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A unidirectional crosslinking strategy for HIV-1 protease dimerization inhibitors

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Abstract—A novel strategy to identify potent HIV-1 protease dimerization inhibitors was developed using 12-aminododecanoic acid as a tether to crosslink interfacial peptides. The directionality of the southern peptide was changed from N→C to C→N as compared to previously reported inhibitors. The terminal amine of the southern peptide and side chains were further diversified to find essential functional groups for dimerization inhibition of HIV-1 protease.

HIV-1 protease (PR) is a pivotal enzyme in viral replication, as it cleaves the polypeptide products of the viral gag and gag/pol genes, which leads to viral maturation.[1] Although six protease inhibitors are now available as therapeutics, drug resistance due to rapid viral mutation rates is a major problem.[2–4] The design of novel inhibitors and inhibition mechanisms is a current focus in anti-HIV research.[5,6] Our strategy is to target the dimerization interface of HIV-1 PR, which is highly conserved.[7,8]

The initial strategy began with crosslinked peptides derived from the interface of HIV protease.[9–11] Since the development of HIV-1 PR dimerization inhibitor **1**, extensive work to improve upon this class of inhibitors has been performed.[9,12,13] When lower molecular weight inhibitors such as **3** were developed from truncation of **1**, the inhibitor lost potency against HIV-1 PR, and more significantly, changed its mode of action from dimerization inhibition to competitive inhibition (Fig. 1a).[13] In an effort to simplify the synthesis of HIV-1 PR

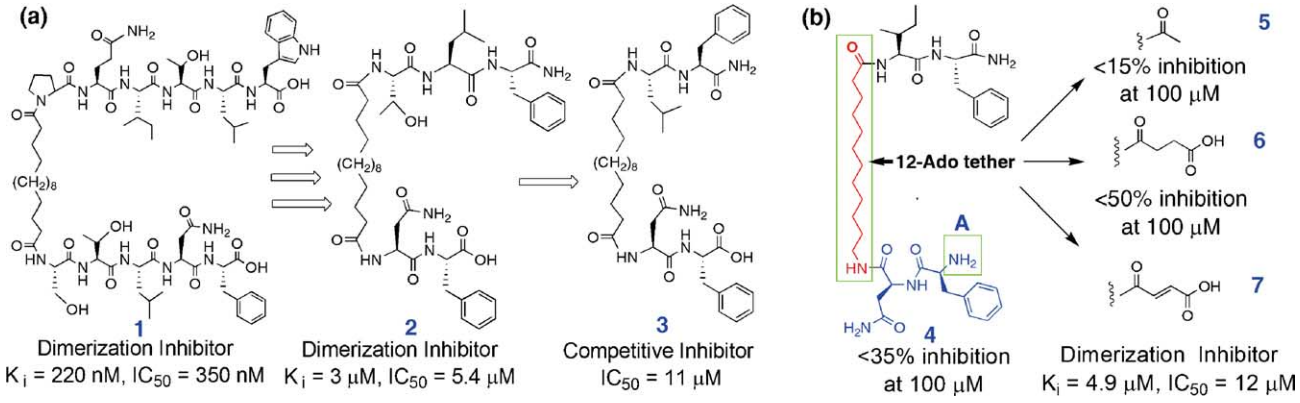

Figure 1. (a) Result of truncation study based on dimerization inhibitor **1**. (b) Result of novel tether strategy and modification at position A.

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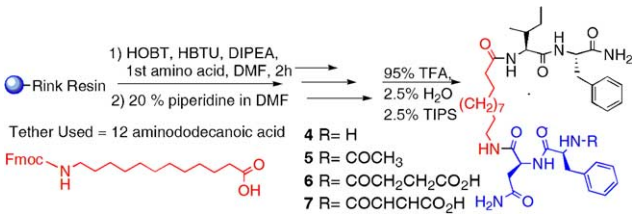


Scheme 1. General synthesis of the designed agents.

