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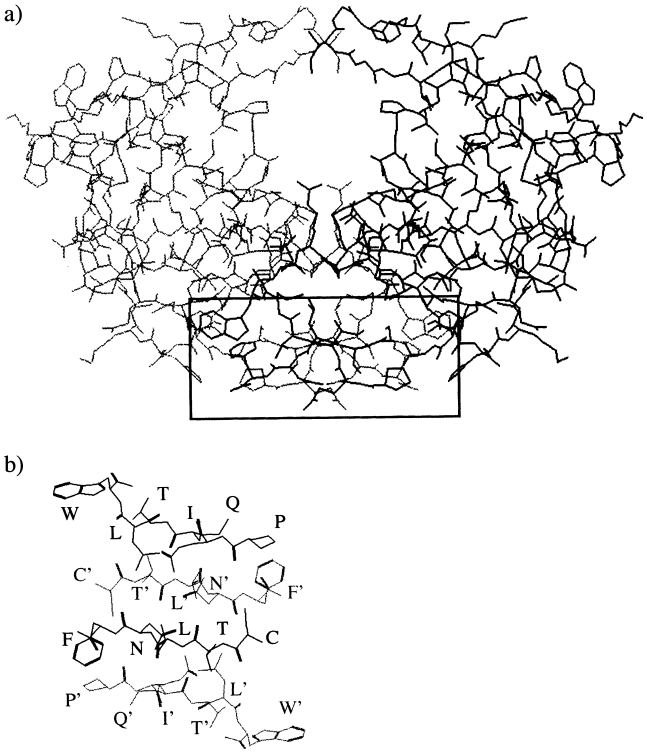

Figure 1. a) Dimeric structure of HIV-1 protease. The area of the N- and C-terminal dimerization interface is indicated by the box. b) The N- and C-terminal dimerization interface of the protease.[10]

Small-Molecule Inhibitors of HIV-1 Protease Dimerization Derived from Cross-Linked Interfacial Peptides**

Michael D. Shultz, Michael J. Bowman, Young-Wan Ham, Xuimin Zhao, George Tora, and Jean Chmielewski*

As more information on macromolecular structure unfolds, it has become obvious that interprotein interactions are a ubiquitous and fundamental aspect of biological activity.[1] Whereas the nature of these protein–protein interactions is becoming better understood, rational approaches to inhibiting these interactions are still in their infancy.[2] Using dimeric HIV-1 protease (Figure 1 a) as a template, we have developed a strategy to inhibit dimerization based on the cross-linking of interfacial peptides derived from the protease.[3] In the current study, we endeavored to identify the essential residues of a cross-linked, interfacial peptide inhibitor of HIV-1 protease, and obtain a minimal structure that maintains the essential features of dimerization inhibition.

The pivotal role of HIV protease in viral replication has made it a prime target for drug design, and there is currently a wide range of potent, active-site inhibitors of HIV-1 protease spanning a number of classes of compounds, including peptide mimetics, C_2-symmetric compounds, and non-peptidic agents.[4] To date five protease inhibitors have been approved by the Food and Drug Administration (FDA) for treatment of HIV infection,[5] but problems of drug resistance due to selection of viruses with specific mutations within protease have been discovered with these inhibitors.[6] Fortunately, results with multidrug approaches made up of protease and reverse transcriptase inhibitors have been promising. Other side effects, however, have also been identified with the current protease inhibitor drugs, including peripheral lipodystrophy,[7] central adiposity,[8] breast hypertrophy in women,[9] hyperlipidemia,[7] and insulin resistance.[7]

The problems that have been identified with the current group of protease inhibitor drugs serve to underscore the need for novel approaches to inhibitor design. One such approach would be to target the dimerization interface of HIV protease.[2] The main dimerization region of HIV-1 protease is found in an interdigitating N- and C-terminal four-stranded, antiparallel β sheet (Figure 1 b).[10] The area centered around the N and C termini accounts for approximately half of the interfacial contact area of the protease homodimer, and the contacts in this area account for greater than 75 % of the free energy of dimerization.[11] This region is also highly conserved among HIV-1 isolates and some HIV-2 isolates, presumably due to the fact that a mutation in the C terminus would necessitate a concomitant mutation in the N terminus to avoid loss of high-affinity dimerization.[12]

