STRUCTURAL BIOLOGY

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 polypeptide processing; hence, this enzyme is an attractive drug target. The 2.7 Å resolution 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 pronounced conformational change of NS2B and 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 endoplasmic reticulum membrane, the site of polypeptide processing.

Revisedly 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 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 ~10.7-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 and furin) and the viral NS2B/NS3 protease into three structural (C, prM/M, and E) and seven non-structural 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 Ser353-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 Gly-Ser-Gly sequence, displays strong peptidolytic activity (5). Crystal structures of the free form of this protease construct (‘NS2B-NS3(500)’ usually reveal an “open conformation” featuring a well-ordered NS3(400) core and a flexible NS2B part that shows only limited interaction with NS3(400), 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-NS3(500) 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 Gly-Ser-Gly 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 Cys55/Ser/Cys75/Ser leads to loss of the disulfide bond, which occurs between Cys55 and 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 Cys55/Ser/Cys75/Ser mutant of ZIKV NS2B-NS3(500) are hyperactive against the standard flavivirus protease substrate benzoyl-norleucine-hydroxybenzamidine (Bz-Nle-Lys-Lys-Arg-AMC), with a very low Michaelis constant ($K_m$) and a specific catalytic efficiency ($k_{cat}/K_m$) 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(500) 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(400) domain as seen previously for other flavivirus proteases, with the NS2B polypeptide...
Fig. 1. Crystal structure of the ZIKV NS2B-NS3pro monomer in complex with cn-716. (A) Overall structure of the complex. NS3pro (light blue) and NS2B (purple) are shown as ribbons, with secondary-structure elements labeled. NS3pro is made up by two β-barrels with strand orders Al-Bi-Cl-ια-De-Elb-Fi and Al-βi-Bilβ-Ci-Dil-Eilla-Elb-Fill. NS2B includes β-strands β1 to β4. The N- and C-termini of NS2B and NS3pro are indicated by letters in italics and nonitalics/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. Asterisk denotes residues from NS2B. (B) The inhibitor cn-716 is embedded in the substrate-binding site of ZIKV NS2B-NS3pro [same view as in (A)]. The surfaces of NS2B and NS3pro are yellow and purple, respectively. A Fobs/Fcalc 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 underlined K54. (C) Chemical structure of cn-716. (D) Schematic drawing and (E) Fobs/Fcalc 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.

wrapped around the NS3pro. The interaction between the two is stabilized by hydrogen bonds between β-strands β1 and Al, β2 and Bi1a, as well as β3 and Bi1b of NS2B and NS3pro, respectively (Fig. 1A). The root mean square deviations between the ZIKV NS2B-NS3pro 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 3UI1 (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-NS3pro with half-maximal inhibitory concentration (IC50) = 0.25 ± 0.02 μM and inhibition constant (Ki) = 0.046 ± 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 and triols, especially if five- or six-membered rings can be formed (10). Our Fobs/Fcalc (observed and calculated structure-factor amplitudes, respectively) 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 IC50 for the boronic acid inhibitor was nearly unchanged (0.20 ± 0.02 μM), but throughester formation with larger, more hydrophobic diols or triols, a prodrg 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 main-chain oxygen of the P2 residue of the inhibitor (Fig. 1E). 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 carbonyl oxygen of Val36 (Fig. 1F). In molecule A, the two ring oxygens (O1 and O3) accept H-bonds respectively from the amide of Gly133 (from the oxygen hole) and from the catalytic His31, 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 Pt-Arg residue of cn-716 forms a salt-bridge with Asp29, a feature conserved in many flavivirus protease complexes. Most probably protonated, the amino group of the 4-aminomethylphenylalanyl residue in the P2 position forms a hydrogen bond with the main-chain oxygen of Ser81 and a salt-bridge with Asp83* of the NS2B polypeptide (Fig. 1B; residues of NS2B are denoted by an asterisk). Asp83* is Asn in WNV and Ser or Thr in DENV 1–4 NS2B-NS3pro (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 Asp83*Asn mutation leads to an approximately twofold increase of Km and a kcat/Km 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 Km and hence the much higher kcat/Km 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) (11). Because ZIKV also permissively infects human DCs (12), we speculate that an increased catalytic activity of ZIKV NS2B/NS3pro could cause more efficient cleavage of STING, leading to an enhanced suppression of the host innate immunity.

