Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 1713–1718

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

PEPTIDE-BASED INHIBITORS OF THE HEPATITIS C VIRUS SERINE PROTEASE

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Received 15 April 1998; accepted 29 May 1998

Abstract: Hexapeptide DDIVPC-OH is a competitive inhibitor of the hepatitis C virus (HCV) NS3 protease complexed with NS4A cofactor peptide. This hexapeptide corresponds to the N-terminal cleavage product of an HCV dodecapeptide substrate derived from the NS5A/5B cleavage site. Structure-activity studies on Ac-DDIVPC-OH revealed that side chains of the P4, P3 and P1 residues contribute the most to binding and that the introduction of a D-amino acid at the P5 position improves potency considerably. Furthermore, there is a strong preference for cysteine at the P1 position and conservative replacements, such as serine, are not well tolerated.

Hepatitis C virus (HCV) infection is an important cause of chronic hepatitis, cirrhosis, hepatocellular carcinoma and liver failure worldwide.[1] The only approved therapies with proven benefit for patients with chronic hepatitis C are various drug regimens of interferon-α(INF_α). These therapies have limited efficacy with a low sustained response rate and frequent side effects.[1] Therefore, there is an urgent need for the development of new therapies for the treatment of HCV infections. HCV is a small enveloped virus containing a single-stranded RNA genome of positive polarity, which encodes a unique polyprotein of approximately 3,000 amino acids.[2] This polyprotein is the precursor of both structural (C, E1, E2, p7)[3] and non structural (NS) (NS2, NS3, NS4A, NS4B, NS5A, NS5B)[4] proteins. The structural proteins are proteolytically processed by host signal peptidases while two virally encoded proteases from the NS2 and NS3 regions process the remaining non structural proteins.

The NS2-3 protease, delineated by a region encompassing the carboxyl terminus of NS2 and the amino terminus of NS3, is responsible for an auto-catalytic cleavage at the NS2/3 junction.[5,6] The NS3 protease, located in the amino-terminal one-third of the NS3 protein, mediates the proteolysis at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B junctions.[5,7,8,9,10] The NS3 protease is the most intensively studied of the two and is a suitable target for antiviral drug discovery. Based on amino acid sequence homology between NS3 and other viral proteases, it was first predicted and then confirmed by structural determination[11,12] that the NS3 protease is a chymotrypsin/trypsin-like serine protease. The NS3 protease activity is enhanced by the NS4A protein, which act as an essential cofactor required for polyprotein maturation.[13,14,15] Structure determination of the NS3 protease complexed with a truncated NS4A cofactor peptide[12] demonstrated that NS4A is an integral structural component of the enzyme.

0960-894X/98/$19.00
PII: S0960-894X(98)00299-6

We developed an enzymatic assay using the NS3 protease complexed with the NS4A peptide cofactor (NS3-4A_{pep} protease). During the implementation of this protease assay for the discovery of inhibitors, we observed that the N-terminal hexapeptide product (DDIVPC-OH) of a substrate derived from the NS5A/5B cleavage site[16] inhibited the enzyme.[17] In this paper, we describe structure activity studies on hexapeptide **1** with the goal of finding the optimal size for inhibition and the contribution of each amino acid residue to binding.

Materials

The synthesis of compounds **24**, **26**, **27**, and **28** was carried out in solution using standard peptide chemistry.[18] All other peptide inhibitors, substrates, and the NS4A-derived peptide needed for the assay[19] were synthesized using standard solid-phase peptide synthesis methodology.[18,20] *N*-Fmoc protected amino acids and resins were obtained from NovaBiochem (USA), Bachem (USA) or Advanced Chemtech (KY, USA). Each peptide was purified by preparative reversed-phase HPLC on a C18 column using an acetonitrile gradient. Satisfactory MS, amino acid analysis and homogeneity data (>90% HPLC) were obtained for all the peptides. ^{1}H NMR spectra were also obtained for all peptide inhibitors. The cloning, expression and purification of the recombinant NS3 protease genotype 1b will be reported elsewhere [Lamarre et al.]. All IC_{50} values reported are the average of at least four separate determinations.

Results and Discussion

Compound **1**, corresponding to the N-terminal cleavage product of a substrate derived from the NS5A/5B cleavage site, inhibits the NS3-4A_{pep} protease with an IC_{50} of 71 μM (Table 1). However, the hexapeptide SMSYTW-OH, corresponding to the C-terminal cleavage product of the same substrate, did not inhibit the enzyme at 1 mM. As shown by Dixon (Fig. 1) and Cornish-Bowden (data not shown) plots, hexapeptide **1** is a competitive inhibitor of the NS3-4A_{pep} protease. An apparent K_i of 14 μM[21] was determined based on the initial inhibitor concentrations and without taking into account the formation of the N-terminal product during the time course of the reaction. Interestingly, this $K_{i\ app}$ value is of the same order of magnitude as the K_m (37 μM)[22] of the substrate from which the N-terminal product is derived.