Taking advantage of the dimeric nature of HIV protease, Craik and co-workers developed mutant forms of HIV protease for use in a dominant-negative strategy of inhibition; incubation of wild-type protease with monomers that have mutated active-site residues (fourfold molar excess) led to

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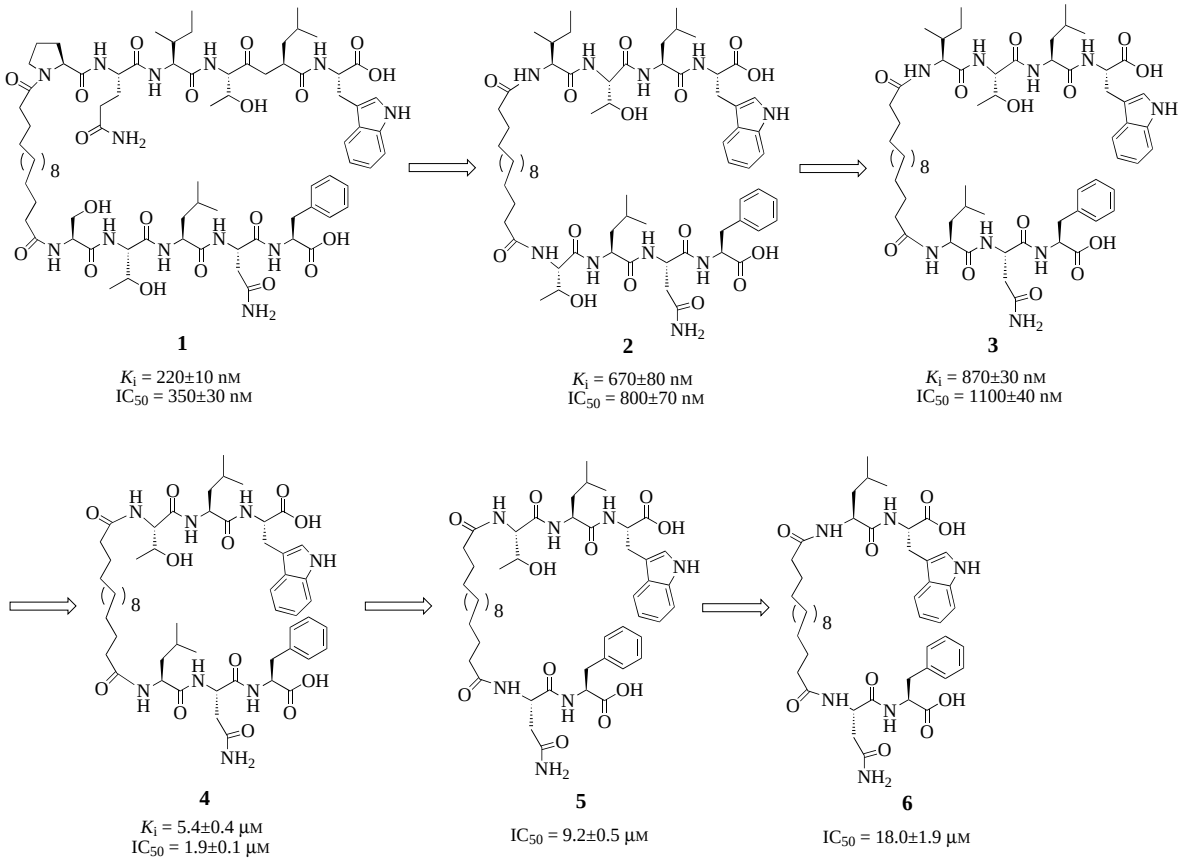
80% reduction in enzyme activity.[13] The features of the dimerization interface of HIV protease have also led to the design of inhibitors that target this portion of the enzyme; interfacial peptides derived from the N and C termini of HIV-1 protease show inhibitory activity,[14] and subunits of the natural product didemnaketal A are protease inhibitors whose kinetic profile suggests a dissociative mechanism of inhibition.[15]

Studies from our group have demonstrated that cross-linking the N termini of interfacial peptides of HIV protease (**1**) can significantly improve the potency of dimerization inhibitors.[3] In the current study we have taken a reductionist approach to developing inhibitors based on agent **1** by removing less essential residues. In this way we hoped to identify a minimal structure that maintains the essential features of dimerization inhibition, and that serves as a core structure for the development of more potent dimerization inhibitors.

