Vol. 180, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1991 Pages 181-186

SUBSTRATE ANALOG INHIBITORS OF HIV-1 PROTEASE CONTAINING PHENYLNORSTATINE AS A TRANSITION STATE ELEMENT

B. Raju and Milind S. Deshpande*

Rational Drug Design, Boston University Medical Center, 88 East Newton Street, Boston, MA 02118

Received August 23, 1991

Summary: *Substrates of HIV-1 protease are classified into three groups (A, B and C) based on the amino acid residues present at P_1' and P_2' sites. Replacement of the scissile amide bond by phenylnorstatine in representative substrate analog sequences from class A, B and C, yielded inhibitors of HIV-1 protease. Of the twelve inhibitors synthesized in this series, class C substrate analog inhibitors are more potent inhibitors (K_i's 3.3-24 μM) than either class A or class B inhibitors. In this series of inhibitors, the (2S,3S) isomer of phenylnorstatine is preferred over the other isomers as a "transition state element" for design of inhibitors of HIV-1 protease.*

Human immunodeficiency virus (HIV), a member of the Lentiviridae subfamily of retroviruses (1), is the causative agent of the acquired immunodeficiency syndrome (AIDS) (2). The primary translation product of the viral genome is a polyprotein. During maturation, this precursor polyprotein is cleaved into functional proteins by an aspartyl protease, HIV-1 protease (3). Cell culture studies using inhibitors of HIV-1 protease have shown that the protease is essential for viral replication (4). Thus, HIV-1 protease is an attractive target for the treatment of AIDS.

Tight binding inhibitors of HIV-1 protease have been synthesized by replacing the P_1-P_1' amide bond in various substrate analogs with nonhydrolyzable isosteres such as reduced amides, phosphinates, difluroketones, hydroxyethylenes, hydroxyethylamines and, statine (5). By taking advantage of the C_2 symmetry of HIV-1 protease, Erickson *et al.* have designed novel inhibitors of HIV-1 protease (6). To date, there are no reports of inhibitors of HIV-1 protease containing 3-amino-2-hydroxy-4-phenylbutanoic acid (AHPBA, or commonly referred to as

*To whom correspondence should be addressed.

Abbreviations
AHPBA: 3-amino-2-hydroxy-4-phenylbutanoic acid. This amino acid is commonly known as phenylnorstatine. Boc: *t*-butyloxycarbonyl.
BOP: benzotriazolyl N-oxytri-dimethylamino-phosphonium hexafluorophosphate.
HEA: hydroxethylamine. LAH: lithium aluminum hydride.

0006-291X/91 $1.50

phenylnorstatine), or any of its analogs as the transition state element. The present work describes the stereochemical requirements for phenylnorstatine to serve as a transition state element when incorporated in substrate analogs, and also explores different substrate sequences as a starting point to develop inhibitors of HIV-1 protease.

Experimental

i) Synthesis: Synthesis of the four possible isomers of Boc-AHPBA was accomplished by a slight modification of the reported procedure (7). N^{α}-(*t*-butoxycarbonyl)-L-phenylalanyl-N-methoxy-N-methylamide, obtained by coupling *t*-Butoxycarbonyl-L-phenylalanine with N,O-dimethylhydroxylamine hydrochloride using BOP, was reduced with LAH in anhydrous THF to give *t*-Butoxycarbonyl-L-phenylalanal (8). The amino aldehyde thus obtained was converted into a mixture of diastereomeric cyanohydrins by reaction with NaCN. After acid hydrolysis and protection of both the amino and carboxyl groups (as Boc- and methyl ester respectively), the diastereomers were separated by column chromatography on silica gel. Basic hydrolysis of the individual diastereomers resulted in N-protected-α-hydroxy-ß-amino acids. The absolute configuration and optical purity of the diastereomers was established by comparing the optical rotations of the α-hydroxy amino acids with reported values (7). The N-protected amino acids were incorporated into peptide sequences by solution phase techniques. Homogeneity of the peptides was determined by TLC, HPLC, ^{1}H-NMR (400 MHz), FAB-MS and amino acid analysis.

ii) HIV-1 protease inhibition assay: The HPLC based assay for inhibition of HIV-1 protease (9) was performed by using cloned HIV-1 protease (10). Reactions were carried out at ambient temperature. The reagents used in the assay were as follows: HIV-1 protease, (0.18 mg/ml) was a gift from The Upjohn company; HIV-1 protease substrate (Val-Ser-Gln-Asn-Phe(4-NO_2)-Pro-Ile-Val), 4.04 mM solution in double distilled water; buffer, 50 mM NaOAc (pH 5.8) containing 1mM EDTA, 2.5 mM DTT, 10% glycerol, 0.2% NP-40.

