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Identification of novel small molecule inhibitors against NS2B/NS3 serine protease from Zika virus

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Abstract

Zika flavivirus infection during pregnancy appears to produce higher risk of microcephaly, and also causes multiple neurological problems such as Guillain–Barré syndrome. The Zika virus is now widespread in Central and South America, and is anticipated to become an increasing risk in the southern United States. With continuing global travel and the spread of the mosquito vector, the exposure is expected to accelerate, but there are no currently approved treatments against the Zika virus. The Zika NS2B/NS3 protease is an attractive drug target due to its essential role in viral replication. Our studies have identified several compounds with inhibitory activity (IC_{50}) and binding affinity (K_D) of ~5–10 μ M against the Zika NS2B–NS3 protease from testing 71 HCV NS3/NS4A inhibitors that were initially discovered by high-throughput screening of 40,967 compounds. Competition surface plasmon resonance studies and mechanism of inhibition analyses by enzyme kinetics subsequently determined the best compound to be a competitive inhibitor with a K_i value of 9.5 μ M. We also determined the X-ray structure of the Zika NS2B–NS3 protease in a “pre-open conformation”, a conformation never observed before for any flavivirus proteases. This provides the foundation for new structure-based inhibitor design.

Graphical abstract

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Keywords

Zika flavivirus; NS2B/NS3 serine protease; Small molecule inhibitor; apo ZIKV NS2B-NS3^{pro} structure

1. Introduction

The Zika flavivirus (ZIKV) is closely related to Dengue, West Nile and Yellow Fever viruses (Kuno et al., 1998); infection results in relatively mild clinical symptoms including mild fever, rash, arthritis, headache, conjunctivitis and edema that seldom result in hospitalization or death (Paixao et al., 2016). ZIKV was isolated in 1947 in the Zika forest in Uganda (Dick et al., 1952), and although *Aedes aegypti* is the main epidemic vector, the virus has been isolated from multiple *Aedes* species that are probably also involved in ZIKV transmission to humans (Paixao et al., 2016). Initially endemic in Africa, ZIKV is now spreading explosively in Latin America, with cases also reported in Europe, Oceania, southeast Asia and throughout the Americas (Dyer, 2015; Gatherer and Kohl, 2016; Gulland, 2016; Imperato, 2016; Malone et al., 2016; Paixao et al., 2016). Infection during pregnancy appears to produce higher risk of microcephaly (Mayor, 2016; Miranda-Filho Dde et al., 2016; Paixao et al., 2016; Vogel, 2016; Weaver et al., 2016), with ZIKV targeting the neural stem cell receptor AXL (Nowakowski et al., 2016). Recent observations also suggest that ZIKV infection can induce Guillain–Barré syndrome (Paixao et al., 2016; Smith and Mackenzie, 2016; Wise, 2016), with indications that ZIKV is highly neurotropic, inducing multiple neuronal effects (Chan et al., 2016; Nowakowski et al., 2016). Although a Zika vaccine is urgently needed, development is likely some years away (Cohen, 2016; Weaver et al., 2016). Thus, alternative therapeutics are needed, both for prophylaxis to prevent or inhibit infection, and for post-infection therapy (Malone et al., 2016; Weaver et al., 2016).

Zika is a small enveloped positive single-stranded RNA virus within the genus *Flavivirus* of the family *Flaviviridae* (Cunha et al., 2016) that also includes the closely related West Nile and Dengue Viruses (Gould and Solomon, 2008). Although there is no evidence yet for highly divergent strains in the current Latin American epidemic (Cunha et al., 2016; Malone et al., 2016), it is likely that ZIKV will be subject to high mutation rates due to the lack of a proof reading function by the NS5 RNA polymerase domain. ZIKV encodes a single polyprotein containing three structural and seven non-structural proteins, two of which form a single essential viral protease complex, the NS2B/NS3 serine protease (Fig. 1A) (Faye et al., 2014). Based on the Dengue and West Nile precedents, the NS2B/NS3 protease is expected to cleave five sites, releasing the resulting non-structural proteins (Sampath and Padmanabhan, 2009). Additionally, the NS2B/NS3 protease has been implicated in immune evasion through cleavage of the human mediator of activation of interferon regulatory factor

3 activator, down regulating the antiviral responses triggered by Dengue infection (Aguirre et al., 2012). Thus, NS2B/NS3 is probably a dual function target, whose inhibition should both inhibit viral replication and protect innate immunity. NS3 contains a protease domain at the N-terminus and an RNA helicase domain at the C-terminus. The protease domain belongs to the trypsin/chymotrypsin protease superfamily, and the catalytic triad is comprised of residues Ser135, His51 and Asp75 (Fig. 1B and C) (Erbel et al., 2006). NS3 requires the NS2B, membrane-bound protein, to position the NS3 catalytic triad and its substrate (Noble et al., 2012). Although the overall structure of the Zika virus has been published (Sirohi et al., 2016) there are as yet no crystal structures of the full-length Zika NS2B/NS3 protein deposited in the PDB. However, structures of the individual protease and helicase domains of NS3 with an inhibitor and ATP, respectively, were recently published (Chen et al., 2016; Lei et al., 2016; Phoo et al., 2016; Tian et al., 2016). The C-terminal region of NS2B contributes to the NS3 catalytic pocket shape, forming part of the recognition site (Aleshin et al., 2007; Erbel et al., 2006; Hammamy et al., 2013; Robin et al., 2009).

