Brief Articles

Development of Low Molecular Weight HIV-1 Protease Dimerization Inhibitors

You Seok Hwang and Jean Chmielewski*

Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, Indiana 47907

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The role of HIV protease in viral replication has made it a significant target for inhibition. The focus of our studies is to target the dimerization interface of HIV-1 protease because disruption of the dimer will inhibit enzymatic activity. The initial strategy began with cross-linked peptides derived from the interface of HIV protease. Herein we describe the design of a focused library of agents based on a minimal pharmacophore for HIV-1 protease dimerization inhibition.

Introduction

Protein−protein interactions are ubiquitous in cellular processes including oligomerization of viral proteins during viral replication and assembly in host cells.[1,2] Such protein assemblies are specific and essential processes for viral replication, which make them attractive targets for antiviral therapeutics.[3] HIV-1 protease (PR) is a homodimer of two identical 99 amino acid subunits and a pivotal enzyme in viral maturation, which makes it a prime target in AIDS chemotherapy.[4] To date, six protease inhibitors targeting the active site of HIV-1 PR are available as therapeutics; however, problems of drug resistance due to rapid viral mutation rates have led to the development of novel inhibitors with new inhibition mechanisms.[5,6] One strategy would be to target the dimerization interface of HIV-1 PR because this region is highly conserved.[7] Targeting dimeric HIV-1 PR, we developed a strategy to disrupt dimerization based on cross-linking of the interfacial peptide inhibitor of HIV-1 PR.[8] Herein we describe the effort to identify the minimal structure necessary for activity and the design of a focused library of agents based on a minimal pharmacophore for HIV-1 PR dimerization inhibition.

Results and Discussion

Since the development of HIV-1 PR dimerization inhibitor **1**, extensive work to improve this class of inhibitors has been performed.[8−10] Although designed agents gained good dimerization inhibitory activity against HIV-1 PR, they suffered from their high molecular complexities. To overcome this drawback, truncation and mutation studies were performed to find the minimal structure necessary for activity. When a truncated inhibitor such as **3** was developed from agent **1**, the inhibitor lost potency against HIV-1 PR and, more significantly, changed its mode of action from dimerization inhibition to competitive inhibition. Previous experiments in our laboratory demonstrated that modification at position 1 of **2** with Chg, Cha, or Ile residues dramatically increased the dimerization inhibition of HIV-1 PR.[11] On the basis of this result, modifications at position 1 of **3** were made in an attempt to modify the mode of inhibition from competitive to dimerization (Figure 1).

The designed inhibitors were synthesized in parallel using a solid-phase approach.[12] The desired resin-bound peptide was cleaved with a TFA cocktail, and the resulting peptide was purified to homogeneity by reversed-phase HPLC and analyzed by mass spectrometry and amino acid analysis (Scheme 1).

The fluorogenic assay developed by Toth and Marshall[13] was used to determine the extent of activity of HIV-1 PR in the presence of these agents. Compounds **4** and **5**, with Chg and Cha mutations, resulted in a dramatic loss of activity, whereas the activity of the agent containing the Ile mutation (**6**) increased in potency by 11-fold compared to **3**. These results are quite distinct from the data obtained with modifications of **2** and may point to a somewhat different binding mode for **3** and the HIV-1 PR monomer. The mechanism of inhibition was determined for **6** using the kinetic method of Zhang and Poorman.[14] Inhibitor **6** was found to function as a dimerization inhibitor because the plots 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 ($V^{1/2}$) with (pink) and without (blue) the inhibitor were parallel (Figure 2), and a K_i of 220 nM was obtained. On the basis of the above results, **6** was chosen as a starting structure for the development of a focused library of agents to find potent low molecular weight dimerization inhibitors.

Previous experiments and our modeling studies indicated that HIV-1 PR has a strong binding interaction with extended aromatic side chains of inhibitors at position 2 and forms a well-defined hydrophobic cavity around the side chain of inhibitors at position 4.[11] The role of hydrophobic groups in positions 2 and 3 and the geometry of these groups were also of interest. Aromatic

* To whom correspondence should be addressed. Phone: 765-494-0135. Fax: 765-494-0239. E-mail: chml@purdue.edu.

