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Inhibition of Hepatitis C Virus NS3 Protease Activity by Product-Based Peptides is Dependent on Helicase Domain

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Abstract—Structure–activity relationships (SARs) of product-based inhibitors of hepatitis C virus NS3 protease were evaluated using an in vitro assay system comprising the native bifunctional full-length NS3 (protease-helicase/NTPase). The results were compared to previously reported data derived from the corresponding NS3 protease domain assay. Shortening the length of the protease inhibitors from hexapeptides to tripeptides revealed that the decrease in potency was much less when determined in the assay system with the full-length NS3 protein. Disagreements in SARs at different positions (P5–P2) were also discovered. Taken together, the results suggest that the impact of the helicase domain upon protease inhibitor binding is substantial.

Introduction

Hepatitis C virus (HCV) is the major etiological agent of post-transfusion hepatitis worldwide. An estimated 3% of the world's population is infected with HCV according to the World Health Organization. HCV infection most commonly results in chronic hepatitis that eventually develops into cirrhosis, hepatocellular carcinoma or liver failure. The current available therapy, using interferon-α and its combination with ribavirin, has limited efficacy and can have severe side effects.[1–5] Thus, there is an urgent need for new therapies.

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 autocatalytic *cis*-cleavage at the NS3/NS4A junction and for *trans* cleavages at the NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions.[3,5–7] Currently, the NS3 protease is being studied extensively and constitutes the major target in the search for effective antiviral pharmaceuticals against HCV.[3,5]

Crystal structures of the NS3 protease domain alone, the NS3 protease domain in complex with the essential cofactor NS4A, and of the NS3 helicase domain alone have been reported previously.[8–13] Thus, the active site of the protease domain has been identified as an extended, shallow, and solvent exposed surface, requiring many interaction points for binding of substrates and inhibitors.[8,10,11,14,15] Recently, Yao et al.[16] published the first crystal structure of the full-length multifunctional NS3, which revealed that the protease and the helicase were segregated as subdomains connected by a flexible strand. The two subdomain folds were found to be similar to the crystal structures of the isolated helicase and protease domain, respectively. However, of particular note, it became clear from the 3D-structure that the NS3 protease active site was oriented towards the interior of the protein, creating a pocket with the helicase domain within close proximity.[16] There is no evidence that proteolytic processing in vivo separates the two domains of NS3. Thus, both local and global conformational changes are required during the *cis* and *trans* cleavages necessary to accommodate a polyprotein substrate.[16] Previously, HCV protease inhibitors have been almost entirely evaluated in in vitro assay systems

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comprising the protease domain of NS3 alone.[15,17–22] NS3 protease is inhibited by its N-terminal cleavage products, as discovered by two groups independently.[17,23] An extensive structure–activity relationship (SAR) study using a combinatorial peptide library, assayed with truncated NS3, delivered hexapeptide inhibitors with affinities in the low nanomolar range.[19]

Although it has been demonstrated that product-based hexapeptide inhibitors derived from the NS4A/NS4B or NS5A/NS5B cleavage sites also inhibit full-length NS3,[24] the important issue of whether SARs differ in the two assay systems (full-length versus truncated NS3) to our knowledge, has not yet been addressed. Therefore, prior to commencing a more extensive program aimed at synthesis of less peptide-like inhibitors we felt prompted to briefly compare the two assay systems with respect to the SARs. Herein we report comparisons of K_i values for a selected series of product inhibitors, based on the NS4A/NS4B cleavage site, previously designed and evaluated in an isolated protease domain assay by Ingallinella et al.[19] with the results from our full-length NS3 assay.

Materials and Methods

Peptide synthesis

The peptide inhibitors **2**–**4**, **6**–**9**, **12**–**14** and **16** were synthesized using standard Fmoc/*t*-Bu solid-phase peptide synthesis methodology.[25,26] The peptide inhibitors **1**, **5**, **10**, **11** and **15** were purchased from Anaspec or Bachem. The *N*-Fmoc protected amino acids were obtained from Senn Chemicals, Alexis Corporation and Nova Biochem. H-Cys(Trt)-2-chlorotrityl resin, obtained from Senn Chemicals, was used as the starting resin. Peptide assembly was performed on a Protein Technologies Symphony/Multiplex™ synthesizer or on a Bodhan MiniBlock™ synthesizer, using *N*-[(1*H*-benzotriazole-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU)/*N*-methylmorpholine (NMM) or *N*-[(1*H*-benzotriazole-1-yl)-(dimethylamino)-methylene]-*N*-methylmethanaminiumtetrafluoroborate *N*-oxide (TBTU)/*N,N*-diisopropylethylamine (DIEA) activation in DMF and a coupling time of 60 min. Acetylation was carried out using acetic anhydride and DIEA. The peptides were cleaved in TFA/H_2O/triethylsilane (90:5:5) and purified by preparative RP-HPLC on a C18 column using an acetonitrile/H_2O gradient with 50 mM NH_4OAc (pH 6.3). All peptides were characterized with plasma-desorption MS and amino acid analysis. The peptide content ranged from 78 to 96%.