There has been very limited research in development of Zika therapeutics to date, although testing and repurposing hepatitis C virus (HCV) therapeutics has been proposed (Malone et al., 2016). A closer precedent is the development of therapeutics for Dengue virus (DENV) and West Nile virus (WNV) that target the non-structural proteins that are essential for viral replication; which is being pursued by many research groups and includes development of inhibitors for the NS2B/NS3 protease, the NS3 helicase domain, and the NS5 methyl transferase and RNA polymerase domains (Bhakat et al., 2014; Lim et al., 2013; Noble and Shi, 2012). Although there is promising progress, particularly for Dengue, most research is still in the early lead phase, with efficacy still far behind the development of therapeutics for the related HCV. The NS2B/NS3 protease is a particularly promising flavivirus therapeutic target, with extensive research on development of inhibitors as therapeutic candidates. Various strategies for Dengue NS2B/NS3 inhibitor development were recently reviewed (Luo et al., 2015), including design of both peptidic (Prusis et al., 2013; Xu et al., 2012) and non-peptidic small molecule inhibitors (Cabarcas-Montalvo et al., 2016; Chu et al., 2015; Deng et al., 2012; Lai et al., 2013a; Lai et al., 2013b; Li et al., 2015; Liu et al., 2014; Tomlinson and Watowich, 2011; Viswanathan et al., 2014; Wu et al., 2015; Zhou et al., 2013). There are already indications that resistance against protease inhibitors develops readily (Yang et al., 2011; Yang et al., 2014), consistent with the HCV protease inhibitor experience (Romano et al., 2010). The high error rate of the Zika polymerase is likely to produce a diversity of viral variants, facilitating resistance development, similar to the HCV protease inhibitor experience (Halfon and Locarnini, 2011). Thus, development of Zika NS2B/NS3 protease inhibitors with activity against potential drug-resistant mutants would be highly desirable for antiviral therapy and prophylaxis. Here, we report multiple inhibitors identified by repurposing our previously identified HCV NS3/NS4A inhibitors (Lee et al., 2013). Inhibitory activity and direct binding of these compounds against Zika NS2B/NS3^{pro} were also explored followed by mechanism of inhibition studies. Additionally, during our attempts to determine the X-ray structure of Zika NS2B/NS3^{pro} in complex with these inhibitors, we identified an apo conformation of the NS2B/NS3^{pro} never observed before for

any flavivirus proteases. The new protein structure is in a “pre-open confirmation” and represents a new targetable folding of the NS2B/NS3^{pro} for structure-based inhibitor design.

2. Materials and methods

2.1. Plasmid construction and purification of Zika NS2B-NS3^{pro}

The gene of ZIKV NS3 protease (residues 1-187) with a His-tag at the N-terminus and NS2B (residues 46-99) were codon-optimized, synthesized (BioBasic Inc.), and cloned into a pET15b vector between NdeI and BamHI. A human rhinovirus (HRV) 3C protease cleavage site was inserted right before NS2B. One liter of Rosetta2(DE3) cells containing the recombinant Zika NS2B-NS3^{pro} gene were grown to an OD₆₀₀ of 0.65- 0.7 at 37 °C in LB medium. The cells were then induced with 0.5 mM IPTG for 16 hours at 25 °C. Harvested cells were lysed by sonication in lysis buffer (1 mg/mL lysozyme in buffer A: 50 mM Tris (pH 8.5), 500 mM NaCl, 20 mM Imidazole, 5% glycerol, and 5 mM β-mercaptoethanol). The His-tag fused ZIKV NS2B-NS3^{pro} was purified by a HisTrap HP column (GE Healthcare) with a stepwise gradient of buffer B (50 mM Tris (pH 8.5), 500 mM NaCl, 500 mM imidazole, 5% glycerol, and 5 mM β-mercaptoethanol). The pooled ZIKV NS2B-NS3^{pro} was then dialyzed with buffer A without imidazole and the His-tag was cleaved by 2 units/mL of HRV 3C protease (Novagen) overnight at 4 °C, producing a ZIKV NS2B-NS3^{pro} with only two extra residues (GP) at the N-terminus. Finally, ZIKV NS2B-NS3^{pro} was again loaded onto the HisTrap column to remove HRV 3C protease, cleaved His-tags, and uncleaved His-tagged ZIKV NS2B-NS3^{pro}. Purified protein with approximately 95% purity was flash frozen in liquid nitrogen for storage at -80 °C.

