Cleavage targets and the D-arginine-based inhibitors of the West Nile virus NS3 processing proteinase

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Mosquito-borne WNV (West Nile virus) is an emerging global threat. The NS3 proteinase, which is essential for the proteolytic processing of the viral polyprotein precursor, is a promising drug target. We have isolated and biochemically characterized the recombinant, highly active NS3 proteinase. We have determined that the NS3 proteinase functions in a manner that is distantly similar to furin in cleaving the peptide and protein substrates. We determined that aprotinin and D-arginine-based 9–12-mer peptides are potent inhibitors of WNV NS3 with K_i values of 26 nM and 1 nM respectively. Consistent with the essential role of NS3 activity in the life cycle of WNV and with the sensitivity of NS3 activity to the D-arginine-based peptides, we showed that nona-D-Arg-NH₂ reduced WNV infection in primary neurons.

We have also shown that myelin basic protein, a deficiency of which is linked to neurological abnormalities of the brain, is sensitive to NS3 proteolysis *in vitro* and therefore this protein represents a convenient test substrate for the studies of NS3. A three-dimensional model of WNV NS3 that we created may provide a structural guidance and a rationale for the subsequent design of fine-tuned inhibitors. Overall, our findings represent a foundation for in-depth mechanistic and structural studies as well as for the design of novel and efficient inhibitors of WNV NS3.

Key words: Dengue virus, flavivirus, furin, peptide inhibitor, proteinase, West Nile virus.

INTRODUCTION

WNV (West Nile virus) is a member of the Flaviviridae family and it was first isolated in 1937 in the West Nile district of Uganda [1]. WNV is transmitted to animals including humans, through mosquito bites. Since 1999, when the virus was identified in the U.S.A., the virus has spread rapidly and has infected in the order of 15 000 people and caused more than 600 deaths. There is currently no effective vaccine or antiviral drug to protect against WNV infection [2].

WNV has a single strand, positive polarity, RNA genome. The 11 kb RNA genome codes for a single polyprotein precursor. The precursor comprises three structural proteins [C (capsid), M (membrane) and E (envelope)] and seven NS (non-structural) proteins arranged in the order C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. Post-translational proteolytic processing of the WNV polyprotein precursor is required to produce the functional viral proteins which assemble a new viral progeny [3]. This processing is performed by host furin-like proprotein convertases and a viral serine proteinase encoded by the N-terminal 184-residue portion of NS3 [4,5]. Host proteinases are responsible for cleavage at the C/prM, prM/E, E/NS1, NS1/NS2A and NS4A/NS4B junctions as well as for cleavage within prM at a late step during virion morphogenesis. Work by several groups suggests that the flaviviral NS2B–NS3 proteinase participates in cleavage at the NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 junctions, as well as at the internal sites within C, NS3 and NS4A [6–13]. The NS3-sensitive cleavage sites in the polyprotein precursor predominantly exhibit arginine at the P1 position, arginine and

lysine at the P2 position, and glycine and serine at the P1' position. These features suggest that NS3 is similar, although less restricted, in its cleavage preference when compared with furin, which cleaves predominantly after an RXR/KR↓ motif. Because mutation of the NS3 cleavage sites in the flaviviral polyprotein precursor abolishes virus infectivity [6,9,13–17], the catalytically active NS3 is essential for the life cycle of WNV and similar flaviviruses. The functional significance of the NS3-mediated processing in the life cycle of WNV makes this proteinase an attractive drug target for antiviral therapeutics [18].

The sequence of the WNV NS3 protease domain with a functional catalytic triad (His⁵¹, Asp⁷⁵, Ser¹³⁵) is approx. 50 % identical with the NS3 of Dengue virus. The spatial structure of the Dengue and hepatitis C NS3 is known, which provides an opportunity for *in silico* modelling of the WNV protease structure [19]. By analogy with the Dengue NS2B–NS3 tandem, the viral protein NS2B is believed to be an activator of the NS3 WNV enzyme. The 40-residue hydrophilic central domain of NS2B is sufficient to activate the catalytic activity of the NS3 protease [8,12,20,21]. Despite the fact that NS3 autocatalytically cleaves at the NS2B/NS3 junction, it remains controversial as to whether NS2B is absolutely required for NS3 activity [18,22]. Although the viral targets of NS3 protease have been studied in substantial detail, it remains unknown whether NS3 also cleaves host proteins, and whether these cleavages contribute independently to cellular injury and pathogenesis.

In the present paper, we describe the enzymatic characteristics of a catalytically potent WNV NS3 proteinase construct and the potential function of the NS2B sequence. Using this information,

Abbreviations used: AMC, 7-amino-4-methylcoumarin; Boc, t-butoxycarbonyl; IPTG, isopropyl β-D-thiogalactoside; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; MBP, myelin basic protein; NS2B and NS3, non-structural viral proteins 2b and 3 respectively; PA63, 63 kDa protective antigen; PA83, 83 kDa protective antigen; Pyr, pyroglutamic acid; R.M.S.D., root mean square deviation; SCG, superior cervical ganglia; WNV, West Nile virus; Z, benzyloxycarbonyl.

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we identified low-nanomolar range inhibitors of the WNV NS3 cleavage activity. Collectively, our experiments provided a foundation for the in-depth studies of WNV NS3 and, potentially, for the development of a broad-range inhibitor. We believe that, following a structural optimization, these inhibitors will also be effective against several other clinically relevant members of the flavivirus family.

