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Crystal structure of Zika virus NS2B-NS3 protease in complex with a boronate inhibitor

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The ongoing Zika virus (ZIKV) outbreak is linked to severe neurological disorders. ZIKV relies on its NS2B/NS3 protease for polyprotein processing; hence, this enzyme is an attractive drug target. The 2.7 Å crystal structure of ZIKV protease in complex with a peptidomimetic boronic-acid inhibitor reveals a cyclic diester between the boronic acid and glycerol. The P2 4-aminomethylphenylalanine moiety of the inhibitor forms a salt-bridge with the non-conserved Asp⁸³ of NS2B, ion-pairing between Asp⁸³ and the P2 residue of the substrate likely accounts for the enzyme's high catalytic efficiency. The unusual dimer of the ZIKV protease:inhibitor complex seen in the crystal may provide a model for assemblies formed at high local concentrations of protease at the endoplasmatic reticulum membrane, the site of polyprotein processing.

Previously considered a rare and mild pathogen for humans (1), Zika virus (ZIKV) infection has recently been found to be responsible for neurological disorders in a significant portion of patients. The infection can trigger Guillain-Barré syndrome (2) and prenatal ZIKV infection is responsible for a dramatically increased number of microcephaly cases in fetuses and newborn children (3). The World Health Organization (WHO) recently declared the association of ZIKV infection with these neurological disorders a Public Health Emergency of International Concern (4). There are no vaccines or antiviral drugs available for protection from or treatment of ZIKV infection.

ZIKV is a member of the genus *Flavivirus* in the *Flaviviridae* family of RNA viruses. Its ~10.7-kb single-stranded RNA genome of positive polarity encodes a single polyprotein, which, by analogy to other flaviviruses, is assumed to be cleaved by host-cell proteases (signalase, furin) and the viral NS2B/NS3 protease into three structural (C, prM/M, and E) and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (fig. S1). Similar to other flavivirus proteases such as those of dengue virus (DENV) and West Nile virus (WNV), the mature form of ZIKV protease consists of the N-terminal domain of NS3, which carries the catalytic triad Ser¹³⁵-His⁵¹-Asp⁷⁵, and the membrane-bound NS2B (see fig. S2 for a sequence alignment). Crystallization of this complex has not been successful so far for any flavivirus protease, but it has been shown that a construct comprising ~40 hydrophilic residues of NS2B and ~185 residues of NS3, covalently linked via a Gly₄-Ser-Gly₄ sequence, displays strong peptidolytic activity (5). Crystal structures of

the free form of this protease construct ("NS2B-NS3^{pro}") usually reveal an "open conformation" featuring a well-ordered NS3^{pro} core and a flexible NS2B part that shows only limited interaction with NS3^{pro}, whereas inhibitor (and presumably substrate) binding induces a pronounced conformational change of NS2B yielding a more compact, "closed" form (6, 7).

We expressed in *E. coli* a DNA construct corresponding to the NS2B-NS3^{pro} coding region of the Brazilian ZIKV isolate BeH823339 (8) (GenBank accession number KU729217.2). This construct codes for residues 49 - 95 of ZIKV NS2B, the C terminus of which is covalently linked via Gly₄-Ser-Gly₄ to the N terminus of the NS3 (residues 1 to 170). The recombinant enzyme obtained is a mixture of monomer, disulfide-linked dimer (here designated "SS-dimer"), and—to a lesser extent—higher oligomers (fig. S3). The double mutant Cys⁸⁰Ser/Cys¹⁴³Ser leads to loss of the disulfide bond, which occurs between Cys¹⁴³ residues of different polypeptide chains, as revealed by the x-ray structure. The SS-dimer and the monomer obtained by reduction with tris(2-carboxyethyl)phosphine (TCEP) (fig. S3) as well as the Cys⁸⁰Ser/Cys¹⁴³Ser mutant of ZIKV NS2B-NS3^{pro} are hyperactive against the standard flavivirus protease substrate benzoyl-norleucine-lysine-lysine-arginine 7-amino-4-methylcoumarine (Bz-Nle-Lys-Lys-Arg-AMC), with a very low *K_m* and a *k_{cat}/K_m* more than 20-times higher than for the WNV enzyme (Table 1).