2.2. Kinetic parameter determination

The activities of both the His-tagged and native ZIKV NS2B-NS3^{pro} enzymes were measured by continuous kinetic assay with AMC substrates Boc-Gly-Arg-Arg-AMC (Bachem Bioscience) that generates a fluorescence signal (excitation λ: 360 nm; emission λ: 450 nm) when aminomethyl-coumarine (AMC) is cleaved. The ZIKV NS2B-NS3^{pro} assays were performed in the assay buffer containing 50 mM Tris, pH 7.5, 1% Chaps, 20% glycerol, and 5 mM reduced glutathione (GSH). A series of substrate concentrations (0 to 250 μM) was prepared in triplicate, and the enzyme reaction was initiated by adding ZIKV NS2B-NS3^{pro} enzyme (50 nM final concentration). The same series of substrate concentrations without any enzyme was also measured as controls. Fluorescence intensity was continuously monitored for at least 10 minutes with a POLARstar OPTIMA microplate reader (BMG LABTECH). All assays were performed in 21 μL final assay volume in 384-well low volume black microplates (Corning Inc.). The Michaelis-Menten constant (K_M) and maximal activity (V_{max}) were calculated using Sigmaplot v12.0 by fitting the data with

a single rectangular hyperbolic equation ($y = \frac{V_{max}x}{K_M + x}$) also known as the Michaelis-Menten equation where y is initial velocity and x is the concentration of substrate.

2.3. Initial compound activity test and IC₅₀ value determination

For initial inhibitor test, 10 mM stock solutions of the 71 compounds (Life Chemicals and synthesized) were prepared in 100% DMSO. 7 μL of 3× ZIKV NS2B-NS3^{pro} (final 100 nM)

was distributed to a 384-well plate, and 7 μL of 3 $\times$ compounds were added followed by 10 min incubation at room temperature. Enzyme reaction was initiated by adding 7 μL of 3 $\times$ substrate (final 50 μM), and fluorescence intensity was continuously monitored for at least 6 min with a POLARstar OPTIMA microplate reader (BMG LABTECH). All compounds were tested in triplicate.

IC_{50} values were measured in the same concentration of enzyme and substrate as in the initial screening with a series of compound concentrations (0 to 200 μM). The enzyme reaction was initiated by adding fluorogenic substrate, and its activity was continuously monitored for at least 10 minutes. The IC_{50} values were calculated by fitting with the three

parameter Hill equation, $y = V_{\max} \left(\frac{x^n}{\text{IC}_{50}^n + x^n} \right)$, with Sigmaplot v12.0 where y is percent inhibition, x is inhibitor concentration, n is the slope of the concentration–response curve (Hill slope), and $V_{\max}$ is maximal inhibition from three independent assays.

2.4. Determination of the dissociation equilibrium constant (K_D) by SPR

The sensor surface was first activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/N-hydroxy succinimide (NHS) mixture. The purified ZIKV NS2B-NS3^{pro} was diluted with 10 mM sodium acetate (pH 4.0) at 50 $\mu\text{g}/\text{mL}$ concentration and immobilized on flow cells 2 and 4 in PBSP (20 mM phosphate, pH 7.4, 2.7 mM KCl, 137 mM NaCl, 0.05% Tween-20) as a running buffer using a Biacore T200 instrument (GE Healthcare) by standard amine coupling. Unmodified surfaces on flow cells 1 and 3 were used as controls. All analyzed compounds were initially prepared as 10 mM stocks in 100% DMSO solutions. Compound solutions with a series of increasing concentrations (0 – 100 μM at 2-fold dilution) were applied to all four channels in SPR binding buffer (PBSP + 0.5 mM TCEP) at a 30 $\mu\text{L}/\text{min}$ flow rate at 25 $^{\circ}\text{C}$. Response unit (RU) values at each concentration were measured during the equilibration phase for steady-state affinity fittings using Biacore T200 evaluation software v3.0 with an embedded equation, $y = y_{\max} \cdot x / (K_D + x)$, where y is the response, $y_{\max}$ is the maximum response and x is tested compound concentration. All data were referenced with blank cell RU values.