MATERIALS AND METHODS

Reagents

Aprotinin, *N*-*p*-tosyl-L-Lys-CHO, the inhibitors of cathepsin B [L-3-*trans*-(propylcarbamoyl)oxirane-2-carbonyl]-L-Ile-L-Pro and [L-3-*trans*-(propylcarbamoyl)oxirane-2-carbonyl]-L-Ile-L-Pro methyl ester, and the Boc-QAR-AMC (Boc is *t*-butoxycarbonyl and AMC is 7-amino-4-methylcoumarin) and Boc-*O*-benzyl-EGR-pNA (pNA is *p*-nitroanilide) peptide substrates were purchased from Sigma. Serpins (α 1-antitrypsin and α 1-antitrypsin variant Portland), the Z-RR-AMC (Z is benzyl-oxycarbonyl) peptide substrate, and the Boc-D-*R*-tosyl-OH and dec-RVKR-cmk (dec is decanoyl and cmk is chloromethylketone) inhibitors were obtained from Calbiochem. The Boc-RVRR-AMC, Pyr-RTKR-AMC (Pyr is pyroglutamic acid) and Boc-AGPR-AMC peptide substrates were acquired from American Peptide. MBP (myelin basic protein) was obtained from Bio-design. Anthrax PA83 (83 kDa protective antigen) precursor and the mature trypsin-nicked PA63 (63 kDa protective antigen) were purchased from List Labs. Furin was purified as described previously [23].

Plasmid construction and cloning

The cDNA fragment of the pathogenic New York strain of WNV was used as a template to generate the NS2B–NS3 construct. WNV cDNA encoding NS2B and NS3 was provided by Dr Richard Kinney (Centers for Disease Control and Prevention, Fort Collins, CO, U.S.A.). NS3 was generated by PCR using 5'-GGCGGGGGAGGTAGTGGTGGAGCGGGGGAGGCGGTG-TTGTGGGACACTCCC-3' and 5'-CAGCATCTCAGGTTTCA-ATCCGGC-3' as direct and reverse primers respectively. The 5'-CACCATGTCAACAGATATGTGGATTGAGAG-3' and 5'-CCCGCCTCCACCACTACCTCCCCGCCCTTCCAAGGTG-CTCCTGGATCATTCATGAGC-3' direct and reverse primers were used to produce a fragment encoding the 40-residue central domain of NS2B. This fragment was flanked with a flexible nona-peptide linker GGGSGGGG (underlined in the primer sequence). The resulting PCR products were amplified to obtain the final 723-bp product encoding for the NS2B–linker–NS3 construct. This construct was cloned into the pET101 Topo cloning vector (Invitrogen). The sequence of the final construct was verified by DNA sequencing. The recombinant plasmid was used to transform *Escherichia coli* BL21 (DE3) Codon Plus cells. Transformed *E. coli* was grown in 1 litre of LB (Luria–Bertani) medium containing 0.1 mg/ml ampicillin at 30°C. Expression of the NS2B–NS3 construct was induced by the addition of IPTG (isopropyl β -D-thiogalactoside) to a final concentration of 0.6 mM. *E. coli* cells were incubated for an additional 16 h at 18°C. The cells were then collected by centrifugation (5000 *g*, 15 min) and used as a source for the isolation of NS2B–NS3.

Enzyme expression and purification

For protein purification, cells were resuspended in 20 ml of lysis buffer (PBS containing 1 M NaCl, 10 mg/ml lysozyme and pro-

tease inhibitor cocktail). The cells were disrupted by sonication on an ultrasound disintegrator. The pellet was removed by centrifugation at 20000 *g* for 30 min. The recombinant NS2B–NS3 construct, C-terminally tagged with a hexahistidine tag, was purified from the soluble fraction by affinity chromatography on a Co²⁺-chelating Sepharose Fast Flow 15 cm column (Amersham Biosciences) equilibrated with PBS, 1 M NaCl and 1 mM PMSF. After washing the column extensively with PBS, 1 M NaCl and 1 mM PMSF, the bound proteins were eluted with 60 ml of a 10–500 mM imidazole gradient. Fractions (1.5 ml) were collected and analysed by SDS/15%-(w/v)-PAGE. The NS2B–NS3-containing fractions were pooled and dialysed against 10 mM Tris/HCl buffer, pH 8.0, containing 0.005 % Brij 35. The dialysed material was then incubated for 16 h at 24°C to allow the autolytic conversion of the NS2B–NS3 construct into the individual NS3 enzyme. The resulting NS3 samples were re-checked by SDS/PAGE to confirm that autolytic conversion had been completed.

Synthesis of peptide inhibitors

The D-arginine peptide inhibitors (hexa-, hepta-, octa-, nona-, deca-, undeca- and dodeca-D-Arg-NH₂) were synthesized by solid-phase peptide synthesis. All peptides were C-terminally amidated, with free N-termini. The actual molar concentration of the peptides was lower because of the presence of water and trifluoroacetate salt in the samples. Based on kinetics and active site titration studies, we estimate that the samples contained approx. 25–35 % of actual peptide. The concentrations reported in the Results section were calculated using these corrections.

Protease assays with fluorogenic peptides

The assay for NS3 protease activity was performed in 10 mM Tris/HCl buffer, pH 8.0, containing 20 % (v/v) glycerol and 0.005 % Brij 35. The substrates and enzyme concentrations, unless indicated otherwise, were 24 μ M and 10 nM respectively. The total assay volume was 0.1 ml. Initial reaction velocities were monitored continuously at λ_{ex} (excitation wavelength) of 360 nm and λ_{em} (emission wavelength) of 465 nm on a Spectramax Gemini EM fluorescence spectrophotometer (Molecular Devices). All assays were performed in triplicate in wells of a 96-well plate. The K_m and k_{cat} values were derived from a double-reciprocal plot of $1/V_0$ against $1/[S]$, using the Lineweaver–Burk transformation: $1/V_0 = K_m/V_{max} \times 1/[S] + 1/V_{max}$, where V_0 is the initial velocity of substrate hydrolysis, $[S]$ is the substrate concentration, V_{max} is the maximum rate of hydrolysis, and K_m is the Michaelis–Menten constant. The concentration of the catalytically active proteinase was measured using the fluorescent assay by titration against a standard aprotinin solution of known concentration. The concentration of active NS3 was close to 100 % when compared with the protein concentration.