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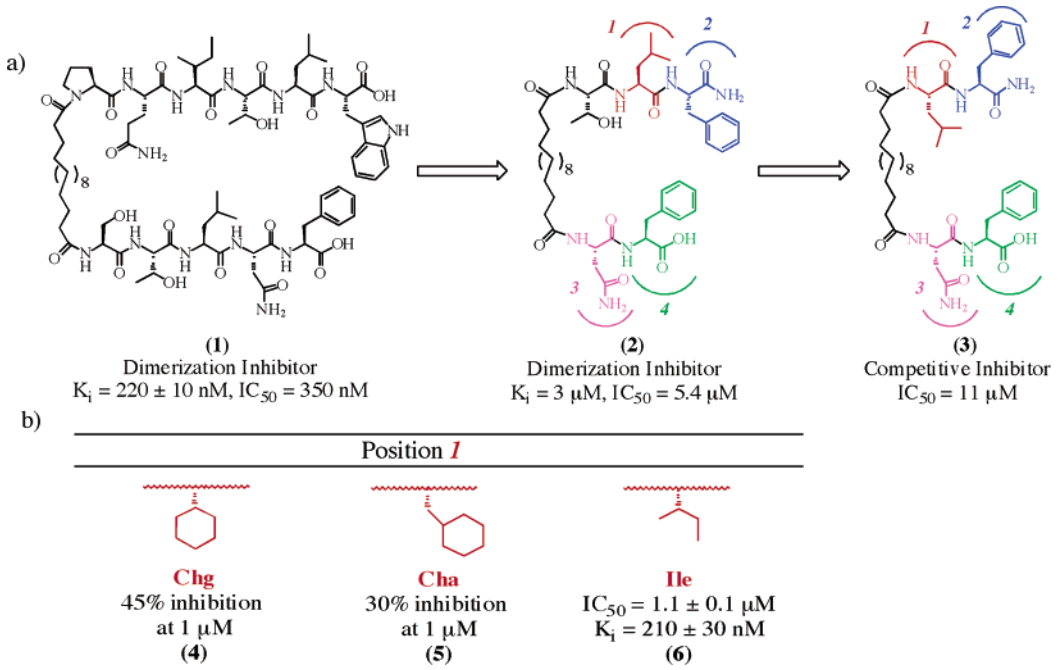
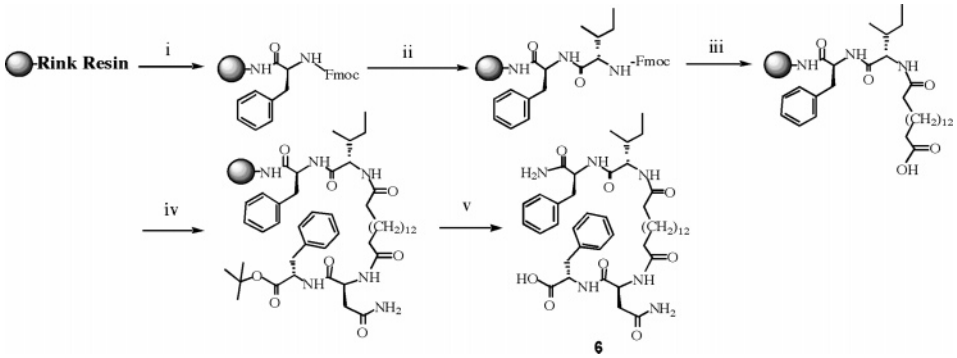


Figure 1. (a) Results of truncation study based on dimerization inhibitor **1**. (b) Side chain modifications at position 1 of **3**.

Scheme 1 Gereral Synthesis of HIV-1 Protease Dimerization Inhibitors[a]



[a] Regents and conditions: (i) (a) 20% piperidine in DMF; (b) HBTU, HOBT, DIEA, Fmoc-Phe-OH; (ii) (a) 20% piperidine in DMF; (b) HBTU, HOBT, DIEA, Fmoc-Ile-OH; (iii) (a) 20% piperdine in DMF; (b) HBTU, HOBT, DIEA, hexadecanedioic acid; (iv) HOBT, HBTU, DIEA, NH_2-Asn-Phe-O*t*Bu; (v) 95% TFA, 2.5% TIPS, 2.5% H_2O.

