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Tetrapeptides as Potent Protease Inhibitors of Hepatitis C Virus Full-Length NS3 (Protease-Helicase/NTPase)

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Abstract—A library of tetrapeptides was evaluated for Hepatitis C Virus NS3 protease inhibitor activity in an in vitro assay system comprising the native bifunctional full-length NS3 (protease-helicase/NTPase) protein. Tetrapeptides with K_i values in the high nanomolar range were identified, for example Suc-Chg-Glu-2-Nal-Cys ($K_i = 0.27 \pm 0.03\,\mu M$) and Suc-Dif-Glu-Glu-Cys ($K_i = 0.40 \pm 0.10\,\mu M$). Furthermore, it was shown that the inhibitory potencies are not affected significantly by assay ionic strength. As suggested by molecular modelling, potential binding interactions of the tetrapeptide inhibitors with the helicase domain might explain the data and structure–activity relationships thus obtained. Hence, we postulate that the full-length NS3 assay is a relevant system for inhibitor identification, offering new opportunities for inhibitor design.

Introduction

Hepatitis C Virus (HCV) infections constitute a global health problem. About 3% of the world's population suffers from chronic hepatitis C, which could ultimately develop into liver cirrhosis, hepatocellular carcinoma or liver failure. The currently available therapies are interferon-α (IFN-α) and its combination with ribavirin and, more recently, pegylated IFN-α. Unfortunately, the success of these therapies is modest and varies depending on the specific HCV genotype.[1–4] Consequently, new and more effective therapies are much needed.

The RNA genome of HCV is translated into the polyprotein NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH, which is proteolytically cleaved into 10 mature viral proteins. The non-structural protein 3 (NS3) is a multifunctional enzyme possessing serine protease activity in the N-terminal third of the protein and RNA helicase/NTPase activity in the C-terminal portion. The NS3 protease is responsible for auto catalytic *cis* cleavage at the NS3/NS4A junction and for *trans* cleavages at the NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions.[5–7] The NS3 protease has been the subject of extensive studies and represents the primary target in the search for effective antiviral treatments against HCV.[2,8–10]

The NS3 protease is inhibited by both classical serine protease inhibitors with an electrophilic serine-trap functionality in the P1 position[11–21] and, uniquely, by its N-terminal cleavage products and derivatives thereof, containing a C-terminal COOH group.[10,12,19,22–26] Furthermore, it was demonstrated with a series of product-based hexapeptide inhibitors that the inhibitory potencies were affected significantly by the ionic strength of the assay medium.[24] Thus, considerably lower activities were reported at physiological NaCl concentrations (~150 mM) than in the absence of salt additives, employing an assay using the protease domain of NS3, that is not the native form of the enzyme.[24]

Recently, we reported that the structure–activity relationships (SARs) of a small series of HCV NS3 protease inhibitors is dependent on the assay used.[27] Most importantly, we found that shortened product-based inhibitors retained much better inhibitory potencies in the native bifunctional full-length NS3 (protease-helicase/NTPase)

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assay, as compared to what had been reported as achieved in the isolated NS3 protease domain assay.[27] It remained to be explored whether the different outcomes of the two assays can be attributed to interactions of the inhibitors not only with the protease domain, but also with the helicase domain. Recent X-ray data suggests that both domains strongly participate in interdomain interactions in addition to interactions with the six C-terminal residues, corresponding to the P-side product of the *cis* cleavage.[28] Hence, it seems likely that additional favourable binding interactions with the helicase may account for a retained inhibitory capacity of smaller peptide inhibitors, although the effect of variations in the enzyme or assay conditions cannot be fully excluded. Moreover, these results emphasize the importance of establishing SARs in an assay based on the native full-length NS3 protein. Previous SARs have been almost exclusively based on inhibitory data obtained from experiments employing the artificial truncated form of the NS3 protease.[14,17–19,22–24,29,30] Consequently, prior to embarking on a more extensive medicinal chemistry program, we felt prompted to perform a more thorough SAR study focused on short peptides, to enable us to determine the structural requirements for protease inhibition of the full-length NS3 (protease-helicase/NTPase) protein.

We herein report the inhibitory potency towards HCV NS3 protease of a small library of tetrapeptides evaluated in a full-length NS3 assay. Potential binding interactions with the helicase domain and the impact of the ionic strength on inhibitor potency are taken into consideration for the rationalization of the SAR data obtained.