For the determination of the K_i value of the inhibitors, NS3 was pre-incubated for 60 min at ambient temperature (18°C) with increasing concentrations of the inhibitors. Following addition of the Boc-RVRR-AMC substrate, the rate of substrate hydrolysis was monitored as described in the preceding paragraph. For the inhibitors with the apparent inhibition constants [K_i (app)] value below the enzyme concentrations, the K_i (app) was derived from non-linear regression fits of a against $[I]_0$, using the following equation:

$$a = 1 - \{([E]_0 + [I]_0 + K_i) - [(E]_0 + [I]_0 + K_i)^2 - 4[E]_0[I]_0\}^{1/2} / 2[E]_0,$$

where $a = V_i/V_0$, V_i is the initial velocity at the inhibitor concentration, V_0 is the initial velocity in the absence of the inhibitor, $[E]_0$ is the total enzyme concentration, and $[I]_0$ is the total inhibitor concentration. For the inhibitors with the K_i (app) value significantly above the $[E]_0$, the IC_{50} was derived by fitting the V_0 against $\log [I]_0$ plots with sigmoidal dose–response curves. For the competitive inhibitors, the inhibition constant (K_i) was derived using the Cheng–Prusoff equation:

$$K_i = IC_{50}/(1 + [S]_0/K_m).$$

Cleavage of protein targets

PA83 (2 μ g, 1.2 μ M) was co-incubated with NS3 (1.7 μ g; 2.5 μ M) for 4 h at 37°C and then analysed by SDS/15% PAGE. MBP (11 μ M) was co-incubated with NS3 (44–440 nM; an enzyme/substrate ratio of 1:25–250) for 60 min at 37°C. Where indicated, dodeca-D-Arg-NH₂ (2–200 μ M) and aprotinin (20–120 μ M) were each added to the reactions. The digested samples were analysed by SDS/PAGE using 4–20% polyacrylamide gels.

Cleavage of peptides and MS analyses

The peptides derived from the sequence of WNV prM (K⁸³TRHSRRSRRSL⁹⁴) which span the furin cleavage site (underlined) and from the anchored capsid C protein sequence (A⁹⁴INRD⁹⁸STKQKKRG¹⁰⁷) that includes the known NS3 cleavage site (underlined), were obtained from GenScript. In the C protein peptide, Asp⁹⁸ (italicized) was substituted for Arg⁹⁸ of the original sequence. Unless the R98D mutation is made, the cleavage of the peptide will occur at both the RR↓S⁹⁹ and the KR↓G¹⁰⁶ sites of the original sequence. The peptides (1 μ g; approx. 30 μ M) were incubated with NS3 (0.7 μ g, 1.25 μ M) for 2 h at 37°C in 20 μ l of 10 mM Tris/HCl buffer, pH 8.0, containing 20% (v/v) glycerol. The molecular mass of the intact peptides and the digest products was determined by MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight)–MS analysis.

Cell-based inhibitor assays and neuronal cultures

Primary sympathetic neuronal cultures were generated from SCG (superior cervical ganglia) of post-natal day 1 C57BL/6J mice [24]. Dissected SCG were treated sequentially with 1 mg/ml collagenase (Worthington) for 30 min at 37°C, and then with trypsin (Sigma) for 30 min at 37°C. Neurons were dissociated by gentle pipetting and were seeded in wells of an eight-well chamber (Nunc) coated with rat tail collagen (6000 cells/well). SCG neurons were cultured for 3 days in AM50 medium, which contained minimal essential medium supplemented with 10% heat-inactivated FBS (foetal bovine serum), 2 mM L-glutamine, 30 μ M fluorodeoxyuridine, 30 μ M uridine, 50 ng/ml NGF (nerve growth factor) (Harlan Bioproducts) and 3.3 ng/ml aphidicolin (Fisher Scientific). Neurons were subsequently maintained in aphidicolin-free medium. Experiments were performed using neurons cultured for 7 days before infection.

Primary SCG neurons were treated with increasing concentrations of nona-D-Arg-NH₂ in serum-free medium for 2 h before infection with the pathogenic New York strain of WNV at a multiplicity of infection of 1 for 1 h at 37°C. Cells were infected in the presence of nona-D-Arg-NH₂. Free virus was then removed by serial washing with PBS followed by a 1 h wash with serum-containing medium. After an additional 2 h of treatment with nona-D-Arg-NH₂ in serum-free medium, serum was added to the cells. Medium samples were taken 24 h after infection. The virus

titre in the samples was determined by plaque assays on BHK21 cells [25].