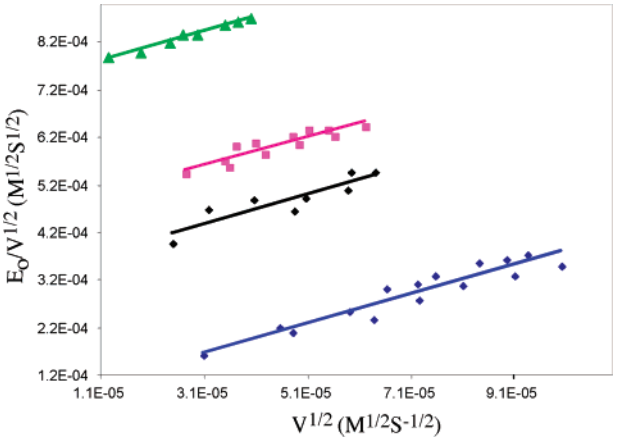


Figure 2. Zhang−Poorman plot for inhibitors **6** (pink), **19** (green), and **21** (black) with uninhibited protease (blue). E_o is the total enzyme concentration, and V is the initial velocity.

side chains with electron-withdrawing groups and hydrogen-bonding functional groups were also designed to determine the role of these groups on the interaction between HIV-1 PR and inhibitors. Having these considerations in mind, we devised a focused library containing 49 single modifications to the parent **3** (Table 1).

Each library entity was tested initially for its inhibitory activity against HIV-1 PR at a single concentration (1.0 μM) using the fluorescent assay of Toth and Marshall.[13] From this assay 38 compounds of the library were chosen for further study. IC_{50} values were obtained using the Toth and Marshall assay,[13] and the mechanism of inhibition was determined for the best inhibitors (23 compounds) using the kinetic analysis of Zhang and Poorman. All of these agents were found to function as dimerization inhibitors by this method. Of the library, about half of the compounds were equipotent or more potent than the parent **3**, and a number of the library components have enhanced inhibitory activity against HIV-1 protease (Figure 3).

Modification at each position revealed invaluable structural information concerning residues necessary for strong interaction with HIV-1 PR, and interesting trends were discerned. For example, at position 1 the nature of the alkyl side chain geometry was found to be significant with the following efficacy trend: *sec*-butyl (Ile) > isopentyl (Hleu) > *n*-butyl (Nle) > *tert*-butyl (Tle)

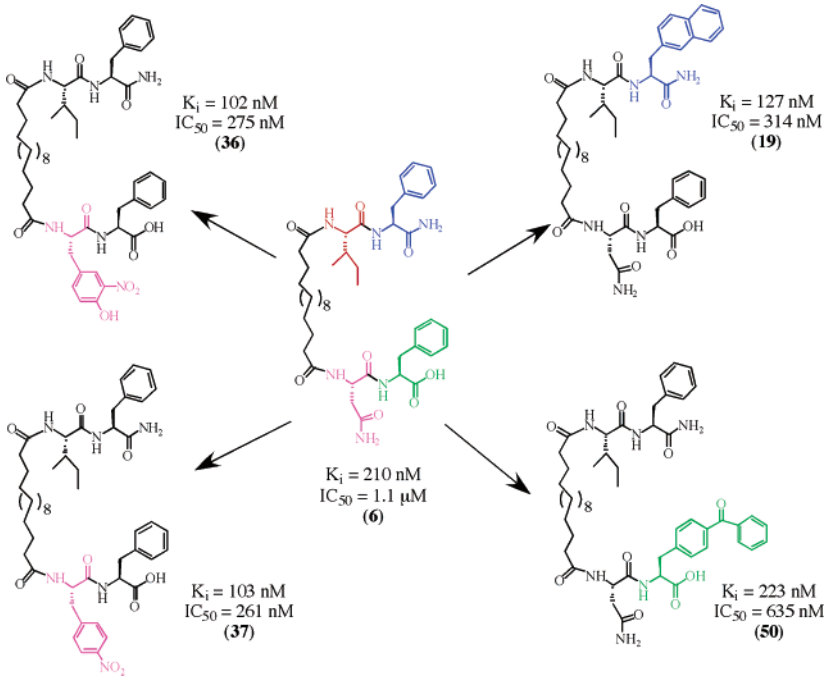


Figure 3. Most potent dimerization inhibitors from the focused library.

