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LETTER TO THE EDITOR

Mechanisms of activation and inhibition of Zika virus NS2B-NS3 protease

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Dear Editor,

Since ZIKV outbreak was first reported in South and Central America and the Caribbean, it has spread to both North America and Asia [1]. Preliminary reports have now estimated as many as 1.3 million cases of infection in this widespread epidemic [2]. Initially it was thought that the ZIKV only causes mild symptoms such as fever, myalgia, and rash; recent studies have revealed that ZIKV can also cause severe neurological pathologies such as neonatal microcephaly and Guillain-Barre syndrome [3]. In response to the spreading epidemic of ZIKV infection, the World Health Organization has declared a Public Health Emergency of International Concern [4]. ZIKV is a mosquito-borne flavivirus, whose NS3 protease (NS3pro) is required for proteolytic processing of its polyprotein to enable viral replication [5], thus making NS3pro an attractive therapeutic target for inhibiting viral proliferation. In the past, successful structure-based drug development against NS3pro of the hepatitis C virus (HCV) has generated a series of United States Food and Drug Administration (FDA)-approved inhibitors with potent efficacy for viral eradication [6]. Flavivirus NS3pro exists predominantly in an inactive state; however, upon association with the NS2B viral protein, its enzymatic activity increases by 3 300-6 600-fold [7]. The recent structure of ZIKV NS2B-NS3pro in complex with a boronate inhibitor has provided some important insights for drug design [8]. However, it remains still largely unknown the exact molecular mechanism of ZIKV NS3pro activation by NS2B, which requires a structure of the apo NS2B-NS3pro complex. An improved understanding of the mechanism of NS3pro activation can lead to identification of novel hot spots that can be targeted in rational drug design.

We designed a single-chain fusion protein of NS2B and NS3pro by connecting the 45-residue core region (residues 48-92) of ZIKV NS2B to the N-terminus of NS3pro (residues 1-170) via a glycine-serine linker (G_4SG_4) (Supplementary information, Figure S1A). The amino acid sequences of NS2B and NS3pro were derived from the Z1106033 ZIKV strain (NCBI ID: KU312312) isolated from Suriname [9] (Supplementary information, Figure S1B). The NS2B-NS3pro fusion protein was purified from *E. coli*, and gel filtration analysis revealed that majority of this complex existed in a monomeric form at various protein concentrations (Supplementary information, Figure S1C). NS2B-NS3pro can be activated readily by adding a widely used synthetic substrate AC-LKKR-AMC [10]. Using Michaelis-Menten kinetics we determined that the K_m and k_{cat} are 85.3 ± 2.1 μM and 1.149 ± 0.069 s^{-1}, respectively (Supplementary information, Figure S1D), which are comparable to the dengue virus (DENV) and the West Nile virus (WNV) NS2B-NS3pros [11, 12], but different from ZIKV NS2B-NS3 (R95A/R29G), which has an increased activity [8]. To discover potential inhibitors of ZIKV, we screened small molecule compounds against ZIKV NS2B-NS3pro. Aprotinin [13], a competitive serine protease inhibitor used to reduce bleeding during complex surgery, was found to be a potent inhibitor of ZIKV NS2B-NS3pro with a K_i of 361 ± 19 nM (Supplementary information, Figure S1E). This finding suggests that ZIKV NS2B-NS3pro may contain a canonical catalytic site of a serine protease that can serve as a drug target.

To investigate the molecular mechanism of NS3pro activation by NS2B, we determined the crystal structure of the ZIKV NS2B-NS3pro at a resolution of 2.6 Å (Figure 1A). Two monomers of NS2B-NS3pro are present in the asymmetric unit. Each monomer is composed of two β-barrels, which is reminiscent of the chymotrypsin-like fold. The catalytic triad (His51-Asp75-Ser135) is located at the cleft between the two β-barrels, and the two β-barrels are surrounded by NS2B. In the absence of an inhibitor or substrate, the apo ZIKV NS2B-NS3pro adopts a "relaxed conformation" similar to other flavivirus NS2B-NS3pros [14]. The root-mean-square deviations between ZIKV NS2B-NS3pro and those of WNV and DENV proteases are ~1.3 Å (for Cα; PDB IDs: 2YOL and 3L6P).

