Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 2431–2436

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

PROBING THE ROLE OF INTERFACIAL RESIDUES IN A DIMERIZATION INHIBITOR OF HIV-1 PROTEASE

Michael D. Shultz and Jean Chmielewski*

Department of Chemistry, Purdue University, West Lafayette, IN 47907, U.S.A.

Received 28 May 1999; accepted 13 July 1999

Abstract: The importance of each side chain of a cross-linked interfacial peptide inhibitor of HIV-1 protease was evaluated using an alanine scanning approach. Whereas the parent inhibitor has an IC_{50} value of 350 nM, values for the mutations reported here range from 280 – 9200 nM. The relative importance or each residue was thus assigned and correlated to the solvent accessible surface area (SASA) exposed upon mutation.

Introduction

The protease (PR) from the human immunodeficiency virus (HIV) has been shown to be essential to the lifecycle of the virus, and, as such, has been the target of many therapeutic agents.[1] PR plays a vital role in the maturation of HIV particles by cleaving the *gag* and *gag-pol* transcription products, resulting in the structural and enzymatic proteins necessary for continuing viral replication.[2] The active PR enzyme has been shown to be a dimer, consisting of two identical 99-amino acid subunits.[3] Current FDA approved protease inhibitors target the active site of PR, but viral mutations arise that render the enzyme resistant.[4] An alternate method of inhibition would be the use of interface mimics that cause the formation of inactive heterodimers (Figure 1).[5] Since the interfacial region of HIV-1 PR has been shown to be relatively free of the mutations found in the substrate binding region of the enzyme,[6] it is therefore an attractive target for the development of anti-HIV compounds.

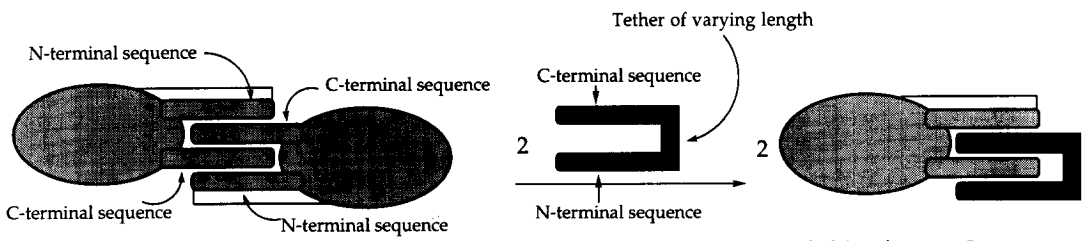

Figure 1. Schematic of dissociative inhibition.

Crosslinked peptides that correspond to the N- and C-terminal strands of HIV-1 PR have been shown to inhibit PR by a dissociative mechanism.[7] Although there has been a significant effort to develop dimerization inhibitors, there is currently insufficient information regarding the forces that govern this inhibition, or how the efficacy of these inhibitors could be improved in a rational way.[7,8] Alanine scanning mutagenesis has been shown to be useful in the identification of 'hot spots' in protein-protein interactions, and we reasoned the same technique could be applied to dissect our protein-inhibitor interactions.[9] Here, we report the use of an alanine scan in identifying the importance of each residue in the dimerization inhibitor **I** to assist in the rational development of more potent analogs (Figure 2).

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PII: S0960-894X(99)00400-X

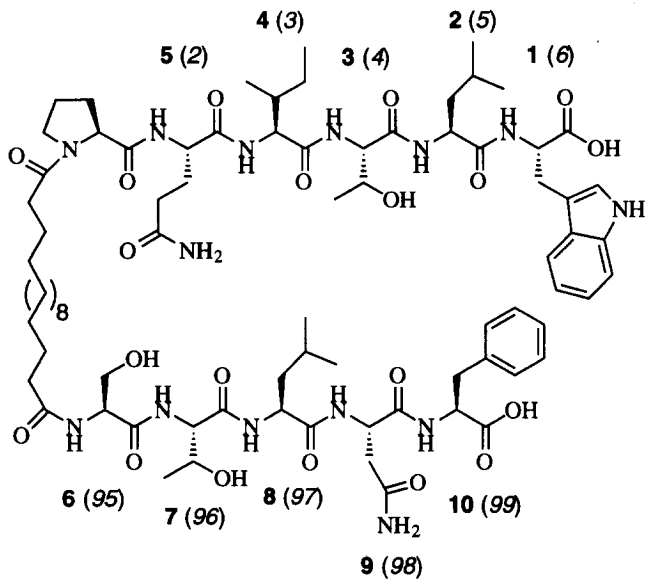

Figure 2. Compound **I** with alanine mutation positions numbered. The numbers in italics correspond to the position of the residue in the complementary protease monomer.