2.5. Mechanism of inhibition

Enzyme activities of ZIKV NS2B-NS3^{pro} were investigated in the same way as the IC_{50} value determination with varying concentration of both substrate (0–250 μM) and inhibitors. The concentration of compounds was varied from 0 to at least 5 $\times$ the IC_{50} value of each compound. The data were fit to four equations (1–4) using SigmaPlot Enzyme Kinetics Module 1.3 in order to determine the best fit inhibition mechanism and kinetic parameters for each compound.

$$\text{Competative inhibition} \quad \nu = \frac{V_{\max}}{\left(1 + \frac{K_m}{[S]}\right) \left(1 + \frac{[I]}{K_i}\right)} \quad (1)$$

Non-competitive inhibition

$$\nu = \frac{V_{\max}}{\left(1 + \frac{[I]}{K_i}\right) \left(1 + \frac{K_m}{[S]}\right)} \quad (2)$$

Uncompetitive inhibition

$$\nu = \frac{V_{\max}}{1 + \frac{[I]}{K_i} + \frac{K_m}{[S]}} \quad (3)$$

Mixed-type inhibition

$$\nu = \frac{V_{\max}}{\left(\frac{K_m}{[S]}\right) \left(1 + \frac{[I]}{K_i}\right) + \left(1 + \frac{[I]}{\alpha K_i}\right)} \quad (4)$$

where ν is the reaction rate, $V_{\max}$ is the maximum rate of the reaction, K_m is the Michaelis-Menten constant for the substrate, $[S]$ is the substrate concentration, $[I]$ is the inhibitor concentration, K_i is the dissociation constant of the inhibitor I to the free enzyme and αK_i is the dissociation constant for the inhibitor I to the ES complex.

2.6. Docking studies

The Protein Preparation Wizard in the Schrödinger Suite (Schrödinger, 2016c) was used to optimize the crystal structure of the ZIKV in complex with a boronate inhibitor (PDB code 5LC0). Since 5LC0 is a crystallographic dimer, a single NS2B-NS3 construct (chain A) was selected for docking. Restrained minimization was performed on hydrogens, and then the RMSD of the heavy atoms was converged to less than 0.30 Å in OPLS3 force field. The 3D structures of compound **3** were created by LigPrep (Schrödinger, 2016b). The OPLS3 force field was applied for ligand geometric optimization with all possible ionization and tautomeric forms created at pH 7.4 by EPIK (Schrödinger, 2016a; Shelley et al., 2007). Default values were utilized for other parameters for protein and ligand preparations. Molecular docking was performed using GOLD v5.2.2 (Verdonk et al., 2003) with the above prepared protein and ligand. The active site for ZIKV was defined as the shell of residues within 10 Å around the catalytic residue Ser135 in the NS3 protease domain. The protein was fixed during docking and other default parameters were used. The best scoring pose for compound **3** was chosen for analysis.

2.7. X-ray data collection and structure determination

Zika NS2B-NS3^{Pro} was concentrated using Vivaspin centrifugal concentrators (GE Healthcare Life Sciences) to 12.8 mg/mL in 1 M Sodium chloride, 0.05 M Tris-HCl pH 8.5. Sitting drop crystallization plates were set up at room temperature and crystals were obtained in the Classics II screen (Qiagen; 1 µL of concentrated protein plus 1 µL of reservoir) in condition H3: 0.24 M Sodium malonate pH=7.0, 20% (w/v) PEG 3350. Harvested crystals were transferred to the reservoir solution before being rapidly cooled in liquid nitrogen. Diffraction data were collected at 100 K at the Life Sciences Collaborative Access Team at the Advance Photon Source, Argonne, Illinois (APS BEAMLINE 21- ID-F).

All structural work was performed by the CSGIK (Anderson, 2009). Data were processed using HKL- 2000 for indexing, integration, and scaling (Otwinowski Z, 1997). The crystal structure of Zika virus NS2B-NS3 protease in complex with a boronate inhibitor (Lei et al., 2016) (PDB code 5LC0) was used for molecular replacement to phase our structure. Structures were refined with Refmac (Murshudov et al., 2011). Models were displayed in Coot and manually corrected based on electron density maps (Emsley and Cowtan, 2004). Structure figures were prepared using PyMOL Molecular Graphics System, Version 1.3 (Schrödinger, LLC). The structure was deposited in the PDB (www.wwpdb.org) with PDB code 5T1V.

