Inhibition of the NS2B-NS3 Protease – Towards a Causative Therapy for Dengue Virus Diseases

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Abstract

The high impact of diseases caused by dengue viruses on global health is now reflected in an increased interest in the identification of drug targets and the rationale-based development of antiviral inhibitors which are suitable for a causative treatment of severe forms of dengue virus infections – dengue haemorrhagic fever and dengue shock syndrome. A promising target for the design of specific inhibitors is the dengue virus NS3 serine protease which – in the complex with the small activator protein NS2B – catalyses processing of the viral polyprotein at a number of sites in the nonstructural region. The NS3 protease is an indispensable component of the viral replication machinery and inhibition of this protein offers the prospect of eventually preventing dengue viruses from replication and maturation. After nearly a decade of mainly genetic analysis of flaviviral replication, recent studies have contributed substantial biochemical information on polyprotein processing including the 3-dimensional structure of the dengue virus NS3 protease domain, the mechanism of co-factor-dependent activation and sensitive in vitro assays which are needed for studies on substrate specificity and the development of high-throughput assays for inhibitor screening. This review discusses recent biochemical findings which are relevant to the design of potential inhibitors directed against the dengue virus NS3 protease.

Keywords: Dengue virus, NS2B/NS3, polyprotein, protease, inhibitor, treatment.

Viral polyprotein processing

Dengue viruses, members of the Flaviviridae family, possess single-stranded, positive sense RNA genomes and generate mature viral proteins by co- and post-translational proteolytic processing of a polyprotein precursor catalysed by host cell and virus-encoded proteases [for review see refs. 1, 2 and references herein]. The genomic RNA of dengue virus serotype 2 contains 10,723 nucleotides and encodes a single polyprotein precursor of 3,391 amino acid residues[3]. Individual viral proteins are arranged in the order C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. Proteolytic cleavages in the N-terminal region of the viral polyprotein are mediated by a host signal peptidase and yields three structural proteins C, prM and E, which constitute the virion[4]. Before the virion exits the cell, prM is cleaved by a cellular furin-type protease.
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prohormone convertase in the post-Golgi acidic compartment to yield the M protein[5]. Cleavages at the NS1/NS2A and NS4A/NS4B junctions are catalysed by a signalase bound to the membranes of the ER[6,7]. Proteolytic cleavages in the nonstructural region of the polyprotein are mediated by a heterodimeric complex of NS3 with the activator protein NS2B which catalyses in cis (intramolecular) cleavages at the NS2A/NS2B and NS2B/NS3 sites and in trans (intermolecular) cleavages at the NS3/NS4A and NS4B/NS5 polyprotein junctions[8-10]. Additional cleavages mediated by the NS3 protease within the C, NS2A, NS4A and within a conserved C-terminal portion of NS3 itself have been described in the literature[11-13].

Figure 1. The 3-dimensional structure of the dengue virus NS3 protease (shown here is a superimposition of a ribbon-presentation of the Ca trace on a space-filling surface model. Residues of the catalytic triad (His51, Asp75, Ser135) are shown as sticks. The figure was generated by Deepview Swiss-Pdb Viewer) Cleavage sites recognized by the NS2B/NS3 protease consist of ‘dibasic’ residues Lys-Arg, Arg-Arg and Arg-Lys at the (nonprime) P1 and P2 positions of the cleavage site sequence followed by short chain residues such as Gly, Ala and Ser at the (prime) P1’ position. The “non-canonical” NS2B/NS3 site contains a Gln residue at the P2 position (Figure 1).

