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Antiviral Research 30 (1996) 155–170

Antiviral Research

The inhibition of human immunodeficiency virus proteases by 'interface peptides'

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Received 19 September 1995; accepted 15 January 1996

Abstract

The active human immuondeficiency virus type 1 (HIV-1) protease has a homodimeric structure, the subunits are connected by an 'interface' β-sheet formed by the NH_2- and COOH-terminal amino acid segments. Short peptides derived from these segments are able to inhibit the protease activity in the range of micromolar IC_{50} values. We have further improved the inhibitory power of such peptides by computer modelling. The best inhibitor, the palmitoyl-blocked peptide Pam-Thr-Val-Ser-Tyr-Glu-Leu, has an IC_{50} value of less than 1 μM. Some of the peptides also showed very good inhibition of the HIV-2 protease. The C-terminal segment of the HIV-1 matrix protein, Acetyl-Gln-Val-Ser-Gln-Asn-Tyr, also inhibits HIV-1 protease. Kinetic studies confirmed the 'dissociative' mechanism of inhibition by the peptides. Depending on the peptide structure and ionic strength, both dimerization inhibition and competitive inhibition were observed, as well as synergistic effects between competitive inhibitors and interface peptides.

Keywords: AIDS; HIV; Proteinase inhibitors

1. Introduction

Human immunodeficiency virus (HIV) requires the viral protease for infectivity, while inhibition of this enzyme blocks viral maturation and replication (Kohl et al., 1988; Seelmeier et al., 1988; Ashorn et al., 1990; Meek et al., 1990). Therefore, the protease is an obvious target for therapeutic intervention in AIDS (Kramer et al., 1986; for a review see Debouck, 1992). The active HIV-1 protease is an aspartyl protease and has the structure of an obligatory homodimer stabilized only by noncovalent interactions (Pearl and Taylor, 1987; Navia et al., 1989; Wlodawer et al., 1989). The full complement of two subunits is needed to form the composite active site. Each subunit con-

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PII S0166-3542(96)00940-0

tributes one active aspartic acid in the conserved segments Asp-Thr-Gly. At the 'interface' of the dimers, an anti-parallel β-sheet is formed by the four N- and C-terminal segments of the protease (residues 1–4 and 96–99, respectively) which connects the two protomers and stabilizes the dimer. Because of this structural peculiarity, a novel method of inhibiting protease activity (and therefore viral replication), by interfering with dimer formation, was suggested and demonstrated (Weber, 1990; Schramm and Schramm, 1990; Zhang et al., 1991; Schramm et al., 1991). In this case of dimerization inhibition (or dissociative inhibition) the inhibitor binds to the C- and N-termini of the protease monomers and prevents the correct assembly of the inactive monomers to the active enzyme.

The strategy of using interface peptides to inhibit enzymatic activity by interfering with the subunit interaction has been applied successfully in the cases of ribonucleotide reductase from herpes simplex virus (HSV) (Cohen et al., 1986; Dutia et al., 1986) and of HIV-1 reverse transcriptase (RT) (Divita et al., 1994). In the first case, a peptidomimetic drug could be developed which is > 10 000-fold more potent than the starting peptide (Liuzzi et al., 1994).

For HIV-1 protease, Zhang et al. (1991) have analyzed the kinetics of dimerization inhibition and found pure dimerization inhibition by the C-terminal tetrapeptide of the protease. This result was supported by sedimentation equilibrium experiments. In these experiments, the apparent molecular weight of the protease was observed to correspond to the monomer or to the dimer, depending on the presence or absence, respectively, of the C-terminal protease peptide Ac-Thr-Leu-Asn-Phe ($K_i = 45$ μM). Furthermore, the inhibition of virus replication by two octapeptides with the natural C- and N-terminal sequences of HIV-1 protease was demonstrated by Schramm et al. (1991) in an MT-4 cell test with ED_{50} values of 27 μM for Ac-Gln^{92}-Ile-Gly-Met-Thr-Leu-Asn-Phe^{99}-NH_2 and 58 μM for Ac-Pro^1-Glu-Ile-Thr-Leu-Trp-Gln-Arg^8-NH_2, respectively. The toxicity was found to be low. In the enzyme test, the two octapeptides gave only inhibitory constants in the mM range (Schramm et al., 1992). Subsequently, more peptides derived from both β-sheet-forming terminal segments were shown to inhibit HIV-1 protease activity (Babé et al., 1992; Schramm et al., 1993; Franciskovich et al., 1993). Other retroviral proteases also show this type of inhibition and even 'cross-inhibition'. For instance, interface peptides derived from HIV protease sequences are also active against HTLV-1 protease human T cell leukaemia virus type 1 (Daenke et al., 1994) and in the F-MuLV cell assay friend murine leukaemia virus (Jurkiewicz et al., to be published).

So far, the main objections to this novel approach of protease inhibition were the limited activity of the peptides and remaining doubts about the actual mechanism of inhibition, mainly caused by reports about a very low K_d of the protease. We have now improved the inhibitory strength of peptides related to natural interface sequences by rational computer design, i.e. by evaluating the binding affinities of mutated peptides to a monomer structure. The best inhibition constants are below 1μM in our (modified) enzyme test. The mode of action of dimerization inhibitors has been confirmed for several peptides by kinetic analysis. The new inhibitor structures allow more reliable conclusions about the structural features required for the dimerization or competitive mode of action, respectively. The observed synergism with competitive protease inhibitors further confirms the mechanism of action and classifies the interface peptides as independent antiviral agents.

2. Materials and methods

2.1. Computer modelling

For the design of peptidic inhibitors, the software package SYBYL6 (TRIPOS Ass.), running on Silicon Graphics or Evans and Sutherland workstations, was used. The X-ray coordinates of Wlodawer et al. (1989), as published in the Protein Data Bank, served as starting data for the modelling of the peptide/monomer complexes. By deletion of amino acids, one of the subunits in the dimer structure was reduced in size to the termi-

nal segment still bound to the unaltered second subunit. Apart from the natural sequence, the best inhibitory segments already published were used as starting structures, e.g. Ac-Thr-Val-Ser-Phe-Asn-Phe. After mutation of single amino acids and energy minimization (including short molecular dynamics runs), the binding energy of the mutated peptides was estimated by ligand docking (DOCK procedure in SYBYL) and by determination of the difference between the energy of the complex and the sum of the peptide chain energies. In addition, the DOCK procedure allowed the helpful discrimination between hydrophobic and electrostatic binding contributions. Alanine substitutions for the single amino acids in the bound peptides were also used for the assessment of side-chain contributions. Peptide sequences with good binding energies to the HIV-1 protease monomer were synthesized and tested.