inhibitor libraries, a new tether, 12-aminododecanoic acid (12-Ado), was developed. The tether, 12-Ado, enabled us to change the directionality of the southern peptide form N→C to C→N in which the terminal amine can be used to incorporate various functional group (Fig. 1b). Furthermore, fast screening for each amino acid residue would be feasible because of the sequential nature of the solid phase synthesis (Scheme 1); compound **3** required a convergent synthesis, as it was not a continuous peptide chain. Herein we describe the design of HIV-1 protease dimerization inhibitors using the 12-aminododecanoic acid tether. Additionally a Leu to Ile modification was employed in the northern peptide, as this has been shown to increase the potency of compounds based on **2**.[13]

The designed inhibitors incorporating the 12-Ado tether could be synthesized on the Rink resin.[14] Using a Fmoc-based approach, each Fmoc-protected amino acid was coupled to the solid support sequentially using HBTU as the coupling reagent (Scheme 1). The desired resin bound peptides were cleaved with a TFA cocktail, and the resulting peptides were purified to homogeneity by reversed phase HPLC, and analyzed by mass spectrometry and amino acid analysis.

Four modifications (agents **4**–**7**) were designed to determine, which functional groups are important at position A (Fig. 1b) with the new tether design. The extent of HIV-1 PR activity in the presence of the agents was determined using the fluorogenic assay developed by Toth and Marshall.[15] Comparing compound **3**–**4**, a significant decrease in inhibitory potency was observed upon reversing the direction of the southern peptides. This may be attributed to a number of factors including deviation from the optimal tether length, loss of the terminal acid functional group, and disruption of the hydrogen bonding network between the agent and HIV-1 PR. Modification at position A resulted in a significant change in inhibitory activity. Modification of the terminal amine (compound **4**) with an acetyl group (compound **5**) resulted in loss of potency. When the terminal acid functional group was recovered in compound **6**, the inhibitory activity was increased slightly as compared to compound **5**, however, the percent inhibition with agent **6** was still less than 50% at 100 μM. Upon incorporation of maleic acid (**7**) at position A, the inhibitory potency was found to increase dramatically as compared to compound **4** and an IC_{50} value of 12 μM was obtained. Our binding model of an inhibitor **7**-PR complex positioned the maleic acid of agent **7** in close proximity to His69 of HIV-1 PR. Under the experimental conditions

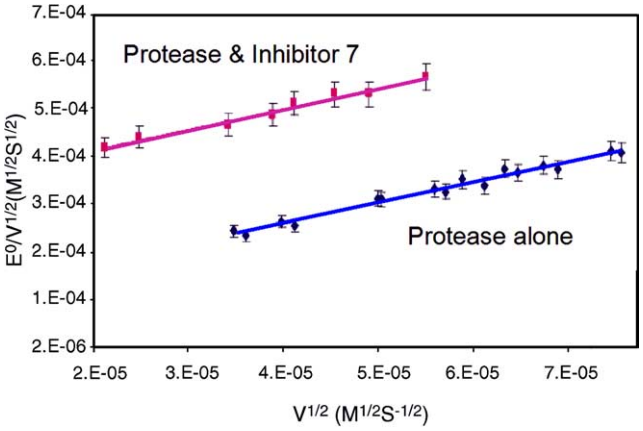


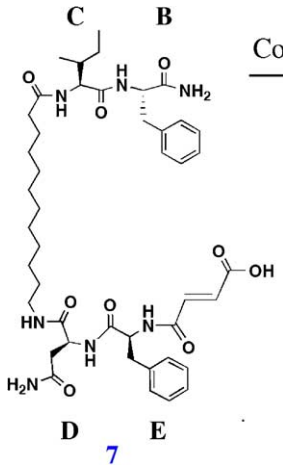
Figure 2. Zhang–Poorman plot for inhibitor **7** (pink, 1 μM) with uninhibited protease (blue).

(pH 5.5), the maleic acid of agent **7** and the side chain of His69 may be associated via hydrogen bonding.

