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STUDIES ON THE C-TERMINAL OF HEXAPEPTIDE INHIBITORS OF THE HEPATITIS C VIRUS SERINE PROTEASE

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Abstract: Replacememt of the C-terminal carboxylic acid functionality of peptide inhibitors of hepatitis C virus (HCV) NS3 protease (complexed with NS4A peptide cofactor) by activated carbonyl groups does not produce any substantial increase in potency. These latter inhibitors also inhibit a variety of other serine and cysteine proteases whereas the carboxylic acids are specific. Norvaline was identified as a chemically stable replacement for the P1 residue of Ac-DDIVPC-OH which was also compatible with activated carbonyl functionalities.

Hepatitis C virus (HCV) is the major etiological agent of post-transfusion and community-acquired non-A non-B viral hepatitis worldwide. HCV infection is the most common cause of chronic hepatitis in the western world, a disease that may lead to cirrhosis and hepatoma.[1] One of the most intensively studied and, therefore, best understood targets for antiviral therapy against HCV is the serine protease of the NS3 protein. The original prediction of NS3 as a chymotrypsin/trypsin-like serine protease on the basis of sequence comparison with other viral and cellular proteases was later confirmed by structural determination.[2–4] The NS3 protease domain that constitutes the amino-terminal one-third of the NS3 protein is responsible for the proteolytic cleavage of non structural proteins NS3, NS4A, NS4B, NS5A, and NS5B.[5–9] The protease activity is enhanced by the NS4A protein, which acts as an essential cofactor required for polyprotein maturation.[10,11] Structure determination of the NS3 protease complexed with a truncated NS4A cofactor peptide[3,4] demonstrated that NS4A is an integral structural component of the enzyme. The crystal structures also revealed the absence of several loops that define the shape of the substrate N-terminal binding pockets in other chymotrypsin-like proteases. The absence of these loops renders the S1 specificity pocket as a shallow, non polar groove formed by the side chains of residues Phe-154, Ala-157, and Leu-135. The aromatic ring of Phe-154 likely interacts with the sulfhydryl group of the cysteine residue found at the P1 position of all the trans-cleavage sites.

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Classical inhibitors of serine proteases are not effective against the NS3 protease or are only effective at high concentrations.[12] This may be due, in part, to the unusual substrate specificity that is quite distinct from that of other serine type proteases.[13] This suggests that it should be possible to design inhibitors with a high degree of selectivity for this viral enzyme. Non peptidic inhibitors of the HCV serine protease have been reported[14–17] that are not specific and do not compete with the substrate. Peptide-based competitive inhibitors of the protease have also been reported.[18–20]

We have previously described inhibition of the NS3 protease complexed with NS4A cofactor peptide (NS3-4A$_{pep}$) by the N-terminal cleavage product of a peptide substrate. We reported hexapeptide Ac-DdIVPC-OH (**1**) as a competitive inhibitor of the NS3-4A$_{pep}$ protease with a K_i of 0.6 μM.[19] Recently, Steinkühler et al.[21] have reported inhibition of the HCV serine protease by the N-terminal cleavage products of substrate peptides corresponding to the NS4A–NS4B, NS4B–NS5B, and NS5A–NS5B cleavage sites. The formation of stable non covalent complexes between serine proteases and peptides containing a C-terminal carboxylic acids have been observed previously in crystal structures.[22–25] In these structures, one of the oxygens of the C-terminal carboxylate group hydrogen bonded with the imidazole ring of the catalytic histidine while the other oxygen occupied the oxy-anion hole. Interestingly, one of these complexes is the Sindbis virus core protein[22] a chymotrypsin-like serine protease that shares the greatest structural similarity with the NS3 protease. In this paper, we describe SAR studies at the P1 position of hexapeptide inhibitors with the goal of replacing the side chain of cysteine with a chemically stable group. The importance of the C-terminal carboxylic acid functionality and its contribution to the binding of these inhibitors to the NS3-4A$_{pep}$ protease is also investigated.