2.2. Peptides

Inhibitory peptides were either prepared at the Max-Planck-Institut für Biochemie, following standard solid-phase procedures or purchased from Neosystems (Strasbourg, France). In our experiments, the peptide synthesizer from either Bachem AG, Bubendorf (Labortec® SP-640), or Applied Biosystems AG (model 431A) were used. The resins used were the P-benzyloxybenzyl alcohol resin (Novabiochem GmbH, Sandhausen) or the Pepsyn-KA® (Milligen-BioSyntech, Hamburg), and the Novasyn-KR® (Novabiochem) for the peptide amides, respectively. The Fmoc(fluorenymethyloxycarbonyl)-technique was used throughout. Amino acid derivatives were purchased from Bachem Biochemica GmbH, Heidelberg, or from Novabiochem. The final acetylation was performed by using acetic acid instead of an Fmoc-amino acid. Peptides were cleaved from the resin with 95% trifluoroacetic acid, with concomitant removal of the side-chain protective groups (e.g. *t*-butyl ethers of Thr). They were purified by gel chromatography on Sephadex® G-25 (Pharmacia, Freiburg) or Fractogel TSK HW-40S (Merck, Darmstadt), or by standard high performance liquid chromatography. The products were analytically characterized by amino acid analysis and fast atom bombardment mass spectrometry.

2.3. Enzymes

HIV-1 protease was kindly supplied by A. Billich, Sandoz Forschungsinstitut, Wien. The protease was also expressed and isolated in our laboratory using the plasmid pET9c-PR and the isolation procedure described by Billich et al. (1990). HIV-2 protease ('purified') was purchased from Bachem, Heidelberg. The stock solution contained 0.1 mg/ml (5 μM) protease in a buffer containing 0.1 M NaOAc (pH 5.5), 0.1 M NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol and was stored in small aliquots at − 80°C. The protein concentration was determined by amino acid analysis.

2.4. Enzyme inhibition assay

The routine activity assay for both HIV proteases was performed using the substrate Lys-Ala-Arg-Val-Nle*(*p*-nitro-Phe)-Glu-Ala-Nle-NH_2 as described by Richards et al. (1990). The chromogenic substrate was synthesized in our laboratory with a purity of 97%. The HIV protease was diluted into assay buffer (0.1 M NaOAc (pH 4.7), 4 mM EDTA, 5 mM dithiothreitol, 0.5 M NaCl) to a final enzyme concentration of 10 nM and preincubated for 5 min with or without inhibitor prior to the addition of the substrate (initial concentration 25 μM). Pepstatin A (Seelmeier et al., 1988) and SDZ PRI 053 (Billich et al., 1995) were used as reference inhibitors.

2.5. Determination of the inhibition mechanism

The determination of the K_i values and the inhibition mechanism of the investigated peptidic protease inhibitors followed the kinetic assay developed by Zhang et al. (1991) with slight modifications. In variation of the original procedure, the chromogenic substrate (the same as in the routine test) was used at an initial concentration of 25 μM, a value in the range of the K_m value. The reaction was started by diluting aliquots of the protease stock solution to yield final enzyme con-

centrations of 2–250 nM in the assay buffer (0.1 M NaOAc (pH 5.0), 1 mM EDTA) containing 25 μM substrate, with or without the inhibitor peptides. The peptides were kept in a 10 mM stock solution in dimethylsulfoxide (DMSO). The total DMSO content in the reaction mixture never exceded 3% (v/v). The variation of the ionic strength in the reaction solution was achieved by addition of NaCl. All measurements were carried out at room temperature. The hydrolysis of the substrate was followed over 6 min by recording the absorbance at 295 nm (1 cm lightpath quartz cuvette) in a Lambda 17 (Perkin-Elmer) spectrophotometer. From the time course, the initial velocities were evaluated using the linear part of a polynomial regression (4th order to the first 120 s of the reaction) with the Origin 3.0 (MicroCal, USA) fitting routine. The data obtained were analyzed with the equation given in Fig. 1a, using the Origin 3.0 and GraFit 3.0 (Erithacus Software. UK) software package.

The test was conceived as a rapid screening procedure to distinguish the two types of inhibitor. Control assays using prolonged enzyme/inhibitor preincubation (20 min) at pH 7.0, or the fluorescence substrate (high ratio K_m/E_0), furnished the same results concerning the inhibition mechanisms.

2.6. Synergism tests

Synergistic effects were analyzed using the three-dimensional method of Prichard and Shipman (1990) and applying the dissimilar site assumption of additivity to calculate the theoretical additive interactions (Fig. 2). The enzyme activity for the individual inhibitors and for mixtures was determined as described above. As in the standard test, the experiments were started by addition of the enzyme to the reaction mixture. The inhibition curves of the individual inhibitors were used to calculate the theoretical additive interactions. Throughout this test, the concentration of HIV-1 protease was 100 nM.

2.7. Electrophoresis

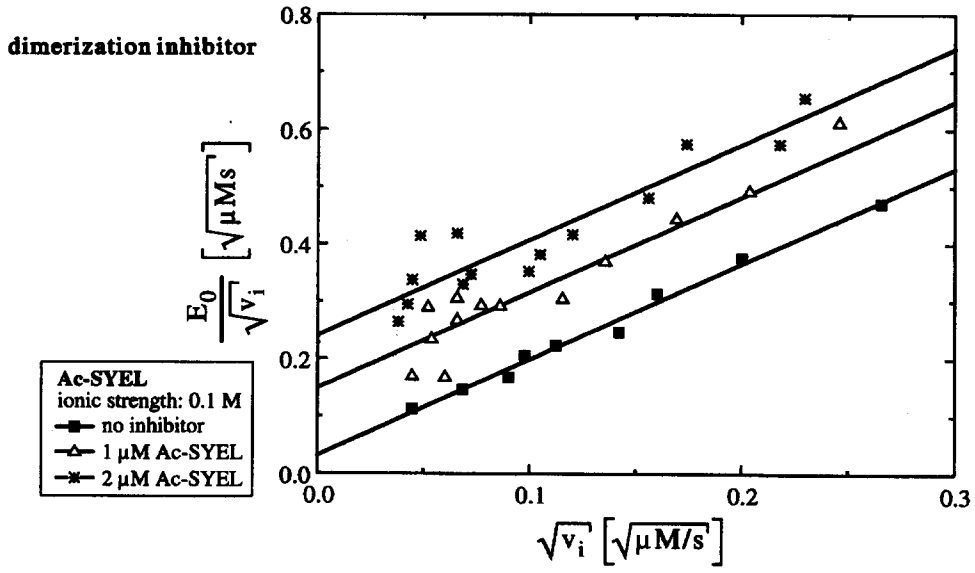
Non-denaturing electrophoresis was performed with a PHAST™ instrument (Pharmacia, Freiburg) using the procedure recommended by the manufacturer ('method 1', buffer: pH 4.2) and polyacrylamide gels (Pharmacia) with 20% cross-linking.

