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Characterisation of ten NS2B-NS3 proteases: Paving the way for pan-flavivirus drugs

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ABSTRACT

Flaviviruses can cause severe illness in humans. Effective and safe vaccines are available for some species; however, for many flaviviruses disease prevention or specific treatments remain unavailable. The viral replication cycle depends on the proteolytic activity of the NS2B-NS3 protease, which releases functional viral proteins from a non-functional polyprotein precursor, rendering the protease a promising drug target. In this study, we characterised recombinant NS2B-NS3 proteases from ten flaviviruses including three unreported proteases from the Usutu, Kyasanur forest disease and Powassan viruses. All protease constructs comprise a covalent Gly_4-Ser-Gly_4 linker connecting the NS3 serine protease domain with its cofactor NS2B. We conducted a comprehensive cleavage site analysis revealing areas of high conversion. While all proteases were active in enzymatic assays, we noted a 1000-fold difference in catalytic efficiency across proteases from different flaviviruses. Two bicyclic peptide inhibitors displayed anti-pan-flaviviral protease activity with inhibition constants ranging from 10 to 1000 nM.

Flaviviruses are arthropod-borne single-stranded RNA viruses that can cause severe illness in humans (Pierson and Diamond, 2020). Prominent examples include the dengue, Zika and Yellow fever viruses. For some flaviviruses effective and safe vaccines protecting from diseases like Yellow fever, Japanese encephalitis and tick-borne encephalitis are available and well established (Heinz and Stiasny, 2012; Ishikawa et al., 2014). Others, for example dengue (dengvaxia), face challenges like antibody-dependent enhancement of disease severity in areas of co-circulating viruses or serotypes (Halstead and Deen, 2002; Wilder-Smith et al., 2019). A next-generation dengue vaccine (TAK-003) raises hopes to overcome previous limitations, demonstrating efficacy and safety against serotypes 1 and 2 in dengue-naïve patients in phase III clinical trials (Biswal et al., 2019; Thomas, 2023; Tricou et al., 2024). Specific anti-flaviviral drugs are not approved to date, highlighting the importance of drug development campaigns (Pierson and Diamond, 2020).

Flaviviruses are composed of three structural and seven non-structural (NS) proteins. The viral replication cycle depends on the catalytic activity of NS2B-NS3, a viral protease which processes a non-functional polyprotein precursor into functional proteins. NS2B is anchored into the membrane of the endoplasmatic reticulum. It constitutes a hydrophilic loop, which extends into the cytoplasm where it interacts with the catalytically active serine protease domain of NS3 (Fig. 1a/b) (Neufeldt et al., 2018). Structural and functional details of NS2B-NS3 have been reviewed previously (Barrows et al., 2018; Nitsche, 2019).

Most constructs used in biochemical assays and structure elucidation rely on the hydrophilic core motif of NS2B (Nitsche, 2019), excluding membrane associated regions and hence avoiding the need for artificial lipid membranes. While reports of these truncated constructs include variations with and without covalent linker between NS2B and NS3 (de la Cruz et al., 2014; Li et al., 2014; Phoo et al., 2016); a Gly_4-Ser-Gly_4 linker between the C-terminus of NS2B and the N-terminus of NS3 is the most frequently used construct due its robustness with respect to *E. coli* expression, purification and overall stability (Lei et al., 2016; Leung et al., 2001; Li et al., 2005; Nall et al., 2004).

In this study we set out to design, express and characterise a library of ten recombinant NS2B-NS3 proteases and assess the prospects for pan-flaviviral protease inhibitors. In order to cover a broad range of well-studied, neglected, mosquito- and tick-borne viruses, we selected Zika (ZIKV) (Voss and Nitsche, 2020), dengue (DENV) (Nitsche et al., 2014), West Nile (WNV) (Voss and Nitsche, 2021), Murray Valley encephalitis