3. Results and Discussion

3.1. Kinetic parameter determination and assay optimization

ZIKV encodes a single essential viral protease complex, the NS2B/NS3 serine protease. A single chain NS2B-NS3^{pro} enzyme of ZIKV was overexpressed in *E.coli* with a linker consisting of GGGGSGGGG between NS2B core region and NS3 protease domain (Fig. 2A). Based on other flavivirus proteases, potential cleavage sites of ZIKV NS2B-NS3 within non-structural protein are summarized in Fig. 2B. Among the commercially available substrates of flaviviruses, we used Boc-GRR-AMC for kinetic parameter determination and assay optimization for testing compounds. The K_M value of ZIKV NS2B-NS3^{pro} was 136 μM (Table 1) with this substrate, which is much larger than the reported K_M value (18.3 μM) with another substrate (Bz-Nle-KRR-AMC) (Lei and Hilgenfeld, 2016). Our substrate, Boc-GRR-AMC, contains similar residues (GKR) of two cleavage sites (NS2A/NS2B and NS2B/NS3), while Bz-Nle-KRR-AMC contains residues (KRR) of one cleavage site between NS4B and NS5 (Fig. 2B). The two K_M values indicate that ZIKV NS2B-NS3^{pro} enzyme has higher affinity to Bz-Nle-KRR-AMC than Boc-GRR-AMC. The k_{cat} value of ZIKV enzyme was 10.0 s^{-1} , and the k_{cat}/K_M value of ZIKV NS2B-NS3^{pro} was 73786 $\text{s}^{-1}\text{M}^{-1}$. Our ZIKV NS2B-NS3^{pro} enzyme was initially over-expressed with a hexahistidine tag with an enzyme cleavage site to yield as native a construct as possible after cleaving the His₆-tag; the K_M value of the His₆-tagged ZIKV NS2B-NS3^{pro} was ~2-fold larger than that of native one, resulting in a slightly smaller k_{cat}/K_M value. Based on the determined K_M value, we decided to use 50 μM substrate for testing potential inhibitors. Other important factors such as reducing agents, detergents, and DMSO tolerance of the Zika enzyme were investigated to determine optimal assay conditions to test a large number of compounds.

3.2. Repurposing of HCV NS3/NS4A as inhibitors of ZIKV NS2B/NS3

From a high throughput screen of more than 40,000 compounds, we previously identified an HCV NS3/NS4A inhibitor with an IC₅₀ value of 2.2 μM against the NS3/NS4A from genotype 1b that is competitive with respect to substrate, indicating direct binding to the protease active site (Lee et al., 2013). The inhibitor was also selective for NS3 from multiple HCV genotypes over two human serine proteases, trypsin and chymotrypsin. Subsequently, we identified 71 HCV protease inhibitors with low μM activity following the overall workflow shown in Fig. 3A. Noting the strong structural similarity of the catalytic region between the ZIKV NS2B/NS3 and the HCV NS3/NS4A proteases, we tested 71 of the promising HCV NS3/NS4A inhibitors, and identified 10 compounds with inhibitory activity

(IC₅₀) values less than 50 μ M (Fig. 3B). SPR analysis of direct binding measurements (K_D) are also generally consistent with IC₅₀ inhibition values, validating them as positive hits. SPR sensorgrams and a steady-state affinity fitting curve of compound **3** are shown in Fig. 3C and 3D as an example. The IC₅₀ value of compound **3** was 4.1 μ M, which was comparable to its determined K_D at 9.0 μ M. Of ten tested compounds, six exhibited both IC₅₀ and K_D values below 20 μ M. These ten compounds were categorized into two different scaffolds, nine of which (compounds **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, and **10**) belong to scaffold 1. Compound **9** is the only one with a different scaffold. Scaffold 1 contains sulfonamide and benzothiazole groups, while scaffold 2 has sulfonamide and thiazole connected adjacent to each other. Compounds **2** and **3**, with IC₅₀ and K_D values below 10 μ M, seem to be the best leads for further studies.

3.3. Mechanism of Inhibition and potential binding pose

Extensive studies have been done for developing inhibitors of HCV NS4A-NS3 and Dengue NS2B-NS3 proteases. There are two main categories of developed inhibitors, catalytic site binding inhibitors and allosteric inhibitors. The allosteric inhibitor-binding site is located between the protease and helicase domains for HCV NS4A-NS3. The only ZIKV NS2B-NS3 protease inhibitor reported to date is a peptidomimetic boronic-acid inhibitor, which binds to the catalytic site as a competitive inhibitor with respect to their substrate (Bz-Nle-Lys-Lys-Arg-AMC) (Lei and Hilgenfeld, 2016). The inhibitory activity of our main lead compounds **2** and **3** exhibited an IC₅₀ values of 5.2 μ M and 4.1 μ M, respectively (Fig. 4A). The mechanism of inhibition was enzymatically tested with varying concentrations of both inhibitor and the substrate (Boc-Gly-Arg-Arg-AMC). Dixon analysis shows that compound **2** is a competitive inhibitor of ZIKV NS2B-NS3 with a K_i value of 9.5 μ M (Fig. 4B). Compound **3** was also determined to be a competitive inhibitor with a K_i value of 13.5 μ M. For an “orthogonal” validation of these results, competition SPR analyses of compounds **2** and **3** were performed in the absence and in the presence of the substrate. The binding affinity (K_D) of compound **2** in the presence of the substrate was ~4-fold weaker than that of compound **2** alone (Fig. 4C), supporting its designation as a competitive inhibitor. For compound **3**, the K_D value was reduced by a factor of 10 when the substrate was present (Fig. 4C), strongly confirming it to be a competitive inhibitor.