Table 1. Activity of a Focused Library from Modifications of Positions 1−4 of Inhibitor **6**[a]

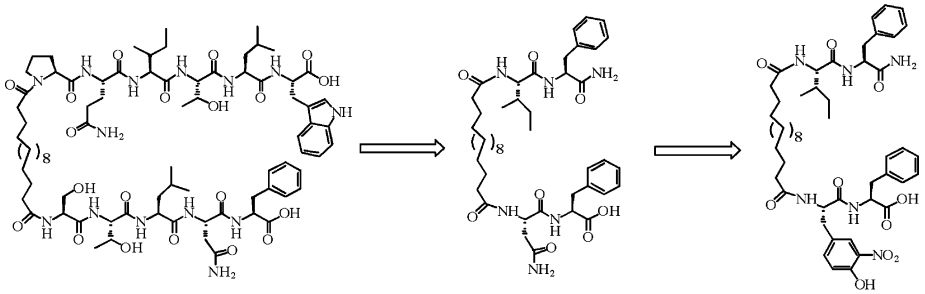
	at position 1			position 2			position 3			position 4	
7	Hleu	502 nM	**19**	2-Nal	127 nM	**36**	Y(3-NO_2)	102 nM	**50**	Bpa	223 nM
8	Hfe	(980 nM)	**20**	Y(3-NO_2)	164 nM	**37**	F(4-NO_2)	103 nM	**51**	Chg	254 nM
9	F(3,4-F_2)	(1.1 μM)	**21**	Bpa	314 nM	**38**	F(3,4-F_2)	106 nM	**52**	2-Nal	267 nM
10	Nle	(1.6 μM)	**22**	Bip	361 nM	**39**	Y(O-Bz)	110 nM	**53**	Hfe	268 nM
11	Phg	(2.1 μM)	**23**	Y(O-Bz)	414 nM	**40**	Phe	112 nM	**54**	Cha	285 nM
12	Tle	(7.7 μM)	**24**	F(4-NO_2)	(590 μM)	**41**	F(4-CN)	140 nM	**55**	F(4-NO_2)	384 nM
13	Cproa	32%	**25**	Y(3,5-I_2)	(740 μM)	**42**	Ile	140 nM	**56**	Tyr	15%
14	Nva	14%	**26**	F(3,4-F_2)	(872 μM)	**43**	Hfe	160 nM			
15	Cpena	13%	**27**	Hfe	(1.3 μM)	**44**	Val	202 nM			
16	5-Avc	12%	**28**	F(4-CN)	(1.4 μM)	**45**	2-Abz	235 nM			
17	Mamb	10%	**29**	F(4-Br)	(1.7 μM)	**46**	Leu	273 nM			
18	Aib	7%	**30**	Tpi	(3.1 μM)	**47**	Tyr	(774 nM)			
			31	Paf	(3.6 μM)	**48**	Paf	(1.2 μM)			
			32	4-Abz	36%	**49**	Y(PO_3H_2)	(2.1 μM)			
			33	Phg	29%						
			34	Cproa	21%						
			35	HSer	8%						

[a] Each position is indicated with activity for the individual modification. Activity is reported as K_i, IC_{50} (in parentheses), and percent inhibition at 1 μM. Values with error bars are in Supporting Information. Unnatural amino acids are the following: Hfe, homophenylalanine; F(3,4-F_2), 3,4-difluorophenylalanine; Hleu, homoleucine; Nle, norleucine; Tle, *tert*-leucine; Cproa, cyclopropylalanine; Nva, norvaline; Phg, phenylglycine; Cpena, cyclopentylalanine; 5-Avc, 5-aminovaleric acid; Mamb, *m*-aminomethylbenzoic acid; Aib, α-aminoisobutyric acid; 2-Nal, 2-naphthylalanine; Bip, biphenylalanine; Bpa, *p*-benzoylphenylalanine; Y(3NO_2), 3-nitrotyrosine, F(4NO_2), *p*-nitrophenylalanine; Y(O-Bz), *O*-benzyltyrosine; Y(3,4-I_2), 3,4-diiodotyrosine; F(4-Br), *p*-bromophenylalanine; Tpi, tryptoline-3-carboxylic acid; Paf, *p*-aminophenylalanine; F(4-CN), *p*-cyanophenylalanine; 4-Abz, 4-aminobenzoic acid; HTyr, homotyrosine; 2-Abz, 2-aminobenzoic acid; Y(PO_3H_2), *p*-phosphonoxytyrosine; Cha, cyclohexyl-alanine; Cyg, cyclohexylglycine.