In order to elucidate the molecular basis of this hyperactivity, and to provide a starting point for structure-based drug design efforts, we have crystallized ZIKV NS2B-NS3^{pro}

in the closed form and determined its X-ray structure at 2.7 Å resolution. Containing two molecules (A and B) per asymmetric unit of the crystal, the structure reveals the same chymotrypsin-like fold for the NS3^{pro} domain as seen previously for other flavivirus proteases, with the NS2B polypeptide wrapped around the NS3^{pro}. The interaction between the two is stabilized by hydrogen bonds between β -strands β 1 and AI, β 2 and BIIa, as well as β 3 and BIIb of NS2B and NS3^{pro}, respectively (Fig. 1A). The r.m.s. deviations between the ZIKV NS2B-NS3^{pro} complex and tetrapeptide aldehyde complexes of WNV and DENV-3 proteases are 0.9 – 1.1 Å [for main-chain atoms; PDB codes 2FP7 and 3U1I (6, 9)]. The capped dipeptide boronic-acid compound **cn-716** (Fig. 1C) was used to obtain the closed conformation of the protease. We found this compound to reversibly inhibit ZIKV NS2B-NS3^{pro} with $IC_{50} = 0.25 \pm 0.02 \mu M$ and $K_i = 0.040 \pm 0.006 \mu M$ (in the presence of 20% glycerol, fig. S4). In the structure of the complex, the boron atom is covalently linked to the side-chain O_γ of the catalytic Ser135 (Fig. 1, B and D to F). The structure also reveals that the boronic acid moiety forms a cyclic diester with glycerol, which was continuously present in our enzyme preparation during purification and crystallization, as well as cryoprotection of crystals. Boronic acids tend to form esters with diols and triols, especially if 5- or 6-membered rings can be formed (10). Our $F_o - F_c$ difference density indicates that a six-membered ring has been formed by reaction of the boronic acid with the terminal hydroxyl groups of glycerol (Fig. 1, B and D to F). The six-membered ring fits neatly into the S1' pocket of the enzyme (Fig. 1B), a site so far rarely addressed by synthetic flavivirus protease inhibitors. In the absence of glycerol, the IC_{50} for the boronic acid inhibitor was nearly unchanged ($0.20 \pm 0.02 \mu M$), but we note that through ester formation with larger, more hydrophobic diols or triols, a prodrug might be obtained that will traverse the cellular membrane more readily than free boronic-acid derivatives.

Due to the ring closure, the tetrahedral geometry of the boron is somewhat distorted. In molecule A, the 6-membered ring assumes a boat-like conformation, with the middle hydroxyl group (O_2) of glycerol in an axial position and donating an intramolecular hydrogen bond to the main-chain oxygen of the P2 residue of the inhibitor (Fig. 1E). In molecule B, the 6-membered ring adopts a somewhat twisted half-chair conformation and the central hydroxyl group, also in an axial position, donates a hydrogen bond to the carbonyl oxygen of Val¹³⁶ (Fig. 1F). In molecule A, the two ring oxygens (O_1 and O_3) accept H-bonds respectively from the amide of Gly¹³³, i.e., from the oxyanion hole, and from the catalytic His⁵¹, whereas the latter interaction is missing in molecule B. These differences between the two inhibitor molecules in the asymmetric unit of the crystal reflect the conformational variability of the cyclic boronates (10).