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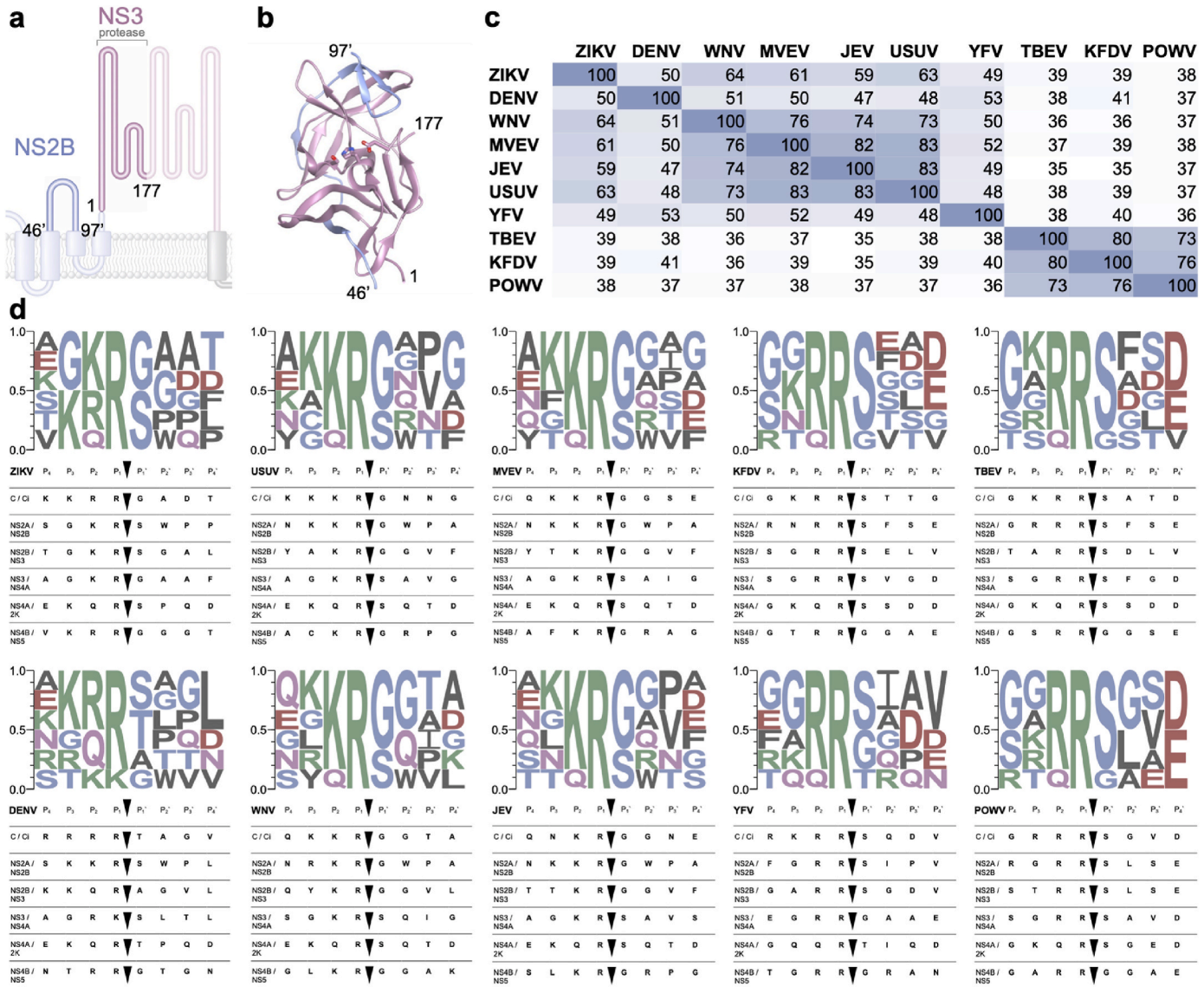

	ZIKV	DENV	WNV	MVEV	JEV	USUV	YFV	TBEV	KFDV	POWV
ZIKV	100	50	64	61	59	63	49	39	39	38
DENV	50	100	51	50	47	48	53	38	41	37
WNV	64	51	100	76	74	73	50	36	36	37
MVEV	61	50	76	100	82	83	52	37	39	38
JEV	59	47	74	82	100	83	49	35	35	37
USUV	63	48	73	83	83	100	48	38	39	37
YFV	49	53	50	52	49	48	100	38	40	36
TBEV	39	38	36	37	35	38	38	100	80	73
KFDV	39	41	36	39	35	39	40	80	100	76
POWV	38	37	37	38	37	37	36	73	76	100

