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Design and Synthesis of New Inhibitors of HIV-1 Protease Dimerization with Conformationally Constrained Templates

Myeong-cheol Song, S. Rajesh, Yoshio Hayashi and Yoshiaki Kiso*

Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, Kyoto Pharmaceutical University, Yamashina-Ku, Kyoto 607-8412, Japan

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Abstract—To inhibit the HIV-1 protease dimerization necessary to exhibit enzymatic activity, we synthesized and evaluated a new β-sheet peptide (compound **1**), containing 4-(2-aminoethyl)-6-dibenzofuranpropionic acid as a conformationally restricted linker and a non-peptidic β-strand mimetic, 2-[3-({2-[(9-fluorenylmethoxy)carbonyl]hydrazino}carbonyl)-4-methoxyanilino]-2-oxoacetic acid (Fmoc-Hao-OH, compound **2**). Kinetic analysis showed that compound **1** inhibited the dimerization of HIV-1 protease by a dissociative mechanism with a K_{id} value of 5.4 μM at 37° C (pH 5.0). However, compound **2** showed a small shift in the slope of the lines in the Zhang–Poorman plot ($K_{id} = 9.1$ μM), suggesting that compound **2** inhibits the dimerization of HIV-1 PR not only through a dissociative mechanism but also through an active-site directed mechanism partly. This is the first study of a non-peptidic inhibitor of HIV-1 protease dimerization.

Introduction

Human immunodeficiency virus type 1 (HIV-1) encodes an aspartic protease that is synthesized as a polyprotein and is responsible for the processing of gag and gag-pol polyproteins to yield mature structural proteins and functional enzymes required in the viral life cycle.[1] Therefore, HIV-1 protease (HIV-1 PR) has been an attractive target for the design of inhibitors for effective antiviral therapy. Recently, many potent protease inhibitors that bind to the active site of HIV-1 PR have been developed and several are used in combination therapies for AIDS.[2] However, the rapid occurrence of resistance against these inhibitors due to the mutation of HIV-1 PR during clinical treatments is one of the major problems.[3] It is, thus, important to develop new protease inhibitors that are less sensitive to mutation causing drug resistance.

Dimerization of HIV-1 PR is one of the essential events to attain the mature structure, which is an enzymatically active C_2-symmetric homodimer.[4] Since the amino acid residues involved in the interfacial region for the dimerization of the HIV-1 PR monomer in numerous resistant strains are conserved, the inhibitors targeted to this region may effectively function against the mutants as well as native HIV-1 PR. In addition, such dimerization inhibitors may decrease the occurrence of mutation itself during long-term clinical treatment.

From the structure-based thermodynamic analysis of the HIV-1 PR molecule, it is suggested that the residues located at the carboxyl (Cys95, Thr96, Leu97, Asn98, and Phe99) and amino terminals (Pro1, Ile3, Leu5) contribute close to 75% of the total binding energy of dimerization.[5] Strategies for developing new inhibitors using interfacial peptides derived from the N- or C-terminal segments have been applied successfully in several studies,[6–8] and the consensus amino acid sequences for the potent inhibition of protease activity were established from the computer modeling of the C-terminal segment composed of the amino acid sequence from 94 to 99.[9] These studies suggested that short peptides derived from these segments are able to interrupt the homodimer formation by non-covalent dissociative inhibition. Recently, it was shown that covalently cross-linked interfacial peptides from each N- and C-terminus of the protease inhibited protease activity and decreased the amount of protease dimer in solution.[10,11] These agents were designed to bridge two peptide chains using flexible tether molecules. One possible drawback of such a flexible tether is its high conformational freedom, which induces an unfavorable entropy term of the interaction energy in the HIV-1 PR–inhibitor complex. To increase the conformational constraint in the tether

*Corresponding author. Tel.: +81-75-595-4636; fax: +81-75-595-4787; e-mail: kiso@mb.kyoto-phu.ac.jp

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part, Bouras et al. recently introduced a more rigid scaffold, so called 'molecular tongs', such as resorcinol, 2,6-pyridinediol and 2,7-naphthalenediol, and inhibition in the range of micromoles to submicromoles was observed by attaching the same C-terminal or modified C-terminal peptides to a molecular tong.[12] However, the conjugation of the C- and N-terminal peptides to such a rigid scaffold has not been carried out.