Results and Discussion

Inhibition of full-length NS3 protease activity

The peptide library was prepared by a parallel approach using standard Fmoc/*t*-Bu solid-phase peptide synthetic methodology.[31,32] The building blocks used are shown in Table 1. These amino acid residues were all selected based on data from previous SAR studies performed by us, using the full-length NS3 assay,[27] and by others, employing the NS3 protease domain assay.[22,24,33]

Table 1. Building blocks used at different positions in the tetrapeptide (and tripeptide) library[a]

Capping group	P4	P3	P2	P1
Ac	Chg	Ile	Cha	Cys
Suc	Dif	Glu	Leu	ACPC
Glt	Leu	Val	Glu	
			2-Nal	
			Phe	
			Bip	

[a]Ac, acetyl-; Chg, cyclohexylglycine; Cha, β-cyclohexylalanine; Suc, succinyl-/3-carboxypropanoyl-; Dif, 3,3-diphenylalanine; ACPC, 1-aminocyclopropane-1-carboxylic acid; Glt, glutaryl-/4-carboxybutanoyl-; 2-Nal, 2-napthylalanine; Bip, β-(4-biphenylyl)-alanine.

Kinetic constants for the enzyme under standard conditions, without NaCl, were calculated to be: $k_{cat} = 0.42$ s^{-1}, $K_m = 0.15$ μM, $k_{cat}/K_m = 2.8$ μM^{-1} s^{-1}.[34] In order to observe trends in the SAR, and to efficiently identify lead structures, the inhibition of the full-length NS3 protein was determined as percent inhibition at certain concentrations. The inhibitory potencies of 37 tetra- and tripeptides, shown in Table 2, were measured in triplicate. For those inhibitors with greater than ~75% inhibition or lower than ~15% inhibition, the determinations were repeated at a more relevant concentration. In addition, most of the inhibitors were tested on different occasions in duplicate to further validate the values obtained. As a control, Ac-Asp-D-Gla-Leu-Ile-Cha-Cys was measured in parallel, yielding an average inhibition of 61±9% at 0.104 μM after more than 100 determinations. Some of the inhibitors, including the most potent, were selected for K_i measurements (Fig. 1).

Structure–activity relationships

As is illustrated in Table 2, the wide range in potency of the peptides revealed trends in the SARs. It should be noted that the inhibitory potency values derived for the two highest concentrations, that is 167 and 83.5 μM are associated with a higher degree of uncertainty, as a result of solubility limitations. Therefore, the discussion below concerning the SARs is predominantly focused on the peptides having Cys in the P1 position and their inhibitory effects at 16.7 and 1.67 μM. Furthermore, in this context it should be emphasized that the response due to the alteration of one individual residue does not entirely reflect the binding interactions of the specific residue in its corresponding protein binding site, but is a function of the amino acid sequence of the tetrapeptide as a whole.

The naturally occurring Cys in the P1 position has, until recently, proved superior to other residues in both substrates and peptide inhibitors,[19,23,24,29,35,36] However, displacement of Cys by 1-aminocyclopropane-1-carboxylic acid (ACPC) could, according to previous reports by Llinàs-Brunet and co-workers, deliver almost equipotent inhibitors, all comprising proline-based residues in the P2 position.[22,33] As is shown in Table 2, the inhibitory effect of the inhibitors used herein is more than ten times greater with Cys in the P1 position than with ACPC, when the full-length NS3 assay is used. Very recently, Narjes et al. reported an elegant design of 2-amino-4,4-difluorobutyric acid as a biochemically stable cysteine mimic, successfully used in potent HCV NS3 protease inhibitors.[12,20] The impact of displacement of Cys for 2-amino-4,4-difluorobutyric acid in the peptides in Table 2 remains to be explored.

Our previous SAR studies on hexapeptides showed that 2-naphtylalanine (2-Nal) and Glu rendered more active inhibitors than cyclohexylalanine (Cha) in the P2 position, which was in contrast to results from the protease domain assay.[27] Therefore, 2-Nal, Glu, Cha, but also Leu, Phe, and β-(4-biphenylyl)-alanine (Bip) were assessed as P2 residues in the present study. In agreement with the previous results from hexapeptides, 2-Nal produced

Table 2. Inhibition of HCV NS3 protease activity by tetra- and tripeptides evaluated in a full-length NS3 (protease-helicase/NTPase) assay[a]