The NS3 protease domain

The existence of a trypsin-like serine protease domain in the N-terminal region of the flaviviral NS3 proteins was originally predicted by sequence comparisons between cellular and virus-encoded proteases[14]. The NS2B-NS3 endopeptidases of the Flavivirus genus which at present comprises at least 68 known members, are now commonly designated as flavivirin (EC 3.4.21.91)[15,16]. The dengue virus 69 kDa NS3 protein is a multifunctional protein with a serine protease domain located within the N-terminal 167 amino acid residues[17] and activities of a nucleoside triphosphatase (NTPase) and RNA helicase in the C-terminal moiety[18]. A catalytic triad consisting of residues His51, Asp75 and Ser135 was identified by site-directed mutagenesis experiments and replacement of the catalytic serine by alanine resulted in an enzymatically inactive NS3 protein[19]. The NS3 protease is an essential component for maturation of the virus and viable virus was never recovered from infectious cDNA clones carrying mutations in the NS3 sequence which abolished protease activity[20]. Interaction of the helicase portion of NS3 with the viral RNA-dependent RNA polymerase NS5 may promote the association of the viral replicase complex to the membranes of the ER[21].
The 3-dimensional structure of the N-terminal 185 residues of the dengue virus NS3 protease domain (NS3pro) was resolved at a resolution of 2.1 Å[22]. The overall folding of the protein resembles the 6-stranded β-barrel conformation typical for chymotrypsin-like serine proteases. Interestingly, the structure of the dengue virus NS3 protease is closer to that of the hepatitis C virus NS3/NS4A co-complex than to the unliganded HCV NS3 protease, an observation which is suggestive of major structural differences in the co-factor-dependent activation mechanism of the two proteases[23]. The substrate binding site of NS3pro is relatively shallow and contourless and specific enzyme-substrate interactions were not predicted to extend beyond the P2 and P2' positions of the substrate peptide in the absence of the NS2B co-factor[22] (Figure 2).

**The NS2B co-factor**

The presence of a small activating protein or co-factor is a prerequisite for optimal activity of the flaviviral NS3 proteases with their natural polyprotein substrates. Although the dengue virus NS3 protease exhibits NS2B-independent activity with model substrates for serine proteases, enzymatic cleavage of dibasic peptides is markedly enhanced with the NS2B-NS3 co-complex and the presence of the NS2B activation sequence is indispensable for the cleavage of polyprotein substrates in vitro[24]. The initial characterization of the co-factor requirement for the dengue virus NS3 protease had revealed that the minimal region necessary for protease activation was located in a 40-residue hydrophilic segment of NS2B[23]. The hydrophobic flanking regions of the 14 kDa NS2B protein are likely to be involved in targeting the protease complex to the membranes of the ER where genome replication occurs. Fusion of the NS2B core sequence to the NS3 protease domain yielded a catalytically active NS2B(H)-NS3p protein, which, upon expression in E. coli and subsequent refolding, displayed autoproteolytic processing at the NS2B/NS3 site conducive to the formation of a non-covalent adduct[24]. Incorporation of a flexible nonamer linker, Gly₄-Ser-Gly₄, between the NS2B core...
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segment and the protease domain resulted in a cleavage-resistant protease with optimized enzymatic activity against hexapeptide substrates representing native polyprotein cleavage junctions. A recombinant construct representing the full-length NS2B co-factor linked to the NS3 protease domain was enzymatically active with peptide substrates derived from the polyprotein; however, this protein was completely resistant to proteolytic self-cleavage.

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An essential requirement for the correct association of the co-factor with the protease is the presence of hydrophobic residues which act as ‘anchor’ for the protease – co-factor interaction. Recently, based on mutagenesis experiments and sequence comparisons of known flaviviral co-factors, the “Φx3Φ” motif was proposed as the common structural element involved in co-factor binding to the protease. The “Φx3Φ” motif is comprised of two bulky hydrophobic residues separated by three unspecified residues and it was hypothesized that additional residues located at the N-terminus of the activation sequence would contribute to the stringent specificity of the protease for the polyprotein substrate. A mutagenesis study with the dengue virus NS2B co-factor had revealed that substitutions of the “Φ” residues (Leu75 and Ile79 in NS2B of dengue virus type 2) by alanine resulted in preponderant effects on the catalytic activity of the NS3 protease rather than on substrate binding. A single residue in the N-terminal region of NS2B, Trp62, was critical for protease activation and replacement of this residue yielded a NS3 protease which was catalytically inactive in autoprotoleolysis and reaction with the synthetic substrate peptide GRR-AMC.

For the HCV NS3-NS4A protease complex, large structural rearrangements leading to a catalytic triad, which is conformationally optimized for proton shuttle during catalysis, were observed as a result of co-factor binding. No 3-dimensional structure is available for the dengue virus NS2B-NS3 co-complex and the precise mechanism of co-factor-dependent activation is not fully elucidated as yet. In particular, it is an open question as to whether binding of the substrate contributes to the formation of an ‘induced fit’ conformation as observed with the HCV NS3 protease.

Substrate specificity

So far, only very limited efforts have been undertaken to analyse the precise substrate requirements and determinants of cleavage efficiency for the dengue virus NS3 protease. This is surprising in the light of the fact that development of inhibitors against serine proteases usually starts with optimal peptide substrates derived from the nonprime side wherein the scissile amide bond is replaced by an electrophile which reacts with the catalytic serine residue.