In the present study, to inhibit HIV-1 PR dimerization, we introduce new conformationally constrained templates such as a 'β-sheet nucleator' that connects the interfacial N-terminal and C-terminal peptide fragments deduced from the protease sequence and an 'unnatural β-strand mimetic', which interferes with the β-sheet interaction between two monomers and inhibits enzymatic activity.

Design and Synthesis of Inhibitors

Based on the design using a conformationally constrained tether that two peptide chains are oriented in an antiparallel β-sheet structure of the HIV-1 PR interfacial region (Fig. 1A), the 'β-sheet nucleator', 4-(2-aminoethyl)-6-dibenzofuranpropionic acid (ABFP), was introduced to connect N- and C-terminal sequences, leading to a β-sheet peptide (Fig. 1B). This rigid linker was introduced to facilitate intramolecular hydrogen bonding between the amides of the flanking α-amino acid residues and to provide a suitable distance between two N- and C-terminal peptides. This hydrogen-bonded hydrophobic cluster is sufficient to nucleate a variety of sequences to fold into a β-sheet conformation in aqueous solution.[13] In addition, based on a consensus sequence of potent peptide inhibitors mentioned above, the wild-type Leu (97) was replaced by Tyr in order to improve their inhibitory potency and the N-terminal sequence was introduced onto the ABFP template in a retro-inverso manner.

The β-sheet peptide **1** (H-Trp-Leu-Thr-Ile-ABFP-Thr-Tyr-Asn-Phe-OH) was synthesized by Fmoc-based solid phase methodology using Fmoc-Phe-Wang resin. Fmoc-protected amino acid residues (3 equiv)[14] were sequentially coupled using the 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/DIEA method. Each coupling was monitored using the ninhydrin test. The protected peptide was cleaved from the resin using a cocktail solution of TFA/EDT/thioanisole/triisopropylsilane/H_2O (85:5:5:4:1, 3 mL) for 2 h at room temperature. After removal of the resin with a glass filter, ether was added to precipitate the cleaved peptide, which was dissolved in 50% acetic acid and then lyophilized. The crude peptide was purified by preparative reverse-phase HPLC (YMC-Pack ODS-AP, 250×10 mm I.D.) using a linear gradient of water/acetonitrile containing 0.1% TFA. The structure was corroborated with the results of mass spectrometry, and purity was assessed by analytical HPLC.[15]

Another approach for the inhibition of the protease was studied. Nowick et al. recently reported that the unnatural amino acid, Hao, which is composed of hydrazine, 5-amino-2-methoxybenzoic acid and oxalic acid groups, mimics the simple tripeptide β-strand, and Hao containing peptides form a β-sheet like hydrogen-bonded dimer by providing an alternating pattern of hydrogen-bond donors and acceptors that matches that of protein.[16] In addition, Rebeck et al. have developed bi- and tricyclic β-strand mimics to inhibit a postulated β-sheet interaction between gp120 and the CD4 receptor.[17] Thus, it is feasible that this β-strand mimic compound binds to N- and C-termini of HIV-1 PR monomer and can interrupt β-sheet homodimer formation. Furthermore, since the addition of hydrophobic moieties to the N-terminal of the short peptide derived from N- and