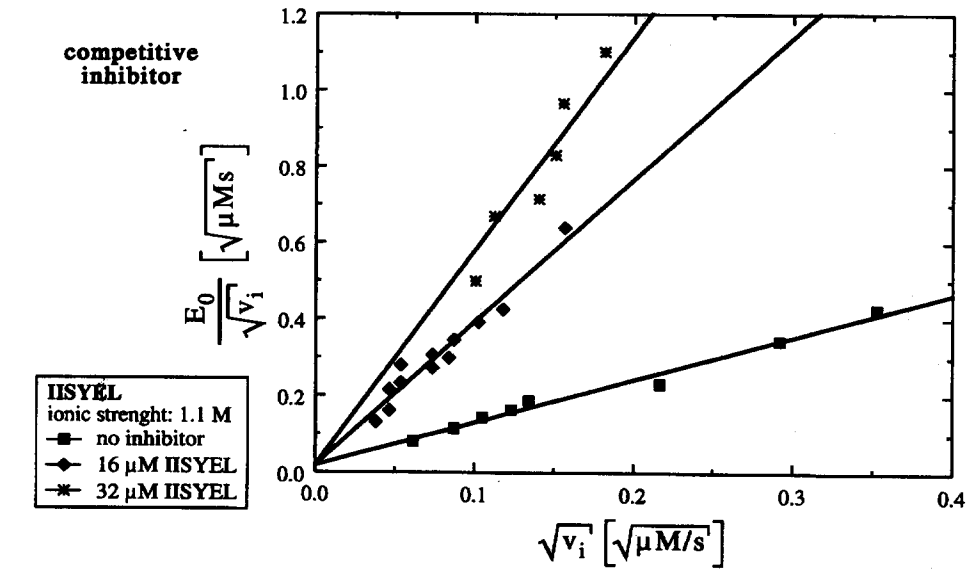
2.8. Cell tests

A rapid screening method for biological activity of protease inhibitors using a recombinant bacterial system was used as described by Sedlácek et al. (1993). The assay allows a rapid identification of possible in vivo inhibitors since only those compounds which are able to penetrate the cell wall and persist as active compounds will protect cell growth. The inhibitors are assumed to counteract the toxic side-effects of HIV protease synthesized in the cells. With our expression system using the plasmid pET9c-PR, the method did not work properly, neither with our peptidic inhibitors, nor with strong active-site inhibitors such as the Sandoz pseudopeptide SDZ PRI 053. Obviously, too much protease was produced in the cells by this system, which was designed for optimal protease yield. Without induction, however, the inhibitors did improve cell growth. This effect is most likely due to the inhibition of a small quantity of toxic HIV protease produced in the cells even without induction by a 'leaky' promotor. For unknown reasons the modified test does not always succeed.

2.9. Biosensor experiments

Binding assays based on surface plasmon resonance were performed in a BIAcore™ instrument (Pharmacia Biosensor, Freiburg). The biotinylated peptides were immobilized on an SA5 chip via streptavidin coupling in 0.1 M sodium acetate solution of pH 4.5. For the binding assays, HBS buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA) with 0.05% P20 surfactant was used at flow rates of 5 μl/min. Kinetic constants were calculated using the BIA evaluation software (version 2.1). A one-to-one model (A + B = AB) is fitted, assuming that the dissociation rate constant is known from the dissociation curve (n = number of binding sites on the surface blocked by the





Eq.1:

$$\frac{E_0}{\sqrt{v_i}} = \frac{K_m\left(1+\frac{I}{K_i^{com}}\right)+S}{Sk_{cat}}\sqrt{v_i} + \sqrt{\frac{K_d K_m}{4Sk_{cat}}}\left(1+\frac{I}{K_i^{dim}}\right)$$

Fig. 1. Kinetic classification of some peptides. (a) Zhang–Poorman Plot for Ac-Ser-Tyr-Glu-Leu. The K_m-value for the substrate is 40 μM at $I = 0.1$ M. The average inhibitor constant obtained by the linear fit is 0.29 μM for dimerization inhibition. The inset shows the Zhang–Poorman equation, E_0 is the total enzyme concentration in the assay, S the initial substrate concentration, I the inhibitor concentration and v_i the initial reaction velocity. (b) Zhang–Poorman Plot for Ile-Ile-Ser-Tyr-Glu-Leu at two different concentrations. The K_m-value for the substrate is 29 μM at $I = 1.1$ M. The unaffected intercept in combination with the systematic variation of the slope indicates a purely competitive inhibition. The average inhibition constant is 3.7 μM.

analyte). For this model $k_{ass}C \gg k_{diss}$ holds. Therefore, the binding curves are only marginally influenced by the dissociation rate k_{diss} which can be determined separately by monitoring the dissociation of the formed complex in the buffer flow. Since there is no analyte ($C = 0$) in this case, the plot $R(t_0)/R(t)$ vs. t will be linear yielding K_d as the slope (Fägerstam et al., 1992).

2.10. Denaturation/renaturation experiments

The experiments were performed according to the method described by Babé et al. (1992). The samples (HIV-2 protease from Bachem, Heidelberg) were denatured in 50% acetic acid at 55°C (3 min), renatured by diluting with 17 volumes of assay buffer in 4 aliquots within 30 min. Solid