Superimposition of the structure of apo ZIKV NS2B-NS3pro (Figure 1A) and the structure of the boro-

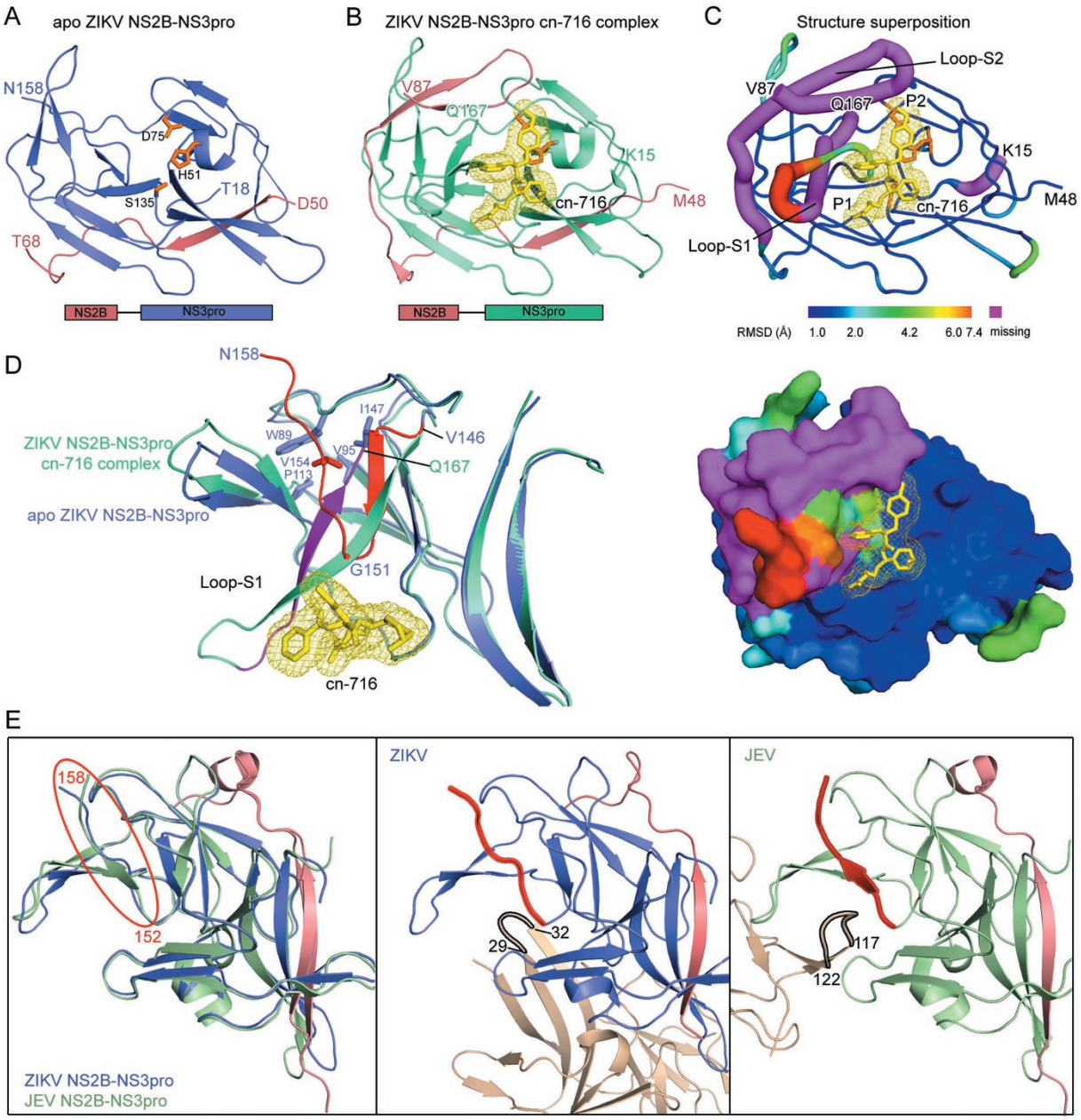

Figure 1 Mechanisms of activation and inhibition of ZIKV NS3pro. **(A)** Overall structure of apo ZIKV NS2B-NS3pro and **(B)** its complex with cn-716 (PDB ID 5LC0). Proteins are shown as ribbons with NS2B and NS3pro colored differently. The N- and C-termini of the proteins are numbered. The catalytic residues are shown as orange sticks. The inhibitor cn-716 is shown as mesh and sticks and colored in yellow. **(C)** Putty (upper panel) and surface (lower panel) representations of the superposition of inhibitor-bound and -unbound structures of ZIKV NS2B-NS3pro. The apo structure is aligned to the cn-716-bound structure (PDB ID 5LC0) using SHP [15]. The color spectrum and the coil thickness represent the root-mean-square deviation (RMSD) of the aligned Cα atoms. Disordered and not observed regions in the apo structure are shown in magenta. Inhibitor is shown as yellow meshes and sticks. **(D)** Close-up view of loop-$S1_{152-167}$. The apo-structure is colored in blue and the inhibitor complex in green. Disordered region in the apo-structure is colored in magenta. The region with significant conformational changes in loop-S1 is highlighted in red. **(E)** "Auto-inhibitory" mode of flavivirus NS3pros. Apo-structures of ZIKV and JEV NS2B-NS3pro (PDB ID 4R8T) are overlaid, demonstrating that the residues 152-158 (circled with red oval) from the loop-$S1_{152-167}$ adopt a similar conformation. NS2B is colored in salmon in both structures. Neighboring molecules in the crystals are shown in wheat in both structures. The loops in the neighboring molecules that are involved in packing interactions are marked and numbered accordingly.

nate inhibitor complex (Figure 1B) reveals that the C-terminal loop$_{69-87}$ of NS2B adopts a distinct conformation in between the canonical apo form and the inhibitor-bound state (Figure 1C). In the apo structure, the C-terminus of NS2B (residues 69-87) is largely unseen, highlighting its high intrinsic flexibility. This is in sharp contrast to DENV apo NS2B-NS3pro, where the C-terminal loop adopts a defined conformation in spite of the discontinuous electron density beyond residue 76 [14]. In the ZIKV inhibitor-bound structure, this region of NS2B (residues 69-87) wraps around NS3pro, participating in interactions that stabilize the NS2B and NS3pro complex. In particular, residues 76-87 are responsible for constituting a negatively-charged S2 subsite, which creates a preference for a basic residue at P2.