The total volume of the reaction mixture was 100 µl. The stock solution of the enzyme was diluted 20-times with the buffer. Twenty µL of the diluted enzyme were mixed with varying concentrations of the inhibitor and the buffer. Stock solutions of inhibitors were prepared by using a mixture of DMSO and double distilled water. The final concentration of DMSO in the assay was less than 8% in all cases. Substrate (20 µl) was added to the reaction mixture. The reaction was quenched after 20 min by addition of 20 µl of TFA. Hydrolysis of the substrate was quantitated by injecting 80 µl of the reaction mixture on a SYSTEM GOLD HPLC. Percent inhibition was plotted against inhibitor concentrations to determine the IC_{50} values. K_i values were calculated form corresponding IC_{50} values by using the equation of Cha *et al.* (11).

Results

The sequences of the twelve inhibitors derived from replacing the scissile amide bond in substrate analogs by phenylnorstatine, and their inhibitory constants are shown in Table II. The K_i's of phenylnorstatine containing inhibitors depend on i) the substrate analog sequence into which the transition state element is incorporated and ii) the stereochemistry of the two chiral centers present in the transition state element. The dependence of potency on the sequence of inhibitors may be illustrated by comparing inhibitors 1-4 with inhibitors 9-12. Inhibitors 9-12,

(K_i's 3.3–24 μM) which possess the Glu–Phe sequence on the C–terminal of the transition state element are at least 12–times more potent than inhibitors 1–4 (K_is 40–387 μM) which have Pro–Ile–Val–OMe on the prime side of the transition state element.

The effect of stereochemistry (of the two chiral centers of phenylnorstatine) on the inhibitory potencies may be evaluated by comparing the K_i's of inhibitors within class A or class C. Within class A inhibitors, inhibitor #1 (K_i = 40 μM) which contains the (2S,3S) isomer of phenylnorstatine, is about 9–times more potent than inhibitors containing the other isomers of phenylnorstatine (K_is 343–387 μM). A similar dependency on stereochemistry is observed within class C type inhibitors. Inhibitor #9 (K_i = 3.3 μM), which contains the (2S,3S) isomer is at least 5–times more potent than the other inhibitors within class C (K_is 17–24 μM).

Under the assay conditions employed for K_i determination, class B inhibitors had limited solubility. Thus, it was not possible to determine accurate inhibitory constants for compounds 5–8. In general, class B inhibitors are weak inhibitors of HIV–1 protease.

Discussion

HIV–1 protease exhibits broadly defined substrate specificity. The substrates of HIV–1 protease (12) may be classified into three groups (A, B and C) based on the residues present at P_1' and P_2' positions (Table I). Class A substrates possess an imino acid (Pro) at P_1' position, while class B and C substrates possess a hydrophobic amino acid at that site. Class C substrates may be distinguished from either class A or class B substrates by the presence of a polar amino acid at P_2' position.

Based on the X–ray structure of HIV–1 protease/inhibitor complex, Erickson *et al.* (6) suggested that residues P_2 – P_2' of the inhibitor are critical for interacting with the enzyme. We thus incorporated Boc–phenylnorstatine into representative substrate analog sequences from class

Table I. Classification of HIV-1 Protease Substrates

	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'
Class A								
	Ser	Gln	Asn	Tyr	Pro	Ile	Val	Gln
	Ser	Phe	Asn	Phe	Pro	Gln	Ile	Thr
	Thr	Leu	Asn	Phe	Pro	Ile	Ser	Pro
Class B								
	Ala	Thr	Ile	Met	Met	Gln	Arg	Gly
	Pro	Gly	Asn	Phe	Leu	Gln	Ser	Arg
	Arg	Gln	Ala	Asn	Phe	Leu	Gly	Lys
	Arg	Lys	Ile	Leu	Phe	Leu	Asp	Gly
Class C								
	Ala	Arg	Val	Leu	Ala	Glu	Ala	Met

