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Targeting the Dimerization Interface for Irreversible Inhibition of HIV-1 Protease

Reena Zutshi and Jean Chmielewski*

Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA

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Abstract—A novel strategy was used to irreversibly inhibit HIV-1 protease. The inhibitor was designed to form a disulfide bond with Cys95, present at the dimerization interface of HIV-1 protease. The inhibitor was shown to be active against HIV-1 protease with $K_{inact} = 3.7\,\mu M$ and $V_{inact} = 0.012\,min^{-1}$.

HIV-1 protease plays a critical role in the life cycle of the human immunodeficiency virus-type 1 (HIV-1). This enzyme is responsible for the post-translational processing of viral polyproteins and subsequent generation of the structural and functional proteins essential for viral infection.[1] Although a number of protease inhibitors are now available as drugs,[2] the onset of resistance to these agents due to mutations in HIV-1 protease warrants identification of new means of inactivation.[3] An alternate yet viable means of inhibiting HIV-1 protease is to target its dimerization interface,[4] which is relatively untouched by mutations.[5]

HIV-1 protease is a homodimeric protein composed of two identical subunits of 99 amino acids.[6] The catalytic site as well as the substrate-binding pocket of HIV-1 protease is formed only when the protease is present in the dimeric state. Hence any agent that acts by sequestering the monomer is expected to inhibit the protease by preventing assembly of the active site (Fig. 1). In our earlier work, we have shown that small tethered peptides (**1**) targeted against the interface of HIV-1 protease do indeed inhibit its dimerization and activity with IC_{50} values in the submicromolar range.[7] In this paper we have extended this concept to the design of irreversible inhibitors, which we envisioned to be active due to their ability to covalently trap the monomer.

Inactivation by covalent modification is an effective inhibition strategy since saturating concentrations of the inhibitor are not required and a single binding event can in time inactivate the enzyme. The irreversible inhibitors designed against HIV-1 protease, to date, have been comprised of epoxide,[8] ynone and α,β-unsaturated ketone derivatives[9] all of which have been directed against the active site aspartate residue. Here, the target in our design of irreversible inhibitors is Cys95, which is present at the dimerization interface. This is a feasible approach since no mutations have so far been observed for Cys95 in HIV-1 protease isolates.

A close inspection of the crystal structure of HIV-1 protease shows that its dimerization interface is a four stranded antiparallel β-sheet composed of interdigitating N- and C-termini from the two monomers.[6] At the interface, the thiol of Cys95 (from one monomer), is separated from the γ-carbon of Phe99′ (from the other monomer) by a distance of approximately 4 Å. It was thought that substitution of the Phe residue in inhibitor **1** with an electrophilic amino acid could generate a covalent bond between the inhibitor and Cys95 of the protease. The initial formation of a protease-inhibitor complex would bring the reactive functional group on the inhibitor into close proximity with the nucleophilic thiol (of Cys95) allowing covalent modification of protease.

In the design of irreversible inhibitors, the Phe residue in agent **1** was replaced by cysteine to generate compound **2** (Fig. 2). Agent **2** was transformed into an electrophile by attaching a 5-thio, 2-nitrobenzoic acid moiety to the free thiol group (**3**), which would promote a facile reaction with Cys95 of HIV-1 protease and generate a disulfide bridge. Agent **3** was synthesized by treating **2** (3.0 μmol) in a 1:1 solution of DMF:1 M Tris–HCl, pH 8.0, with 6.0 μmol of Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) in 1 M Tris–HCl,

*Corresponding author. Tel.: +1-765-494-0135; fax: +1-765-494-0239.

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pH 8.0 for 2 h at 25 °C. The product, **3**, was characterized by mass spectrometry and amino acid analysis.[10]

HIV-1 protease was incubated with differing concentrations of agent **3** (0.25–4 μM) in 100 mM phosphate buffer containing 0.5 M NaCl and 1 mM EDTA at pH 7.3, with 10% DMSO. The activity of the preincubated solutions was measured over 300 min by a fluorescence based assay.[11] K_{inact} (inhibitor concentration resulting in half maximal inactivation) and V_{inact} (maximum inactivation rate) were determined from the double reciprocal plot of k_{obs} against the inhibitor concentration (Fig. 3). The values of K_{inact} and V_{inact} for agent **3** were calculated to be 3.7±0.3 μM and 0.012±0.001 min^{-1}, respectively.

In addition to Cys95, HIV-1 protease contains another cysteine residue, Cys67, which is approximately 8 Å away from Phe99′ in the crystal structure.[6] To determine if covalent modification of HIV-1 protease was occurring at the two Cys residues with inhibitor **3**, HIV-1 protease (700 nM) was treated with **3** (0.2–15 μM) for 1 h in a 50 mM phosphate buffer at pH 7.5 (Fig. 3c). The concentration of DMSO was kept constant at 10% in the reaction mixture. Visualization of covalent modification was achieved on a 20% SDS-polyacrylamide gel with silver staining. A calibration curve was generated by standard molecular weight markers (BSA, carbonic anhydrase, cytochrome C, and aprotinin) and used to determine the molecular weights of the different species on the gel. While the

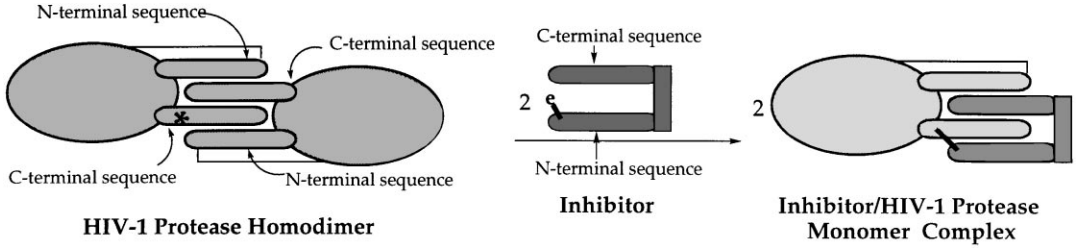

Figure 1. Strategy for irreversible inhibition of HIV-1 protease; * marks the site of Cys95 and e shows the position of the electrophilic disulfide within **3**.