Compound	Capping group	P4	P3	P2	P1	% Inhibition at 167 μM	% Inhibition at 83.5 μM	% Inhibition at 16.7 μM	% Inhibition at 1.67 μM
1	Suc	Chg	Ile	Cha	Cys			82	29
2	Glt	Chg	Ile	Cha	Cys			69	
3	Suc	Leu	Ile	Cha	Cys	91		30	
4	Suc	Chg	Glu	Cha	Cys			51	
5	Suc	Chg	Glu	Leu	Cys	97		71	6
6	Suc	Chg	Glu	Bip	Cys			61	
7	Suc	Chg	Ile	Glu	Cys	82		48	
8	Suc	Chg	Val	Glu	Cys	68		36	
9	Suc	Chg	Glu	2-Nal	Cys	100		90	50
10	Glt	Chg	Glu	2-Nal	Cys			88	37
11	Ac	Chg	Glu	2-Nal	Cys			74	8
12	Suc	Chg	Glu	Glu	Cys			86	11
13	Glt	Chg	Glu	Glu	Cys	94		91	13
14	Ac	Chg	Glu	Glu	Cys	74		50	
15		Chg	Glu	Glu	Cys	0			
16	Suc	Dif	Glu	Glu	Cys	100		97	41
17	Suc	Dif	Glu	2-Nal	Cys		74	42	3
18	Suc	Dif	Glu	Cha	Cys			25	
19	Suc	Dif	Glu	Phe	Cys			31	
20	Suc	Dif	Glu	Bip	Cys			13	
21	Suc	Leu	Glu	Glu	Cys	100		82	17
22	Suc		Glu	Glu	Cys			32	
23	Glt		Glu	Glu	Cys			29	
24	Ac		Glu	Glu	Cys			15	
25	Suc	Chg	Ile	Cha	ACPC	70	38		
26	Suc	Leu	Ile	Cha	ACPC	7–19	7		
27	Glt	Leu	Ile	Cha	ACPC	12	3		
28	Suc	Chg	Glu	Leu	ACPC	17	3		
29	Suc	Chg	Ile	Glu	ACPC	28	14		
30	Suc	Chg	Val	Glu	ACPC	30	14		
31	Suc	Chg	Glu	Glu	ACPC	54	31		
32	Ac	Chg	Glu	Glu	ACPC	27	18		
33	Suc	Dif	Glu	Glu	ACPC	23	22		
34	Glt	Dif	Glu	Glu	ACPC	24	27		
35	Suc	Leu	Glu	Glu	ACPC	21	14		
36	Suc		Glu	Glu	ACPC		11		
37	Glt		Glu	Glu	ACPC	24	17		

[a]Abbreviations as in Table 1. For assay details see Experimental. Ac-Asp-D-Gla-Leu-Ile-Cha-Cys (D-Gla, D-γ-carboxyglutamic acid) was measured in parallel yielding an average inhibition of 61 ± 9% at 0.104 μM after more than hundred determinations. The inhibitory potency values derived for the two higher concentrations, that is 167 and 83.5 μM are associated with a higher degree of uncertainty, as a result of solubility limitations.

more active compounds than Cha, in otherwise identical sequences (**4**, **9**, **17** and **18**, Table 2). Suc-Chg-Glu-2-Nal-Cys **9** ($K_i = 0.27$ μM, Fig. 1) is in fact the most active inhibitor identified in the present study. However, displacement of cyclohexylglycine (Chg) by diphenylalanine (Dif) in position P4 makes Glu rather than 2-Nal the preferred residue in P2 (**4**–**6**, **9**, **12** and **16**–**20**, Table 2). Thus, Suc-Dif-Glu-Glu-Cys **16** exhibits a K_i of 0.4 μM (Fig. 1).

We have demonstrated previously that the acidic Glu in contrast to the lipophilic Ile in the P3 position delivered a more active hexapeptide inhibitor, when assayed in the full-length NS3 assay.[27] In the present study, Glu in the P3 position is more favourable than both Ile and Val in one tetrapeptide sequence, that is Suc-Chg-P3-Glu-Cys. However, Ile gives a better inhibitor when the more lipophilic sequence Suc-Chg-P3-Cha-Cys is considered, which illustrates the difficulties in activity predictions (**1**, **4**, **7**, **8** and **12**, Table 2).

In position P4, Dif gave the best results for hexapeptides in the full-length assay.[27] Llinàs-Brunet et al. reported that substitution with Chg at P4 systematically produced 3- to 5-fold more potent inhibitors than Ile, the latter being almost equipotent with Leu, when assayed against the protease domain alone.[22,33] Thus, this encouraged us to examine the impact of both Dif and Chg as P4 residues in short peptide inhibitors. As shown in Table 2, Chg emerged as a promising P4 residue. It is present in the most potent inhibitor **9** and works well in many different sequences. The effect of the more bulky Dif, on the other hand, is highly influenced by the P2 residue and a favourable outcome is only accomplished in combination with Glu in P2 (**4**–**6**, **9**, **12** and **16**–**20**, Table 2).