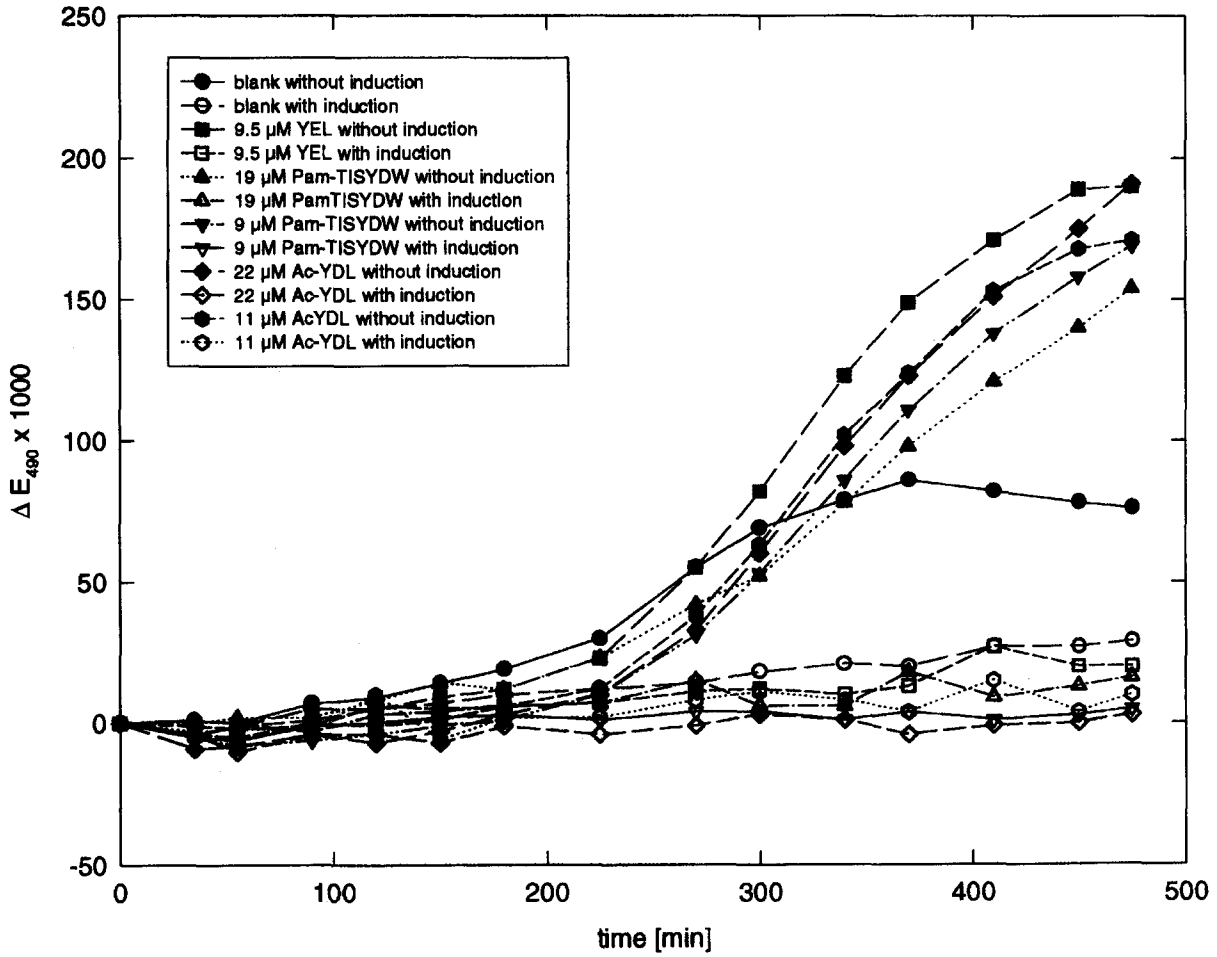


Fig. 2. Synergistic inhibition of HIV-1 protease. Combined effects of SDZ PRI 053 and Pam-Thr-Val-Ser-Tyr-Glu-Leu on the inhibition of the HIV-1 protease. The difference surface between the measured and the theoretical inhibition is shown. The theoretical additive interactions were calculated from the inhibition curves of the individual inhibitors, applying the dissimilar site assumption of additivity for mutually exclusive inhibitors. Positive values in the difference surface indicate synergy, negative values antagonism. The data clearly reveal regions of synergistic interactions at higher Pam-Thr-Val-Ser-Tyr-Glu-Leu molarity.

Table 1
Inhibition of HIV proteases by interface peptides

Peptide	Terminus	IC_{50} (μM)	
		(HIV-1)	(HIV-2)
S-F-N-L	C	900	–
S-Y-N-L	C	440	–
S-Y-E-L	C	80	–
Ac-S-Y-E-L	C	55	–
S-Y-E-Y	C	75	–
S-Y-E-W	C	75	–
(w)-Y-D-L	C	12	–
(w)-Y-E-L	C	27	–
Y-E-L	C	75	–
Ac-Y-E-L	C	34	–
Bio-Y-E-L	C	8	–
Y-E-W	C	88	–
Ac-Y-E-W	C	21 [a]	–
Y-D-L	C	9 [a]	–
Ac-Y-D-L	C	9 [a]	–
Y-S-Y-E-L	C	5.5	–
I-S-Y-E-L	C	4.5, 12.2 [b]	–
Ac-I-S-Y-N-L	C	70	–
I-S-Y-E-Y	C	37	20
V-S-Y-E-C(SEt)-G	C	120	–
Ac-T-V-S-F-N-F [c]	C	140	–
Ibu-T-V-S-F-N-F	C	<62	–
Pam-T-V-S-F-N-F	C	15	20
T-V-S-Y-E-L	C	12	4
Ibu-T-V-S-Y-E-L	C	6	–
Bio-Cap-T-V-S-Y-E-L	C	3	–
Pam-T-V-S-Y-E-L	C	0.5	1.3
Ibu-T-V-S-Y-E-L-NH_2	C	175	–
Ac-T-V-S-Y-E-L-N	C	100	–
Bio-T-V-S-Y-E-L	C	5	–
T-I-S-Y-E-L	C	6	1.5
I-I-S-Y-E-L	C	8	–
L-Q-I-T-L-W	N	37	–
L-Q-I-T-L-(w)	N	140	–
L-Q-I-T-C(SEt)-W	N	12	–
Ac-N-R-G-L-A-A [d]	C	>1000	375
Ac-Q-V-S-Q-N-Y [e]	C	100	–
Pepstatin A	–	0.6	–

Inhibition of the HIV proteases by peptides derived from interface peptides (standard test at 0.6 M ionic strength). All peptides were at least 90% pure as estimated by HPLC and/or mass spectroscopy. Ibu-, isobutyroyl-; Cap-, ε-aminocaproyl-; Pam-, palmitoyl-; Bio-, biotinyl-; SEt, -*S*-ethyl; (w), D-Trp; –, not determined.

[a] Test at 0.1 M ionic strength.

[b] K_i value (Daenke et al., 1994).

[c] C-terminal segment of HIV-1 p6* protein.

[d] C-terminal segment of HIV-2 p6* protein.

[e] C-terminal segment of HIV-1 matrix protein.

NaCl was added to obtain a molarity of 275 mM NaCl. The solution was concentrated in a centricon 3000 tube (Amicon Inc., Beverly, USA) before native electrophoresis (detection with silver stain).

3. Results

3.1. Interface peptides derived from the C-terminus.

Starting from already known inhibitory sequences (such as Thr-Val-Ser-Phe-Asn-Phe, (Schramm et al., 1993) and Thr-Ile-Ser-Tyr-Glu-Leu, (Arnold et al., 1994)) or from natural sequences, peptides with 3–7 residues were modelled and computer-docked into their natural binding site, the interface cleft of a monomeric protease subunit. In this step, the structural motif of two parallel β-strands is transformed into an anti-parallel β-sheet with three (or four) strands.

The best-fitting peptides were synthesized by solid-phase methods and tested in our modified enzyme assay (Table 1). The best inhibitory constant is now less than 1 μM, i.e. comparable to pepstatin A. Already tri- and tetrapeptides derived from the positions 96–99 or 97–99 showed comparatively strong inhibition. The addition of a residue in position 100 did not improve binding. The good activity of palmitoyl-peptides should be emphasized. Obviously, the long aliphatic side chains bestow the peptidic parts with further unspecific affinity for the protease monomer which has large hydophobic patches.

Although the single calculations were not always comparable, there was a good overall correlation (data not given) between the obtained docking energies (e.g. in the DOCK procedure) and the K_i or IC_{50} values in the test, with the exceptions of some charged peptides and some peptides with extensions of the 95–99 segment. It was not intended (or possible) to study this correlation in detail. The fact that the inhibition constants could be improved so much (about 1000-fold) gives support that the basic notion of dimerization inhibition holds well for the β-sheet moiety of the peptides. It also confirms that this relatively simple kind of rational design is valid and appropriate.

Table 2
Inhibition mode of some of the inhibitory peptides

Inhibitor								Ionic strength (M)	Inhibition constants (μM)	
	94[a]	95	96	97	98	99			K_i^{dim}	K_i^{com}
C-terminal peptides										
				Y	E	L		0.1	2.35 ± 0.12 [b]	–
			(w)	Y	D	L		0.1	–	2.16 ± 0.13
			(w)	Y	E	L		0.1	–	2.11 ± 0.23
			S	Y	E	W		0.1	0.32 ± 0.066	–
			S	Y	E	L		0.1	1.27 ± 0.25	–
		Ac	S	Y	E	L		0.1	0.29 ± 0.017	–
		Ac	S	Y	E	L		0.6	8.7 ± 1.1	12.6 ± 0.8
		Ac	S	Y	E	L		1.1	16 ± 2.5	37.6 ± 0.7
		I	S	Y	E	L		0.1	0.39 ± 0.05	–
	Ac	I	S	Y	E	L		0.1	0.27 ± 0.003	–
Pam	T	V	S	Y	E	L		0.1	0.16 ± 0.026	–
	I	I	S	Y	E	L		0.1	–	0.55 ± 0.05
	I	I	S	Y	E	L		1.1	–	3.7 ± 0.3
N-terminal peptides										
		L	Q	I	T	L	W	0.1	0.59 ± 0.03	–
		L	Q	I	T	C(SEt)	W	0.1	–	5.2 ± 0.07
		L	Q	1	T	F	(y)	0.1	–	4 ± 0.1
Peptides containing C- and N-terminal sequences										
					M 2/3 [c]			0.1	0.2 ± 0.03	0.37 ± 0.02
					M 2/3			1.1	–	1.3 ± 0.08