The NS3 protease reacts with small model substrates for serine proteases such as N-α-benzoyl-L-arginine-p-nitroanilide (BAPA) and activity of the unliganded NS3 protease towards this substrate is higher than that of the NS2B-NS3 co-complex, a finding which suggests that substrate recognition in the complex requires additional interactions extending beyond the P1 side for optimal activity. A number of fluorogenic tripeptides containing dibasic residues at the P1 and P2
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positions are cleaved by NS2B(H)-NS3pro protease and the best substrate identified in these experiments was GRR-AMC, which had a $K_m$ value of 180 $\mu$M, a $k_{cat}$ value of 0.031 s$^{-1}$ and a catalytic efficiency expressed as $k_{cat} / K_m$ of 172 s$^{-1}$ M$^{-1}$ [24].

Chromogenic $p$-nitroanilide peptides representing hexameric sequences of the native polyprotein cleavage junctions were tested in a photometric assay and the most efficiently cleaved substrate derived from the NS4B/NS5 site (Ac-TTSTRR-$p$NA) had a $K_m$ of 346 $\mu$M, $k_{cat}$ of 0.095 s$^{-1}$ and a $k_{cat} / K_m$ of 275 s$^{-1}$ M$^{-1}$ [26].

Recently, we have shown by a HPLC-based assay with fluorometric detection that the NS2B-NS3pro protease incorporating a full-length NS2B co-factor could cleave N-terminally dansylated 12mer peptides mimicking native polyprotein junctions in the absence of microsomal membranes[28]. However, this protein was completely inactive in autocleavage and the efficiency of this recombinant protease with the peptide substrate was markedly reduced when compared to constructs incorporating the 40-residue NS2B core sequence, likely due to structural distortions induced by the flanking regions of NS2B and inefficient activation of the protease.

Currently, a detailed study on substrate specificity of the dengue virus NS3 protease is in progress, which uses combinatorial libraries of internally quenched fluorogenic peptides labelled with the aminobenzoyl / $p$-nitrotyrosine reporter pair. For a substrate peptide based on the NS3/NS4A cleavage site, Abz-AAGRK?SLTLY(NO$_2$)R-NH$_2$ (? denotes the cleavage site), a $K_m$ value of 141 $\mu$M, a $k_{cat}$ of 0.18 s$^{-1}$ and a $k_{cat} / K_m$ of 1262 s$^{-1}$ M$^{-1}$ was found, which is approximately 10-fold better than the best commercially available substrate tested so far, GRR-AMC [J. Wikberg, unpublished data]. In the near future, these investigations will likely lead to the identification of NS3 substrates with optimized sequence length and improved binding affinities, which can be applied as sensitive probes for enzyme activity in high-throughput inhibitor screenings.

**Perspectives for inhibitor development**

Principally, every step of viral morphogenesis, from cell-entry, uncoating, replication and assembly of new virus particles, is a potential target for antiviral inhibitors. However, the molecular events in the infectious cycle of flaviviruses such as dengue virus are characterized only to a very limited extent, making the design of specific inhibitors an adventurous task. In contrast, proteases and their inhibitors have been intensively studied because of their potential for the development of selective antiviral compounds. Although tremendous progress in the field is indicated by the design and clinical use of antiviral drugs against HIV (AIDS) and hepatitis C virus proteases, the potential of dengue and related flaviviral proteases for inhibitor discovery is largely unexploited. In response to the global problem of the dengue virus epidemics, considerable efforts are now being devoted to the development of drugs which will eventually be suitable for a chemotherapeutic intervention in acute dengue diseases, not only by academic institutions but also by pharmaceutical companies (for example see http://www.nitd.novartis.com).
In a first step towards a rational inhibitor design for the dengue virus NS3 serine protease, inhibition by synthetic peptides mimicking uncleavable transition state isosteres of the P6-P2' residues of the native polyprotein sites, was demonstrated. The peptides with an α-keto amide in place of the scissile amide bond acted as competitive inhibitors of the NS3 protease with $K_i$-values in the micromolar range. Replacement of the P1'-P2' residues by a carboxyl-terminal aldehyde in the NS3/NS4A-derived peptide (Ac-FAAGRRCHO) yielded a competitive inhibitor with a $K_i$ of 16 $\mu$M. For the dengue virus protease, the hexapeptides displayed $K_i$ -values which were only 2 to 6 fold lower than the $K_m$ -value for the corresponding substrate, a feature which discriminates dengue virus NS3 from the HCV protease, where product inhibitors had binding affinities which were one order of magnitude lower than those for the substrates.