Acidic functionalities like Asp and Glu in the P6 position, and to some extent also in the P5 position, are important for good inhibitory potency in the NS3 protease domain assay.[23,24,37] However, AcAsp in P6 could successfully be substituted by succinic acid (Suc) or glutaric acid (Glt). In pentapeptides, the substitution of AcAsp/AcGlu in P5 by Suc and Glt delivered less potent inhibitors, although the corresponding tetrapeptides which have Suc and Glt instead of acetyl (Ac) were

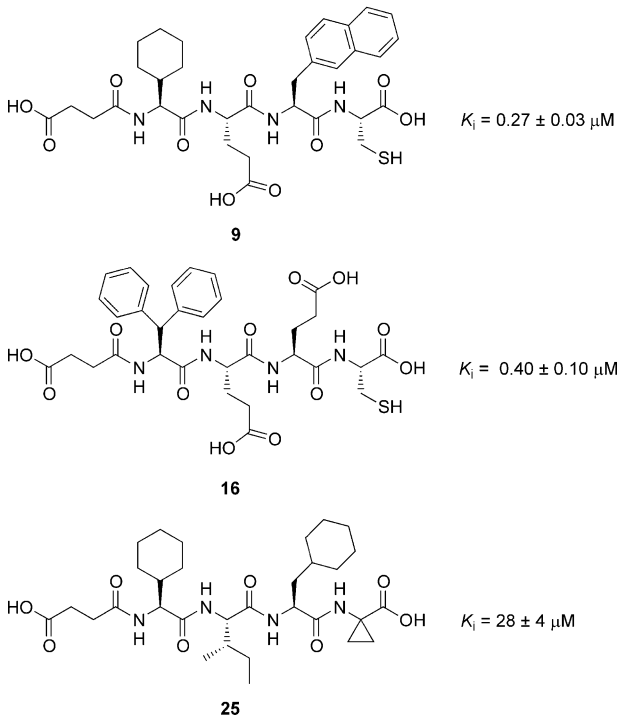

Figure 1. Structures and K_i values of the two most potent inhibitors (**9** and **16**) obtained, and of the most potent ACPC containing inhibitor (**25**).

more active.[23,24] Hence, we found it relevant to explore the impact of different capping groups with tetra- and tripeptides in the full-length NS3 assay. In addition, one tetrapeptide **15** lacking a capping group, that is with a free amine group, was made as a control. Results from the present library show that capping with Suc or Glt consistently produced more potent inhibitors than acetylation (**9**–**11**, **12**–**14** and **22**–**24**, Table 2). Inhibitory potency is lost with a free amine group present.

Molecular modelling

The active site of the NS3 protease is, after the *cis* cleavage event, oriented towards the interior of the protein with the helicase domain in close proximity: as supported by a crystal structure of the bifunctional NS3 (protease-helicase/NTPase) protein (as an scNS3-NS4A construct) reported by Yao and co-workers.[28] Thus, both the protease and helicase domains of NS3 participate in interactions with the six C-terminal residues, corresponding to the P-side product derived from the *cis* cleavage. Furthermore, Yao et al. postulated that local and global structural changes in NS3 would be required to displace the *cis* cleavage product and to subsequently accommodate the successive polyprotein recognition sequences.[28] Our previous observations demonstrated that the structural requirements for inhibition of the protease of full-length NS3 are different from those for the artificial truncated form of the NS3 protease.[27] We therefore hypothesize that the NS3 protein, after accommodation of a substrate (or an inhibitor as addressed herein) in line with other proteolytic enzymes, for example HIV-protease, re-establishes the original conformation, and thereby recovers favourable interdomain interactions.[38] Thus, not only interactions with the protease part of NS3 but also those with the helicase part will influence the binding affinities of the inhibitors.

An analysis of the X-ray model by Yao depicted in Figure 2 suggests that there are some potential sites for inhibitor recognition where the helicase is engaged. Firstly, there is a hydrogen bond from His528 of the helicase domain to the carbonyl oxygen of the P4 residue. Secondly, there is a large S2 pocket separated from the S4 pocket by the side chain of Gln526. Thirdly, Phe531 and/or Phe438 may be sufficiently close to Leu627, the P5 residue in the NS3 C-terminal, for hydrophobic interactions. Docking of peptide inhibitors into the active site, in line with the suggested hypothesis, has been achieved (see Experimental). Both tautomeric forms of His528 have been used (Fig. 3a and b). As is illustrated for Suc-Chg-Glu-2-Nal-Cys **9** in Fig. 3, two of the features described above for the crystal, the hydrogen bond from His528 (either to the carbonyl of P4 as in the crystal or to the amine of P4) and the large S2 pocket delimited by Gln526 are also seen in the resulting inhibitor–protein complexes. In addition, Gln526 was found to form a hydrogen bond to the P4 carbonyl in the model, considering protonation at His-N4 (Fig. 3a). Consequently, we suggest that the retained inhibitory capacity of smaller peptides observed in the full-length NS3 assay could be explained by additional favourable binding interactions with the helicase domain. Such interactions can also help us rationalize why SARs are somewhat different in the full-length NS3 assay as compared to the protease domain assay.[27] For example, the preference for 2-Nal in P2 in the full length NS3 assay as compared to Cha in the protease domain assay, might be attributed to an interaction between the aromatic residue and the amide of Gln526, as suggested by one of the models (Fig. 3b).[39,40] The large S2 site located at the protease/helicase interface explains why bulky P2 residues such as 2-Nal can easily be accommodated (Fig. 3).

