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A Modular Approach to HIV-1 Proteinase Inhibitor Design

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HIV-1 proteinase represents a promising target for antiviral chemotherapy. We have designed, synthesized, and tested modular inhibitors combining an active-site inhibitor tethered to a structure targeted to the dimerization domain of the enzyme. At pH 5 the parent active site inhibitor, the equimolar mixture of active site and dimerization inhibitors, and the best compound from our series of modular inhibitors show the same inhibition activity. At neutral pH, however, the combination of the dimerization and active-site inhibitors shows a synergistic effect. Moreover, the modular inhibitor has an IC_{50} value 5× lower than the parent active site inhibitor and 2× lower than the equimolar mixture of the two parent inhibitors. The Lineweaver-Burk plot for modular inhibitors corresponds to a pattern for mixed type inhibition.

Human immunodeficiency virus is the causative agent of AIDS. HIV-proteinase (PR) plays an important role in the viral life cycle—cleaving *gag* and *gag/pol* polyproteins into functional proteins during viral maturation. The importance of HIV PR for maturation of viral particles makes it a suitable target for antiviral chemotherapy (for review see [1]). HIV PR is functional as an obligatory homodimer. Each monomer subunit contributes one aspartate unit to the active site. Most to date reported inhibitors are transition state mimics and bind to the active site of the enzyme (for review see [1]); several peptide inhibitors targeted to the dimerization domain of monomer subunits have also been reported [2].

Our work represents a new approach towards HIV PR inhibitor design, where the active site directed inhibitor is tethered to an inhibitor targeted against the dimerization domain. In principle, a similar approach has been chosen by Maraganore *et al* [3] who studied inhibitors binding by one part of the molecule to the catalytic site of thrombin and by another part to the anion-binding exosite.

We hypothesize that these modular inhibitors may have several advantages. First, development of resistance to such compounds might be restricted, because nonlethal parallel mutations in two essential domains of the enzyme (in the active site and in the dimerization domain) might be less probable. Second, dimerization inhibitors may be able to bind the PR domain in uncleaved *gag/pol* polyprotein prior to particle assembly. These polyproteins form densely packed immature particles together with other viral components in the budding process. During this process, modular inhibitors may be transported together with viral proteins because of their dimerization inhibitor segment. The relatively high local concentration of the inhibitor within the particle may then improve the inhibitory potential of the active site directed segment. Finally, we hypothesize that a bivalent proteinase inhibitor might have an advantage over the simple mixture of corresponding parent compounds due to possible stronger interaction of a single inhibitor molecule with two domains of the enzyme(s).

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Abbreviations: Ac, acetyl; AIDS, acquired immunodeficiency syndrome; Boc, t-butoxycarbonyl; Bzl, benzyl; DCM, dichlormethane; DIC, diisopropylcarbodiimide; DMF, dimethylformamide; EDTA, ethylenadiminetetracetic acid; FAB MS, fast atom bombardment mass spectrometry; HIV, human immunodeficiency virus; HOB, 1-hydroxybenztriazole; PR, proteinase; RP HPLC, reverse phase high performance liquid chromatography.

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METHODS

All peptides as well as pseudo peptides with reduced amide bond were synthesized by solid phase methodology on Merrifield and benzhydrylamine resins. Synthesis was performed by standard protocol (DIC-HOBt in DCM/DMF) with Boc, Bzl protection [4]. Reduced amide bonds were synthesized as published by Souček and Urban [5]. Substrate, 2-aminobenzoyl-TINleF(pNO$_2$)QR-NH$_2$, was prepared as published [6]. Completed peptides were cleaved from the resin with the HF/anisole (9:1) mixture. After lyophylization the Boc- group was joined to the N-termini of crude compounds I, II, III using standard method of preparing N-protected amino acids [4]. Peptides were purified and their identity was confirmed by RP HPLC, amino acid analysis and FAB mass spectrometry.

High-level expression of HIV-1 proteinase (PR) was achieved in an adapted T7RNA polymerase/promotor system and purified as described by Konvalinka *et al.* [7]. Concentration of HIV-1 PR was determined by active site titration using a specific peptide inhibitor as described [8].

Spectrophotometric measurements were carried out as follows: The hydrolysis of the substrate, 2-aminobenzoyl-TINleF(pNO$_2$)QR-NH$_2$, in 1 ml of assay buffer at 37°C, in presence of various concentrations of inhibitors was monitored at spectrophotometer Aminco DW2000 at 284 nM for 180s. The volume fraction of DMSO as partial denaturant did not exceed 0.6%. The initial rates of enzyme reaction were graphically expressed. The IC_{50} values were determined from the plots of the ratio of initial rate of the inhibited reaction and the rate of the reaction without inhibitor vs. concentration of the tested compound using Enzfitter program. IC_{50} values for the equimolar mixture of A (Boc-Fψ[CH$_2$NH]FEF-NH$_2$) and D (Ac-TLNF-OH) inhibitors is expressed as the concentration of A required to reduce the enzyme activity by one half.