An analysis of the contribution of each amino acid within **1** toward inhibition demonstrated the importance of the C-terminal residues, Trp1 and Phe11.[16] Since these residues of **1** were determined to be essential for inhibition, truncated agents (**2**–**6**; see Scheme 1) were prepared in which the peptides were shortened from the N termini. These compounds were synthesized by either a solution- or solid-phase strategy as previously described.[3]

The extent of HIV-1 protease inhibition by the truncated and cross-linked interfacial peptides was evaluated using the fluorogenic substrate developed by Toth and Marshall [17] and the kinetic analysis developed by Zhang et al.[14a] As was anticipated, removal of residues from the N termini of the peptides of **1** led to reduced potencies, with the largest losses observed with removal of Ile4 and Leu9, two hydrophobic residues whose importance was also identified from our alanine scanning experiments (Scheme 1).[16] Overall, a 25- and 50-fold loss in potency was observed from truncation of **1** (11 amino acids) to **5** (five amino acids) and **6** (four amino acids), respectively. More significantly, however, truncation to **5** and **6** led to a change in the mechanism of inhibition.

Dimerization inhibitors are easily identified using a Zhang–Poorman plot as they provide lines that are parallel to those for data with protease alone, whereas competitive and noncompetitive inhibitors provide nonparallel lines.[14a] The parent inhibitor (**1**) displayed a parallel line in the Zhang–Poorman plot (Figure 2a), indicating that this compound inhibited the dimerization of HIV-1 protease with a K_i value of 220 nM. Truncation of the parent compound **1** by three residues had no effect on the mechanism of inhibition, as parallel lines were also obtained for the truncated inhibitor **2**. As more residues were truncated from the N termini of the peptides, there was a small shift in the slope of the lines for compounds **3** and **4** (Figure 2b), indicating that inhibition was not occurring through a purely dissociative mechanism. In contrast, larger shifts in the slope were obtained with the smallest inhibitors **5** and **6** (Figure 2c), indicating a change in the mechanism of inhibition. Since compounds **5** and **6** did not inhibit HIV-1 protease through a dissociative mechanism, a double reciprocal plot was used to probe their inhibitory



Scheme 1. Results of truncating the N-terminal residues from the peptides of dimerization inhibitor **1**.

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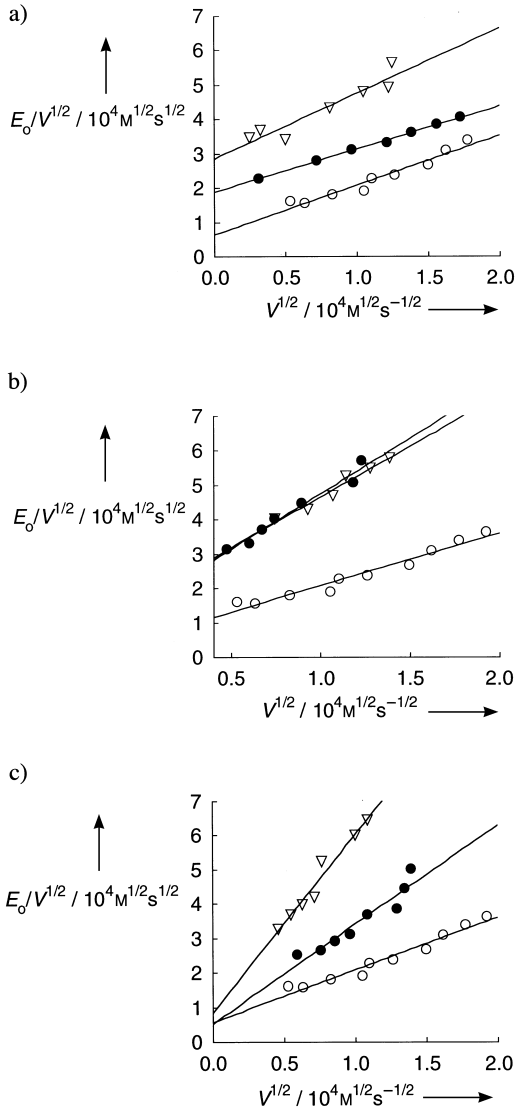
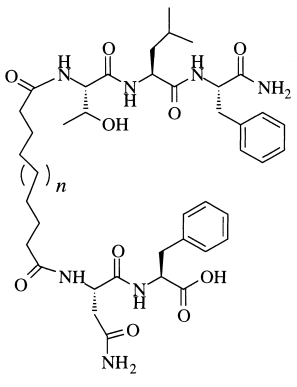