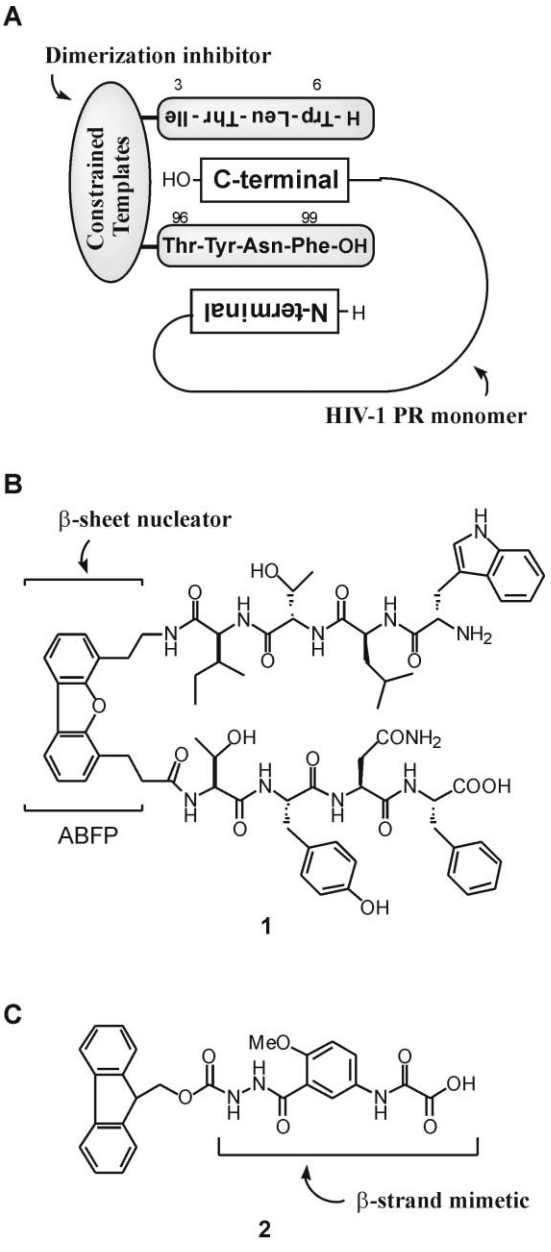

Figure 1. (A) Approaches to inhibit the HIV-1 PR dimerization using the β-sheet nucleator as a conformationally restricted linker; (B) structure of compound **1**; (C) structure of compound **2**.

C-terminal peptides exhibited about 20-fold enhancement of protease inhibitory activity,[8] we decided to introduce the hydrophobic fluorene structure which is similar to the ABFP core structure, and thus synthesized compound **2** (Fmoc–Hao–OH, Fig. 1C), which consisted of the hydrophobic Fmoc group and non-peptidic β-strand mimetic. Compound **2** was synthesized using a similar procedure as described by Nowick et al.[16,18]

Enzyme Inhibition

HIV-1 PR inhibition was determined by monitoring the fluorescence change (305 nm, $\lambda_{ex} = 275$ nm) associated with the cleavage of the fluorogenic substrate, H-Lys-Ala-Arg-Val-Tyr*Phe(p-NO_2)-Glu-Ala-Nle-NH_2. Reactions were carried out in 0.1 M sodium acetate buffer (final volume: 50 μL, pH 5.0) containing 1 mM EDTA, 1 M NaCl, and 5% DMSO at 37 °C. For the kinetic analysis according to Zhang et al.,[7] the substrate concentration was 25 μM, the enzyme concentrations and the inhibitor concentrations ranged from 3.2 to 23.8 nM and from 2.5 to 15 μM, respectively. Plots of $[E]_o/\sqrt{v_i}$ versus $\sqrt{v_i}$ constructed for the two compounds studied gave straight lines with similar slopes as without inhibitors. As shown in Figure 2A, the plot yields a straight line with a positive slope and a positive intercept. From the slope and the intercept of the fitting line, we determined the dissociation constant (K_d) of the dimer as 4.95 nM. The value was similar to that obtained by Schramm ($K_d = 4.0 \pm 2$ nM).[9] The value of inhibition constant (K_{id}) with a mode of dimerization inhibition was calculated from the intercepts in the presence and absence of inhibitor. Compound **1,** which exhibited moderate inhibitory activity with a K_{id} of 5.4 μM ($IC_{50} = 12$ μM), altered the y-intercept value, but had no effect on the slope (Fig. 2A). This kinetic analysis indicated that **1,** having a β-sheet nucleator connected to N- and C-terminal peptide segments of the protease monomer, acted as an inhibitor of HIV-1 PR dimerization through disruption of the interfacial region. Modification of the peptide fragments of **1** may improve the potency. A Zhang–Poorman plot was also used to examine the mechanism of inhibition with compound **2** (Fig. 2B). There was a small shift in the slope of the lines for **2**, indicating that inhibition occurred not only through a dissociative mechanism, but through a complex manner. The K_{id} constant of **2** was 9.1 μM ($IC_{50} = 30$ μM), which was approximately 2-fold less potent than β-sheet peptide **1**. Interestingly, it was reported that the interfacial peptides may also be partly active-site directed.[11] Therefore, it is suggested that compound **2** functions as a *'bifunctional inhibitor'*, which can bind to the active site as well as the interfacial region. Although the inhibitory potency of compound **2** was slightly weaker than those of other peptidic dimerization inhibitors reported earlier,[10] this is the first study of the inhibitor with a non-peptidic structure and it will be a lead compound for developing more potent non-peptidic inhibitors. It is suggested that modification of the Fmoc group or the carboxylic acid part in compound **2** will lead to more potent inhibitors of HIV-1 PR. Ongoing work is being directed towards improving the potency of the inhibitors.