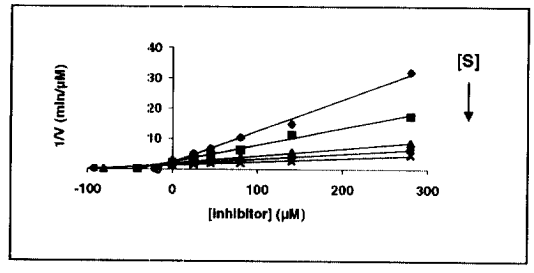


Figure 1. Dixon plot for hexapeptide

Knowing that peptide **1** is a competitive inhibitor of NS3-4A_{pep} protease, we studied the inhibition of this enzyme by N-terminal product analogs of varying size (Table 1).

Table 1. N-Terminal studies.

Compound	Sequence	IC_{50} (μM)
1	DDIVPC-OH	71
2	GEAGDDIVPC-OH	52
3	Ac-DDIVPC-OH	28
4	DAD-DIVPC-OH[a]	33
5	Ac-DIVPC-OH	130
6	DAD-IVPC-OH	830

[a] DAD-= 3-Carboxypropanoyl.

Increasing the length of hexapeptide **1** to ten residues (decapeptide **2**) slightly increased the potency of the inhibitor. However, a larger increase in potency was observed by capping the N-terminus of hexapeptide **1** with an acetyl group (compound **3**). 3-Carboxypropanoyl pentapeptide **4** was equipotent to Ac-hexapeptide **3** indicating that the N-terminal acetamide in **3** does not contribute to the binding of the inhibitor. However, replacement of the 3-Carboxypropanoyl-capping group in **4** by an acetyl group, as in compound **5**, produced a 4-fold decrease in potency indicating the importance of the carboxylic acid residue at P6. In contrast to hexapeptide **3**, the N-terminal acetamide of pentapeptide **5** does contribute to binding as evidenced by the poor inhibition displayed by tetrapeptide **6**.

Next, we studied the contribution of the side chains to potency by replacing each residue of hexapeptide **3** in turn by either an alanine or its own enantiomer (D-AA) (Table 2). The alanine scan identified the side chains of P4 (Ile), P3 (Val), and P1 (Cys) as making the strongest contributions to binding. Replacement of any of these three residues with alanine (compounds **9**, **10** and **12**) gave significantly less potent inhibitors. These results parallel published studies with substrates in which the P3 and P1 side chains were found to be important determinants for substrate binding as reflected by K_m determinations.[23,24]

Table 2. Alanine and D-amino acid scan on hexapeptide lead compound **3**

Compound	Alanine Scan	IC_{50} (μM)	Compound	D-Amino Acid Scan[a]	IC_{50} (μM)
3	Ac-DDIVPC-OH	28	**3**	Ac-DDIVPC-OH	28
7	Ac-**A**DIVPC-OH	200	**13**	Ac-**d**DIVPC-OH	40
8	Ac-D**A**IVPC-OH	140	**14**	Ac-D**d**IVPC-OH	4
9	Ac-DD**A**VPC-OH	580	**15**	Ac-DD**i**VPC-OH	800
10	Ac-DDI**A**PC-OH	890	**16**	Ac-DDI**v**PC-OH	830
11	Ac-DDIV**A**C-OH	93	**17**	Ac-DDIV**p**C-OH	>1000
12	Ac-DDIVP**A**-OH	710	**18**	Ac-DDIVP**c**-OH	330

[a] Lower case letters denote D-amino acid.

Replacement of the L-amino acid by the isomeric D-counterpart was not well tolerated in the P1 to P4 positions (compounds **15**, **16**, **17**, and **18**). However, replacement of the L-Asp at P6 by the D-Asp was tolerated, resulting in only a slight decrease in potency (compound **13**). Interestingly, the introduction of D-

Asp at P5 resulted in a *7-fold increase in potency* (compound **14**). Encouraged by this increase in potency, we explored further substitutions at the P5 position (Table 3).

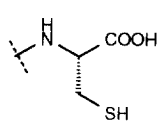
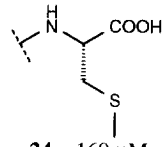
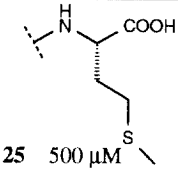
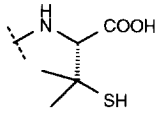
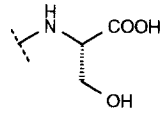
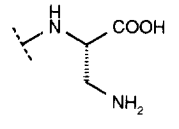
Table 3. P5 Substitutions.

Compound	L-amino acid	IC_{50}(μM)	Compound	D-amino acid[b]	IC_{50} (μM)
3	Ac-DDIVPC-OH	28	**14**	Ac-DdIVPC-OH	4
19	Ac-D-TBG-IVPC-OH[a]	20			
20	Ac-DVIVPC-OH	52	**21**	Ac-DvIVPC-OH	7.5
4	DAD-DIVPC-OH[c]	33	**22**	DAD-dIVPC-OH[c]	76
7	Ac-ADIVPC-OH	200	**23**	Ac-AdIVPC-OH	200

[a] TBG = *tert*-Butylglycine.
[b] Lower case letters denote D-amino acids.
[c] DAD-= 3-Carboxypropanoyl.