Besides the potential binding interactions with the helicase domain, the fact that the P5 and P6 residues are strongly involved in electrostatic interactions with the electropositive S5–S6 area of the protease domain provides an significant feature for understanding factors of importance for the binding of hexapeptides.[37] The impact of the arginine and lysine rich domain that is fully solvent exposed in the truncated protease, should be of less importance in the full-length NS3 assay, in particular when short tetrapeptides are considered.[37] Thus, since we expected that the binding of tetrapeptides to the full-length NS3 protein should be less affected by the ionic strength of the assay, we found it adequate to examine its importance for binding affinities.

Influence of ionic strength on inhibitor activity

Alteration of the ionic strength may provide information on the forces involved in the interaction between proteins and ligands. In general, increased ionic strengths weaken electrostatic interactions and strengthen interactions of hydrophobic origin. Ingallinella et al. clearly

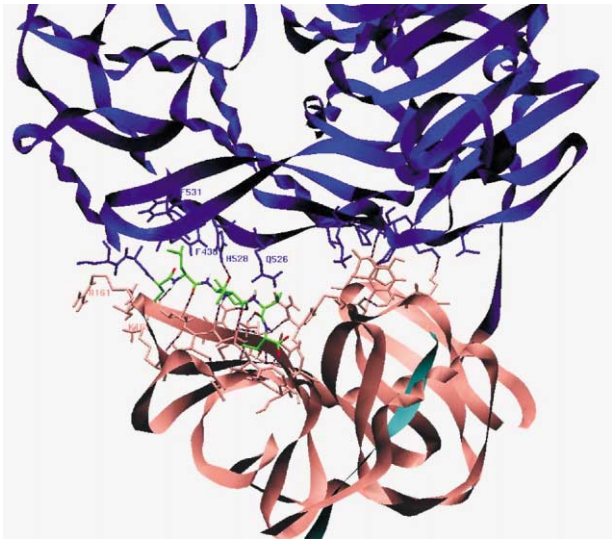

Figure 2. Picture of the crystal structure of the full-length scNS3-NS4A protein (PDB ID: 1CU1).[28] Protease residues 3–181 depicted in pink, helicase residues 182–625 in blue, NS4 in turquoise and the C-terminal residues 626–631 are color-coded with all carbons in green, oxygens in red, nitrogens in blue, and hydrogens in gray. Only residues close to the C-terminal residues (within 4.0 Å and Arg161) and those involved in the interactions between the protease and the helicase part are drawn as sticks.[28] Hydrogen bonds are indicated.

demonstrated that the ionic strength has a pronounced impact on the inhibition constants of hexapeptide HCV protease inhibitors containing carboxylate groups when evaluated in an isolated protease assay. The observed effect on potency correlated well with the number of carboxylate groups. Thus, not only the binding contributions from the P6 and P5 residues but also those from the carboxylate functions in closer proximity to the C-terminus were affected by salt addition.[24] We performed inhibition measurements of full-length NS3 in an assay buffer with 150 mM NaCl. The inhibitors included in the study were the hexapeptide Ac-Asp-D-Gla-Leu-Ile-Cha-Cys and four different tetrapeptides comprising various numbers of carboxylate groups as shown in Table 3.

The acidic hexapeptide produced 70% inhibition of the full-length NS3 protease at an inhibitor concentration of 0.104 μM in the buffer without NaCl, and 44% inhibition in the buffer with 150 mM NaCl. Consequently, for Ac-Asp-D-Gla-Leu-Ile-Cha-Cys a two-fold decrease in percent inhibition is observed in the full-length NS3 assay, which should be compared to a 27-fold decrease of the IC_{50} value in a protease domain assay, as previously reported for the same compound.[24] The difference expressed in percent inhibition actually represents a greater difference in inhibitory potency, since the affinity of the substrate is also affected by NaCl concentration: reflected in an observed change in K_m. Thus, in coherence with an assay containing the protease domain only,[37,41] an increase in K_m (3-fold) and slight decrease of k_{cat} (1.36-fold) was found by addition of 150 mM NaCl. The actual difference in inhibitory potency, affected by the presence of NaCl, is more accurately reflected by K_i values of the inhibitor in both of the buffers. Hence, Ac-Asp-D-Gla-Leu-Ile-Cha-Cys provided K_i values of 15 and 60 nM in the buffer without and with NaCl, respectively, which is then approximately a four-fold difference (Table 3).