Fig. 1. Overview of flavivirus NS2B-NS3 proteases used in this study. (a) Model of NS2B-NS3 as part of the flaviviral polyprotein embedded into the membrane of the endoplasmic reticulum (ER). NS2B is shown in dark blue and NS3 is magenta. The hydrophilic loop of NS2B (residues 46′–97′(WNV)) and the protease domain of NS3 residues 1–177 are highlighted. b) X-ray crystal structure of the West Nile virus protease NS2B-NS3 in absence of its co-crystalised ligand (PDB ID: 2YOL) (Hammamy et al., 2013). The figure was generated using USCF Chimera (Pettersen et al., 2004) c) Sequence similarity analysis of truncated NS2B (46′–97′) and NS3 (1–177). Sequence similarities in (%) were calculated using T-COFFEE (Di Tommaso et al., 2011). Values (%) are visualised as heatmap: blue =high sequence similarity; white =low sequence similarity. d) Analysis of polyprotein cleavage sites processed by NS2B-NS3. Residues covering P_4–P_4' are shown. Full-length sequences from human isolates reported in the NCBI protein data bank were analysed (Brister et al., 2015). The consensus sequence for each cleavage site is shown. The overall consensus sequence over all cleavage sites was plotted using WebLogo (Crooks et al., 2004).

(MVEV) (Joy et al., 2010), Japanese encephalitis (JEV) (Junaid et al., 2012), Usutu (USUV) (Cle et al., 2019), Yellow fever (YFV) (Kondo et al., 2011), tick-borne encephalitis (TBEV) (Akaberi et al., 2021), Kyasanur forest disease (KFDV) (Holbrook, 2012), and Powassan (POWV) (Kemenesi and Banyai, 2019) viruses.

Although individual flaviviruses may differ in their pathogenesis, we hypothesise that the underlying replication machinery, particularly the viral protease, is highly conserved. Thus, this study aims to characterise ten flavivirus proteases and explores the possibility of broad-spectrum protease inhibitors for flaviviruses. While many previous studies and drug discovery campaigns mainly focused on single viruses (often even limited to a single serotype, e.g., DENV serotype 2) or on a limited subset of prominent examples like dengue, Zika or West Nile viruses, this study follows a more comprehensive approach, taking the bigger picture into account.

We first aligned full length NS2B and NS3 protein sequences of each virus with known constructs of ZIKV, DENV-2 and WNV proteases to identify regions of interest (Chen et al., 2014; de la Cruz et al., 2011, 2014; Mahawaththa et al., 2017; Su et al., 2009a, 2009b). This alignment indicated that the hydrophilic core motif of NS2B corresponds to residues 45'/46′ to 96'/97' of NS2B and residues 1 to 177 of the NS3 protease domain (Fig. 1a, Fig. S1). We then selected the same regions for each virus to ensure a consistent construct architecture for direct comparison. In order to develop protein constructs that could later be used for X-ray crystallography, we decided to place a His_6 affinity tag at the N-terminus followed by a TEV (tobacco etch virus) protease cleavage site.

Next, we examined sequence similarities of the truncated NS2B and NS3 residues (Fig. 1c). The results suggest two major groups which align with reported phylograms (Kuno et al., 1998). The first group includes WNV, MVEV, JEV and USUV with similarity of 73–83%. All four are mosquito-borne and share the same clade. The second group comprises

Table 1
Experimental parameters of NS2B-NS3 constructs linked by a G_4SG_4 linker.