Figure 2. Zhang–Poorman plots[14a] for a) compounds **1** (●, 0.5 μM) and **2** (▽, 3.0 μM) with uninhibited protease (○), b) compounds **3** (●, 2.0 μM) and **4** (▽, 4.0 μM) with uninhibited protease (○), and c) compounds **5** (●, 20 μM) and **6** (▽, 40 μM) with uninhibited protease (○). E_o is the total enzyme concentration, and V is the initial velocity.

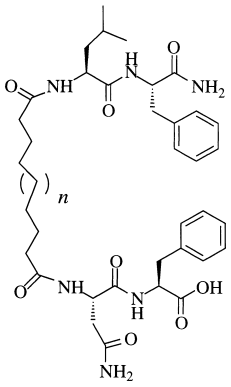
pathway. In both cases linear reciprocal plots were obtained that intersected on the $1/V$ axis, pointing to purely competitive inhibition.

Previous experiments in our laboratory had found that replacing the Trp-OH residue of **1** with Phe-NH_2 led to a threefold increase in HIV-1 protease inhibition.[18] This modification was also incorporated into compounds **5** and **6** in an attempt to increase potency (**7d** and **8d**, respectively). With both compounds this change led to approximately a twofold increase in inhibition (Table 1). A Zhang–Poorman plot was again used to probe the mechanism of inhibition by **7d** and **8d**. In this case parallel lines were obtained with compound **7d**, indicating dissociative inhibition ($K_i = 3.0 \pm 0.1$ μM), whereas nonparallel, intersecting lines were obtained with compound **8d**, indicating that inhibition did not occur by disruption of the dimerization of HIV-1 protease. A double reciprocal plot was used to determine the mechanism of

Table 1. HIV-1 protease inhibition with modified inhibitors.



7a-e (n=3, 6-9)



8a-e (n=3, 6-9)

Compound	Tether length[a]	IC_{50} [μM]
7a	9	54 ± 4
7b	12	28 ± 2
7c	13	9.7 ± 0.3
7d	**14**	**5.9 ± 0.5**
7e	15	5.0 ± 0.8
8a	9	101 ± 5
8b	12	18 ± 2
8c	13	14.1 ± 1.5
8d	**14**	**11 ± 1**
8e	15	8.2 ± 1.4

[a] Number of (CH_2) units.

protease inhibition by **8d**, and the compound was found to act as a competitive inhibitor as had been found for its analogue **6**.

The optimum tether length for inhibitor **1** was found to be 14 methylene units,[3] and further studies pointed to a potential binding interaction between the hydrophobic tether of **1** and a hydrophobic cleft formed by Phe99 and Leu97 in the interfacial region of a protease monomer.[19] While amino acid residues were removed from the N termini of compound **1**, the $(CH_2)_{14}$ tether was maintained since molecular modeling predicted that the distance to be spanned between the amino termini of the peptides in **1** and **5**, for instance, was fairly constant (9.3 Å and 9.8 Å, respectively). To test this prediction, the optimum tether length for compounds **7** and **8** was evaluated (Table 1). In both cases, decreasing the tether length to nine methylene units (**7a** and **8a**), a length that molecular modeling predicted to be too short for the two peptides to bind within the β sheet dimerization interface, led to approximately a tenfold decrease in inhibitor potency as compared to the $(CH_2)_{14}$ tether (**7d** and **8d**). Increasing the tether length to 12–15 methylene units (**7b–e**, **8b–e**) resulted in increased inhibition, although in both cases the $(CH_2)_{12}$ tether had the lowest activity, as had been observed with compound **1**.[3]