The P1-Arg residue of **cn-716** forms a salt-bridge with Asp¹²⁹, a feature conserved in many flavivirus protease complexes. Most probably protonated, the amino group of the 4-aminomethyl-phenylalanyl residue in the P2 position forms a hydrogen bond with the main-chain oxygen of Ser^{81*} and a salt-bridge with Asp^{83*} of the NS2B polypeptide (Fig. 1B; residues of NS2B are denoted by *). Asp^{83*} is Asn in WNV and Ser or Thr in DENV 1–4 NS2B-NS3^{pro} (fig. S2), i.e., unable to form an ion-pair interaction with the P2 residue of the inhibitor or the substrate, Bz-Nle-Lys-Lys-Arg-AMC. The Asp^{83*}Asn mutation leads to a ~2-fold increase of K_m and a k_{cat}/K_m reduced by 55%, compared to WT enzyme (Table 1). The Asp residue in this position provides an at least partial explanation for the significantly lower K_m and hence, the much higher k_{cat}/K_m of ZIKV protease compared to the WNV and DENV enzymes (Table 1). DENV NS2B/NS3 protease has been shown to counteract the type-I interferon response via digesting the stimulator of interferon genes (STING) in human dendritic cells (DCs) (11). As ZIKV also permissively infects human DCs (12), we speculate that an increased catalytic activity of ZIKV NS2B/NS3^{pro} could cause more efficient cleavage of STING, thereby leading to an enhanced suppression of the host innate immunity.

In the crystal, ZIKV NS2B-NS3^{pro} forms an unusual dimer with non-crystallographic, quasi-twofold symmetry (Fig. 2A) that has not been seen with other flavivirus proteases. This tight dimer has to be distinguished from the labile SS-dimers seen in solution (see above). In the tight dimer, the substrate-binding sites of the two monomers, along with the bound inhibitor, face each other (Fig. 2B). The dimer has openings at both sides, which upon some “breathing” would allow access of substrate to the two active sites located at the center (Fig. 2A). The tight dimer in the asymmetric unit of the crystal is connected to neighboring dimers through two labile disulfide bonds linking Cys¹⁴³ of monomer A to the same residue of monomer B in a symmetry-related dimer, and vice versa, giving rise to disulfide-mediated polymers of tight dimers (Cys¹⁴³ is indicated in Fig. 2A). This disulfide bond is responsible for the formation of the “SS-dimer” apparent in the SDS-PAGE (fig. S3). After a few seconds of the crystal in the x-ray beam, this exposed disulfide appears to be reduced as a consequence of irradiation, although the presence of the disulfide seems to be essential for crystallization of the ZIKV NS2B-NS3^{pro}, as we failed to obtain crystals of the Cys⁸⁰Ser/Cys¹⁴³Ser variant.

Formation of the tight dimer in the asymmetric unit buries ~1240 Å² of the surface of each of the two monomers and the shape complementarity (Sc) index (13) is 0.64 [for a large set of well-characterized homodimeric proteins, the mean Sc was 0.69 ± 0.07 ; (14)]. If we include the two inhibitor molecules in the calculation, ~1500 Å² of molecular surface are buried per monomer. Both the large surface area

buried and the shape complementarity indicate that dimer formation is likely of biological relevance. Although we failed to observe this dimer in solutions of the ZIKV NS2B-NS3^{pro} complex with **cn-716** up to a concentration of 133 μ M, we detected it by electrospray ionization (ESI) mass spectrometry in the presence but not in the absence of the boronic acid inhibitor (fig. S6). The structure suggests that the closed form of the enzyme has the potential of forming well-defined dimers at higher concentrations, as they may occur (and perhaps be promoted by the membrane-embedded parts of NS2B which are lacking in the present structure) at the endoplasmic reticulum membrane where polyprotein processing and viral replication take place.

Peptide boronic acids have previously been tested as drugs, and the proteasome inhibitor bortezomib (Velcade) has been approved for the treatment of multiple myelomas (15). A tetrapeptide-boronic acid was reported as a potent inhibitor of the DENV-2 NS2B-NS3^{pro}, but not studied further (16). Peptide boronic acids are usually not cytotoxic to Huh7 cells, which is what we observe with compound **cn-716** (fig. S7). The structure presented here forms a good starting point for the design of more specific anti-ZIKV drugs.