Flavivirus	Yield (mg/l)[a]	T_m (°C)[b]	k_{cat} (s^{-1})[c]	K_M (μM)[d]	k_{cat}/K_M (M^{-1} s^{-1})[e]	K_i (μM)[f]		
						BPTI[g]	Cpd. 1	Cpd. 2
ZIKV	123	53.0	0.630 [h]	19.1[h]	33,000 [h]	0.017 [h]	0.009 [h]	0.026 [h]
DENV	45.0	55.1	0.016 [h]	81.9 [h]	197 [h]	0.063 [h]	0.424 [h]	1.066 [h]
WNV	52.7	52.6	0.562 [h]	59.8 [h]	9,390 [h]	0.065 [h]	0.100 [h]	0.050 [h]
MVEV	42.2	51.8	0.353 [h]	76.1 [h]	4,562 [h]	1.055 [h]	0.061[h]	0.170 [h]
JEV	21.2	48.9	0.051 [h]	25.3 [h]	2,012 [h]	0.245 [h]	0.087 [h]	0.093 [h]
USUV	9.1	44.4	0.005 [h]	160 [h]	33.8 [h]	0.195 [h]	0.084 [h]	0.260 [h]
YFV	47.6	46.0	0.005 [h]	36.1 [h]	125 [h]	0.067 [h]	0.227 [h]	0.209 [h]
TBEV	23.8	46.7	0.002 [i]	8.9 [i]	191 [i]	0.033 [i]	0.106 [i]	0.132 [i]
KFDV	31.2	45.7	0.001 [i]	16.2 [i]	24.7 [i]	0.171 [i]	0.164 [i]	0.283 [i]
POWV	35.8	49.9	0.006 [i]	10.9 [i]	550 [i]	0.186 [i]	0.233 [i]	0.252 [i]

[a] Expression yields are based on single experiments from 1 litre cell culture.
[b] Denaturation midpoint determined by differential scanning fluorimetry.
[c] Turnover number.
[d] Michaelis-Menten constant.
[e] Catalytic efficiency.
[f] Inhibition constant.
[g] Estimated inhibition constants of bovine pancreatic trypsin inhibitor (BPTI) calculated from IC_{50} values using the Cheng-Prusoff equation.
[h] Boc-Gly-Lys-Arg-AMC was used as substrate.
[i] Bz-Nle-Lys-Arg-Arg-AMC was used as substrate.

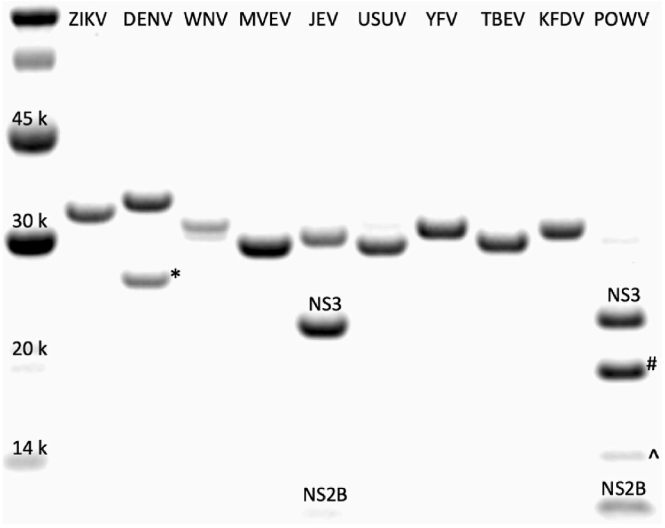

Fig. 2. SDS-PAGE of recombinant covalently linked N2SB-NS3 proteases. The asterisk (*) indicates the autoproteolytic cleavage product of DENV NS3 (Fig. S3). The hash (#) indicates truncated NS3 cleavage product of POWV following autoproteolysis. The caret (^) indicates elongated autoproteolytic cleavage product of NS2B (including the G_4SG_4 linker and residues 1–16 of NS3) of POWV (Fig. S11).

TBEV, KFDV and POWV with sequence similarities of 73–80%. These three tick-borne viruses, which also share a clade, align only to a lesser extend (35–41%) with the remaining proteases, indicating their distant origins. The proteases of ZIKV, DENV and YFV do not appear to clearly align with either of these clusters which agrees with phylogenetic studies in which neither one shares a clade with any other selected species.