Determination of dimerization constant HIV-1 PR was carried out as published [9]. An aliquot of the substrate solution was diluted with the assay buffer in a 3 ml cuvette at 37°C. The reaction was started by adding of the enzyme. The fluorescence intensity (337 nm excitation and 410 nm emission) was recorded for 600s—1300s with the 1s step on the spectrofluorimeter Spex Fluorolog 2 connected to a personal computer. Transferred data were fitted to the relation derived by Kuzmič [9] for the concentration jump experiment

$$\alpha = \sqrt{K_d(K_d + 8E)}, \qquad \beta = K_d + 4E,$$

$$\alpha_0 = \sqrt{K_d(K_d + 8E_0)}, \qquad \beta_0 = K_d + 4E_0,$$

$$\gamma = \frac{E(\beta_0 - \alpha_0) - E_0(\beta + \alpha)}{E(\beta_0 - \alpha_0) - E_0(\beta - \alpha)},$$

$$\ln(S) = \ln(S_0) - \frac{\beta + \alpha}{8}\frac{k_{cat}}{K_m}t + \frac{k_{cat}}{4k_1K_m}\ln\frac{\gamma\exp(\alpha k_1 t) - 1}{\gamma - 1}, \qquad [1]$$

where $S \ll K_m$, K_d is dimerization constant, E final enzyme concentration, E_o enzyme concentration before dilution, k_{cat} rate constant for the hydrolysis product formation, S actual substrate concentration, S_0 is the initial substrate concentration, K_m Michaelis constant, and k_1 is the rate constant for dimer association. The unknown parameters in this relation were determined by the least squares method using direct minimalization of the S^2 function [10].

RESULTS

We have designed modular inhibitors (see Table 1), where the inhibitor segment targeted to the active site is tethered to an inhibitor interfering with the dimerization domain of HIV-1 PR. We used an active site inhibitor (compound A) [8] and the C-terminal tetrapeptide of HIV-1 PR sequence which has been shown to function as a dimerization inhibitor [2] (compound D) as building blocks. Compounds I, II and III (Table 1) possess the sequence of the active site inhibitor on their N-termini and the sequence of the dimerization inhibitor on their C-termini, differing only in the length of the linker consisting of 1, 3 or 5 methylene groups, respectively. Another modular inhibitor (compound IV) has the sequence of a dimerization inhibitor on its N-terminus followed by the peptide linker PISG. The sequence -TLNFPIS- represents the natural cleavage site between PR and reverse transcriptase in the *gag/pol* polyprotein. The C-terminus of inhibitor IV corresponds to an active site inhibitor domain.

IC_{50} values of the parent active site inhibitor, the dimerization inhibitor and modular inhibitors were determined at pH 5.0 ($K_m = 30\mu M$, $k_{cat} = 10.1\ s^{-1}$ for the substrate used) which is near to enzyme pH optimum, and at 7.0 ($K_m = 64\mu M$, $K_{cat} = 4.6\ s^{-1}$) which is near to pH expected *in vivo.* At pH 5.0 the inhibitor I exhibits the same IC_{50} value (12nM) as the parent active site inhibitor A

TABLE 1
Comparison of Inhibition Activities of Active Site, Dimerization, and Modular Inhibitors of HIV-1 Proteinase at pH 5.0[a] and pH 7.0[b]

Symbol	Sequence	IC_{50}[nM] pH 5	IC_{50}[nM] pH 7
A	Boc-Fψ[CH_2NH]FEF-NH_2	12 ± 1	1178 ± 57
D	AcTLNF-OH	76,350 ± 416	29,482 ± 384
I	BocFψ[CH_2NH]FEF-NH-CH_2-CO-TLNF-OH	12 ± 1	256 ± 20
II	BocFψ[CH_2NH]FEF-NH-$(CH_2)_3$-CO-TLNF-OH	19 ± 1	328 ± 22
III	BocFψ[CH_2NH]FEF-NH-$(CH_2)_5$-CO-TLNF-OH	21 ± 1	567 ± 51
IV	AcTLNFPISGFψ[CH_2NH]FEF-NH_2	43 ± 2	8,518 ± 231
A + D[c]		12 ± 1	470 ± 38