In silico modelling

A homology search for the WNV NS3 sequence from the MEROPS Peptidase Database at <http://merops.sanger.ac.uk> (accession number MER00288) was performed using PSI-BLAST on the NCBI BLAST server (<http://www.ncbi.nlm.nih.gov/BLAST/>). A multiple sequence alignment was performed using the NS3 sequence of Kunjin virus (accession number MER00287), Murray Valley encephalitis virus (accession number MER00291), Japanese encephalitis virus (accession number MER00293), Dengue type 1 virus (accession number MER04078), Dengue type 2 virus (accession number MER00282), Omsk haemorrhagic fever virus (accession number MER35167), tick-borne encephalitis virus (accession number MER02975), Louping ill virus (accession number MER02434), Alkhurma virus (accession number MER16976), Langat virus (accession number MER04116), Powassan virus (accession number MER04117), Deer tick virus (accession number MER25135), yellow fever virus (accession number MER00290) and Usutu virus (GenBank® identifier gi45378910). The alignment was performed using the T-COFFEE software (T-COFFEE, version 1.37; Swiss Institute of Bioinformatics; <http://www.ch.embnet.org/software/TCoffee.html>). The resulting alignment was plotted using the GeneDoc software (GeneDoc, version 1.0, Free Software Foundation; <http://www.psc.edu/biomed/genedoc/>). The results of the multiple sequence alignment were also used to generate a phylogenetic tree. The tree was created using Mega-2 software (Molecular Evolution Genetics Analysis, Version 2.1, Penn State University; <http://www.megasoftware.net/>). An initial distance estimation was performed using the Kimura correction. Tree topology was derived using the neighbour-joining method. The values obtained from each bootstrap analysis are shown for the branches.

Modelling of WNV NS3 was performed using the Modeller and Swiss PDB Viewer software, and the Dengue NS3 protease (PDB code 1DF9) as a template. The model was validated using PSQS (Protein Structure Quality Score; <http://www1.jcsq.org/psqs>) and ProCheck (version 3.5.4, EBI, Cambridge; <http://biotech.ebi.ac.uk:8400/>). The model total energy score is -0.0379 (local = -0.0524 , burial = -0.0013 , contact = 0.0158), which is consistent with the feasible molecular models. The R.M.S.D. (root mean square deviation) value of the model is 0.21 Å (1 Å = 0.1 nm). This value is an indication of a highly reliable model. The structural images of WNV NS3 and furin (PDB code 1P8J) were prepared by using PyMOL (version 0.95, DeLano Scientific; <http://pymol.sourceforge.net/>). Surface residues of WNV NS3 were determined using a surface-mapping analysis [26].

RESULTS

Purification and autolytic conversion of NS2B–NS3 into NS3

Previous studies showed that the presence of either the full-length NS2B sequence or the 40-residue central NS2B domain linked to the N-terminus of NS3 significantly enhanced the accumulation of the soluble recombinant NS3 in *E. coli* [18,22]. We used a similar approach to express the WNV NS3. The 40-residue central portion of NS2B (short NS2B) was linked with NS3 via a GGGGSGGGG linker. To facilitate its isolation, the NS2B–NS3 construct was C-terminally tagged with a His₆-tag. To characterize the catalytic activity of NS3, we expressed the short NS2B–NS3 construct in *E. coli*. After induction with IPTG, large amounts

Figure 1 Constructs and purification of the NS3 proteinase of WNV

(A) The 40-amino-acid-long central portion of NS2B (short NS2B) was linked with NS3 via a GGGGSGGGG linker. A construct was C-terminally tagged with a His₆-tag. After its isolation, the short NS2B-NS3 construct autolytically cleaved the K¹⁵↓G¹⁶ scissile bond (numbering starts from the N-terminus of NS3) and generated the individual NS3 proteinase. The constructs had a C-terminally tagged His₆-tag. The presence of the His₆-tag facilitated the purification of the constructs by metal-chelating chromatography. (B) The His₆-tagged NS2B-NS3 construct was cloned in the pET101 plasmid and expressed in *E. coli*. The soluble construct was purified from *E. coli* by Co²⁺ metal-chelating chromatography. The construct was eluted with a 50–500 mM imidazole gradient, pH 7.5. The pooled NS2B-NS3 fractions, which contained a 10 mM residual imidazole concentration, were re-chromatographed on a Co²⁺-column to generate the homogenous samples. (C) Autolytic conversion of the NS2B-NS3 precursor into NS3. The purified material (samples 1, 2 and 3) was incubated overnight (0.02–0.2 mg/ml; pH 8) at 24 °C to generate the autolytically processed NS3 proteinase. (D) The representative purified sample of NS3. An antibody against the His₆-tag was used in Western blotting. After the purification and autolysis steps, the yield of the purified NS3 enzyme was approx. 5 mg of protein/litre of *E. coli* culture. (E) Aprotinin and nona-D-Arg-NH₂ inhibit autolysis of the NS2B-NS3 construct. The purified NS2B-NS3 sample (2.5 μM) was incubated alone or with aprotinin (25 μM; a 10-fold molar excess) and nona-D-Arg-NH₂ (40 μM; a 16-fold molar excess) each for 16 h at 24 °C. Molecular-mass sizes are given in kDa.

of NS2B-linker-NS3 were produced as a soluble protein. The soluble fraction was loaded on to a pre-equilibrated Co²⁺-agarose affinity column. The NS2B-NS3 protein was then eluted with a 10–500 mM gradient of imidazole concentrations (Figure 1). The pooled, partially purified, NS2B-NS3 fractions were dialysed and then re-chromatographed on a Co²⁺-agarose column to acquire the homogenous NS2-NS3 samples. During purification, we observed an autolytic conversion of the 36 kDa NS2B-NS3 construct into the 30 kDa NS3 proteinase. To facilitate the autolytic conversion, the purified samples (0.02–0.2 mg/ml) were incubated overnight at pH 8 and 24 °C to generate the autolytically processed individual NS3 proteinase. Under these experimental conditions, complete conversion was accomplished yielding NS3 (Figure 1). The resulting NS3 samples retained the C-terminal His₆-tag as the NS3 moiety was readily recognized by anti-His₆-tag antibody (Figure 1). We determined that, in the course of an autolytic conversion, NS3 cleaved the EYKK¹⁵↓G¹⁶DT sequence (numbering starts from the N-terminus of NS3) that generated the individual NS3 proteinase commencing from the N-terminal Gly¹⁶ (Figure 1). The catalytically inert NS2B-NS3 mutant with the H51A mutation of the active site His⁵¹ had approx. 0.1–1 % of enzymatic activity when compared with that of the autolytically activated NS2B-NS3 construct, and, accordingly, this mutant was not autolytically cleaved into NS3 (results not shown). Inhibitors of NS3 completely inhibited autolysis of the NS2B-NS3 construct and its conversion into NS3 (Figure 1). The catalytically inert NS2B-NS3 mutant has already been crystallized, and the crystallographic analysis is currently in progress (A. Aleshin, S. A. Shiryayev, R. C. Liddington and A. Y. Strongin, unpublished work). According to the results of size-exclusion