From both enzymatic studies and competition SPR binding analyses, compounds **2** and **3** are competitive inhibitors with respect to the substrate, indicating they bind within the substrate binding region, adjacent to the catalytic site of ZIKV NS2B-NS3. Docking studies were done to visualize the potential binding pose of compound **3** in the catalytic site. The best fit docking pose is shown in Fig. 5 with two main contributing interactions, H-bond and Pi-Pi interactions. The docked sulfonamide of compound **3** can form two H-bonds with the sidechain OH of S135 (Fig. 5B) and the backbone NH of G133. In addition, the phenyl ring in the middle of compound **3** can form a Pi-Pi stacking interaction with H51, and the benzothiazole ring can form a Pi-Pi stacking interaction with Y161.

3.4. The pre-open conformation of Zika NS2B-NS3 protease

The crystal structure of the NS2B-NS3 protease without any inhibitor or ligand was determined in the space group P4₁2₁2 at 3.1 Å with an R_{factor} of 22.3% and an R_{free} of

28.7% and good stereochemistry (Table 2; PDB: 5T1V). Containing two molecules (chain A and B) in the asymmetric unit, the structure has the typical chymotrypsin-like fold, as has been observed for the same protease in complex with a boronate inhibitor (PDB:5LC0) and for the proteases from other flaviviruses (Lei et al., 2016). The structure is made up by two β -barrels, each formed by six β -strands, with the catalytic triad (Ser135, His51 and Asp75) located in the cleft between the two β -barrels (Fig. 6A) (Erbel et al., 2006). The apo structure reported here, 5T1V, and the structure of the complex with the boronate inhibitor, 5LC0, are very similar, having an RMSD of 0.53 Å for 134 C α positions (Fig. 6B). Modeled residues span the sequence 46-66/17-158 in chain A and 46-62/20-157 in chain B (Fig. 6A). 19 residues of NS2B at its C terminus are disordered and consequently not included in the model (Fig. 6B). Those residues in the 5LC0 inhibitor bound structure form the β -hairpin β 3- β 4, that is essential for shaping the catalytic active site in a closed conformation (Noble et al., 2012; Noble and Shi, 2012). The closed conformation is observed when substrate-like inhibitors are co-crystallized with any of the flavivirus NS2B-NS3 proteases, suggesting that the C-terminus of NS2B becomes ordered upon binding to substrate or substrate-like molecules and explains why the C-terminus of NS2B is required for catalytic activity *in vitro* (Noble et al., 2012; Noble and Shi, 2012). Comparing it to the available structures for the flavivirus NS2B-NS3^{pro}, 5T1V is instead in an open-like conformation, a conformation in which the C-terminus of NS2B is away from the active site of NS3^{pro}.

The apo structure reported here additionally has a unique conformation of the NS3^{pro} C-terminus that also makes it different from previously reported open structures. Indeed, while the β -hairpin β -EIIB/ β -FII is seen in the open conformations of other flavivirus NS3^{pro} (i.e., Dengue Virus NS2B-NS3^{pro} PDBcode 2FOM) in 5T1V NS3^{pro} has a shorter β -EIIB and does not form the β -strand β -FII (Fig. 6C) (Erbel et al., 2006; Noble et al., 2012; Noble and Shi, 2012; Robin et al., 2009). In the 5T1V structure the C-terminus of NS3^{pro} moves away from the substrate binding site, making instead hydrophobic interactions with the strand connecting the two β -barrels of NS3^{pro} (Fig. 6C). This structural feature is described here as a 'pre-open conformation' and has never before been observed in other structures of flavivirus NS2B-NS3 proteases.

Having both the β -hairpins β -EIIB/ β -FII and β 3- β 4 missing in the pre-open conformation, the altered structure of the NS3^{pro} C-terminus in 5T1V coincides with a somewhat different orientation of the two chains in the crystallographic dimer with respect to 5LC0 (Fig. 6D). As described for 5LC0, the two independent subunits of 5T1V may represent a dimer in the asymmetric unit, with the substrate-binding sites of the two monomers facing each other. In 5T1V the buried surface area is less than in 5LC0 (~800 Å² in 5T1V compared to ~1200 Å² in 5LC0). However, the calculated ΔG (PDBePIS: <http://www.ebi.ac.uk/pdbe/pisa/>) is similar (~10 Kcal/mol of apo form compared to ~11 Kcal/mol of 5LC0), suggesting the possibility that the dimer might form independent of substrate binding, and if so, that the substrate can access the binding site through the two openings to the side of the dimer, as previously suggested by Lei *et al.* (2016). In our structure, the individual subunits may be connected to neighboring molecules in the crystal lattice through disulfide bonds linking the Cys143 of monomer A to the same residue of B in a symmetry related molecule. However, the resolution of the structure does not allow us to be conclusive about the presence of the

SS bonds between the two cysteines (C α distance of 5.6 Å). They may be occurring between some, but not all, molecules in the crystal lattice.