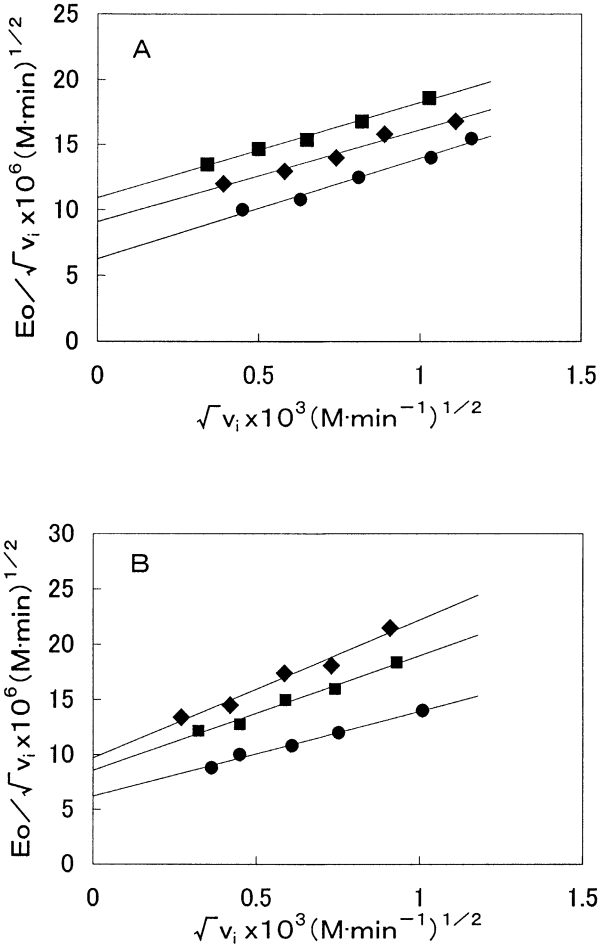

Figure 2. Plot of the $[E]_o/\sqrt{v_i}$ versus $\sqrt{v_i}$ for hydrolysis of the fluorogenic substrate by HIV-1 PR at 37 °C (pH 5.0) in the absence (●) and presence of (A) 2.5 μM (◆), 5 μM (■) compound **1**; (B) 4 μM (■), 6 μM (◆) compound **2**. E_o is the total enzyme concentration and v_i is the initial velocity.

In conclusion, the new HIV-1 PR inhibitors based on two conformationally constrained templates were designed and synthesized. These inhibitors targeted the dimerization interface region of the protease. The β-strand mimic structure of compound **2** is probably a useful lead for developing new types of potent non-peptidic inhibitors of HIV-1 PR with further understanding of the interactions involved in dimerization of HIV-1 PR. In addition, they might be able to overcome the drug resistance depending on the mutation of HIV-1 PR.

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15. Compound **1**: FABHRMS calcd for $C_{70}H_{87}O_{15}N_{11}Na$ $(M+Na)^+$ 1344.6281, found 1344.6276.
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18. Compound **2**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.70 (s, 1H), 9.76 (s, 1H), 9.41 (s, 1H), 8.07 (d, $J=1.8$ Hz, 1H), 7.84 (d, $J=7.5$ Hz, 2H), 7.79 (dd, $J=2.1$ Hz, 8.7 Hz, 1H), 7.69 (d, $J=7.2$ Hz, 1H), 7.6 (br s, 1H), 7.37 (dd, $J=7.8$ Hz, 6.9 Hz, 2H), 7.28 (dd, $J=7.5$ Hz, 6.9 Hz, 2H), 7.08 (d, $J=9.0$ Hz, 1H), 4.3 (d, $J=6.6$ Hz, 2H), 4.21 (t, $J=6.3$ Hz, 1H), 3.77 (s, 3 H); FABHRMS calcd for $C_{25}H_{22}O_7N_3$ $(M)^+$ 476.1458, found 476.1453.