Kinetic studies developed by Zhang et al. were used to determine the mode of inhibition of our inhibitors.[16] In the Zhang–Poorman analysis, a plot of the total enzyme concentration over the square root of the initial velocity ($E^0/V^{1/2}$) versus the square root of the initial velocity ($V^{1/2}$) will provide a straight line for HIV PR (Fig. 2). If an agent is a dimerization inhibitor, it will have a line parallel to the data with protease alone; nonparallel lines indicate competitive or noncompetitive inhibition.[16] The data for compound **7** provided parallel lines in the Zhang–Poorman plot, indicating that the inhibition observed occurred by the disruption of the protease dimer with a K_i value of 4.9 μM (Fig. 2). Using the 12-Ado tether and incorporating the maleic acid moiety in compound **7** re-established the ability to inhibit HIV-1 PR through a dissociative mechanism. More potent inhibitory activity as compared to compound **3**, which is a competitive inhibitor, was also obtained. In this way we identified a minimal structure that maintained the essential features of dimerization inhibition that may serve as a core structure for the development of more potent dimerization inhibitors.

The structural features of inhibitor **7** were further investigated using D-amino acids, which may improve metabolic stability and bioavailability since D-amino acid peptides are resistant to proteolysis.[17,18] The IC_{50} values obtained showed that incorporating unnatural amino acids (compounds **8**–**10**), and elongating the tether length (compound **11**) in the agents did not significantly alter inhibition (Fig. 3). These data demonstrate that D-amino acids can be used for the development of HIV-1 PR dimerization inhibitors without severely compromising activity.

Further modifications of compound **7** were designed to improve its biological activity by incorporating various modification at positions B, D, and E (Fig. 4a). Previous experiments in our laboratory using hexadecanedioic acid (compound **3**) as a tether found that replacing Phe-NH_2 (B position) with Tyr(3-NO_2), Asn-OH (D



Compound	Modification at Position **D** and **E**	IC_{50} (μM)
7	(L) Asn-(L) Phe	12.1 ± 0.4
8	(D) Asn-(L) Phe	14.2 ± 0.3
9	(L) Asn-(D) Phe	18.8 ± 0.2
10	(L) Asn-(L) Phe	9.7 ± 0.4
11	Gly-(L) Asn-(L) Phe	16.8 ± 0.2

Figure 3. Compounds with unnatural amino acids and their biological activity.