In contrast to what is observed with the hexapeptide, no significant drop in the percentage inhibitory potencies of the tetrapeptides in Table 3 were encountered at higher NaCl concentration. Notably, not even the binding of

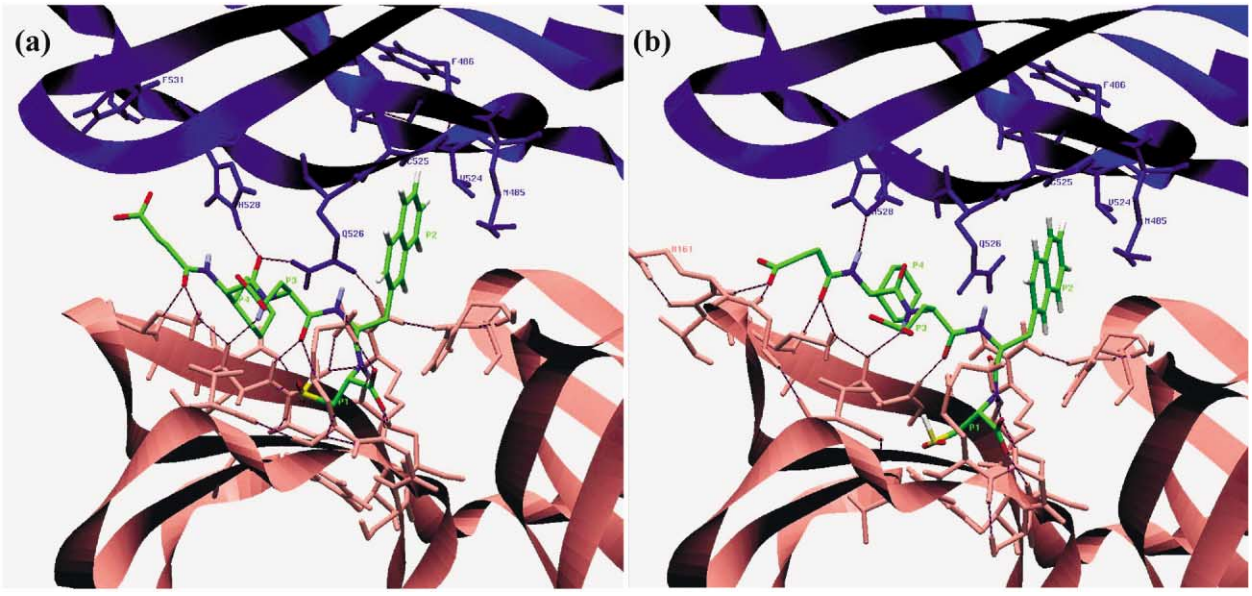

Figure 3. Picture of the most successful tetrapeptide Suc-Chg-Glu-2-Nal-Cys **9** docked into the protease active site of the full-length scNS3-NS4A protein. Protease residues 3–181 depicted in pink, helicase residues 182–631 in blue, NS4 in turquoise and the inhibitor color-coded with all carbons in green, oxygens in red, nitrogens in blue, sulphur in yellow (lone pairs in red), and hydrogens in gray. Only residues close to the inhibitor (within 4.0 Å) are drawn as sticks. Hydrogen bonds are indicated. Both possible tautomeric forms of His528 in the enzyme are considered: (a) protonation at His-N4, (b) protonation at His-N2.

Table 3. NS3 protease inhibitory potencies of peptides measured in absence and presence of 150 mM NaCl (in parentheses) in the full-length NS3 assay[a]

Capping group	P6	P5/Capping group	P4	P3	P2	P1	% Inhibition at 16.7 μM	% Inhibition at 1.67 μM	% Inhibition at 0.104 μM
Ac	Asp	D-Gla	Leu	Ile	Cha	Cys			70[b] (44[c])
		Ac	Dif	Ile	Cha	Cys	77 (75)		
		Suc	Chg	Ile	Cha	Cys	82 (83)		
		Suc	Chg	Glu	2-Nal	Cys		50 (43)	
		Suc	Dif	Glu	Glu	Cys		36 (33)	

[a]Abbreviations as in Table 1; D-Gla, D-γ-carboxyglutamic acid. For assay details see Experimental. Both measurements were performed in parallel.
[b]$K_i = 0.015 \pm 0.001$ μM.
[c]$K_i = 0.060 \pm 0.006$ μM.

the tetrapeptide containing four carboxylate groups was affected significantly by salt addition (Table 3). This could be attributed to a shielding effect by the helicase domain.