4. Conclusion

Although multiple protease inhibitors have been approved for Hepatitis C virus (HCV), and others proposed for the more closely related Dengue and West Nile viruses, inhibitor design against these proteases is still challenging, as the active site is shallow, solvent exposed, and flexible. Moreover, analogous to HCV protease inhibitors, resistance can be expected to arise for Zika protease inhibitors. In this study, building on a foundation of our prior work in identifying inhibitors with activity against both multiple genotypes and the most common resistance mutations of the related Hepatitis C virus NS3/NS4A, we have identified multiple compounds with inhibitory activity against the Zika NS2B/NS3 protease. There has been only one peptidomimetic boronic-acid inhibitor reported against this protease to date. Our non-peptidic small molecule inhibitors can be categorized into two different scaffolds, two of which were competitive inhibitors. These scaffolds provide a good starting point to develop low molecular weight NS2B/NS3 inhibitors with potentially broad-spectrum activity and limited side effects.

We also determined the crystal structure of the NS2B-NS3 protease in a pre-open conformation, a conformation never observed before for flavivirus proteases. In this conformation the active site is missing both the β -hairpin β -EIIb/ β -FII and the β -sheet β 3- β 4 that are fundamental structural features of the enzymatically active closed conformation (Erbel et al., 2006; Noble et al., 2012; Noble and Shi, 2012; Robin et al., 2009). This new finding suggests a sequence of mechanistic events in the dynamics of the enzyme active site (Fig. 7) for the NS2B-NS3^{pro} flavivirus:

- i. the pre-open conformation, which has a short β -EIIb and the rest of the C-terminus is stabilized by hydrophobic interactions between the two β -barrels of NS3, while the C terminus of NS2B is disordered, In this state the binding site is not well developed and the enzyme is not able to bind the substrate (Fig. 7A and D).
- ii. the open conformation –i.e. the structure of Dengue Virus NS2B-NS3 proteases (PDBcode 2FOM)- in which the β -hairpin β -EIIb/ β -FII is formed and is stabilized by the interaction with β -strand β -2b of NS3^{pro}. In this state the binding site is structured and can bind the substrate (Fig. 7B and E);
- iii. the closed conformation, in which the binding of substrate or substrate-like molecules involves the C-terminus of NS2B that reorganizes as the β -hairpin β 4/ β 5 and the β -hairpin β 4/ β 5 is stabilized by the β -EIIb/ β -FII β -hairpin at the C-terminus of NS3^{pro} (Fig. 7C and F).

The structure of the Zika virus NS2B-NS3 protease in this pre-open conformation provides new possibilities to design new classes of antiviral compounds.

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Abbreviations

ZIKV	Zika flavivirus
HCV	hepatitis C virus
DENV	Dengue virus
WNV	West Nile virus
SPR	surface plasmon resonance
HTS	high-throughput screening

Highlights

- We identified several small molecule inhibitors of the Zika NS2B-NS3 protease.
- Two lead compounds were determined to be competitive inhibitors by both enzymatic and competition SPR binding analyses.
- We determined the X-ray structure of the apo Zika NS2B-NS3 protease in a “pre-open conformation”.

Fig. 1. NS2B/NS3 serine protease

(A) Schematics of the ZIKV and HCV polyproteins with cleavage sites. (B) Aligned X-ray structures of Zika NS2B-NS3^{pro} (PDB:5LC0) and HCV NS4A-NS3^{pro} (PDB:1CU1). The catalytic triad residues (S135, H51, and D75) of Zika NS2B-NS3^{pro} are well aligned with those of HCV (S139, H57, and D81). (C) Sequence alignment of NS2B/NS3 proteases from ZIKV, WNV, DENV2, and HCV. Catalytic site residues are shown in green.

Fig. 2. ZIKV NS2B-NS3^{pro} construct and cleavage sites of ZIKV

(A) A schematic of NS2B-NS3^{pro} construct used with residue numbers of ZIKV. (B)

Cleavage sites of ZIKV NS2B-NS3^{pro} within non-structural proteins.

Fig. 3. Compound test and hit validation

(A) Schematic of compound test and hit validation process. (B) Bar graphs of 10 selected compound inhibitory activity (IC_{50}) and binding affinity (K_D) to ZIKV NS2B-NS3 protease. (C) Sensorgrams of compound 3 at a series of increasing concentrations. (D) Steady-state fitting curve of compound 3 using steady-state affinity equation embedded in Biaevaluation v3.0.