[a] 100 mM acetate buffer, pH 5.0, 1 mM EDTA, 5% v/v glycerol; 37°C, 39 μM substrate, 17 nM HIV-1 PR.
[b] 20 mM McIlvaine buffer (Na_2HPO_4–citric acid), pH 7.0, 1 mM EDTA, 5% v/v glycerol; 37°C, 79 μM substrate, 17 nM HIV-1 PR.
[c] IC_{50} for the equimolar mixture of the of A and D inhibitors is expressed as the concentration of the A required to reduce the enzyme activity by one-half.

and the equimolar mixture of both parent inhibitors. Note that the IC_{50} value of the equimolar mixture of the two starting inhibitors is calculated as the concentration of A in this mixture needed to decrease the enzyme activity by one half. The other inhibitors show weaker binding and their corresponding IC_{50} values increase with the length of the spacer (see Table 1).

At pH 7.0 the mixture of active site inhibitor A and dimerization inhibitor D inhibits 2.5× better than the parent inhibitor A. The modular inhibitor I exhibits an IC_{50} value of 256nM, inhibiting fivefold more tightly than the parent active site inhibitor A and twice more than the equimolar mixture of active site A and dimerization inhibitor D. Compounds II and III inhibit more weakly.

Lineweaver-Burk plots for the starting active site inhibitor A and for the compound IV corresponds to the pattern for competitive inhibition; straight lines of this plot intersect at a point on the positive semi-axis $1/v$ at $1/S = 0$ (data not shown). Interestingly, lines of the Lineweaver-Burk plots for the modular inhibitors (see Figure 1) intersect in the second quadrant corresponding to mixed type inhibition (i. e. where both the maximal velocity of the reaction and apparent Michaelis constant are influenced in the presence of the inhibitor). The same pattern was observed for the dimerization inhibitor D, as well as for compounds II and III (data not shown).

The dimerization constant K_d, defined as the ratio of the square of the monomer concentration and dimer concentration, was measured at various pH. Our results indicate that the K_d increases with increasing pH (see Figure 2) from $K_d = 19$nM at pH 4.5 to $K_d = 304$nM at pH 7.6. The equilibrium concentration of active enzyme dimer thus decreases with increasing pH. The half-times for reestablishing of new monomer-dimer equilibrium obtained from our data are 50–182s.

DISCUSSION

The aim of this study was to analyze the feasibility of a modular approach to the design of HIV PR inhibitors which is based on the fact that PR molecule possesses two functional domains as possible inhibitor targets. Bifunctional inhibitors should be able to bind by their segments to both enzyme domains—to the active site and to the dimerization domain. Since the K_i values for active site inhibitors with methylenamine isostere [Weber J.: unpublished results], Michaelis constant [11] and dimerization constant of HIV-1 PR are significantly pH dependent, we decided to analyze the inhibition at two different pH values.

Measurements of the dimerization constant showed (see Figure 2) that the concentration of the enzyme active dimer significantly decreases with increasing pH. Comparison of our results

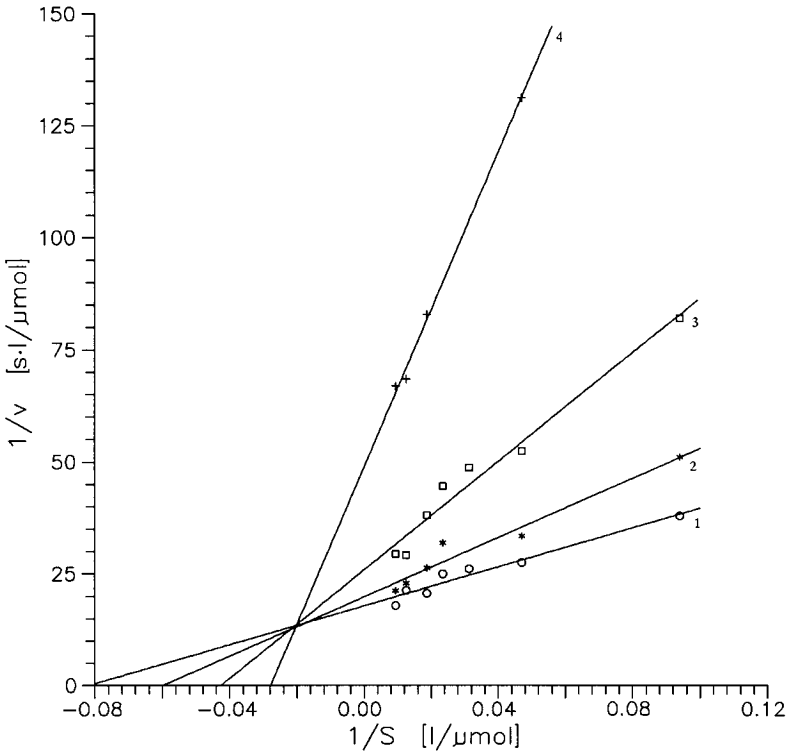

FIG. 1. Lineweaver-Burk plot for the modular inhibitor I. The hydrolysis of 10–100 μM substrate by 17 nM HIV-1 PR in 100 mM acetate buffer, pH 5.0, containing 1 mM EDTA and 5% v/v glycerol was monitored spectrophotometrically as described under Methods. The inhibitor concentration was 0, 1.9, 9.5, and 19.8 nM for measurements in the series 1, 2, 3 and 4, respectively.