Conclusion

HCV NS3 protease inhibitors with K_i values in the high nanomolar range have been obtained and new SAR data for the native bifunctional full-length NS3 (protease-helicase/NTPase) protein have been elucidated from a focused library of tetrapeptides. Furthermore, we have shown that the inhibitory potency is not affected significantly by the presence of 150 mM NaCl. We believe that favourable binding interactions with the helicase domain might account for the observed data as supported by molecular modelling. Hence, the full-length NS3 assay seems not only a more relevant system to use for inhibitor identification, it also creates new opportunities for inhibitor design.

Experimental

General

Peptide assembly was accomplished in a Bodhan Mini-Block™ synthesizer. RP-HPLC was performed on a Gilson-Finnigan ThermoQuest AQA system in ESI mode, on a Zorbax Stable Bond C8 column (20×50 mm or 4.6×50 mm), using an acetonitrile/H_2O gradient with 50 mM NH_4OAc (pH 6.3) or an acetonitrile/H_2O gradient with 0.05% HCOOH, using UV (214, 255 nm) and MS detection. Plasma desorption mass spectroscopy (PD-MS) was carried out on an Applied Biosystems (Uppsala, Sweden) BIOION 20 instrument. Amino acid analyses were performed at the Department of Biochemistry, Uppsala University, Sweden, on 24 h hydrolysates (on oxidized samples of Cys containing peptides) with an LKB 4151 alpha plus analyser using ninhydrin detection.

Materials

The *N*-Fmoc protected amino acids were obtained from Senn Chemicals, Alexis Corporation and Nova Biochem. H-Cys(Trt)-2-chlorotrityl and 2-chloro-tritylchloride resins were obtained from Senn Chemicals and Alexis Corporation, respectively. *N*-[(1*H*-benzotriazole-1-yl)-(dimethylamino)methylene] - *N* - methylmethanaminium tetrafluoroborate *N*-oxide (TBTU) were obtained from Richelieu Biotechnologies and *N,N*-diisopropylethylamine (DIEA), redistilled, 99.5% from Aldrich. DMF (Aldrich, 99.9 + %, HPLC grade) was stored over molecular sieves (4 Å) and dichloromethane (DCM) (Riedel-deHaën) was freshly distilled from calcium hydride. Our control substance Ac-Asp-D-Gla-Leu-Ile-Cha-Cys was purchased from Bachem.

Solid-phase peptide synthesis (SPPS)

The peptide inhibitors (**1**–**37**) were synthesized by standard Fmoc/*t*-Bu solid-phase peptide synthesis techniques using a parallel approach.[31,32] H-Cys(Trt)-2-chlorotrityl resin and H-ACPC-2-chlorotrityl resin were used as the starting resins. Loading of Fmoc-ACPC was performed using a slightly modified standard procedure,[42] from 2-chloro-tritylchloride resin (1.47 mmol/g, 500 μmol), Fmoc-ACPC-OH (300 μmol) and DIEA (1.2 mmol) by stirring for 60 min in DCM (5 mL). End-capping was performed by washing with DCM/MeOH/DIEA (17:2:1) followed by DCM, DMF, and DCM. Peptide assembly was performed on a 33 μmol scale using Fmoc-AA-OH (0.13 mmol), TBTU (0.13 mmol) and DIEA (0.26 mmol) in DMF (0.5 mL) with a coupling time of 60 min. Removal of the Fmoc group was achieved by reaction with 25% piperidine in DMF for 1 + 10 min. Terminal acylation was carried out using acetic anhydride (0.33 mmol), succinic anhydride (0.33 mmol) or glutaric anhydride (0.33 mmol), and DIEA (0.33 mmol) in DMF (0.5 mL) for 60 min. The peptides were cleaved from the resin in 0.5 mL TFA/H_2O/triethylsilane (18:1:1) for 60 min, and the resin was subsequently filtered off. The TFA was partially evaporated followed by precipitation of the crude peptide by addition of cold diethyl ether. The products were washed several times with fresh diethyl ether, air-dried, and purified by preparative RP-HPLC. The peptides were analysed by MS and by analytical RP-HPLC. In every instance the observed molecular weight was within ±0.2% mass unit of the calculated ($[M+H]^+$ for HCOOH buffer, $[M+NH_3+H]^+$ for peptides purified in the NH_4OAc containing buffer). Peptide content was determined by amino acid analysis. The ratios of the amino acids (Leu, Ile, Glu, Val, Chg, Cha, Cys) were correct within the precision of the method, that is ±3%, with exception of the peptides containing the Chg-Ile sequence,

which is difficult to hydrolyse under the conditions used.