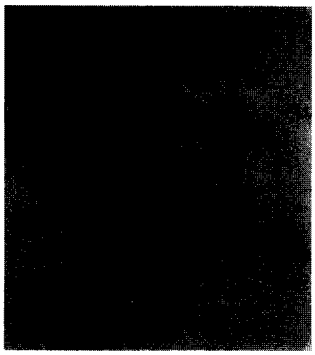
[a] Corresponding position in the C-terminal HIV-1 protease sequence (GCTLNF[99]).
[b] Mean and standard deviation.
[c] Leu-Glu-Ile-Thr-Leu-Gly-Glu-Arg-Gly_5-Asp-Arg-Ile-Ser-Tyr-Gln-Leu (Arnold et al., 1994). Other abbreviations as in Table 1.

The inhibition constants in an HIV-2 protease test were also determined using the same substrate. Some peptides gave better inhibition constants in the HIV-2 assay. Peptides with a large C-terminal amino acid, as Trp 99 or Phe 99, are relatively weak inhibitors, however. This can be explained by the smaller pocket available in the HIV-2 protease monomer for the side chain of residue 99.

3.2. N-terminal interface peptides

Peptides derived from the N-terminal segment of the protease usually show poor inhibition in comparison to C-terminal peptides. One complication is that the bond Leu[5]-Trp[6] is cleaved by the protease itself (Babé et al., 1991). The best N-terminal peptide is Leu-Gln-Ile-Thr-Cys(SEt)-Trp with an IC_{50} of 12 μM. In the 'natural' (N-terminal) binding mode, the -S-S-ethyl group of this peptide might have allowed in situ formation of a covalent bond by disulfide exchange with Cys[95] of the monomeric protease ('affinity labelling'). However, the peptide turned out to be active-site directed in our test (Table 2), raising further questions about the usefulness of N-terminal sequences. Computer docking of the N-terminal sequence Leu-Gln-Ile-Thr-Leu-Trp (IC_{50} = 37 μM)—a confirmed dimerization inhibitor (Table 2)—into the 'C-terminal' binding site (the cleft between the N- and C-termini of a free subunit, with Leu[1] to Trp[6] in position 95–100) showed stronger binding than in the 'natural' (N-terminal) binding mode. A synergism between N-terminal and C-terminal peptides could also not be found. It can be assumed, therefore, that at least some of the 'N-terminal' peptides or parts of bifunctional peptides (Babé et al., 1991; Arnold et al., 1994) preferentially bind to the 'C-terminal' binding site, or to the active site.

The lower inhibitory potency of peptides derived from the N-terminal segments can be



1 HIV-2 PR + DMSO
2 HIV-2 PR + Ac-TVSYEL-OH
3 HIV-2 PR + M2/3
4 HIV-2 PR + Pam-TVSYEL-OH
5 HIV-2 PR 1:1
6 Cytochrom C

1 2 3 4 5 6

Fig. 3. Denaturation/renaturation of HIV-2 protease. The figure shows a native electrophoresis plot of HIV-2 protease denatured with 50% acetic acid and renatured in the presence of peptidic inhibitors (see Materials and methods). The inhibitor Pam-Thr-Val-Ser-Tyr-Glu-Leu with $IC_{50} = 0.5$ μM and 'M2/3', the bifunctional 20mer peptide Leu-Glu-Ile-Thr-Leu-Gly-Glu-Arg-$(Gly)_5$-Asp-Arg-Ile-Ser-Tyr-Glu-Leu with $IC_{50} = 1$ μM (Arnold et al., 1994), prevent renaturation of the protease under standard conditions, while the short peptide Ac-Thr-Val-Ser-Tyr-Glu-Leu (IC_{50} = ca. 2 μM) does not prevent renaturation. The bands visible in lanes 3 and 4 are aggregated peptides.

expected, since these peptides are attached to the outside of the protease β-sheet and form only one hydrogen bond per peptide bond. A weaker binding of the N-terminal segment is in fact necessary for the liberation of the protease during auto-processing of the precursor polypeptide. The up-stream p6* domain (Partin et al., 1991; Zybarth et al., 1994) is cleaved off after folding of the protease domain by insertion of the flexible N-terminal protease segment—still connected to the p6* peptide—into the active-site tunnel (Louis et al., 1994).

3.3. Influence of viral proteins on HIV proteases

The strong inhibitory potency of the C-terminal p6* sequence Ac-Thr-Val-Ser-Phe-Asn-Phe ($IC_{50} = 28(50)$ μM against HIV-1(HIV-2) protease is already known (Schramm et al., 1993). The corresponding C-terminal segment of the HIV-2 p6* polypeptide Ac-Asn-Arg-Gly-Leu-Ala-Ala was found to be inactive against HIV-1 protease (against HIV-2 protease: $IC_{50} = 375$ μM). However, the C-terminal segment of the HIV-1 matrix protein Ac-Gln-Val-Ser-Gln-Asn-Tyr ($IC_{50} = 100$ μM) showed moderate inhibition of HIV-1 protease. It is remarkable in this context that this segment—after phosphorylation of the terminal Tyr by a virion-associated cellular kinase—is also involved in a key regulatory process (Gallay et al., 1995), bestowing the matrix protein with karyophilic properties. The result suggests a complicated interaction between viral proteins, e.g. during viral assembly and maturation.

3.4. Prevention of renaturation of protease by peptides

Denaturation/renaturation experiments were performed according to the method described by Babé et al. (1992). The renaturation of acetic acid-denatured protease was successfully prevented only by the 'long' inhibitor 'M2/3', the 20mer Leu-Glu-Ile-Thr-Leu-Gly-Glu-Arg-$(Gly)_5$-Asp-Arg-Ile-Ser-Tyr-Glu-Leu ($IC_{50} = 1$ μM, Arnold et al., 1994) and the palmitoyl-blocked peptide Pam-Thr-Val-Ser-Tyr-Glu-Leu ($IC_{50} =$ 0.5 μM), not, however, with the short peptide Ac-Thr-Val-Ser-Tyr-Glu-Leu ($IC_{50} = 8$ μM), as demonstrated in non-denaturing electrophoresis (Fig. 3).

After treatment of the protease with DMSO (to 20% v/v), which abolishes activity almost completely, the reactivation by dilution with the 10-fold amount of buffer is nearly instantaneous (Fig. 5).