Table 1 NS3 cleaves fluorescent peptide substrates of furin

pNA, *p*-nitroanilide.

Substrate	$V_{\max}$ (μM/s)	K_m (μM)	k_{cat} (s ⁻¹)	K_{cat}/K_m (M ⁻¹ ·s ⁻¹)
Z-RR-AMC	0.0031	146	0.062	424
Boc-RVRR-AMC	0.0037	41	0.073	1791
Pyr-RTKR-AMC	0.05	43	1	23000
Boc-AGPR-AMC			No cleavage	
Boc-QAR-AMC			No cleavage	
Boc-O-benzyl-EGR-pNA			No cleavage	

chromatography (results not shown), the NS2b remains non-covalently bound with the NS3 moiety after the autocatalytic cleavage of the NS2B-NS3 construct.

NS3 cleaves furin-like motifs in protein substrates

In the viral polyprotein precursor, NS3 predominantly cleaves the sequence regions with positively charged amino acid residues at both the P1 and P2 positions [12]. This cleavage preference resembles that of cathepsin B and furin [5,27]. In our cleavage tests, WNV NS3 efficiently cleaved the furin fluorescent substrates Boc-RVRR-AMC and Pyr-RTKR-AMC, while the cleavage of the cathepsin B substrate Z-RR-AMC was significantly less efficient (Table 1). The cleavage of the peptide substrates followed Michaelis-Menten kinetics. The Lineweaver-Burk plot was linear over the range of the substrate concentrations

Figure 2 NS3 mimics furin and nicks anthrax PA83

The furin cleavage motif in the anthrax PA83 sequence is underlined. An arrow indicates an Arg¹⁶⁷-Ser¹⁶⁸ scissile bond in the PA83 peptide sequence (top). The NS3 protease cleaves the RKKR↓S furin motif. This cleavage converts PA83 into the mature PA63 (bottom). Molecular-mass sizes are indicated in kDa.

(results not shown). The $V_{\max}$ and k_{cat}/K_m values for the substrates are summarized in Table 1. The presence of any residue at P2, other than positively charged arginine or lysine, made the substrate resistant to the NS3 cleavage.

Consistent with its furin-like cleavage preference, the NS3 proteinase cleaved the RKKR¹⁶⁷↓S¹⁶⁸TS furin cleavage motif in the sequence of anthrax PA83 [28]. Similar to furin, this cleavage converted PA83 into the mature PA63 (Figure 2). There are no additional sequence motifs in PA83 which fit the cleavage preference of NS3.

To corroborate the ability of NS3 to process the substrates of furin, we analysed the cleavage of the peptide K⁸³TRHSRRSRRSL⁹⁴ that spans the furin-cleavage site in WNV prM. During the secretory export of flavivirus particles, a viral envelope glycoprotein prM is cleaved by furin [11,29]. This cleavage generates the M protein which is required for the subsequent rearrangement of E-glycoprotein and for virus infectivity. We subjected the K⁸³TRHSRRSRRSL⁹⁴ peptide to NS3-mediated proteolysis. The mass of the digested products was identified by MALDI-TOF-MS (Figure 3). In our *in vitro* cleavage experiments, catalytic quantities of NS3 cleaved the 1540 Da peptide and generated a 941 Da K⁸³TRHSRR digest product, thus indicating that cleavage takes place at the R⁸⁹↓S⁹⁰ site. As a control for NS3 cleavage, we used the A⁹⁴INRD⁹⁸STKQKKR↓GG¹⁰⁷ peptide that spans the known NS3 KR↓GG¹⁰⁷ cleavage site in the anchored capsid C protein [13]. The peptide A⁹⁴INRD⁹⁸S sequence was modified, and then compared with the C protein sequence (A⁹⁴INRRS), to prevent multiple cleavages of the peptide. As anticipated, the NS3 proteinase efficiently cleaved the A⁹⁴INRD⁹⁸STKQKKR↓GG¹⁰⁷ peptide at the expected R¹⁰⁵↓G¹⁰⁶ site.