Enzyme

Details of the cloning, expression and purification of the enzyme used are described elsewhere.[43] Briefly, cDNA coding for the full-length NS3 protein (amino acids 1027–1657) from a 1a strain of HCV was introduced into a pBAD/HisA vector and transformed into *Escherichia coli* TOP10 cells. The cells were grown in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) containing 90 μg/mL ampicillin at 37 °C until $OD_{600} \approx 0.6$. The culture was cooled down to 30 °C and 100 μM $ZnCl_2$ and 0.002% L(+)-arabinose were added. The culture was incubated for another 4 h at 30 °C under gentle shaking before harvesting by centrifugation. The cells were resuspended in 25 mM HEPES pH 7.6, 0.3 M NaCl, 20% glycerol, 10 mM β-mercaptoethanol and 0.1% CHAPS and lysed in a French pressure cell, whereupon 20 μg/mL DNAseI was added and insoluble material was precipitated by centrifugation. The supernatant was loaded onto a Ni^{2+}-Chelating Sepharose Fast Flow column equilibrated with 50 mM HEPES pH 7.6, 0.3 M NaCl, 26% glycerol, 10 mM β-mercaptoethanol, 0.1% CHAPS (Buffer A) and washed with buffer A supplemented with 50 mM imidazole. Bound proteins were eluted with buffer A supplemented with 200 mM imidazole. Fractions containing active protein were pooled and concentrated by ultrafiltration, and thereafter desalted using PD10 columns equilibrated with 25 mM HEPES pH 7.6, 0.2 M NaCl, 20% glycerol, 10 mM β-mercaptoethanol and 0.1% *n*-octyl-β-D-glucoside (Buffer B). The protein solution was loaded onto a 2.5 mL polyU Sepharose 4B column equilibrated with buffer B and bound protein was eluted with buffer B containing 1 M NaCl. The purified enzyme was frozen and stored in aliquots at −80 °C.

Inhibition measurements

The activity of the protease was measured at 30 °C by a fluorescent assay, using Ac-DED(Edans)EEAbuψ[COO]ASK(Dabcyl)-NH_2 (AnaSpec, San José, USA) as substrate and the peptide KKGSVVIVGRIVLSGK (Åke Engström, Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden) as cofactor.[29] The enzyme was incubated in 50 mM HEPES pH 7.5, 10 mM DTT, 40% glycerol, 0.1% *n*-octyl-β-D-glucoside, 3.3% DMSO with 25 μM cofactor and inhibitor at 30 °C for 10 min, the reaction was initiated by addition of substrate. Any modifications of the standard assay buffer are indicated in the text. Kinetic constants were determined with substrate concentrations from 0.25 to 4 μM and enzyme concentrations from 0.5 to 1 nM, inhibition measurements were performed with 1 nM enzyme, 0.5 μM substrate, and a final concentration of 3.3% DMSO (see Results and Discussion). Inhibitors were dissolved in DMSO, a mixture of DMSO and assay buffer, or buffer alone, sonicated for 30 s and vortexed. The solutions were stored at −20 °C between measurements. Corrections for inner filter effects were made according to published procedures.[44] Non-linear regression analysis was used to determine kinetic parameters (GraFit, Erithacus Software, Staines, MX, UK). Measurements were performed in at least triplicates, and K_i values were determined using at least five different inhibitor concentrations.

Molecular modelling

Molecular modelling studies were performed with MacroModel (versions 7.0 and 7.1) using the new Maestro interface (versions 1–3).[45] All molecular calculations used the Amber* force field with the united atom charge set.[46,47] The effect of water as solvent was modelled by the GB/SA solvation method.[48] The crystal structure 1CU1 (chain A) was fitted to the force field by stepwise optimisation after addition of the essential hydrogens.[28] The Zn atom was disregarded in the calculations, since it is far from the inhibitor binding site. However, the amino acids involved in the Zn-binding site were restricted with an extra force of at least 200 kcal/mol/Å^2 in all calculations in order to keep the stabilizing effect. Residues 626–631 were removed from the active site by rearranging residues 615–631 into a helical structure as was found for the NS3-helicase.[49] A stepwise geometry optimisation of the resulting structure, considering only residues close to the C-terminal, was performed. Both possible tautomeric forms of His528 in the enzyme were taken into account, which gave two structures for the docking studies. Docking was performed in two steps: Potential inhibitors were put in the place of the original residues 626–631, taking care to use the same backbone conformation. First only the side chains of the inhibitor, the residues of the protein within 7.0 Å of the inhibitor and the active triad His57, Asp81 and Ser139 were optimised whereas the backbone and all residues within 12.0 Å were restricted by a force of 100 kcal/mol/Å^2. No other atom was allowed to move. In a second step, all residues within 7.0 Å were optimised whereas those within 12.0 Å were still restricted as above.

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