Fig. 4. Mechanism of inhibition

(A) IC₅₀ fitting curve of compounds 2 and 3 using the Hill equation embedded in Sigmaplot 12.0. (B) Dixon plot for competitive inhibition of compound 2. Determined IC₅₀ and K_i values of compound 2 were 5.2 μM and 9.5 μM, respectively. (C) Bar graphs of the dissociation equilibrium constants (K_D) of compounds 2 and 3 in the absence (black) and in the presence (green) of the substrate (Boc-Gly-Arg-Arg-AMC). K_D values were determined by Surface Plasmon Resonance (SPR), and data were fitted with steady-state affinity equation embedded in Biaevaluation v3.0.

Fig. 5. Docking pose of compound 3

(A) Calculated docked conformation of compound 3 in the active site of ZIKV NS2B-NS3^{pro} (PDB: 5LC0). (B) The two main interactions, H-bond and Pi-Pi, between compound 3 and ZIKV NS2B-NS3^{pro} are shown.

Fig. 6. Crystal structure of NS2B-NS3^{pro}

(A) Ribbon representation of NS2B-NS3^{pro} without inhibitor with the strand of NS2B (46-66) in red and NS3^{pro} (17-158) in yellow. The catalytic triad H51-D75-S135 is shown as green sticks. (B) Superimposition of the NS2B-NS3^{pro} without inhibitor (colored as in A) with the NS2B-NS3^{pro} determined in complex with the boronate inhibitor (PDB code 5LC0; NS2B/49-86 in blue; NS3^{pro}/18-170 in cyan; compound as stick in gray). (C) Enlarged view of the binding site from the superimposition as in B. The figure shows the large differences at the C terminus between the structures with and without the inhibitor. The rest of the foot domain and the boronate inhibitor is semi-transparent. (D) Comparison of the crystallographic dimers in 5T1V and 5LC0.

Fig. 7. Comparison of the pre-open, open and closed conformations of NS2B-NS3^{pro}
(A) Surface of the Zika NS2B-NS3^{pro} in pre-open conformation (5T1V) with the surface of NS2B in black and the surface of the C terminus of NS3 in red. **(B)** Surface of the Dengue Virus NS2B-NS3^{pro} in open conformation (2FOM) with the surface of the NS2B in black and the surface of the C terminus of NS3 in red. **(C)** Surface of the Zika virus NS2B-NS3^{pro} in closed conformation in complex with a boronate inhibitor (5LC0) with the surface of the NS2B in black and the surface of the C terminus of NS3 in red. **(D)** Electrostatic surface of the Zika NS2B-NS3^{pro} in pre-open conformation (5T1V). **(E)** Electrostatic surface of the Dengue Virus NS2B-NS3^{pro} in open conformation (2FOM). **(F)** Electrostatic surface of the Zika virus NS2B-NS3^{pro} in closed conformation in complex with a boronate inhibitor (5LC0).

Table 1

Comparison of kinetic parameters of His6-tagged and native NS2B-NS3^{pro} from ZIKV.

Protease	k_{cat} (s ⁻¹)	K_M (μM)	k_{cat}/K_M (s ⁻¹ M ⁻¹)
NS2B-NS3 ^{pro}	10.0 ± 1.3	136 ± 20	73786
His ₆ -NS2B-NS3 ^{pro}	10.4 ± 1.1	312 ± 30	33171

Kinetic parameters were determined with the substrate Boc-Gly-Arg-Arg-AMC, and the K_M values were determined from three independent assays.

Table 2
Data collection and Refinement statistics

<i>NS2B-NS3 (PDB code 5TIV)</i>	
Data collection	
Space group	P4 ₁ 2 ₁ 2
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	55.09, 55.09, 249.20
<i>a</i> , <i>b</i> , <i>c</i> (°)	90.00, 90.00, 90.00
Resolution (Å)	29.90-3.10 (3.21-3.10) *
<i>R</i> _{sym} or <i>R</i> _{merge}	0.115 (0.732) *
<i>I</i> / <i>σ</i> <i>I</i>	17.6 (2.7) *
Completeness (%)	99.79 (100.0) *
Redundancy	6.8 (6.7) *
CC 1/2	(0.925) *
Refinement	
Resolution (Å)	29.90 – 3.10
No. reflections	6875 (666) §
<i>R</i> _{work} / <i>R</i> _{free}	0.223/0.287
No. atoms	2,442
Mean B Value	93.35
R.m.s. deviations	
Bond lengths (Å)	0.006
Bond angles (°)	1.25
Ramachandran plot	
Favoured region (%)	92.9
Allowed region (%)	7.1
Disallowed region (%)	0

* Values in parentheses are for highest-resolution shell.

§ Values in parentheses are for reflection used for *R*_{free}