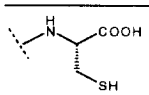
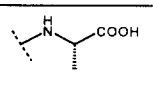
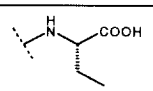
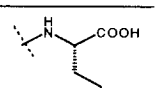
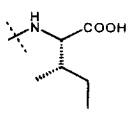
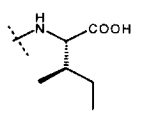
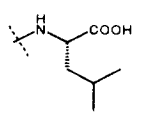
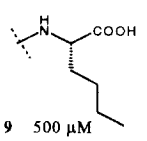
Materials and Methods

The synthesis of compounds **1** to **10** and **19** to **22** was accomplished by standard solid-phase or solution-phase peptide chemistry.[26,27] The synthesis of compounds **12** to **14** and **16** to **18** was carried out using previously reported methods.[28] Aldehydes **11** and **15** were prepared by Dess–Martin oxidation[29] of the corresponding alcohol. All peptides were purified by preparative reverse-phase HPLC on a C18 column using a gradient of 0–60% acetonitrile in water. Satisfactory MS, ^{1}H NMR spectra, amino acid analysis and homogeneity data (>90% by HPLC) were obtained for all inhibitors. The enzymatic assay was performed as reported previously.[19] All IC_{50} values reported are the average of at least four separate determinations.

Results and Discussion

We have previously reported[19] on replacements of cysteine at the P1 position of hexapeptide **2** (Table 1) that resulted in a dramatic loss of potency. Other natural and unnatural amino acids containing a heteroatom in the side chain produced hexapeptide analogs that were, at best, poor inhibitors of the enzyme. Table 1 shows the potency of hexapeptides containing various lipophilic side chains at the P1 position. Alanine derivative **3** is a poor inhibitor of the enzyme. However, when the length of the alkyl side chain is increased to ethyl (compound **4**) or to *n*-propyl (norvaline derivative **5**), peptide inhibitors with moderate potency were obtained. A further increase in length to an *n*-butyl group (norleucine derivative **9**) decreased potency. Norvaline derivative **5** is five times less potent than the cysteine containing analog **2**, but chemically more stable. Having identified the optimal length for the P1 side chain to be *n*-propyl, we investigated the effect of branching on the alkyl chain. Introduction of an *(S)*-methyl group at the β-carbon produced isoleucine derivative **6**, which was a much poorer inhibitor of the enzyme. However, introduction of the isomeric *(R)*-methyl group, as in *allo*-isoleucine derivative **7**, was well tolerated. When the methyl group was introduced at the γ-position, as in leucine derivative **8**, a loss in binding affinity was observed. In summary, of the amino acid studied, norvaline is the best overall replacement for cysteine producing a moderate inhibitor of the protease. The introduction of norvaline into the sequence Ac-DdIVP-**X** produced a low micromolar potency inhibitor of the NS3-4A$_{pep}$ protease (compound **10**, Table 2).

Table 1. P1 Side chain study

Ac-DDIVP-**X** Compound	IC_{50}
 **2**	28 μM
 **3**	750 μM
 **4**	250 μM
 **5**	150 μM
 **6**	780 μM
 **7**	190 μM
 **8**	800 μM
 **9**	500 μM

Peptide analogues containing an electrophilic carbonyl group are, in general, good inhibitors of serine proteases.[30] These activated carbonyl derivatives are believed to form a tetrahedral hemiacetal with the active site serine hydroxyl group. Therefore, we studied the effect of introducing an electrophilic carbonyl on the potency of inhibitors **5** and **10** (Table 2). Aldehyde derivatives **11** and **15** bind 15-fold better than the corresponding acid analogs **5** and **10**, respectively. Fluorine containing carbonyl derivatives such as trifluoromethylketone (TFMK) derivatives **12** and **16** and pentafluoroethylketone (PFEK) derivatives **13** and **17** displayed potencies comparable to the corresponding carboxylic acids. α-Ketoamide derivatives **14** and **18** were the best inhibitors of the NS3-4A$_{pep}$ protease. Overall, in contrast to what is observed with other serine proteases, peptide sequences containing electrophilic carbonyls were only marginally more potent (with the

exception of **14** and **18**) inhibitors of the HCV serine protease than the corresponding carboxylic acids. The better inhibition observed for compounds **14** and **18** maybe due to additional binding in the S1' pocket.

Table 2. Peptide analogs containing an activated carbonyl group at the C-terminus.

Compound		IC_{50} (μM)	Compound		IC_{50} (μM)
5	Ac-DDIVP-Nva-**OH**	150	**10**	Ac-DdIVP-Nva-**OH**[1]	17
11	Ac-DDIVP-Nva-**H**	10	**15**	Ac-DdIVP-Nva-**H**	1.1
12	Ac-DDIVP-Nva-**CF_3**	160	**16**	Ac-DdIVP-Nva-**CF_3**	22
13	Ac-DDIVP-Nva-**C_2F_5**[2]	79	**17**	Ac-DdIVP-Nva-**C_2F_5**[2]	12
14	Ac-DDIVP-Nva-**CONHBn**[2]	2.0	**18**	Ac-DdIVP-Nva-**CONHBn**[2]	0.64

1. Lower case letters denote D-amino acids.
2. The α-carbon of norvaline is racemic.

One of our concerns with the introduction of electrophilic carbonyls in the peptide sequence was the specificity of these inhibitors against other proteases. We investigated the inhibitory activity of these compounds against a variety of serine/cysteine proteases. As shown in Table 3, carboxylic acid derivative **10** was highly specific and did not inhibit other serine/cysteine proteases at 1 mM. However, electrophilic carbonyl-containing derivatives **14** to **18** also inhibited other serine proteases such as human leucocyte elastase (HLE) or porcine pancreatic elastase (PPE) very effectively.