Enzyme

The full-length HCV-NS3 protein (amino acids 1027–1657 of the HCV polyprotein) was heterologously expressed with an N-terminal tag (38 amino acids including a stretch of six histidines) from a construct in the pBAD-HisA plasmid. The HCV isolate used here is related to the HCV H77 strain, which is a subtype of the hepatitis C virus type 1a. The sequence, cloning, expression and purification will be published elsewhere.[27]

Inhibition measurements

The activity of the protease was followed continuously over time on a fluorescence plate reader as described by Poliakov et al.[27] Briefly, the depsipeptide Ac-DED(Edans)EEAbuψ[COOASK](Dabcyl) (AnaSpec, San José, USA) was used as substrate and the central part of NS4A was used as activator, synthesized with two N-terminal lysines for solubilization (KKGSVVIVGRIVLSGK, peptide 2K-NS4A). Stock solutions of 2K-NS4A and inhibitors were prepared in DMSO. Measurements were performed at 30 °C in 50 mM Hepes pH 7.5, 10 mM DTT and 40% glycerol including a final concentration of 3.33% DMSO, 16.6 mM NaCl, 15 μM 2K-NS4A and 2 μM substrate (final reaction volume 300 μL). The enzyme (≤1 nM) was preincubated with 2K-NS4A for 10 min on the plate, followed by addition of the inhibitor and a second incubation for 15 min. Thereafter the reaction was initiated by addition of the substrate. For each inhibitor, enzyme activity was determined using at least five different inhibitor concentrations (in triplicate or quadruplicate). IC_{50} values were determined by using the GraFit program version 4.0 (Erithacus Software, Staines, UK) and the four parameter logistic equation therein. K_i values were either calculated using the relation $K_i = IC_{50}/(1 + S/K_m)$, where K_m is 1.25 μM (Poliakov et al.),[27] or fitting to the equation $v = V_{max}S/(K_m(1 + I/K_i) + S)$.

Results and Discussion

Inhibition of full-length NS3 by 16 product-based HCV NS3 protease inhibitors (**1**–**16**) was determined and compared to literature data derived from an assay using the isolated NS3 protease domain.[19] Results are shown in Table 1.

Two series (section A in Table 1) were used to investigate the possibility of shortening the inhibitors. Remarkably, with both series the decrease in potency observed with shortened inhibitors was much less pronounced using the full-length NS3 assay. In the first series (compounds **1**–**4**) deletion of the P6 to P4 residues reduced the potency by a factor of 100 as compared to 9000 observed with the truncated NS3 assay. In the second series (compounds **5**–**7**) P6/P5 truncation resulted in a 10-fold decrease in potency observed in the full-length NS3 assay, whereas a 1700-fold decrease is seen with the truncated enzyme. Notably, the tripeptides retained a reasonable inhibitory effect and might therefore be exploited as starting points in a drug discovery process. These results imply the existence of important interactions between the protease inhibitors and the helicase domain, or an inhibitor-dependent conformational reorientation of the protease-helicase/NTPase, that is an induced fit, similar to that postulated for the protease domain alone.[28,29] The exact mechanism can only be ascertained by crystallographic or NMR studies of a short inhibitor inside the active site of a full-length NS3 protein.

The effects of amino acid exchange in position P5–P2 of the hexapeptide inhibitors are shown in section B-E in

Table 1. Inhibition of NS3 protease activity by product-based inhibitors evaluated in assay systems comprising the full-length NS3 protein or the isolated NS3 protease domain