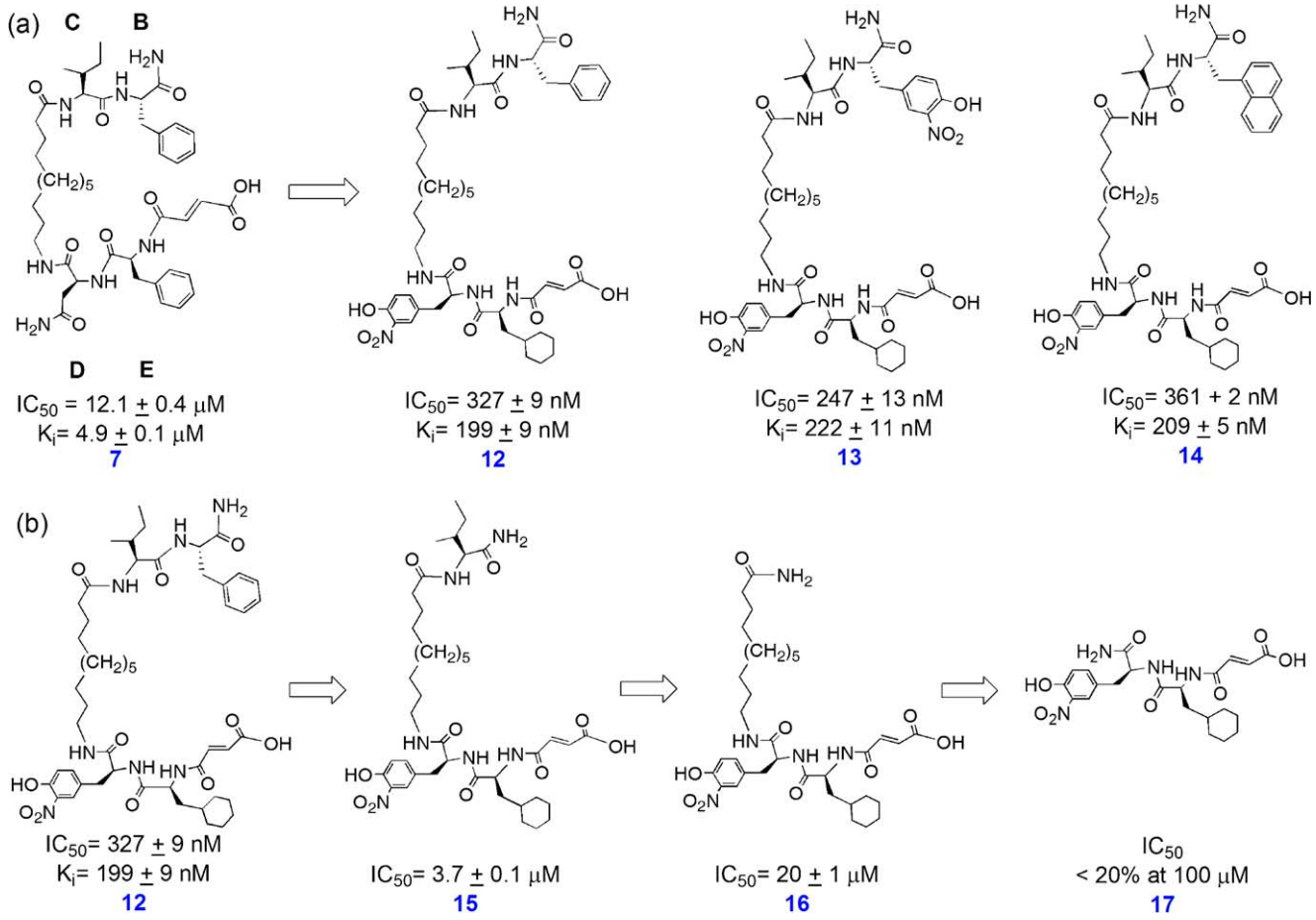


Figure 4. (a) Development of potent small-molecule dimerization inhibitors of HIV-1 PR from compound **7**. (b) Truncated inhibitors with their biological activity.

position) with 1-naphthylalanine, and Phe-OH (E position) with cyclohexyl-alanine led to approximately a threefold increase in potency with each change.[19] A combination of these modifications was incorporated into compounds **12**–**14** in an attempt to increase potency. With all compounds this change led to a greater than 20-fold increase in dimerization inhibition (Fig. 4a). Compounds **12**–**14** demonstrated that combined mutations could have cumulative effect on the inhibitor activity. Most importantly, compounds **12**–**14** are more active than compound **1** despite having one-third of amino acids. This dramatic increase in potency

clearly demonstrates that side chain interactions of inhibitors play an important role in determining the biological activity against HIV-1 PR.

Finally, a reductionist approach was taken to develop inhibitors with even lower molecular weight based on compound **12** by sequentially removing northern amino acid and tether residues (Fig. 4b). Truncating the C-terminal Phe residue (compound **15**) resulted in loss of inhibitory activity by 11-fold. Further truncation of agent **15** led to an additional fivefold decrease in inhibition. Compound **17** demonstrated that the aliphatic tether, 12-Ado, was pivotal for inhibitor potency (Fig. 4b) as has been observed using alternative crosslinking strategies.[12]

In this study we demonstrated that a new tether, 12-Ado, can be used as a structural motif to develop potent, low molecular weight HIV-1 PR dimerization inhibitors. Although inhibitory activity was lost in the case of compounds **4**–**6**, further modifications at positions A–E, including the maleic acid moiety and unnatural amino acids, restored potent dimerization inhibition against HIV-1 PR. Having the terminal amine available for fuctionalization, one may ultimately design an irreversible inhibitor by forming a covalent bond with the Cys95 of HIV-1 PR, which is in a close proximity.[20,21]

Acknowledgements

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