Table 3
Biochemical interaction analysis: HIV-1 protease with biotinylated interface peptides

Peptide	K_i^{dim}	K_i^{com} (μM)	k_{ass} [a] (M/s)	k_{diss} (/s)	K_d [a] (nM)
Biotinyl-Y-E-L	–	2.3	1.0×10^3	8.3×10^{-4}	830
Y-E-L	2.4 μM	–	–	–	–
Biotinyl-T-V-S-Y-E-L	–	1.14	1.9×10^3	6.9×10^{-4}	372
Biotinyl-Cap-T-V-S-Y-E-L	–	0.47	2.8×10^3	2.7×10^{-4}	96
Biotinyl-Cap-T-V-S-Y-E-L plus streptavidin	180 nM	–	–	–	–
Palmitoyl-T-V-S-Y-E-L	160 nM	–	–	–	–

Kinetic constants of streptavidin binding of biotinylated peptides compared with the K_i values from the enzyme test (see also Table 2 and Section 3.). Cap-, ε-aminocaproyl-.
[a] The molecular mass of the monomeric protease is used for the calculation.

3.5. Mechanism of inhibition

A method which allows dimerization inhibitors to be distinguished from competitive inhibitors has been established by Zhang et al. (1991). The analysis uses the equation given in Fig. 1. The $E_0/\sqrt{v_i}$ vs. $\sqrt{v_i}$ plot of HIV-1 protease allows the dissociation constant K_d of the dimer to be estimated from the positive y-intercept value. A dimerization inhibitor (with K_i^{dim}) alters the y-intercept value by a factor of I/K_i^{dim}, but has no effect on the slope; this results in a series of parallel lines for the different inhibitor concentrations [I]. On the other hand, a competitive inhibitor (with K_i^{com}) alters the slope and leaves the y-intercept unaffected[1]. In plots $E_0/\sqrt{v_i}$ vs. $\sqrt{v_i}$, a competitive inhibitor is represented by lines that cut the ordinate at the same point. This kinetic analysis allows a reliable identification of dimerization inhibitors.

With our modification of the original method, dissociation constants of $K_d = 4.0 \pm 2$ nM for HIV-1 protease at pH 5.0, and $K_d = 57$ nM at pH 7.0 (20 min preincubation of the diluted enzyme), respectively, were obtained. These values are in good agreement with the K_d values of 3.6 nM (pH 5.0) and 50 nM (pH 7.0) obtained by Zhang et al. (1991) and Cheng et al. (1990), respectively. The K_i of Ac-Thr-Leu-Asn-Phe (32 μM) was also similar to the value obtained by Zhang (45 μM). This shows that the different K_m values and S_0/K_m quotients for the fluorogenic and chromogenic substrates used in the two different tests do not influence the assay within a reasonable experimental error. The chromogenic substrate, however, is considerably cheaper and the determination easier.

The inhibition curves (Fig. 1a) obtained for Ac-Ser-Tyr-Glu-Leu at ionic strength 0.1 M exhibit a systematic variation of the intercept indicating inhibition of dimerization. The equation yields an average K_i^{dim} of 0.29 μM, a good value for a short tetrapetide. On the other hand, the data for the peptide Ile-Ile-Ser-Tyr-Glu-Leu indicate competitive inhibition represented by a bundle of lines with the same intercept (Fig. 1b). From the slope variation, a K_i^{com} value of 3.7 μM was calculated. The variation of the ionic strength from 1.1 M (mostly used in other publications) down to 0.1 M leads to a significant increase of protease inhibition (Table 2) by the peptides, approximately by a factor of 4–6 for competitive inhibitors and up to 55 for dimerization inhibitors.

The ionic strength of the reaction solution can not only influence the K_i values but also the inhibition mechanism. In 0.1 M solution, for instance, Ac-Ser-Tyr-Glu-Leu acts as a genuine dimerization inhibitor at all protease concentrations, while at 1.1 M ionic strength a combined mechanism prevails. This may be partly due to the known salt stabilization of the dimers but also due to the larger contribution of hydrophobic interactions for peptides bound to the active site. The lower ionic strength is, however, more related to natural conditions.

Short β-sheet peptides exhibit a prevalent affinity to the interface and the inhibition constants

[1] A careful analysis of the data shows a small effect of the competitive inhibitors on the y-intercept. This is due to the stabilization of the dimers by the inhibitors, similar to the substrate stabilization of HIV protease (Kuzmic, 1993).

are relatively high. Extentions of the 'canonical' β-sheet segment (amino acids 96–99) are possible without change in the inhibition mode: e.g. Ac-Ile-Ser-Tyr-Glu-Leu and Pam-Thr-Val-Ser-Tyr-Glu-Leu are good dimerization inhibitors. C-terminal peptides containing D-tryptophan in position 96 or a biotinyl extention at the N-terminus act as competitive inhibitors. Surprisingly, the elongation of Ile-Ser-Tyr-Glu-Leu by only one additional amino acid changes the mechanism completely: Ile-Ile-Ser-Tyr-Glu-Leu is a genuine competitive inhibitor (Fig. 1) at both molarities ($I = 0.1$M and $I = 1.1$ M).

3.6. Biosensor experiments

Binding assays performed in a BIAcore™ instrument showed strong binding of HIV-1 protease to biotinylated peptides immobilized on a streptavidin chip (Table 3). The peptides switch from dimerization inhibition to competitive inhibition after biotinylation, indicating an active-site-directed component of biotin binding. As expected, the same biotinyl peptides change back to the dimerization inhibition mode in the presence of streptavidin which blocks the biotin moiety. This means that the impressive low K_d values (last column) must be related to interface binding. These values are not easily comparable to those of free peptides, however, since non-specific interaction between streptavidin and protease may contribute to binding. For optimal binding to the chip, a spacer between biotin and the peptide seems to be required.

3.7. Synergism studies

For the determination of a possible synergistic action between competitive inhibitors and interface peptides, active-site-directed pseudopeptides (e.g. Sandoz Inhibitor SDZ PRI 053; $K_i = 9.5$ nM (Billich et al., 1995) and benzyloxycarbonyl-Ala-Ala-NH- $CH((CH_2)_3CH_3)$-CH(OH)-$CH((CH_2)_3CH_3)$-NH-Ala-Ala-benzyloxycarbonyl; $K_i = 3.2$ μM (König et al., 1995)) were used together with dimerization inhibitors (e.g. Pam-Thr-Val-Ser-Tyr-Glu-Leu; $K_i = 0.16$ μM and Ac-Ser-Tyr-Glu-Leu; $K_i = 0.3$ μM). The proteolytic activity in the presence of different mixtures of both inhibitor types shows distinct synergistic behaviour in all cases (Prichard and Shipman, 1990). In a the three-dimensional synergy analysis between SDZ PRI 053 and Pam-Thr-Val-Ser-Tyr-Glu-Leu is shown. The data indicate that a higher concentration (> 2 μM) of the palmitoyl-peptide may still improve the inhibition, while the optimal concentration for synergistic action of the active-site inhibitor is 0.01 nM. The poor solubility of the palmitoyl-peptide, however, may prevent much higher concentrations. Mixtures of C- and N-terminal peptides were also tested in the standard test assay, but no synergism was found. This casts further doubt on the interpretation that N-terminal peptides are able to bind as surrogates of 'N-terminal' protease segments (see above).