Potential host cell proteins with NS3 cleavage sites

After the maturation of the viral proteins and the formation of the virion, the NS3 proteinase is released from the infected dead host cells [30]. After its release, the catalytically active NS3 can engage host cell targets and contribute independently to WNV pathogenesis. To identify the proteins which may be useful as the substrates of NS3 for the subsequent *in vitro* studies, as well as to narrow the number of the putative cleavage targets, we used the known cleavage preference of NS3 to construct a probabilistic cleavage profile of the NS3 protease. For these purposes, we

Figure 3 MS analysis of the NS3 cleavage peptides

The K⁸³TRHSRR↓SRRSL⁹⁴ peptide (A) that spans the furin cleavage site (underlined) in prM and the A⁹⁴INRD⁹⁸STKQKKR↓GG¹⁰⁷ peptide (B) that includes the known NS3 cleavage site (underlined) in the anchored protein C sequence are shown. After cleavage by NS3, the mass of the digest products was determined by MALDI-TOF-MS. The molecular mass of the intact peptides and the cleavage products are shown. There was no difference between the calculated and estimated masses of the peptides. Left-hand panels show the undigested peptides, right-hand panels show the digest products. An arrow indicates the cleavage site. In the C protein peptide, Asp⁹⁶ (italicized) substituted for the Arg⁹⁸ of the original sequence. Unless the R98D mutation is made, the cleavage of the peptide will occur at both the RR↓S⁹⁹ and the KR↓G¹⁰⁶ sites of the original sequence.

used PoPS, a system for the Prediction of Protease Specificity (<http://pops.csse.monash.edu.au>). Recently, a PoPS analysis of the human proteome was used successfully in our laboratory for an *in silico* prediction of the potential cleavage targets of membrane type-1 matrix metalloproteinase [31].

To perform PoPS searches, the protein sequences known to be susceptible to NS3 proteolysis were used to generate a position-specific scoring matrix (on a scale of −5.0 to +5.0) for the P4–P4' cleavage positions of the known NS3 protein and peptide substrates. This software uses the scoring matrix rather than the sequence motif in searching for cleavage targets [32]. PoPS was then used to search for the presence of the NS3 cleavage profile in the human proteome (28 796 proteins). Because, in our search motif, the amino acid residues other than positively charged arginine and lysine occupied the P4 position, the substrates of furin (which require the presence of positively charged residues at P4) would not be identified in our searches. To filter these predictions further, the programs PSIPRED and NCOILS (integrated in the PoPS system) were used to predict the secondary structure of the potential protein targets and to search for cleavage sites that are located in unstructured regions.

This analysis returned a score for each identified site, based on the weighted matrix. The analysis revealed 28 top scoring hits with the score of 46–48 in the human proteome, as shown in Table 2. The identified hits were predominantly represented by the hypothetical and predicted proteins and by the proteins without any obvious physiological relevance to the NS3 function. The analysis also identified two brain proteins, which may be relevant to the biology of WNV: MBP and myelin protein zero (PoPS score of both was 46). These proteins are required for the normal functioning of neurons and their absence or mutation is associated with neuron degeneration and neurological pathologies [33–36].

Table 2 PoPS search identifies MBP and myelin protein zero as the potential NS3 proteolytic targets

The putative physiologically relevant targets of NS3 are shown in bold.

Accession number	Protein	Maximum score	Protein size (residues)
XP_373106.2	Predicted: similar to Tb-291 membrane-associated protein	49	647
NP_542390.1	Hypothetical protein MGC14839	49	123
XP_499219.1	Predicted: hypothetical protein XP_499219	48	98
NP_115923.1	KIAA1862 protein	47	1030
NP_008827.2	Homeobox protein A7	47	230
NP_002376.1	MBP isoform 2	46	186
NP_000521.1	Myelin protein zero	46	258
XP_497672.1	Predicted: similar to ribosomal protein S2	46	863
XP_498795.1	Predicted: hypothetical protein XP_498795	46	263
XP_376350.2	Predicted: Rap guanine nucleotide-exchange factor (GEF) 2	46	1487
XP_498160.1	Predicted: similar to transitin	46	604
XP_374600.2	Predicted: similar to Grid2ip protein	46	1485
XP_380128.2	Predicted: hypothetical protein XP_380128	46	300
XP_292796.3	Predicted: similar to ret finger protein-like 1	46	835
NP_004088.1	Empty spiracles homologue 1	46	257
NP_689553.1	Hypothetical protein FLJ39075	46	120
XP_379507.2	Predicted: hypothetical protein XP_379507	46	300
NP_000200.1	Insulin promoter factor 1, homeodomain transcription factor	46	283
NP_055448.1	SH2 (Src homology domain 2) domain-binding protein 1	46	1173
NP_003474.1	High-mobility group AT-hook 2	46	109
NP_006764.3	DEAD (Asp-Glu-Ala-Asp) box polypeptide 18	46	670
NP_056239.3	Chordin-like 2	46	451
NP_653187.2	LY6/PLAUR domain-containing 1	46	165
NP_644804.1	A-kinase anchor protein 1 isoform 2 precursor	46	593
NP_003031.2	Erythrocyte membrane protein band 3-like 1	46	1241
NP_061174.1	Hypothetical protein DKFZp547A023	46	639
NP_056175.2	Superkiller viralicidic activity 2-like 2	46	1042

To confirm the validity of PoPS prediction, we evaluated the ability of NS3 to cleave MBP *in vitro*. As predicted, MBP was highly sensitive to specific cleavage by WNV NS3 (Figure 4).

D-Arginine-based peptides and serpins are the NS3 inhibitors

D-Arginine-based peptides and serine protease inhibitors (serpins) are potent inhibitors of furin [23,37]. Based on our data that show that NS3 has furin-like activity, we tested whether similar peptide and protein inhibitors would inhibit WNV NS3. The 6–12-mer D-arginine peptides were effective at a nanomolar range against the WNV NS3 proteinase *in vitro* (Table 3). Consistent with these findings, NS3 was also sensitive to a well-known inhibitor of furin, α 1-antitrypsin variant Portland [38]. Among the protein inhibitors examined, aprotinin was most effective in inhibiting NS3 ($K_i = 26$ nM). Aprotinin and D-arginine peptides were efficient both in the inhibition of the cleavage of the fluorescence peptide model substrates by NS3 and in protecting the protein substrates from the NS3 cleavage. Thus both aprotinin and D-arginine-based peptides efficiently inhibit NS3 proteolysis of MBP *in vitro* (Figure 4).