All proteases were expressed in *E. coli* BL21(DE3) and were purified from the soluble fraction of the cell lysate using Ni-NTA affinity chromatography. For most constructs, we observed high expression yields in *E. coli* ranging from 20 to 50 mg protein per litre cell culture (Table 1). While the ZIKV protease overexpressed exceptionally well (120 mg/l), USUV NS2B-NS3 showed an exceptionally low expression level (9 mg/l). Identity of all protease samples was confirmed by intact protein mass spectrometry (Figs. S2–S12). Analysis by SDS-PAGE revealed that the constructs for ZIKV, WNV, MVEV, USUV, YFV, TBEV and KFDV proteases yielded exclusively the expected covalently linked recombinant protein (Fig. 2), whereas the proteases of JEV and POWV showed a tendency for autoproteolysis at the junction of NS2B and NS3 as confirmed by mass spectrometry (Figs. S6, S11, S12). In both cases, the resulting unlinked construct represents the dominant population. In contrast to these autocleavages at the junction of NS2B and NS3, mass spectrometry analysis also revealed autoproteolysis within the NS2B domain of DENV and the NS3 domain of POWV (Figs. S3, S11, S12).

We analysed the conservation of substrate sequences across flaviviruses to inform the generation of pan-flavivirus proteases inhibitors. We examined the peptide sequence covering P_4 to P_4' residues (Schechter and Berger, 1967) of full-length human isolates (Fig. 1d). The data reveal full conversion of arginine in P_1 across all ten proteases and a strong preference for a second basic residue (lysine/arginine) in P_2 (except for the NS4A/2K cleavage site). Based on the P_2 preference for either lysine or arginine, three groups can be identified, which correspond to our sequence similarity studies. The first group includes WNV, MVEV, JEV and USUV which all demonstrate a clear preference for lysine in P_2. The second group shows a preference for arginine in P_2 and is composed of TBEV, KFDV, POWV and, in contrast to our sequence similarity analysis, also YFV. The third group is comprised of ZIKV and DENV which tolerate both lysine and arginine in P_2. In the prime site, only P_1' shows a noticeable preference for small residues like glycine or serine. The overall high conversion of the major recognition motif P_2–P_1' across all examined sequences is remarkable and raises hopes that pan-flaviviral protease inhibitors can be generated.

Based on our cleavage site analysis, we selected the established fluorescent substrates Boc-Gly-Lys-Arg-AMC and Bz-Nle-Lys-Arg-Arg-AMC (Nle, L-norleucine; AMC, 7-amino-4-methylcoumarin) for enzymatic assays (Steuer et al., 2009). We first examined proteolytic activities of all ten proteases using Boc-Gly-Lys-Arg-AMC. The tick-borne species TBEV, KFDV and POWV, which all indicated a preference for arginine in P_2, displayed no measurable activity with this substrate (data not shown). These examples required the longer substrate Bz-Nle-Lys-Arg-Arg-AMC with arginine residues in P_1 and P_2 to display measurable proteolytic activity with K_M values between 9 and 16 μM (Table 1). Michaelis-Menten constants (K_M) for the other seven proteases ranged from 20 to 80 μM except for USUV (160 μM). Given significant differences in observed turnover numbers, the overall catalytic efficiencies among flavivirus proteases differ by three orders of magnitude, with ZIKV protease demonstrating highest fidelity as previously described (Lei et al., 2016). At the other end of the spectrum is the protease of USUV, which showed a 1000-fold lower catalytic efficiency

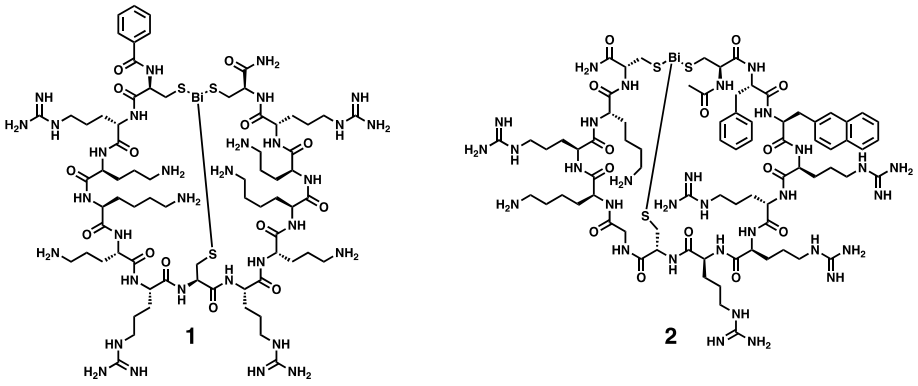

Fig. 3. Chemical structures of bicyclic peptide inhibitors **1** and **2**.

than ZIKV for the identical substrate and with similar cleavage preference (Tables S3 and S4, Figs. S26–30).