3.8. Inhibition of cell growth

The tests using the screening method of Sedlácek et al. (1993) in a modified form yielded surprisingly good cell protection by our inhibitors, e.g. the small peptide Ac-Tyr-Asp-Leu-OH (Fig. 2). Despite the differences in membrane structure between *Escherichia coli* cells and lymphocytes, the results indicate good cell permeation and activity. The toxicity of the compounds is low, since only a slight growth impairment of the host cells (without the plasmid) could be observed (data not shown).

4. Discussion

The dimers of HIV protease dissociate spontaneously (the K_d value is probably between 5 and 50 μM and still controversial; see Holzman et al., 1991; Kuzmic, 1993; Kuzmic et al., 1993; Pargellis et al., 1994; Darke, 1994). The free monomers are then partly blocked by the inhibitory peptides which compete with the corresponding terminal segments of other free subunits. This shifts the monomer/dimer ratio towards the monomers and causes the enzymatic inhibition by dimer depletion. Alternatively, a partial dissociation of the dimeric structure—there are four terminal segments in the four-stranded interface—with subse-

quent attachment of the peptides to the still dimeric but partly unfolded protease at the β-sheet interface might also interfere with the catalytic activity, since amino acid residues of both subunits contribute to the active-site region. The kinetic analysis of Zhang–Poorman indicates an influence of the C-terminal interface peptide Ac-Thr-Leu-Asn-Phe on the dissociation of the dimer, due to the formation of the monomer–inhibitor complex, but not on the substrate binding. Furthermore, ultracentrifugal studies identified monomers in the presence of this peptide. These two results clearly indicate a dissociative mode of inhibition.

The Zhang–Poorman equation has been derived assuming fast equilibria between all species, including enzyme–substrate and enzyme–inhibitor complexes and a slower final catalytic step. This is the simplest way to derive an equation which fits the observed data. However, it is also possible to apply the steady-state assumption for the concentrations of the monomer, monomer–inhibitor, dimer–inhibitor and dimer–substrate complexes, respectively. The resulting, rather complex equation can be simplified by assuming the decay of the dimer–substrate complex to be faster than the final catalytic step. The obtained simplified formula can be shown to be identical to the Zhang–Poorman equation derived under less restrictive conditions. It is therefore assumed that, although the dissociation process was found to be slow (Darke et al., 1994; Pargellis et al., 1994), the experimental data can be evaluated by applying the Zhang equation. The analysis supplies a potent and rapid analytical tool for the discrimination of the two (competitive/dissociative) mechanisms, allowing a safe identification of interface inhibitors. It has not been possible so far to isolate monomer complexes. However, the good correlation of the inhibition constants with the docking affinities resulting in a considerable improvement of the inhibition constants of the peptides (about 1000-fold) also confirms the basic notion of this type of inhibition. The results in Table 2 show that most peptides derived from the 'canonical' β-sheet of HIV protease exhibit genuine dimerization inhibition of the enzyme (Table 2).

The interface peptides, however, may also be partly active-site directed, since the terminal segments from which they are derived are cleavage segments of HIV protease. Our modified peptides, therefore, should also have some remaining affinity for the subsites. Indeed, only small changes in structure or ionic strength induce a switch in the binding mode of the inhibitory peptides (Table 2). Some peptides even seem to possess similar affinities for both sites. This suggests that short peptidic or non-peptidic 'active-site inhibitors' of HIV-1 protease should be scrutinized in a suitable assay to ascertain that they are competitive inhibitors. Even established active-site inhibitors with confirmed X-ray structures of the complexes (crystallized at high ionic strength) may act as dimerization inhibitors at more physiological conditions (i.e. at low ionic strength), for instance against the unprocessed protease domain within the Gag-Pol precursor polyprotein.

The reason for the influence of the ionic strength on the K_i values and the inhibition mechanism of some of the peptides may be salt stabilization of the protease dimers. This is also a reason for the strongly differing K_d values which were measured under very different buffer conditions. Another reason for the salt influence is probably the larger proportion of hydrophobic contacts for peptides bound to the hydrophobic subsites of the catalytic center in comparison to those of interface peptides. The lower ionic strength, however, which is favourable for dimerization inhibition is far more related to natural conditions than the unphysiological 1 M (up to 3 M) ionic strength usually applied in enzyme tests.

Bifunctional inhibitory peptides which contain both affinities (for active site and interface, respectively) in different but connected segments should also be possible. The fact that only some of the (larger) peptides prevent refolding after denaturation (Fig. 3) is inconclusive with respect to the mechanism, but it shows that an interaction of some of the peptides with the unfolded protease occurs.

The physical attachment of interface peptides to the protease was confirmed in biosensor experiments by the strong binding of HIV-1 protease to