Additional conformational differences between the inhibitor-bound and the apo structures are found in loop$_{152-167}$ of NS3pro (Figure 1D). In the absence of the inhibitor, residue 151G induces a sharp kink, causing the subsequent residues 152-158 to pack between β-strand$_{123-126}$ and β-strand$_{147-150}$. Besides the main chain interactions, 154V snugly inserts into a hydrophobic pocket formed by the side chains of 89W, 95V, 113P and 147I in NS3pro. The rest of the residues (159-167) are not observed in the structure, possibly due to their exposure to solvent. In contrast, in the inhibitor-bound structure, residues 152-167 constitute a loop that is involved in S1 subsite formation. This indicates that the substrate/inhibitor binding is required to induce the appropriate configurations of these two loops (loop-S1: residues 152-167; loop-S2: residues 69-87) to form the mature S1 and S2 subsites in ZIKV NS2B-NS3pro. Both S1 and S2 subsites are essential for catalysis thus deletion of either loop-S1$_{152-167}$ or loop-S2$_{69-87}$ abolishes enzymatic activity (Supplementary information, Figure S1F).

Further analysis of loop-S1$_{152-167}$ in the apo structure of NS2B-NS3pro suggests that this loop is a molecular switch between the inactive and activate state of NS3pro. Since residues 152-167 are the key components for S1 subsite, their conformation is critical for enzymatic activity. The configuration of this loop in our structure may represent an auto-inhibitory mode (Figure 1E), which is distinct from DENV and WNV apo NS2B-NS3pro structures [12, 14]. In our crystal structure, this loop (in molecule A) is pushed away by residues 29-32 from the neighboring molecule (molecule B) (Figure 1E, middle panel), resulting in a conformation that is unfavorable for catalysis. Interestingly, a similar phenomenon was observed in the structure of Japanese encephalitis virus (JEV) NS2B-NS3pro (Figure 1E, right panel), where the same loop adopts a surprisingly identical conformation although the crystal-packing environment differs substantially (Figure 1E, left panel). The conformational changes observed in our apo structure demonstrate how loop-S1$_{152-167}$ can potentially act as a switch between the active and inactive (auto-inhibition) states. This region may represent a novel target site for rational drug design of small molecules that can "lock" this loop in the inactive conformation.

In conclusion, we present the crystal structure of the apo ZIKV NS2B-NS3pro complex in a monomeric form. Two loops, loop-S1$_{152-167}$ and loop-S2$_{69-87}$, which are critical for the formation of S1 and S2 subsites in NS2B-NS3pro, undergo large conformational changes upon substrate/inhibitor binding. Out structure reveals a molecular mechanism for ZIKV NS3pro inhibition and identifies a new target for rational drug design against flavivirus.

The atomic coordinates and structure factors for the structure have been deposited in the Protein Data Bank (PDB) with the accession code 5GXJ.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)