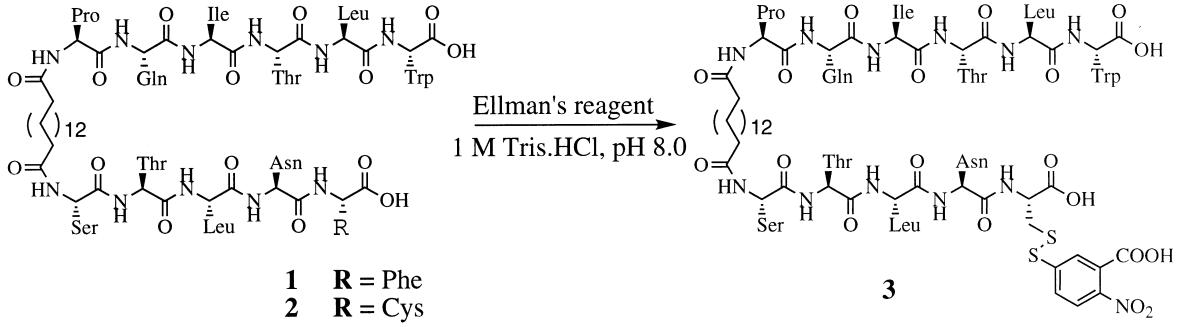

Figure 2. Synthesis of the irreversible dimerization inhibitor **3** from compound **2**. The design of agent **3** was based on the lead compound **1**.

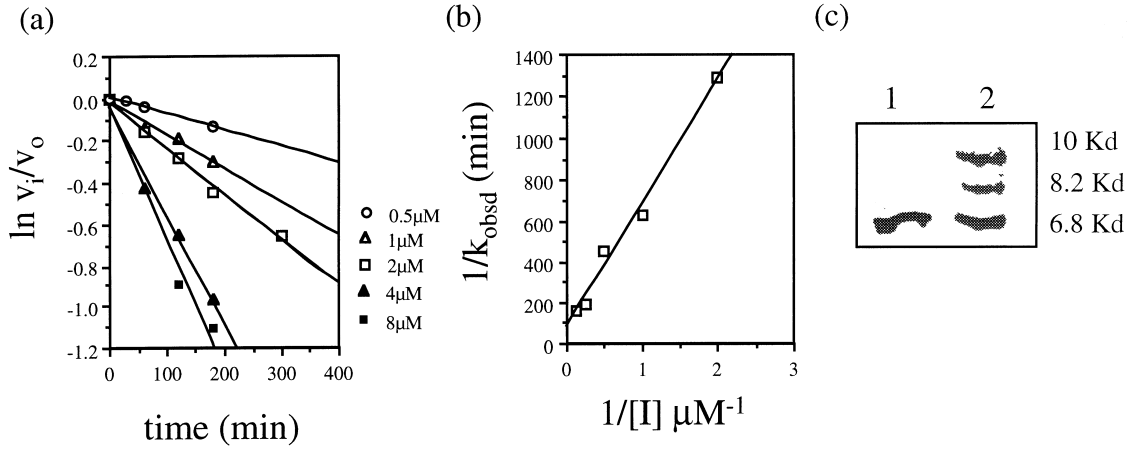

Figure 3. (a) k_{obsd} was determined from the plot of ln v_i/v_o against time; (b) K_{inact} was calculated from the double reciprocal plot of k_{obsd} against the concentration of agent **3**; (c) demonstration of covalent modification of HIV-1 protease by SDS PAGE: lane 1, HIV-1 protease; lane 2, HIV-1 protease (700 nM) and compound **3** (5 μM).

protease monomer migrated with a molecular weight of approximately 6.8 kDa, the reaction of HIV-1 protease with **3** resulted in the formation of two protease adducts with molecular weights of 8.2 and 10 kDa. The crosslinking studies suggest that **3** reacted with both cysteine residues in HIV-1 protease. Since the two cysteine residues, Cys95 and Cys67, are close in space to the electrophilic moiety (4 and 8 Å, respectively), it is not surprising that the disubstituted adduct was formed.

In this paper, we have chosen a novel means of irreversibly inhibiting HIV-1 protease (by targeting a Cys residue at its dimerization interface). In doing so, we have succeeded in developing a potent molecule, agent **3** having K_{inact} of 3.7 μM. Although **3** reacted with both Cys95 and Cys67, it proved to be a better inhibitor in comparison with the well characterized active site Fmoc-based, *cis*-epoxide inhibitor (K_{inact} of 65 μM, V_{inact} of 0.009 min^{-1}).[8d] Interestingly, these two highly conserved Cys residues have been found to regulate HIV-1 protease activity during viral maturation; oxidation of these residues with H_2O_2 or diimide inhibited Gag processing of wild-type virions.[12] Our studies also point to loss of protease function as these residues are modified with a dimerization inhibitor. Ongoing work in this area is being directed towards improving the potency and specificity of the inhibitors, in addition to identifying crucial residues for protease inhibition.

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10. Mass spectrometry of **3** by FABMS (NBA matrix): 1780 ($M+K^+$). Amino acid analysis: Pro 1.0 (1.0), Glx 1.0 (1.0), Ile 0.9 (1.0), Thr 1.8 (2.0), Leu 2.2 (2.0), Ser 0.7 (1.0), Asx 1.2 (1.0).
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