Results

Synthesis of Inhibitors. Compound **I** was prepared as previously described.[7a] Alanine mutants of **I** were prepared in parallel. In a typical synthesis, the peptide containing the alanine mutation was prepared with a solid phase approach using the Wang resin[10] and an Fmoc-based strategy. The resin bound peptide (1 equiv) was treated with a mixture of the di-N-hydroxysuccinimide ester of hexadecanedioic acid (1.2 equiv) and a solution of the non-mutant peptide strand (1.2 equiv) in DMSO at 60 °C for 24 h . The resin was washed twice with DMSO, CH_2Cl_2, EtOH and MeOH, and dried in vacuo. Cleavage from the resin was accomplished with 95% trifluoroacetic acid, 2.5% ethanedithiol, 1.5% thioanisole, and 1% anisole. The mixture was filtered from the resin, and the solvent was removed under reduced pressure. The residue was triturated with ether, centrifuged, washed with ether (2x) and dried in vacuo. The resulting compounds were purified using reverse phase HPLC and characterized by plasma desorption mass spectrometry. The experimentally determined masses were in agreement with the calculated mass for each compound.[11] Yields of inhibitors were, on average, 20%.

Evaluation of inhibitors. Inhibitor concentrations were determined by UV absorbance for Trp or Phe (ε = 5500 M^{-1} or 200 M^{-1}, respectively) in 6 M guanidine HCl. The fluorogenic substrate developed by Toth and Marshall was used to monitor PR activity.[12] The inhibitors were incubated with HIV-1 PR (purchased from Bachem) at room temperature for 1 h prior to the start of the assay. Final concentrations in the enzyme reaction were as follows: 25 nM PR, 60 μM substrate, 10% glycerol, 14% DMSO, 1 mM EDTA, 0.1% CHAPS, 1 mM

DTT, pH 5.5, at 30 °C. Inhibition was determined by comparison of the time taken to achieve an increase of 1000 fluorescence units (arbitrary) in the presence and absence of inhibitor. IC_{50} values were obtained from a plot of the percent inhibition vs. concentration of inhibitor (Table 1).

Table 1. Inhibition of HIV-1 protease[a] with alanine mutants.

Compound	IC_{50} (nM)	Relative to **I**
Trp1Ala	9200 +/-160	26
Leu2Ala	550 +/-60	1.6
Thr3Ala	1160 +/- 20	3.3
Ile4Ala	960 +/- 50	2.7
Gln5Ala	280 +/- 30	0.8
Ser6Ala	400 +/- 90	1.1
Thr7Ala	1170 +/- 12	3.3
Leu8Ala	1590 +/- 260	4.5
Asn9Ala	670 +/- 110	1.9
Phe10Ala	1240 +/- 90	3.5

[a] HIV-1 protease concentration was 25 nM

Discussion

The results of the assays indicated that certain side chains were critical for inhibition while others were less important. Mutations on the peptide derived from the N-terminus of the PR enzyme were on the whole less deleterious than on the peptide corresponding to the C-terminus. From the change in IC_{50} values after mutation, Trp1 was determined to be most important, followed by Leu8, Phe 10, with Thr3 and Thr7 of roughly equal importance. The remaining residues were judged to be minimally important as they lost less than threefold of their activity when replaced by alanine.

The Trp1Ala mutation of **I** caused a 26-fold loss in inhibitor potency. This result was initially perplexing because the Trp residue does not lie completely within the four-stranded anti-parallel β-sheet region of the dimerization interface, and, therefore, was not included in other attempts at dimerization inhibitors.[7e] The indole ring of Trp(*6*) in HIV-1 PR does, however, make extensive contacts with Thr4', Thr91', Gln92', and Gly 94' (' denotes the corresponding PR monomer). All four of these residues have been shown to be critical to the stability of the PR dimer.[13] We propose that the removal of the indole ring would expose these residues to solvent, thereby reducing much of the stabilization energy between the inhibitor and the PR monomer. Indeed, it is well known that burial of solvent accessible surface area (SASA) is the major factor in stabilizing oligomeric proteins, and it seems reasonable that the same intermolecular forces would be at work with these inhibitors.[14]

With the inhibition data in hand for the alanine mutants, we sought to examine whether compound **I** bound to a protease monomer in an analogous fashion to the complementary protease monomer. To address this question, we evaluated the relative amount of SASA exposed within the protease dimer when a single residue within the dimerization interface was mutated to Ala, and compared this value to the relative change in inhibition

within **I** upon mutation of single residues to Ala (Figure 3).[15] We found that there is a strong correlation between these two variables for the Trp1(*6*), Leu2(*5*), Ser6(*95*) and Leu8(*97*) mutations. The correlation is quite striking in certain cases, for example, the Trp1Ala mutant is 5.8 times less potent than the Leu8Ala mutant, while 5.7 times more surface area is exposed to solvent with the Ala mutation of Trp(*6*) as compared to Leu(*97*) in the protease dimer. The Thr3(*4*), Ile4(*3*), Thr7(*96*), and Asn9(*98*) mutations also show a good correlation between IC_{50} and SASA, whereas the Gln5(*2*) and Phe10(*99*) mutations do not correlate as well.