Table II. Substrate Analog Inhibitors and their K_i Values

Inhibitor#	Sequence	K_i(μM)
Class A		
1	Boc-(2S,3S)AHPBA - Pro - Ile - Val - OCH_3	40
2	Boc-(2R,3S)AHPBA - Pro - Ile - Val - OCH_3	359
3	Boc-(2S,3R)AHPBA - Pro - Ile - Val - OCH_3	387
4	Boc-(2R,3R)AHPBA - Pro - Ile - Val - OCH_3	343
Class B*		
5	Boc-(2S,3S)AHPBA - Phe - Leu - OCH_2CH_3	NI[1]
6	Boc-(2R,3S)AHPBA - Phe - Leu - OCH_2CH_3	NI[2]
7	Boc-(2S,3R)AHPBA - Phe - Leu - OCH_2CH_3	NI[3]
8	Boc-(2R,3R)AHPBA - Phe - Leu - OCH_2CH_3	NI[4]
Class C		
9	Boc-(2S,3S)AHPBA - Glu - Phe	3.3
10	Boc-(2R,3S)AHPBA - Glu - Phe	17
11	Boc-(2S,3R)AHPBA - Glu - Phe	22
12	Boc-(2R,3R)AHPBA - Glu - Phe	24

AHPBA = 3-Amino-2-hydroxy-4-phenylbutanoic acid
*K_i values for compounds 5-8 could not be determined due to limited solubility of these compounds.
[1] no inhibition at 40 μM
[2] no inhibition at 27 μM
[3] no inhibition at 33 μM
[4] no inhibition at 70 μM

A, B and C with the intention of mapping at least $S_2 - S_2'$ subsites of HIV-1 protease. Rich *et al.* (5b) have pointed out that the stereochemistry of the transition state element necessary for maximal inhibitory activity is dependent upon the peptide framework. Thus, all four isomers of phenylnorstatine were incorporated into substrate analog sequences to yield a series of twelve peptides. The sequences of inhibitors obtained are shown in Table II.

Inhibitors based on class A and B substrate analogs have been previously reported. Rich *et al.* (5b) incorporated hydroxyethylamine as a transition state element into class A substrate analogs to obtain potent inhibitors of HIV-1 protease. As seen from Table II, incorporation of phenylnorstatine into class A substrate analogs produced inhibitors with K_i's ranging from 40–343 μM. It may be possible to increase the affinity of class A inhibitors (1–4) by mapping additional binding sites towards the N-terminus of the inhibitor.

Recently, Vacca *et al.* (5e) reported a series of hydroxyethylene tripeptide inhibitors based on class B substrate analogs. Most of these inhibitors span the region between P_2 and P_2' positions. It was postulated that replacement of the hydroxyethylene transition state element by phenyl-norstatine should yield inhibitors of HIV-1 protease. In our studies, compounds 5–8 (Table II), were found to be weak inhibitors of HIV-1 protease. Also, these compounds have limited solubility under the assay conditions. Thus, phenylnorstatine containing class B substrate analogs do not appear to be a good starting point for the design of HIV-1 protease inhibitors.

Inhibitors based on class C substrates have higher affinity towards HIV-1 protease than either class A, or class B substrate analogs. There is no apparent explanation for the preference of a polar residue (Glu) in the inhibitory sequence. A close examination of the X-ray structures of HIV-1 protease/inhibitor complexes reported to date, does not reveal any possible interactions between the glutamic acid residue present in the inhibitor, and the protease.

It is of interest to note that the preferred stereochemistry of the hydroxyl group in phenylnorstatine inhibitors is *S*. This is consistent with the observation by Rich *et al.* (5b) that the preferred stereochemistry of the hydroxyl group in smaller HEA inhibitors (inhibitors lacking P_4, P_3 and, P_3' subsites) is *R* (equivalent to *S* in phenylnorstatine). In HEA inhibitors, the stereochemical preference changes from *R* to *S* in longer inhibitors. Syntheses of longer phenylnorstatine inhibitors to acheive greater potency and, to address the stereochemical preference of the hydroxyl group are in progress.