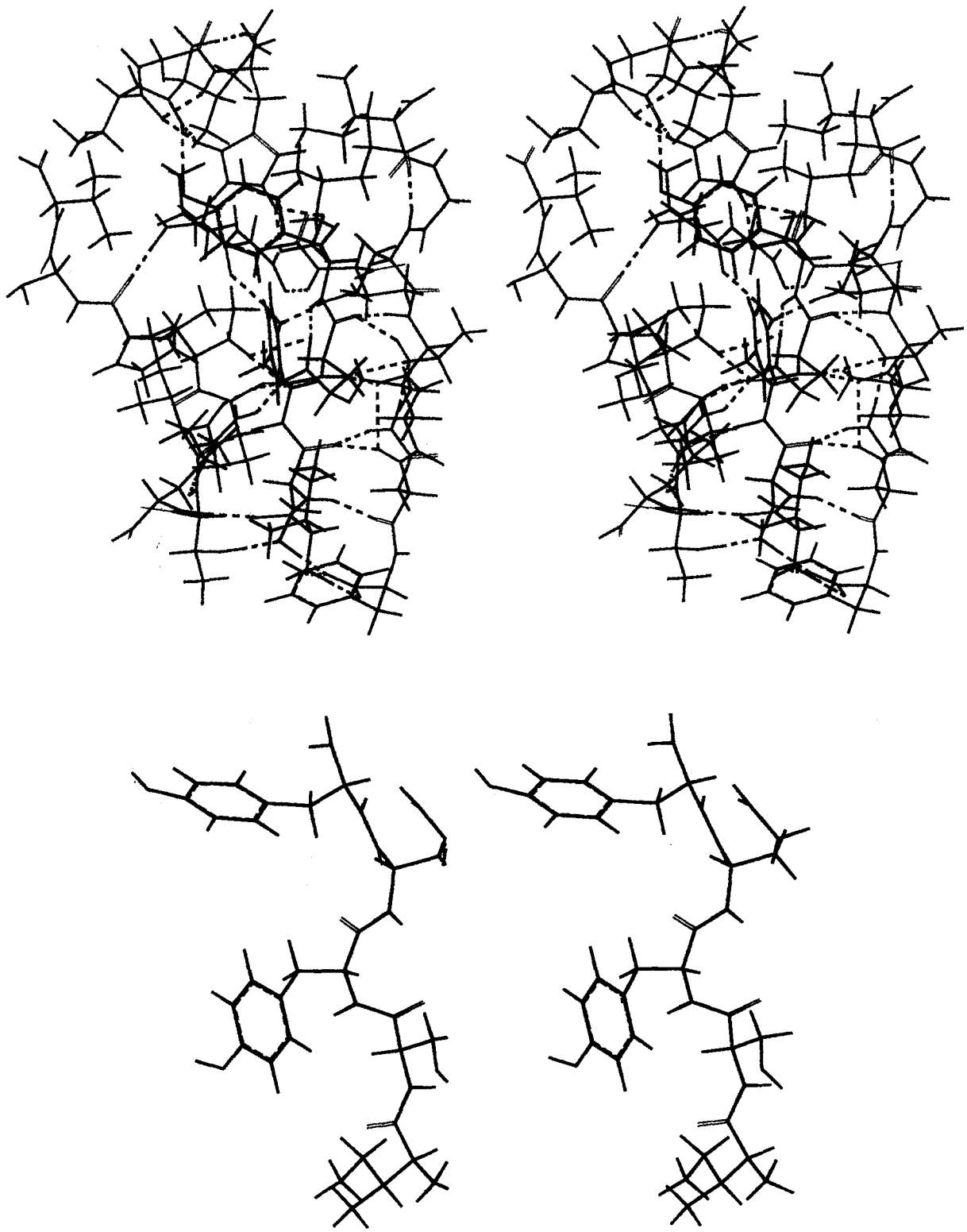


Fig. 4. The dimer interface of HIV protease with the bound peptide Ile-Ser-Tyr-Glu-Tyr. The distances are: C of terminal CO_2 to C-OH of Tyr^{99}, 0.51 nm; C of terminal CO_2 to C-OH of Tyr^{97}, 1.06 nm; C of terminal CO_2 to C of CO_2 Glu^{98}, 0.50 nm; C of CO_2 Glu^{98} to C-OH of Tyr^{97}, 1.13 nm; C of CO_2 Glu^{98} to C-OH of Tyr^{99}, 0.85 nm.

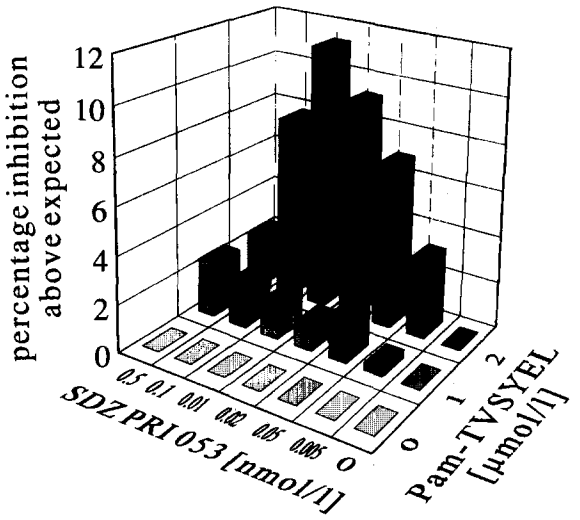


Fig. 5. Synergistic inhibition of HIV-1 protease. Combined effects of SDZ PRI 053 and Pam-Thr-Val-Ser-Tyr-Glu-Leu on the inhibition of the HIV-1 protease. The difference surface between the measured and the theoretical inhibition is shown. The theoretical additive interactions were calculated from the inhibition curves of the individual inhibitors, applying the dissimilar site assumption of additivity for mutually exclusive inhibitors. Positive values in the difference surface indicate synergy, negative values antagonism. The data clearly reveal regions of synergistic interactions at higher Pam-Thr-Val-Ser-Tyr-Glu-Leu molarity.

the peptides biotinyl-Tyr-Glu-Leu, biotinyl-Thr-Val-Ser-Tyr-Glu-Leu and biotinyl-6-aminocaproyl-Thr-Val-Ser-Tyr-Glu-Leu (all bound to streptavidin chips). Because of the different methods and test conditions (surface, ionic strength), the K_d values and inhibition constants found in the the two assays are not comparable; however, they correlate well (Table 3). The full binding affinity becomes evident only if a long spacer separates the two binding moieties, the heterocycle and the peptide. In the Zhang–Poorman assay, it turned out that the bifunctional biotinyl peptides are active-site directed. If the biotin moiety is blocked by streptavidin (in solution or immobilized on the chip), however, the intrinsic β-sheet propensity of the peptide shows up again: a striking demonstration of the 'amphitropic' behaviour of the interface peptides.

The test results allow a summarizing formulation of a 'consensus sequence' of potent peptidic inhibitors for the positions 94–99 of the protease:

H,Ac,Pam–Thr^{94}-Val,Ile^{95}-Ser,Thr^{96}-Tyr^{97}-Asp,Glu,Asn^{98}-Leu,Tyr,Trp^{99}–OH.

The summary does not supply one optimal sequence, it identifies, however, a 'pharmacophore' structure which binds to the interface β-cleft of protease monomers. The main binding part is obviously the -Tyr-Glu-Leu-OH structure (and similar sequences as -Tyr-Asp-Phe-OH etc.) with two carboxyl groups (in Glu^{98} and the free C-terminus of Leu^{99}), the deeply inserted Tyr^{97} with a hydrogen-bonded phenolic -OH group, and the large (or very large, even biphenyl should fit) hydrophobic side chain of amino acid 99. Amino acid 96 may be a small hydrophilic residue with β-sheet propensity and 95 a residue with a medium or large hydrophobic side chain. A three-dimensional representation of one of these possible structures (Ile-Ser-Tyr-Glu-Tyr, $IC_{50} = 37$ μM) is given in Fig. 4.

The knowledge of the characteristic structural properties of potent protease inhibitors is a prerequisite for the development of AIDS therapeutics, e.g. by the design of small-size peptide mimetics or by the preparation of constructs for intramolecular expression. While much information on the structural properties of active-site inhibitors has been gained from X-ray structures, the presented structural details ('consensus structure') should be helpful for better defining the interface interactions and for developing inhibitors of this alternative type. In contrast to active-site inhibitors, dimerization inhibitors may also act on the Gag-Pol precursor polyprotein (Kräusslich, 1991), thus preventing—rather than inhibiting—protease activity and viral infectivity. This may be the reason why the above-mentioned octapeptides (Schramm et al., 1991) produce a greater effect in the cell test than in the enzyme test.

Synergistic action allows the application of lower concentrations in mixtures of drugs with toxic side effects. While synergism between N- and C-terminal peptides could not be found, this

enhancement of action was apparent in all experiments using pairs of dimerization and competitive inhibitors. This result again confirms the presence of a second binding site and the proposed mode of action. Because of their synergistic action, dimerization inhibitors may become valuable constituents of therapeutic 'cocktails' of active-site inhibitors and RT inhibitors. Such an approach is likely to succeed, since a reduction of protease activity by only 50-fold completely prevents the formation of infectious particles (Rosé et al., 1995) (Fig. 5).

Experiments with cell cultures are just beginning. However, the delay of viral replication in T4 lymphocytes by some of the newly synthesized dimerization inhibitors for 5 days and longer (Gürtler et al., in preparation) demonstrates their antiviral potential.

Acknowledgements

We gratefully acknowledge the donation of HIV-1 protease, the plasmid pET9c-PR and the inhibitor SDZ PRI 053 by A. Billich and P. Lehr, Sandoz Forschungsinstitut, Wien. We also thank W. Schäfer for the mass spectroscopy of the peptides, F. Siedler for some of the peptides and the Deutsche Forschungsgemeinschaft and Armour Co. for grants.

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