Product inhibition of the HCV NS3 protease by cleavage-site derived peptides led to the discovery of very potent inhibitors of this enzyme with IC$_{50}$ -values in the nanomolar range by cyclic optimization of the inhibitor structures. Recently, we have shown that peptides representing non-prime-side residues of the dengue virus NS3 protease act as competitive inhibitors of the enzyme, whereas prime-side peptides appeared to have negligible effects on enzyme inhibition at concentrations >1.0 mM. (S. Chanprapaph, unpublished data). $K_i$ -values for hexapeptides derived from all 4 dengue virus cleavage sites were in the low micromolar range and the best inhibitor was based on the NS2A/NS2B site (Ac-RTSKKR-CO$NH_2$) and gave a $K_i$ of 12 $\mu$M. In analogy to the HCV NS3 protease, these findings suggest the existence of a high-affinity binding site in the non-prime region of the enzyme and offer the prospect of developing effective inhibitors against the dengue virus protease by combinatorial optimization based on the structure(s) of native polyprotein cleavage site peptides. However, in general, peptide-based inhibitors exhibit poor pharmacokinetic properties and usually the conversion of these structures into less “peptide-like” compounds (“peptidomimetics”) is required to generate drug-like entities.

Inhibitor discovery for the dengue virus NS3 protease is currently limited by the lack of a 3-dimensional structure for the NS2B-NS3 co-complex; however, it can be expected that crystallographic studies and NMR-experiments will provide more insight into the structure and catalytic mechanism of the enzyme in the near future. In addition, powerful computer modelling approaches exist which may help to obtain information required for a rational drug design even in the absence of crystallographic structures.

Proteochemometric modelling (PCM) is currently explored as a tool to analyse the structural and physicochemical properties which are necessary for the interaction of potential inhibitors with the dengue virus NS3 protease target structure. Preliminary data obtained by this approach suggest that the proteochemometric models are valid and useful for the accelerated design of novel inhibitors. This approach does not only circumvent the traditional erratic drug discovery process with its high attrition rates, but also allows to incorporate potential resistance of the target and the development of ‘drug resistance – resistant’ compounds as an initial consideration in the design process. The existence of large conformational
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ensembles is particularly challenging in the case of rapidly mutating viral enzymes, where a design against a moveable target would require large sets of corresponding inhibitors. In the future, these problems are likely to be addressed by ‘shotgun approaches’ to the structure of enzyme-inhibitor complexes and the identification of hot spots of ‘interaction flexibility’ by the use of fast, high-resolution methods such as NMR. Detailed accounts on this strategy are given in Reference 36.

Conclusions

Substantial progress has been made over the past few years in the biochemical characterization of the dengue virus NS2B-NS3 two-component protease. The data, which are available now, make the dengue virus NS3 protease a valid molecular target for the development of antiviral compounds. A large repertoire of powerful methods for inhibitor development and evaluation exists which includes state-of-the-art technology in organic synthesis and computer-aided molecular design. Although there is no suitable animal model available for dengue virus diseases, initial screening of potential antiviral compounds would be facilitated by well-established insect and mammalian cell culture systems which are useful to monitor the effects of anti-NS3 inhibitors on the propagation of the virus.

Moreover, alternative drug targets which are present on the dengue virus NS3 protein can be exploited for inhibitor development. These include the binding site of the NS2B co-factor to the NS3 protease, the NS3 NTPase / helicase portion and the interaction surface of NS3 with the NS5 replicase. The presence of multiple biomedical targets in the dengue virus polyprotein would even make a therapy feasible, which uses combinations of different inhibitors and therefore could minimize the risk of rapid resistance development.

It can also be expected that progress for inhibitors against the dengue virus protease will be of large benefit for drug design against related human pathogens of the flavivirus complex such as yellow fever virus, Japanese encephalitis virus and West Nile virus.

However, in order to bring an effective anti-dengue drug from the ‘bench to the bedside’, several questions and limitations need to be addressed. These include the evaluation of prognostic markers for disease severity, the pathobiology of dengue haemorrhagic fever and shock syndrome, the problem of selectivity against pharmacologically relevant human proteases such as furin and the risk of adverse effects. Potential complications may also arise from the presence of four related dengue serotypes in the case that their NS3 proteases show marked differences in their inhibition profiles. Intensive efforts and sustained multidisciplinary research is required in the future to cope with the challenging task of a causative treatment for dengue virus diseases.

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