> *n*-propyl (Nva). Incorporation of various cycloalkanes at position 1 of the inhibitor was strongly disfavored by HIV-1 PR. These results demonstrate that the side chain at position 1 should not be constrained, bulky, or contracted for better inhibitory efficacy.

Mutations at position 2 of **6** clearly demonstrated the importance of extended aromatic side chains for stronger dimerization inhibition. In our modeling, the side chain at position 2 of inhibitors occupies an extended hydrophobic surface. Mutations at position 2 with nonaromatic side chains such as cyclopropylalanyl (**34**), and hydroxyalkyl chains (**35**) demonstrated significantly decreased potency. Constrained side chains such as tryptoline (**30**) also resulted in poor binding with HIV-1 PR. Contracted side chains (**32**) at position 2 have a negative effect on inhibitor efficacy. Electron-withdrawing groups substituted on the aromatic side chain, such as fluorine (**26**) and nitro (**20** and **24**) at position 2, increased inhibitory potency. Extended aromatic side chains such as naphthyl (**19**) also had a good effect on inhibitor potency; mutation at position 2 from Phe (**6**) to 2-Nal (**19**) led to approximately a 3-fold increase in potency. It should be noted that **19** (MW = 856 Da) is 1.7-fold more potent than **1** (K_i = 220 nM, MW = 1750 Da) but at half the molecular weight.

Modifications at position 3 of **6** led to a number of inhibitors that are more potent than the initial inhibitor **6**. Replacement of the asparagine in position 3 with a range of aromatic side chains led to inhibitors that are approximately 2-fold more potent than **6**. Alkyl groups



(1) $K_i = 220 \pm 10$ nM **(6)** $K_i = 210 \pm 30$ nM **(36)** $K_i = 102 \pm 1$ nM

Figure 4. Development of potent low molecular weight HIV-1 PR dimerization inhibitors.

were also well supported; the nature of the alkyl side chain geometry at position 3 was found to be significant with the efficacy trend *sec*-butyl (Ile) > *sec*-propyl (Val) > isobutyl (Leu). Modifications at position 3 with aromatic side chains with an electron-withdrawing group (**36**−**38**) demonstrated significant increases in potency (Table 1), whereas electron-donating groups on the aromatic ring decreased potency.

Although only a small number of modifications were attempted in this study at position 4 of **6** because of synthetic difficulties, in general no large changes were observed in potency when extending the aromatic moiety. Similarly, the use of more bulky cycloalkyl side chains also displayed little difference in potency. The one exception, however, was the Phe4Tyr mutation that resulted in significant loss of inhibitory activity (15% inhibition at 1 μM).

Conclusions

In conclusion, this study demonstrated that minor modifications in HIV-1 PR dimerization inhibitors improved inhibitory activity significantly. A number of agents demonstrated better inhibitory efficacy than the full length, cross-linked interfacial peptides (**1**) with about the half of the molecular weight (Figure 4). Previous studies in our group demonstrated that multiple modifications based on the best individual changes in dimerization inhibitors led to a significant increase in potency against HIV-1 PR.[11] It will be worthwhile to design an extensive library containing multiple mutations for the development of low molecular weight, extremely potent HIV-1 protease dimerization inhibitors based on this series of compounds.

Experimental Section

Enzyme Assay. For standard inhibition determination, 50 μL of HIV-1 protease (50 nM in aqueous buffer A; 20 mM phosphate (KH_2PO_4), 10% glycerol, 0.1% CHAPS, 1 mM EDTA, 1 mM DTT, pH 5.52 (adjusted using Na_2PO_4))[10] was incubated with 10 μL of the inhibitor solution in DMSO for 1 h. The solution was added to 40 μL of the substrate[13] 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. All experiments were performed in triplicate.

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Supporting Information Available: Synthesis of key intermediates and their NMR values, inhibition constants with error bars, amino acid analysis results, reverse-phase HPLC conditions, and mass spectrometry results. This material is available free of charge via the Internet at http://pubs.acs.org.

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