Our final objective was to demonstrate pan-flaviviral protease inhibition. We used two inhibitors, **1** and **2**, available in our laboratory which were previously discovered as ZIKV and WNV NS2B-NS3 inhibitors (Voss et al., 2022, 2023). Both compounds are bicyclic peptides that utilise the binding of bismuth(III) to three cysteines in a peptide chain to constrain its structure. This chemical modification can result in remarkable potencies likely related to the reduced entropic penalty associated with preorganisation. Polybasic substrate analogues are known inhibitors of several NS2B-NS3 proteases (Boldescu et al., 2017). Hence, compounds **1** and **2** contained different polybasic sequences (including arginine, lysine and ornithine) that were hypothesised to fit well into the substrate binding pockets (Fig. 3). We determined inhibition constants (K_i) of **1** and **2** (assuming competitive inhibition models) for all ten proteases by recording Michaelis-Menten kinetics at different inhibitor and substrate concentrations (Tables S5-S8, Figs. S31-S40). Compound **1** inhibited all proteases with sub-micromolar inhibition constants ranging from 0.01 to 0.4 μM (Table 1). Compound **2** showed slightly higher inhibition constants between 0.03 and 1.1 μM.

Additionally, we measured half-maximal inhibitory concentrations of the bovine pancreatic trypsin inhibitor (BPTI; aprotinin), known as a potent inhibitor of NS2B-NS3 and other serine proteases (Chen et al., 2014). The estimated inhibition constants, calculated based on the Cheng-Prusoff equation (Cheng and Prusoff, 1973), are within the same order of magnitude as previously reported inhibition constants for dengue (0.026 μM), Zika (0.076 μM) and West Nile (0.016 μM) viruses NS2B-NS3 (Mueller et al., 2007; Phoo et al., 2016). The data suggest that BPTI is a potent inhibitor of all tested proteases (K_i = 0.02–0.25 μM), except for MVEV ($K_i \sim 1.1$ μM), which is a remarkable observation given the broad activity of BPTI across serine proteases (Tables S9 and S10, Figs. S41–S45).

All inhibition data are summarised in Table 1. Interestingly, compounds **1** and **2** as well as BPTI displayed strongest inhibition against ZIKV protease, which is also the most active flavivirus protease reported. Overall, there appears to be a loose relationship between K_M values of substrates and K_i values of inhibitors (Table 1), indicating substrate-like binding. While these results establish the groundwork for the development of pan-flaviviral protease inhibitors, certain limitations are inherent in the exact quantification of inhibition constants through the measurement of Michaelis-Menten kinetics at various inhibitor concentrations (Figs. S31–S40). In certain instances, the lowest inhibitor concentration approaches or falls below the enzyme concentration, for which tight binding kinetics might apply (Morrison, 1969).

In conclusion, we report three new recombinant NS2B-NS3 protease constructs of Usutu, Kyasanur forest disease and Powassan viruses. Alongside these three recombinant proteases, we expressed seven additional proteases based on an identical linked construct architecture, making this the most comprehensive direct comparison of NS2B-NS3 proteases reported. We further conducted a comprehensive cleavage site analysis which assisted in the selection of suitable substrates for enzymatic assays. We showed that all constructs yielded proteolytically active proteins and report their kinetic parameters. Finally, we explored the avenue of pan-flaviviral protease inhibition by demonstrating that highly basic substrate-based peptides can display nanomolar activity against most flavivirus proteases.

Our findings may contribute to the development of protease inhibitors against Usutu, Kyasanur forest disease and Powassan viruses. The comprehensive collection of NS2B-NS3 proteases from various flaviviruses may also assist in the development of pan-flavivirus protease inhibitors, which seem within reach. The rational approach described herein, which capitalised on two decades of intensive research into flavivirus proteases, may also allow for the rapid development of recombinant NS2B-NS3 variants against emerging flaviviruses for future screening campaigns.

CRediT authorship contribution statement

Saan Voss: Conceptualization, Data curation, Methodology, Validation, Visualization, Writing – original draft. **Jörg Rademann:** Resources, Supervision. **Christoph Nitsche:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data are available in the attached Supporting Information file (Supplementary Material).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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