Calorimetric experiments with HIV-1 protease previously identified the four residues that contribute the most to the stability of the protease dimer: Phe99, Asn98, Leu5, and Leu97.[11] Interestingly, three of these residues occur in the smallest dimerization inhibitor identified in this study, **7d** (Phe5, Asn4, and Leu2). The current truncation strategy,

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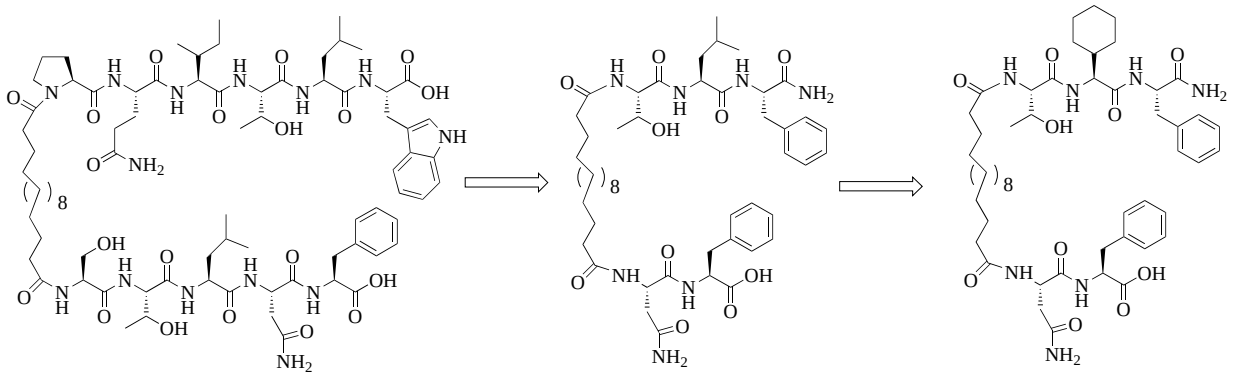

1 K_i = 220 ± 10 nM (MW=1586) **7d** K_i = 3000 ± 120 nM (MW=907) **9** K_i = 310 ± 4 nM (MW=933)

Scheme 2. The development of small-molecule dimerization inhibitors of HIV-1 protease derived from the cross-linked interfacial peptide inhibitor **1**.

therefore, has identified the minimal structure (**7d**) that maintains the essential features of dimerization inhibition, and this information correlates well with known stability data with HIV-1 protease. Using **7d** as a starting structure for molecular modeling with HIV-1 protease, we identified a compound (**9**) that was predicted to have increased interactions with HIV-1 protease within the binding pocket normally occupied by the isobutyl group of Leu2 (Scheme 2). The Zhang–Poorman analysis confirmed that **9** inhibited HIV-1 protease through a dissociative mechanism with a K_i value of 310 ± 4 nM ($IC_{50} = 680 \pm 10$ nM). Interestingly, compound **9** was an order of magnitude more potent than its precursor, **7d**, and was only 1.4-fold less potent than the full-length inhibitor, **1**, the compound that was the starting point for the truncation study. These data, therefore, confirm that the core structure, **7d**, is an ideal starting point for our efforts at identifying more potent agents through a focused library approach. The β sheet motif is also commonly found at dimerization interfaces, and the inhibition strategy developed with HIV-1 protease could be extended to a wide range of dimeric protein systems.

Experimental Section

Enzyme assay: For standard inhibition determination, 50 μL of HIV-1 protease (50 nM in buffer A)[3] was incubated with 10 μL of the inhibitor solution in DMSO for 1 h. The solution was added to 40 μL of the substrate[17] solution (buffer A with 10% DMSO) to yield a final substrate concentration of 60 μM. The final concentration of DMSO was kept constant at 14%. The change in fluorescence at 430 nm ($\lambda_{ex} = 360$ nm) was monitored at 30 °C. For the determination of K_i values, all parameters were identical with the following exceptions: enzyme concentrations ranged from 5 to 160 nM and the final substrate concentration was kept constant at 25 μM. For the determination of K_m and V_{max}, the substrate concentration ranged from 5 to 200 μM and the final enzyme concentration was kept constant at 25 nM. All experiments were performed in triplicate.

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