Lineweaver–Burk plots of the D-arginine-based inhibitors demonstrated strictly competition-type inhibition (results not shown). A series of D-arginine inhibitors with a chain length of six to twelve residues was synthesized and tested for inhibitory potency against NS3 and furin. In agreement with the earlier data [23,37], Table 3 shows that the K_i of the D-arginine peptide for furin increased from 36 to 100 nM as the chain length decreased from nine to six residues. In contrast, the K_i of hexa-D-Arg-NH₂ for NS3 was 80–100-fold higher when compared with that of nona-D-Arg-NH₂ peptide. These findings suggest that there is a significant difference in the binding mode of the positively charged inhibitors with furin and with NS3. To identify this

difference, we built a spatial model of WNV using a known structure of Dengue virus NS3 as a template [19,39].

Modelling of the spatial structure of NS3

Because the level of homology between the WNV and Dengue NS3 is high (above 50%), we could produce the model of WNV NS3 (R.M.S.D. = 0.21 Å) (Figure 5). This model was used to guide our studies until the actual crystallographic structure of NS3 becomes available. The *in silico* model of the NS3 proteinase shows a low density of negatively charged residues in the proximity of the catalytic triad and this structural relationship emphasizes the importance of the two aspartate residues (Asp⁷⁵ and Asp¹²⁹) (Figure 5). According to this model, there are no negatively charged residues, except for Asp⁷⁵ and Asp¹²⁹, in the active-site groove of WNV NS3. We suspect that these two residues of NS3 interact with the positively charged arginine-based antagonists. In contrast, the number of negatively charged residues is high in furin's catalytic site region [27,40].

Cell-based assays confirm the inhibitory effect of D-arginine-based peptides

Although our *in vitro* data suggested that D-arginine-based peptides had the capacity to inhibit NS3 proteinase activity, it was important to confirm the relevance of this finding. To assess this, we treated primary neurons with increasing concentrations of nona-D-Arg-NH₂ and then infected them with the pathogenic New York strain of WNV. At a 10–50 μ M range of peptide, a several log reduction of virus production was observed (Figure 6). There was no significant non-specific toxicity in the assays. The relatively high concentrations of D-arginine peptides that were required (compared with the K_i) may be explained by the poor

Figure 4 MBP is a putative cleavage target of the NS3 proteinase in the brain

(A) Arrows indicate the identified NS3 cleavage sites in the human MBP sequence. (B) The sequence of the matrix metalloproteinase-26 cleavage site. The first, N-terminal, NS3 cleavage site (PKR⁵⁵↓G⁵⁶SG) is conserved in mammals and birds. The second, C-terminal, NS3 cleavage site (KGR¹⁴⁴↓G¹⁴⁵LS) is also strictly conserved in humans, monkeys, horses, dogs, mice, rats, birds and many other animals (results not shown). (C) MBP (18.5 kDa) is highly susceptible to the catalytic amounts of NS3 *in vitro* (an enzyme/substrate ratio of 1:25–250). Molecular-mass sizes are given in kDa. (D) Aprotinin (left) and dodeca-D-Arg-NH₂ (right) inhibit the cleavage of MBP by NS3 *in vitro*. NS3 is highly sensitive to inhibition by dodeca-D-Arg-NH₂ ($K_i = 1\text{--}2\text{ nM}$) and by aprotinin ($K_i = 25\text{ nM}$; Table 3). The data show that NS3 (2 μM) is fully inhibited by dodeca-D-Arg-NH₂ in a 2–200 μM range. The data also show that NS3 (2 μM) is fully inhibited by aprotinin at a 20–120 μM range and is partially inhibited at a 1–5 μM range.

penetration of charged peptides across the neuronal membrane. Nevertheless, cell-based assays, in agreement with the essential role of the NS3 activity in the life cycle of WNV, confirmed that D-arginine-based peptides, which inhibited NS3 activity in the *in vitro* cleavage tests, reduced viral infection in a relevant cell type.

DISCUSSION

In the present study, we purified and partially characterized the NS3 processing proteinase from West Nile flavivirus. Our purified NS3 samples were homogeneous as shown by SDS/PAGE and demonstrated a high proteolytic activity against model peptide substrates of furin. In contrast with an opposing theory that the resulting NS3 is inactive if the activator NS2B sequence is absent [18,21,22], our studies suggest that the NS2B sequence represents a prodomain, rather than a cofactor or activator, of the 30 kDa NS3 proteinase. It appears that the NS2B sequence functions as an autolytically cleaved prodomain, which is essential for the proper folding and, consequently, the activity of the NS3 proteinase.

Table 3 Peptidic and protein inhibitors of the NS3 serine proteinase of WNV

NI, no inhibition. The K_i value of the inhibitors was determined using Boc-RVRR-AMC as a substrate. Note the dramatic difference in the efficiency of inhibition of furin and NS3 by nona-D-Arg-NH₂ and hexa-D-Arg-NH₂.