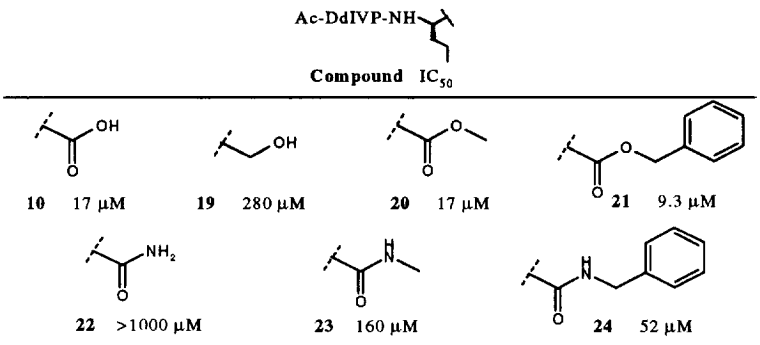
Table 3. Activity of various inhibitors of HCV Protease against other serine and cysteine proteases.

Compound	IC_{50} (μM)			
	HLE	**PPE**	**BPC**	**cat-B**
10	>1000	>1000	>1000	>1000
14	0.03	<0.06	10	100
15	6.7	2.2	>900	9.5
16	<0.06	0.19	4.5	>900
17	0.06	<0.06	18	416
18	0.10	<0.06	30	172
21	>1000	>1000	29	>1000
24	120	206	>1000	>1000

HLE = Human Leukocyte Elastase.
PPE = Porcine Pancreatic Elastase.
BPC = Bovine Pancreatic α-Chymotrypsin.
Cat-B = Human Liver Cathepsin B.

Because of the high specificity displayed by the C-terminal carboxylic acids, we decided to further investigate the contribution of this functionality to the potency of the inhibitors (Table 4). The importance of the carboxylic acid was evidenced by the significant decrease in potency of the corresponding primary alcohol **19** and primary amide **22**. Interestingly, the methyl and benzyl ester derivatives **20** and **21** displayed similar potencies to the carboxylic acid derivative **10**. Since peptides containing an ester linkage at the scissile bond have been reported as substrates for the HCV serine protease,[31] we hypothesized that these esters were hydrolyzed by the enzyme. Incubation of esters **20** and **21** with the NS3-$4A_{pep}$ protease under the enzymatic assay conditions in the absence of substrate followed by HPLC analysis of the reaction products confirmed that both esters were hydrolyzed to the corresponding carboxylic acid **10**. However, in contrast to carboxylic acid **10**, benzyl ester **21** inhibited significantly bovine pancreatic α-chymotrypsin (BPC) (IC_{50} = 29 μM, Table 3).

Table 4. C-terminal carboxylic acid modifications.



1. d = D-Aspartic Acid.

Whereas replacement of the C-terminal carboxylic acid with the primary amide **22** resulted in at least 50-fold loss in potency, the methyl and benzyl amide derivatives **23** and **24** showed some inhibition. No enzymatic hydrolysis of these amides was observed under the same conditions used to study the hydrolysis of the esters. The specificity profile of these amide derivatives was also investigated since it has been reported that *N*-alkylamides are weak inhibitors of serine proteases.[32] We observed that benzylamide **24** displayed similar potency against NS3-4A_{pep}, HLE and PPE (Table 3).

Conclusions

The hexapeptide inhibitor **2**, based on the *N*-terminal cleavage product of a dodecapeptide substrate, contains a cysteine residue at P1. We have been able to identify a chemically more stable replacement for cysteine. Norvaline derivative **10** is a low micromolar and very specific inhibitor of the HCV serine protease. Introduction of electrophilic carbonyls at the C-terminus produces only a moderate increase in potency (with the exception of compounds **14** and **18**) with loss of specificity. The carboxylic acid functionality at the C-terminus contributes considerably to potency and imparts great specificity to these peptide-based inhibitors of the HCV serine protease.

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