manuscript in preparation), we report here on kinetic and binding studies of the interaction of a selected compound with HIV proteinases and related enzymes. We have chosen SDZ-283471 (**2**), the iodo derivative of **1**, since a labeled variant can be easily prepared by radioiodination. The compound has a lower K_i-value (3 nM) than the parent compound **2** (8.1 nM at pH 4.7).

First, we noted that a preincubation to reach the equilibrium between enzyme and inhibitor prior to the start of the enzymatic reaction by addition of substrate is not required. Thus, the compounds are not slow-binding inhibitors as has been observed for a variety of compounds including pepstatin A in the case of pepsin[28] and, more recently, for phosphinates with HIV-1 proteinase[29].

SDZ-283471 was found to be a pure competitive inhibitor as expected for a statine-based transition-state inhibitor. Similar inhibition (K_i) and dissociation constants (K_D) were determined which indicates that these parameters describe the same equilibrium, namely that between free enzyme, free inhibitor and enzyme-inhibitor complex.

Monophasic association and dissociation curves were observed, indicating that the inhibitor binds by a simple bimolecular reaction. This is supported by the ratio of k_{-1}/k_{+1} being close to the K_D-value obtained from equilibrium saturation studies. Thus, we do not have an indication of an isomerization step required to proceed from an initial collision intermediate to a secondary enzyme-inhibitor complex, as has been described in the case of pepstatin binding to pepsin[30]. For HIV-1 proteinase, Furfine *et al.*[12] reported an apparent single-step binding for pepstatin and for the *R*-isomer of quinoline-2-carbonyl-Asn-Phe-Ψ[CH(OH)CH_2N]Pro-O-tBu (compound **7**), but a two-step mechanism for the *S*-isomer; Rodriguez *et al.*[13] also observed one-step binding in the case of Ala-Ala-Phe Ψ[CHOHCH_2[Gly-Val-Val-OMe (compound **8**).

The kinetic association constant found here for SDZ-283471 ($4.1 \cdot 10^7$ $M^{-1} \cdot min^{-1}$) is in the same order of magnitude as the values reported for pepstatin and inhibitors **7** and **8** (4.8 to $18.6 \cdot 10^7$ $M^{-1} \cdot min^{-1}$ [12,13]). By contrast, the dissociation rate constant (k_{-1}) of **8** was 20-fold higher and that of pepstatin and **7** was 500- and 100-fold higher, respectively, than of SDZ-283471 (0.07 min^{-1}). Thus, considerable differences in the half-lifes of proteinase-inhibitor complexes exist which may be of significance for the antiviral efficacy of different compounds.

Our studies of inhibitor binding to other aspartic proteinases show that SDZ-283471 has appropriate specificity for the proteinases of HIV-1 and HIV-2. This result was

also confirmed in enzyme assays where the compound failed to inhibit cathepsin *D*, renin or pepsin (data not shown).

We have compared a series of derivatives containing the benzylamino-statine moiety and differing by one amino acid (see Table 2); IC_{50}-values obtained from competitive binding experiments and K_i-values from kinetic measurements are in good agreement. Thus, the competitive binding assay provides a means to directly compare the relative potency of HIV-proteinase inhibitors, as has been described before in the case of renin inhibitors[14].

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