Peptide and protein inhibitor	K_i (nM)	
	NS3	Furin
(D)RRRRRR-NH ₂ ; hexa-D-Arg-NH ₂	478 ± 20	100 ± 10 (106*)
(D)RRRRRR-NH ₂ ; hepta-D-Arg-NH ₂	41 ± 18	68*
(D)RRRRRR-NH ₂ ; octa-D-Arg-NH ₂	17 ± 11	61*
(D)RRRRRRRR-NH ₂ ; nona-D-Arg-NH ₂	6 ± 1	36 ± 7 (42*)
(D)RRRRRRRR-NH ₂ ; deca-D-Arg-NH ₂	2 ± 1	
(D)RRRRRRRRRR-NH ₂ ; undeca-D-Arg-NH ₂	1 ± 1	
(D)RRRRRRRRRRR-NH ₂ ; dodeca-D-Arg-NH ₂	1 ± 1	
Boc-(D)-R-(Tosyl)-OH	2450 000 ± 35 000	
Dec-RVKR-CMK	380 ± 59	2*
Cathepsin B inhibitor [L-3- <i>trans</i> -(propylcarbamoyl)-oxirane-2-carbonyl]-L-Ile-L-Pro		NI
Cathepsin B inhibitor [L-3- <i>trans</i> -(propylcarbamoyl)-oxirane-2-carbonyl]-L-Ile-L-Pro methyl ester		NI
<i>N</i> - <i>p</i> -tosyl-L-Lys-CHO		NI
Aprotinin	26 ± 4	
α 1-Antitrypsin	590 ± 65	> 500*
α 1-Antitrypsin variant Portland	274 ± 17	1.4*

* Data taken from [23,37,38].

As with bacterial proteinases (subtilisin and thermolysin) and mammalian furin [27,41–44], the viral NS2B, most probably stabilizes the folding intermediates [45–48] on the path to the fully folded/active catalytic domain of NS3. Because the individual NS3 was catalytically active in the peptide and protein cleavage assays, we believe that the presence of the covalently bound NS2B sequence is not required for the enzymatic activity of NS3.

Our studies determined that NS3 exhibited furin-like, albeit less restricted, cleavage preferences. These distant relationships between furin and NS3 led us to hypothesize that there are common inhibitors of these proteinases. This hypothesis facilitated the identification of the inhibitors of NS3. Thus the D-arginine-based peptides are nanomolar range inhibitors of NS3 *in vitro*.

Based on the modelling of the three-dimensional structure of WNV NS3, we suggest that the negatively charged Asp⁷⁵ and Asp¹²⁹ residues, which are 20 Å apart, appear to be important for the interactions with the D-arginine-based inhibitors, which should span approx. 20 Å to reach both Asp⁷⁵ and Asp¹²⁹. It is obvious that D-arginine-based inhibitors will interact most efficiently with the aspartate residues and especially with the partially protonated Asp⁷⁵ of the catalytic triad only at high pH. These considerations were taken into account in our inhibitory studies, which were performed at pH 8.

We hypothesized that the presence of Asp⁷⁵ and Asp¹²⁹ could explain why short arginine peptides are less efficient inhibitors of NS3 as compared with furin. The synthesis and the testing of these structure-based inhibitors are currently in progress. Because we have already prepared the crystals of the catalytically inert H51A NS2B–NS3, we will soon know the exact role of and the precise distance between Asp⁷⁵ and Asp¹²⁹. We believe that these studies at atomic resolution level will shed light on the mechanism of NS3 inhibition by the positively charged arginine peptides as well as guiding the design of the synthetic, non-peptidic, inhibitors.

Knowledge of the substrate preference of NS3 led us to search for possible host targets of NS3 proteolysis. Using a prediction program, we identified MBP as an NS3 cleavage target. Experimental studies confirmed that MBP was specifically

Figure 5 Model of the WNV NS3 proteinase

The numbering corresponds to the WNV NS3 sequence. The residues of the catalytic triad are purple. The surface residues conserved in all of mosquito-borne flaviviral NS3 proteases are light blue. The residues conserved in the WNV, Usutu virus, Japanese encephalitis virus, Murray Valley encephalitis virus and Kunjin virus NS3 are red. These residues are not conserved in Dengue virus and yellow fever virus NS3. The corresponding residues of the Dengue virus NS3 are shown in parentheses. Modelling was performed using the Dengue NS3 protease (PDB code 1DF9) as a template. The model total energy score is -0.0379 (local = -0.0524 , burial = -0.0013 , contact = 0.0158), which is consistent with the feasible molecular models. The R.M.S.D. of the model is 0.21 Å. (B) Close-up of the active-site region of furin (PDB code 1P8J) and WNV NS3. The catalytic triad residues (histidine, aspartate and serine) are purple, the residues which are near the catalytic triad and which are involved in the substrate binding, are light blue. The Dec-RVKR-cmk (dec is decanoyl and cmk is chloromethylketone) inhibitor, co-crystallized with furin, is red. In the NS3 structure, the substrate-binding residues, which are conserved in the mosquito-borne WNV, Usutu virus, Japanese encephalitis virus, Murray Valley encephalitis virus, Kunjin virus, yellow fever virus and Dengue 1 and 2 virus NS3 proteinases are yellow, and the residues conserved only in WNV and Dengue virus are light blue. The light-blue and purple arrows point to Asp¹²⁹ and Asp⁷⁵ respectively, which are expected to be 20 Å apart and which, we suspect, interact with the D-arginine-based inhibitors.

Figure 6 Nona-D-Arg-NH₂ reduces WNV infection in primary neurons

SCG neurons from wild-type mice infected with WNV at a multiplicity of infection of 1. Neurons were treated with increasing concentrations of nona-D-Arg-NH₂ 2 h before infection. At 24 h after infection, supernatants were harvested, and the amount of infectious WNV was titrated by viral plaque assay on BHK21 cells. The assay was performed in triplicate. Results are means $\pm$ S.E.M.

cleaved by NS3. Despite the fact the physiological relevance of the MBP–NS3 interactions in the course of viral infection is currently unknown, MBP may be used as a convenient, sensitive and inexpensive, albeit functionally irrelevant, protein target in the biochemical cleavage and inhibitory tests. We suspect that a link between MBP and NS3 warrants additional studies.

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