20. D. Q. Fuller, G. Wilcox, R. G. Allaby, Endoplasmatic reticulum membrane, the site of polyprotein processing.  

ACKNOWLEDGMENTS
This paper is a product of the Palaeogenome Analysis Team (PAT).

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7 April 2016; accepted 5 July 2016
Published online 14 July 2016
10.1126/science.aaf7943

RESEARCH | REPORTS

Structural Biology
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, Rolf Hilgenfeld

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 angstrom 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 nonconserved 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), ZIKV infection has recently been found to be responsible for neurological disorders in a substantial 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-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 and furin) and the viral NS2B/NS3 protease into three structural (C, prM, and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and 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 ser355-his51-asp75, and the membrane-bound NS2B (a sequence alignment is available in fig. S2). 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 GlySer-Glyx sequence, displays strong peptidolytic activity (5). Crystal structures of the free form of this protease construct (NS2B-NS3pro) usually reveal an “open conformation” featuring a well-ordered NS3pro core and a flexible NS2B part that shows only limited interaction with NS3pro, whereas inhibitor (and presumably substrate) binding induces a pronounced conformational change of NS2B yielding a more compact, “closed” form (6, 7).

We expressed in Escherichia coli a DNA construct corresponding to the NS2B-NS3pro-coding region of the Brazilian ZIKV isolate BeH823339 (GenBank accession number KU729217.2) (8). This construct codes for residues 49 to 95 of ZIKV NS2B, the C terminus of which is covalently linked via GlySer-Ser-Gly4 to the N terminus of 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 Cys39Ser/Cys53Ser leads to loss of the disulfide bond, which occurs between Cys39 and Cys53 residues of different polypeptide chains, as revealed by our x-ray structure. The SS-dimer and the monomer obtained by reduction with tris(2-carboxyethyl)phosphine (TCEP) (fig. S3) as well as the Cys39Ser/Cys53Ser mutant of ZIKV NS2B-NS3pro are hyperactive against the standard flavivirus protease substrate benzoyl-norleucine-hydroxamic acid (Bz-Nle-Lys-Lys-Arg-AMC), with a very low Michaelis constant (Km) and a specific catalytic efficiency (kcat/Km) 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-NS3pro 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 NS3pro domain as seen previously for other flavivirus proteases, with the NS2B polypeptide
wrapped around the NS3\textsuperscript{pro}. The interaction between the two is stabilized by hydrogen bonds between \(\beta\)-strands \(\beta1\) and \(\beta1\), as well as \(\beta3\) and \(\beta1\) of NS2B and NS3\textsuperscript{pro}, respectively (Fig. 1A). The root mean square deviations between the ZIKV NS2B-NS3\textsuperscript{pro} complex and tetrapeptide aldehyde complexes of WNV and DENV-3 proteases are 0.9 to 1.1 Å [for main-chain atoms; Protein Data Bank (PDB) codes 2FP7 and 3U11 (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\textsuperscript{pro} with half maximal inhibitory concentration (IC\(_{50}\)) = 0.25 ± 0.02 μM and inhibition constant (K\(_I\)) = 0.040 ± 0.006 μM (in the presence of 20% glycerol) (Fig. S4). In the structure of the complex, the boron atom is covalently linked to the side-chain Oy 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 or triols, a prodrug might be obtained that will traverse the cellular membrane more readily than will free boronic acid derivatives.

Because of the ring closure, the tetrahedral geometry of the boron is somewhat distorted. In molecule A, the six-membered ring assumes a boat-like conformation, with the middle hydroxyl group (O2) of glycerol in an axial position and donating an intramolecular hydrogen bond to the carbonyl oxygen of Val36 (Fig. 1F). In molecule B, the six-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 carboxyl oxygen of Val36 (Fig. 1F). In molecule A, the two ring oxygens (O1 and O3) accept H-bonds respectively from the amide of Gly133 of the inhibitor (Fig. 1E). In molecule B, the six-membered ring interacts with the inhibitor and is indicated by underlined K54. (C) Chemical structure of cn-716. (D) Schematic drawing and (E) F\(_{obs}\)-F\(_{calc}\) difference density contoured (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.