($K_d = 112$ nM at pH 6.4) with those obtained by Kuzmič [9] ($K_d = 440$ nM) shows fourfold difference. We attribute this difference to the buffer composition. Our unpublished results show that ethylene glycol (used by Kuzmič in as much as 20% v/v) strongly inhibits the activity of HIV-1 PR and might also affect the K_d determination.

From this data it is conceivable to assume that at higher pH the lower concentration of available PR dimer would favour the inhibition of the enzyme by a dimerization inhibitor. Indeed, at pH 5.0 the IC_{50} values for the equimolar mixture of parent inhibitors A and D, for the active site inhibitor A alone and for the modular inhibitor I are identical. At this lower pH the difference between inhibition activity of the dimerization inhibitor D and the active site inhibitor A is several orders of magnitude and thus the contribution of the 'dimerization module' is not significant. At the higher pH, the inhibitory activity of our parent inhibitors differs only by one order of magnitude and even the mixture of the two inhibitors inhibits better than would correspond to total concentration of both parent inhibitors. To our knowledge, this is a first observation of a synergy between HIV PR inhibitors directed towards different domains of the enzyme. Note that several theoretical models for mixtures of various types of inhibitors describes their synergy—among them the model for a mixture of competitive and mixed type inhibitors that are mutually exclusive [12]. Interestingly, at pH 7.0 compound I exhibits an IC_{50} value twice lower than the equimolar mixture of both parent compound A and D (see Table 1). This result not only indicates a synergistic effect of individual segments of the inhibitor molecule to the overall inhibitory activity; it also seems to suggest additional positive effect of the linkage of both inhibitors to a single molecule in comparison to the simple mixture of compounds. There are two possible explanations of this phenomenon. First, one

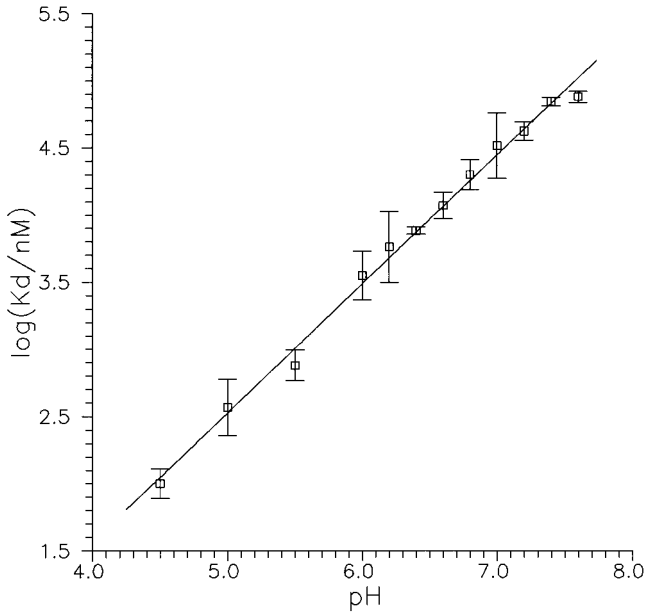

FIG. 2. The pH dependence of HIV-1 PR dimerization constant. The experimental setup is described under Methods. A typical concentration of the substrate was 1.7 μM. Final PR concentration was 11.3 nM, diluted from the stock solution 3.4 μM. Buffers used were 20 mM McIlvaine buffer (Na_2HPO_4—citric acid) with 1 mM EDTA, and 5% v/v glycerol of appropriate pH.

molecule of the modular inhibitor might bind to the active site and to the dimerization domain of the same PR dimer. Alternatively, one can speculate that the modular inhibitors bind to the active site by the appropriate segment inhibitor and the linker with the rest of the molecule might be pointing outwards from the enzyme dimer in such an orientation that favours its binding to the dimerization domain of another PR molecule. The dependence of the inhibition activity on the length of linker appears to be connected with the relative flexibility of the two parts of the inhibitor molecule (compare inhibitors I, II, and III in Table 1); too flexible hinge might enable unfavourable nonspecific interactions.

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