Section	Compound	Sequence[a]	K_i (μM)[b]±SD NS3 Full-length	K_i (μM)[c] NS3 Protease domain
A: Truncation				
	1	AcAsp-Glu-Dif-Glu-Cha-Cys	0.041±0.006	0.025
	2	AcGlu-Dif-Glu-Cha-Cys	0.33±0.06	0.7
	3	AcDif-Glu-Cha-Cys	1.1±0.2	15
	4	AcGlu-Cha-Cys	4.1±1.1	230[d]
	5	AcAsp-Glu-Dif-Ile-Cha-Cys	0.22±0.04	0.03
	6	AcGlu-Dif-Ile-Cha-Cys	1.0±0.2	1.2
	7	AcDif-Ile-Cha-Cys	2.1±0.7	50
	8	AcIle-Cha-Cys	25±5	
B: P5				
	9	AcAsp-D-*Glu*-Leu-Glu-Cha-Cys	0.18±0.03	0.023
	10	AcAsp-*Glu*-Leu-Glu-Cha-Cys	0.49±0.10	0.06
	11	AcAsp-D-*Gla*-Leu-*Ile*-Cha-Cys	0.012±0.002	0.00075
C: P4				
	1	AcAsp-Glu-*Dif*-Glu-Cha-Cys	0.041±0.006	0.025
	12	AcAsp-Glu-*Met*-Glu-Cha-Cys	1.04±0.24	0.18
	10	AcAsp-Glu-*Leu*-Glu-Cha-Cys	0.49±0.10	0.06
D: P3				
	1	AcAsp-Glu-Dif-*Glu*-Cha-Cys	0.041±0.006	0.025
	5	AcAsp-Glu-Dif-*Ile*-Cha-Cys	0.22±0.04	0.03
	13	AcAsp-Glu-Dif-*Lys*-Cha-Cys	1.24±0.26	8% inhib at 6 μM
E: P2				
	12	AcAsp-Glu-Met-Glu-*Cha*-Cys	1.04±0.24	0.18
	14	AcAsp-Glu-Met-Glu-*Nal*-Cys	0.44±0.09	0.4[e]
	15	AcAsp-Glu-Met-Glu-*Glu*-Cys	0.36±0.09	0.5
F: Acetylation				
	16	Asp-D-Glu-Leu-Glu-Cha-Cys	1.54±0.28	
	9	*Ac*Asp-D-Glu-Leu-Glu-Cha-Cys	0.18±0.03	0.023

[a]Dif, 3,3-diphenylalanine; Nal, 2-napthylalanine; Cha, β-cyclohexylalanine; D-Gla = D-γ-carboxyglutamic acid.
[b]The K_i values are converted from IC_{50} values using $K_i \approx 0.4IC_{50}$ as described in Materials and Methods.
[c]Ingallinella et al.[19] The K_i values are converted from IC_{50} values using their reported recalculation factor: $K_i \approx 0.5IC_{50}$.
[d]Cicero et al.[14]
[e]Pessi et al.[30]

Table 1. At the P5 position it was confirmed that D-Glu is preferred over L-Glu and that an additional acidic functionality enhances the activity, as was previously shown in the truncated NS3 test.[19,23] However, it should be noted that the overall potency was somewhat lower in the full-length NS3 assay. Dif was best accepted at the P4 position, but the full-length NS3 was less tolerant towards Met and Leu. The same behavior was observed at the P3 position, where Glu was preferred and the full-length NS3 showed less tolerance towards Ile. However, at the P2 position Glu and Nal were preferred over Cha in the full-length NS3 assay, whereas the opposite was true in the assay comprising the NS3 protease domain alone. It is noteworthy that the inhibitory potency of the natural NS4A/NS4B cleavage product Ac-Asp-Glu-Met-Glu-Glu-Cys **15** is nearly the same in both assay systems.

Acetylation of the N-terminal of the inhibitor **16** improved potency as shown in section F, Table 1. This agrees with previous data derived from the NS3 protease domain reported by Llinàs-Brunet et al.[23]

Results herein show that the SARs of product-based HCV NS3 protease inhibitors depend considerably on the test systems used. As compared to the assay used by Ingallinella et al.,[19] the presently used assay differs in more respects than the length of the enzyme. Nineteen amino acids differ in the protease domain of the two enzyme strains, but none of these residues is within 5 Å of the catalytic serine residue (PDB ID: 1CU1).[16] Also, the N-terminal tag used here is predicted to be flexible and solvent exposed (based on the protease domain structure by Kim et al.[8]). Finally, the differences in the sequence of the cofactor are not expected to have a direct effect on the inhibition. We therefore believe that the observed structure–activity differences for this series of product-based inhibitors can primarily be attributed to the influence of the helicase domain.

Conclusions

We have found differences in the SARs of product-based HCV NS3 protease inhibitors when evaluated in an in vitro assay system comprising the native bifunctional full-length NS3 (protease-helicase/NTPase) protein compared to that of the NS3 protease domain alone. Most importantly it was found that shortened product inhibitors retain a much better inhibitory potency in the full-length NS3 assay. Thus, tri- or tetrapeptides might be used as starting points in the search for smaller non-peptidic HCV NS3 protease inhibitors. Additionally, investigations at the P5–P2 position revealed disparities. Overall, the present results show that the influence from the helicase domain is substantial.

This emphasizes the importance of using the native full-length NS3 protein for evaluation of potential inhibitors and a more thorough SAR study is now required to determine the structural requirements for efficient inhibition of the NS3 protease activity.

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