The Pt-Arg residue of cn-716 forms a salt-bridge with Asp\textsuperscript{289}, a feature conserved in many flavivirus protease complexes. Most probably protonated, the amino group of the 4-aminomethylphenylalanine residue in the P2 position forms a hydrogen bond with the main-chain oxygen of Ser\textsuperscript{286} and a salt-bridge with Asp\textsuperscript{83X} of the NS2B polypeptide (Fig. 1B; residues of NS2B are denoted by an asterisk). Asp\textsuperscript{289} is Asn in WNV and Ser or Thr in DENV 1–4 NS2B-NS3\textsuperscript{pro} (Fig. S2), 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\textsuperscript{289}Asn mutation leads to an approximately twofold increase of \(k_{cat}/K_m\) and a \(k_{cat}/K_m\) reduced by 55%, as compared with the wild-type (WT) enzyme (Table 1). The Asp residue in this position provides an at least partial explanation for the lower \(K_m\) and hence the much higher \(k_{cat}/K_m\) of ZIKV protease as compared with 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) (7). Because ZIKV also permissively infects human DCs (12), we speculate that an increased catalytic activity of ZIKV NS2B/NS3\textsuperscript{pro} could cause more efficient cleavage of STING, leading to an enhanced suppression of the host innate immunity.

In the crystal, ZIKV NS2B-NS3\textsuperscript{pro} forms an unusual dimer with noncrystallographic, 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. In the tight dimer, the substrate-binding sites of the two monomers,
The presence of the disulfide seems to be essential for crystallization of the ZIKV NS2B-NS3pro, 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-NS3pro complex with cn-716 up to a concentration of 133 μM, we detected it by means of electrospray ionization 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 are perhaps 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-NS3pro but not studied further (16). Peptide boronic acids are usually not cytotoxic to HuH7 cells, which is what we observed with compound cn-716 (fig. S7). The structure presented here forms a good starting point for the design of more specific anti-ZIKV drugs.

Table 1. Kinetic parameters of variants of ZIKV NS2B-NS3 protease, in comparison to a similar WNV NS2B-NS3pro construct.

<table>
<thead>
<tr>
<th>Protease</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{\text{cat}}/K_m$ (s$^{-1}$·M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZIKV NS2B-NS3pro</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monomer (wt)</td>
<td>44.6 ± 1.0</td>
<td>18.3 ± 1.6</td>
<td>2,440,000 ± 215,000</td>
</tr>
<tr>
<td>SS-dimer (wt)</td>
<td>28.5 ± 0.6</td>
<td>5.9 ± 0.5</td>
<td>4,850,000 ± 429,000</td>
</tr>
<tr>
<td>Cys80Ser/Cys143Ser</td>
<td>28.8 ± 0.5</td>
<td>5.1 ± 0.5</td>
<td>5,620,000 ± 546,000</td>
</tr>
<tr>
<td>Asp83Asn (monomer)</td>
<td>38.5 ± 1.4</td>
<td>35.3 ± 4.2</td>
<td>1,091,000 ± 136,000</td>
</tr>
<tr>
<td>WNV NS2B-NS3pro</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>8.7 ± 0.1</td>
<td>77.4 ± 3.6</td>
<td>112,000 ± 5000</td>
</tr>
<tr>
<td>(G)</td>
<td>—</td>
<td>—</td>
<td>37,000 ± 7000</td>
</tr>
<tr>
<td>DENV-2 NS2B-NS3pro</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt (G)</td>
<td>—</td>
<td>—</td>
<td>30,000 ± 7000</td>
</tr>
</tbody>
</table>

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 symmetrically 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-polyacrylamide gel electrophoresis (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-NS3pro, as we failed to obtain crystals of the Cys80Ser/Cys143Ser variant.

Fig. 2. The tight, noncrystallographic 2:2 dimer of quasi-twofold symmetry formed by the ZIKV NS2B-NS3pro-inhibitor complex in the asymmetric unit of the crystal. (A) Front view and back view. The surfaces of NS2B and NS3pro (of molecule A are shown in light blue and orange, respectively; those of molecule B are dark blue and beige. Labels of molecule B residues are underlined. Residues of NS2B are marked by an asterisk. Residues Leu30 (purple/green) and Leu35 at the tip of the A1-B1 loop (Fig. 1A) form a hook, making hydrophobic contacts with the opposing monomer. The Cys345 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 (dark blue) covalently bound to the inhibitor molecules. The color code is the same as in (A). Inhibitor molecules are colored purple and red. A schematic illustration of the interactions across the dimer interface is provided in fig. S5.
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Science 353 (6298), 503-505.
DOI: 10.1126/science.aag2419 originally published online July 7, 2016

Zooming in on the Zika virus protease

The lack of a vaccine or antiviral drugs to combat the Zika virus has scientists scrambling to identify and better characterize potential drug targets. One attractive candidate is the NS2B/NS3 viral protease, which, together with host cell proteases, cleaves the viral polyprotein into the individual proteins required for viral replication. Lei et al. report the crystal structure of this protease bound to a peptido-mimetic inhibitor. The structure reveals key interactions that probably contribute to the high catalytic efficiency of this enzyme relative to other flaviviruses, indicating promising starting points for drug design. Science, this issue p. 503