Acknowledgments

This work was supported by a grant from National Institutes of Health (AI29895-01). We thank Drs. C.W. Smith and J.O. Hui of the Upjohn company for providing cloned HIV-1 protease, and Dr. James Burton for helpful discussions.

References

1. Dickson, C., Eisenman, R., Fan, H., Hunter, E. and Teich, N. In RNA Tumor Viruses, Molecular Biology of Tumor Viruses; Weiss, R., Teich, N. and Coffin, J., Eds.; Cold Spring Harbor: New York, 1984; pp 513-648.
2. Ratner, L., Haseltine, W., Patarca, R., Livak, K.J., Starcich, B., Josephs, S.F., Doran, E.R., Rafalski, J.A., Whitehorn, E.A., Baumeister, K., Ivanoff., L. Petteway, S.R., Jr. Pearson, M.L., Lautenburger, J.A., Papas, T.S., Ghrayeb, J., Chang, N.T., Gallo, R.C. and Wong-Staal, F. (1985) Nature 313, 277.
3. McQuade, T., Tomasselli, A., Liu, L., Karacostas, V., Moss, B., Sawyer, T., Heinrikson, R., Tarpley, W. (1990) Science 247, 454.
4. Meek, T.D., Lambert, D.M., Dreyer, G.B., Carr, T.J., Tomaszek, T.A., Moore, M.L., Strickler, J.E., Debouck, C., Hyland, L.J., Mattews, T.J., Metcalf, B. and Petteway, S.R. (1990) Nature 343, 90.
5. (a) Debouck, C. and Metcalf, B.W. (1990) Drug Development Research 21, 1. (b) Rich, D.H., Sun C.Q., Prasad, J.V.N.V., Pathiasseril, A., Toth, M.V., Marshall, G.R., Clare, M., Mueller, R.A. and Houseman, K. (1991) J. Med. Chem. 34, 1222. (c) Ashorn, P., Mcquade, T.J., Thaisrivongs, S., Tomasselli, A.G., Tarpley, W.G., Moss, B. (1990) Proc. Natl. Acad. Sci. U.S.A 87, 7472. (d) Grobelny, D., Wondrak, E.M., Galardy, R.E. and Oroszlan, S. (1990) Biochem. Biophys. Res. Commun. 169, 111. (e) Vacca, J.P., Guare, J.P., deSolms, S.J., Sanders, W.M., Giuliani, E.A., Young, S.D., Darke, P.L., Zugay, J., Sigal, I.S., Schleif, W.A., Quintero, J.C., Emini, E.A., Anderson, P.S. and Huff, J.R. (1991) J. Med. Chem. 34, 1228. (f) Sham, H.L., Betebenner, D.A., Wideburg, N.E., Saldivar, A.C., Kohlbrenner, W.E., Vasavanonda, S., Kempf, D.J., Norbeck, D.W., Zhao, C., Clement, J.J., Erickson, J.E. and Plattner, J.J. (1991) Biochem. Biophys. Res. Commun. 175, 914.

6. Erickson, J.E., Neidhart, D.J., VanDrie, J., Kempf, D.J., Wang, X.C., Norbeck, D.W, Plattner, J.J., Rittenhouse, J., Turon, M., Wideburg, N.E., Kohlbrenner, W.E., Simmer, R., Helfrich, R., Paul, D. and Knigge, M.F., (1990) Science 249, 529.
7. Nishizawa, R., Sanio, T., Takita, T., Suda, H., Aoyagi, T. and Umezawa, H. (1977) J. Med. Chem. 20, 510.
8. Fehrentz, J.A. and Castro, B. (1983) Synthesis 676.
9. Deshpande, M.S., Raju, B. and Manly, S.P. Int. J. Pep. Protein Res. (Submitted for publication).
10. Tomasselli, A.T., Olsen, M.K., Hui, J.O., Staples, D.J., Sawyer, T.K., Heinrikson, R.L. and Tomich, C–S.C. (1990) Biochemistry 29, 264.
11. Cha, S., Agarwal, R.P. and Parks, R.E. Jr. (1975) Biochemical Pharmacology 24, 2187.
12. Skalka, A.M. (1989) Cell 56, 911.