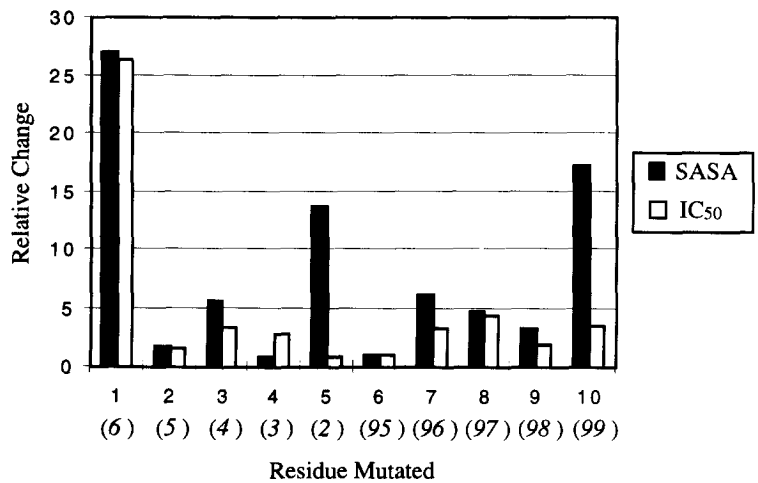

Figure 3. Correlation between the relative change in IC_{50} value with Ala mutation in **I** and the amount of solvent accessible surface area exposed upon mutation of the complementary residue in PR to Ala.[15] The italicized numbers in parentheses correlate to the position in PR.

Of the Ala mutants two residues demonstrate a weak correlation between IC_{50} values and SASA exposed upon mutation: Phe10(*99*) and Gln5(*2*). The side chain of Phe(*99*) makes extensive contacts with the PR monomer in a cavity surrounded by Ile3', Val11', Leu24', Ile66', Cys67', Ile93', Cys95', and His96'. The removal of the phenyl group should generate a deep cavity exposed to the aqueous environment. However, the change in SASA does not correlate strongly to the change in IC_{50} observed in Phe10Ala. It is possible that the hydrophobic residues mentioned above could reorganize in the absence of the phenyl moiety, thereby collapsing the cavity that would otherwise have formed, and exposing less SASA than was calculated. The side chain of Gln(*2*) lies on the exterior of the PR enzyme, and contacts between Gln(*2*) and Thr96' and Asn98' are hydrophilic in nature. In solution, these interactions are not expected to contribute much to the stability of the complex, and as the Gln5Ala illustrates, the efficacy remains essentially unchanged under our experimental conditions. The discrepancy between exposed SASA and change in IC_{50} with this residue, therefore, is likely due to the hydrophilic nature of the SASA generated.

Conclusion

This study has demonstrated the relative importance of the sidechain functionality in compound **I** for effective inhibition of HIV-1 PR, and has also highlighted the importance of including the interfacial Trp residue in future dimerization inhibitor designs. The good correlation observed between inhibition with Ala mutants of **I** and the SASA exposed upon Ala mutation within a monomer of PR (with the exception of Phe10(*99*) and Gln5(*2*)) suggests the possibility that the inhibitor binds to a protease monomer in an analogous fashion as the complementary protease monomer. In future designs it is possible that the side chains least affected by alanine mutation could be changed or deleted, while Trp1, Leu8, and Phe10 could be modified to optimize hydrophobic contacts. This work should ultimately aid in the development of more potent dimerization inhibitors of HIV-1 protease by providing a starting point for rational mutations and deletions.

Acknowledgments: We gratefully acknowledge funding from the NIH (R01 GM52739).

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11. Mass spectral data for compounds:

Compound	(M + H) expected	(M+H) Found
Trp1Ala	1474	1474
Leu2Ala	1547	1547
Thr3Ala	1558	1559, 1581(+ Na)
Ile4Ala	1545	1547
Gln5Ala	1530	1530, 1554(+ Na), 1568(+ K)
Ser6Ala	1572	1572, 1595(+ Na)
Thr7Ala	1558	1559, 1582(+ Na), 1599(+ K)
Leu8Ala	1546	1546
Asn9Ala	1545	1545
Phe10Ala	1512	1512

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