We found *tert*-butylglycine derivative **19** to be equipotent with the aspartic acid analog **3**, indicating that the carboxylic acid at P5 did not contribute to potency. Similarly, valine derivative **20** was well tolerated, displaying only a 2-fold loss in potency. As in the case of aspartic acid, replacement of L-Val at P5 in compound **20** by D-Val (compound **21**) also resulted in a 7-fold increase in potency. However, when D-Asp was introduced at the P5 position of compound **4**, containing a 3-Carboxypropanoyl group at P6, or compound **7**, containing an Ac-Ala at P6, to give compounds **22** and **23**, respectively, no increase in potency was observed. Therefore, the increase in potency observed by the introduction of a D-amino acid at the P5 position is dependent on the nature of the P6 substitution. Taken together these results suggest an orienting role for the P5 residue, causing the Ac-Asp residue at P6 to make a more favorable interaction with the enzyme.

Table 4. P1 amino acid replacements

Ac-DDIVP-AA		
Compound IC_{50}		
		
3 28 μM	**24** 160 μM	**25** 500 μM
		
26 630 μM	**27** >1000 μM	**28** >1000 μM

Next, we investigated the importance of the Cys residue at the P1 position of our inhibitors. It is well known that for peptide-based inhibitors of serine proteases, the P1 substitution contributes considerably to the potency and to the specificity of these inhibitors.[25] In the case of the HCV NS3 protease, Phe-154 defines the bottom of the S1 pocket and is the main determinant for substrate specificity.[11,12] The strong requirement for cysteine at P1 for substrate specificity may be due to a favorable interaction between the sulfhydryl group of the P1 cysteine and the aromatic side chain of Phe-154.[26] The importance of cysteine at the P1 position of our hexapeptide inhibitors is illustrated in Table 4.

Removal of the sulfhydryl group of the cysteine in P1 gave the alanine derivative **12** (IC_{50} = 710 μM, Table 2), which is a weak inhibitor of the enzyme. Methylation of the sulfhydryl group (*S*-methylcysteine derivative **24** and methionine derivative **25**) or β-dimethylation (penicillamine derivative **26**) was also detrimental to potency. Serine derivative **27** and β-aminoalanine derivative **28** did not inhibit the enzyme at 1 mM even though these residues, in principle, can still interact with Phe-154. In summary, a cysteine residue at P1 is very important for the potency of these peptide-based inhibitors.

Conclusions. We have found that DDIVPC-OH (**1**), corresponding to the N-terminal cleavage product of a NS5A/5B derived peptide substrate, inhibits the NS3-4A_{pep} protease. Hexapeptide **1** is a competitive inhibitor of the enzyme with a $K_{i\ app}$ of 14 μM. Structure-activity studies on **1** led to inhibitors with IC_{50} values in the low micromolar range (compounds **14** and **21**). Hexapeptide **14** (IC_{50} = 4 μM, Table 2) is the most potent inhibitor of the NS3-4A_{pep} protease identified in this study. We have also determined that **14** is a competitive inhibitor of the enzyme with a $K_{i\ app}$ of 0.6 μM. A study on the importance of the C-terminal carboxylic acid functionality of these peptide-based inhibitors will be reported shortly.

Acknowledgment. We thank E. Wardrop, G. McKercher, S. Lefebvre, and the analytical department for technical assistance. We also thank R. Déziel, J. Kelland, G. Kukolj and P. Lavallée for critical review of the manuscript. Finally, we thank P. Anderson and M. Cordingley for encouragement and support.

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19. **Protease assay**: The enzymatic assay was performed in 50 mM Tris-HCl, pH 7.5, 30% glycerol, 1 mg/mL BSA, 1 mM TCEP. 25 μM of the substrate DDIVPCSMSYTW [16], ~1 nM biotin-DDIVPC-SMSY[^{125}I]TW and various concentrations of inhibitor were incubated with 25 nM of protease and 2.5 μM of the NS4A-derived peptide KKGSVVIVGRIILSGRK [15] for 40 min at 23°C. The final DMSO concentration did not exceed 6.4%. The reaction was terminated with the sequential addition of 1 M MES, pH 5.8, and 0.5 N NaOH. The separation of substrate from products was performed by adding avidin-coated agarose beads to the assay mixture followed by filtration. A non-linear curve fit using the Hill model was then applied to the % inhibition-concentration data and 50% effective concentration (IC_{50}) was calculated through the use of SAS (Statistical Software System, SAS Institute Inc., Cary, N.C.).
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21. **K_i determination**. The initial velocity of the inhibited reaction was determined under the conditions described for the protease assay at several fixed inhibitor concentrations in presence of 25, 50, 100, 200 and 400 • M substrate. Calculations were performed by non-linear regression analysis of the velocity data using the GraFit software (version 3.0, Erithacus Software Ltd, Staines, UK).
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