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S7

Table S1

References (17–28)

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Fig. 1. Crystal structure of the ZIKV NS2B-NS3^{pro} monomer in complex with cn-716. (A) Overall structure of the complex. NS3^{pro} (cyan) and NS2B (purple) are shown as ribbons, with secondary-structure elements labeled. NS3^{pro} is made up by two β -barrels with strand order AI-BI-CI- α I-DI-EIa-EIb-FI and AII-BIIa-BIIb-CII-DII-EIIa-EIIb-FII. NS2B includes β -strands β 1 - β 4. The N- and C- termini of NS2B and NS3^{pro} are indicated by letters in *italics* and *non-italics/underlined*, respectively. The inhibitor **cn-716** is shown with carbon atoms in purple and boron in yellow. Residues of the catalytic triad are in dark blue. * denotes residues from NS2B. (B) The inhibitor **cn-716** is embedded in the substrate-binding site of ZIKV NS2B-NS3^{pro} (same view as in (A)). The surfaces of NS2B and NS3^{pro} are yellow and purple, respectively. An F_o-F_c difference density contoured at 2.5σ is shown for **cn-716**. Lys⁵⁴ from molecule B of the dimer interacts with the inhibitor and is indicated by K54. (C) Chemical structure of **cn-716**. (D) Schematic drawing and (E), F_o-F_c difference density (2.5σ) for the cyclic diester and its environment in molecule A. (F) Difference density (2.5σ) for the cyclic diester and its environment in molecule B.

Fig. 2. The tight, non-crystallographic 2:2 dimer of quasi-twofold symmetry formed by the ZIKV NS2B-NS3^{pro}:inhibitor complex in the asymmetric unit of the crystal. (A) Front view and back view. The surfaces of NS2B and NS3^{pro} of molecule A are shown in cyan and orange, resp., those of molecule B are dark blue and wheat. Labels of molecule B residues are underlined. Residues of NS2B are marked by *. Residues Leu³⁰ (purple/green) and Leu³¹ at the tip of the AI-BI loop (Fig. 1A) form a hook making hydrophobic contacts with the opposing monomer. The Cys¹⁴³ residues forming labile disulfide bonds in the SS-dimer are yellow and pink. (B) A slice through the interior of the dimer, showing the S135 side-chains (blue) covalently bound to the inhibitor molecules. The color code is the same as in (A). Inhibitor molecules are colored purple and red. See fig. S5 for a schematic illustration of the interactions across the dimer interface.

Table 1. Kinetic parameters of variants of ZIKV NS2B-NS3 protease, in comparison to a similar WNV NS2B-NS3^{pro} construct. Data are for the cleavage of the flavivirus protease substrate Bz-Nle-Lys-Lys-Arg-AMC. “Monomer (wt)” [See supplementary materials (p. 4) for details, including definition of “wt” (p. 2)] and “SS-dimer (wt)” indicate enzyme preparations corresponding to the monomer (in the presence of TCEP) and the SS-dimer fraction from gel permeation chromatography. The kinetic parameters for the ZIKV protease with Asp⁸³* replaced by Asn are also included. “WNV NS2B-NS3^{pro} (wt)” is our recombinant preparation of the WNV protease. For comparison, the k_{cat}/K_m values for WNV and DENV-2 NS2B-NS3^{pro} and with the substrate Bz-Nle-Lys-Arg-Arg-AMC reported in (6) are given. (dash indicates not reported). All values in this table are obtained at pH 8.5.

Protease	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat}/K_m (s ⁻¹ M ⁻¹)
ZIKV NS2B-NS3^{pro}			
Monomer (wt)	44.6 ± 1.0	18.3 ± 1.6	2,440,000 ± 215,000
SS-dimer (wt)	28.5 ± 0.6	5.9 ± 0.5	4,850,000 ± 429,000
Cys ⁸⁰ Ser/Cys1 ⁴³ Ser	28.8 ± 0.5	5.1 ± 0.5	5,620,000 ± 546,000
Asp ⁸³ *Asn (monomer)	38.5 ± 1.4	35.3 ± 4.2	1,091,000 ± 136,000
WNV NS2B-NS3^{pro}			
wt	8.7 ± 0.1	77.4 ± 3.6	112,000 ± 5000
wt (6)	—	—	37,000 ± 7000
DENV-2 NS2B-NS3^{pro}			
wt (6)	—	—	30,000 ± 7000

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Crystal structure of Zika virus NS2B-NS3 protease in complex with a boronate inhibitor

Jian Lei, Guido Hansen, Christoph Nitsche, Christian D. Klein, Linlin Zhang and Rolf Hilgenfeld (July 7, 2016)
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Editor's Summary

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