In the crystal, ZIKV NS2B-NS3pro 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,
After a few seconds of the crystal in the x-ray beam, 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 Leu19 at the tip of the A-E loop (Fig. 1A) form a hook, making hydrophobic contacts with the opposing monomer. The Cys43 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.

Table 1. Kinetic parameters of variants of ZIKV NS2B-NS3pro protease, in comparison to a similar WNV NS2B-NS3pro construct. Data are for the cleavage of the flavivirus protease substrate Bz-Nle-Lys-Lys-Arg-AMC. “Monomer (wt)” (details, including definition of “wt”, are provided in the supplementary materials), 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-NS3pro (wt)” is our recombinant preparation of the WNV protease. For comparison, the $k_{cat}/K_m$ values for WNV and DENV-2 NS2B-NS3pro and with the substrate Bz-Nle-Lys-Arg-Arg-AMC reported in (6) are given. Dashes indicate “not reported.” All values in this table are obtained at pH 8.5.

<table>
<thead>
<tr>
<th>Protease</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}/K_m$ (s⁻¹ M⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>ZIKV NS2B-NS3pro</td>
<td></td>
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<tr>
<td>Monomer (wt)</td>
<td>44.6 ± 1.0</td>
<td>18.3 ± 1.6</td>
<td>2.440 ± 215.000</td>
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<tr>
<td>SS-dimer (wt)</td>
<td>28.5 ± 0.6</td>
<td>5.9 ± 0.5</td>
<td>4.850 ± 429.000</td>
</tr>
<tr>
<td>Cys80Ser/Cys143Ser</td>
<td>28.8 ± 0.5</td>
<td>5.1 ± 0.5</td>
<td>5.620 ± 546.000</td>
</tr>
<tr>
<td>Asp83*Asn (monomer)</td>
<td>38.5 ± 1.4</td>
<td>35.3 ± 4.2</td>
<td>1.091 ± 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>112.0 ± 5000</td>
</tr>
<tr>
<td>(6)</td>
<td></td>
<td></td>
<td>37.0 ± 7000</td>
</tr>
<tr>
<td>DENV-2 NS2B-NS3pro</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt (6)</td>
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<td>30.0 ± 7000</td>
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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 Cys43 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 (Cys43 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.

Formation of the tight dimer in the asymmetric unit buries $\sim$1240 Å² of the surface of each of the two monomers, and the shape complementarity ($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, $\sim$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.

REFERENCES AND NOTES


ACKNOWLEDGMENTS

We thank S. Zoske and Y. Gül for assistance, S. Anemüller for recording the mass spectra, L. Weigel for the synthesis of precursors of cn-716, and D. Graf for testing this compound for cytotoxicity. We are indebted to the staff of synchrotron beamlines BL 14.1 (BESSY, Berlin) and P11 (DESY, Hamburg) for support. The corresponding coordinates and structure factors are available from the PDB under accession code 5LCO. Funding from the German Center for Infection Research (DZIF-17TU01, grant 8701801911) is gratefully acknowledged. C.D.K. thanks the Deutsche Forschungsgemeinschaft for financial support (KL-1356/3-1).

SUPPLEMENTARY MATERIALS

www.sciencemag.org/cgi/content/353/6298/503/suppl/DC1

Figs. S1 to S7

Table S1

References (17–28)

28 May 2016; accepted 30 June 2016

Published online 7 July 2016
10.1126/science.aag2419
Crystal structure of Zika virus NS2B-NS3 protease in complex with a boronate inhibitor
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Science 353 (6298), 503-505,
DOI: 10.1126/science.aag2419originally 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