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Discovery, X-ray Crystallography and Antiviral Activity of Allosteric Inhibitors of Flavivirus NS2B-NS3 Protease

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Abstract

Flaviviruses, including dengue, West Nile and recently emerged Zika virus, are important human pathogens, but there are no drugs to prevent or treat these viral infections. The highly conserved Flavivirus NS2B-NS3 protease is essential for viral replication and therefore a drug target.

Compound screening followed by medicinal chemistry yielded a series of drug-like, broadly active inhibitors of Flavivirus proteases with IC₅₀ as low as 120 nM. The inhibitor exhibited significant antiviral activities in cells (EC₆₈: 300-600 nM) and in a mouse model of Zika virus infection. X-ray studies reveal that the inhibitors bind to an allosteric, mostly hydrophobic pocket of dengue NS3 and hold the protease in an open, catalytically inactive conformation. The inhibitors and their binding structures would be useful for rational drug development targeting Zika, dengue and other Flaviviruses.

Graphical Abstract

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Supporting Information. Experimental section, Figure S1-S11 and Table S1-S3. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

Allosteric Inhibitor; X-ray Crystallography; Flavivirus NS2B-NS3 Protease; Zika virus; Antiviral

Dengue (DENV), West Nile and recently emerged Zika (ZIKV) viruses belong to the genus *Flavivirus* in the *Flaviviridae* family of RNA viruses. These viruses are transmitted primarily by *Aedes* mosquitoes. Four serotypes of DENV infect ~400 million people each year with 100 million developing dengue fever. ~500,000 cases develop serious dengue hemorrhagic fever, causing ~22,000 deaths each year¹. Moreover, patients recovered from one serotype are still susceptible to other serotypes with an increased likelihood of a more severe disease due to existing antibodies². ZIKV has caused three major outbreaks in Pacific Ocean islands (2007 and 2013), Brazil and other American countries (2015-2016), in which >1 million infections were reported and a large number of patients sought medical treatment³. More seriously, ZIKV infection has been correlated with a 20-fold increased incidence of serious neurological disorders, including Guillain-Barré syndrome⁴ and >4,000 cases of microcephaly in newborns^{5, 6}. Since 2015, ZIKV has quickly spread to 48 pan-American countries. Recently, ZIKV was found to be transmitted through sex or body fluids⁷. Despite these serious outcomes as well as possible future outbreaks, there have been no antiviral drugs to prevent or treat ZIKV and DENV infections. A licensed dengue vaccine, Dengvaxia, has raised concerns about efficacy and increased risk of severe disease for seronegative people during clinical trials⁸.

ZIKV/DENV contain a single-stranded, positive-sense RNA with ~10,800 nucleotides, encoding a viral polyprotein. The polyprotein is site-specifically cleaved by the viral NS2B-NS3 protease and several host proteases to produce functional proteins (Supporting Information Fig. S1)^{1, 3}. The NS2B-NS3 protease is essential for viral replication and, therefore, a promising drug target^{1, 9, 10}. A number of peptide-based covalent inhibitors of Flavivirus proteases have been reported^{1, 11, 12}, but they did not demonstrate significant antiviral activities in cells or animal models due to low cell permeability and metabolic stability. Non-peptidic inhibitors have also been reported, but their inhibitory activities are relatively weak and how these compounds bind to the protease is unknown^{1, 13, 14}.

Homology analysis showed Flavivirus proteases are evolutionally conserved (Fig. S2) and highly stable (Fig. S3). NS3 contains an N-terminal serine protease domain, but complexation with NS2B is required to become an active enzyme. Previous X-ray^{11, 15-17}

and NMR^{18–20} studies show the protease can adopt a “closed” or “open” conformation. In the closed state that is catalytically active, NS2B is fully tied around NS3 (Fig. S4), and becomes part of the active site. In the open and inactive conformation, NS2B is partially bound to NS3 and far from the active site.

We produced a Gly₄-Ser-Gly₄ linked¹¹ and binary²¹ form of recombinant ZIKV protease (ZVpro), containing NS2B (47-95) and NS3 (1-170). ~1,200 compounds in our laboratory that were synthesized targeting histone modifying enzymes including lysine specific demethylase 1 (LSD1)²² were screened against the linked-ZVpro. Compounds **1** and **2** were identified to be novel inhibitors with IC₅₀ of 21.7 and 3.1 μ M (Table 1).

Scheme 1 shows the general synthesis for medicinal chemistry studies. 6-Chloro-2-aminopyrazine (**10**) was selectively iodized using *N*-iodosuccinimide, and the 2-amino group was converted to a hydroxyl, which was alkylated using a Mitsunobu reaction to give **12** with a protected piperidin-4-yl-methoxy group. Two selective Suzuki reactions were performed to introduce different aryl groups R⁵ and R⁶ to produce, after deprotection, compounds **3-5**, **7** and **9**. Mono-substitution of 1,6-dibromo-pyridine or -pyrazine (**15**) with (*N*-Boc-piperidin-4-yl)methylamine, followed by iodination produced the intermediate **16**. Selective replacement of the 5-iodo and 6-bromo substituent using a Suzuki reaction gave compound **6** or **8**.

Compound **9** was found to be a potent inhibitor of the linked- and binary-ZVpro with IC₅₀ of 200 and 220 nM, respectively (Fig. S5). Tables 1 and S1 summarize the inhibitory activities of selected analogs **3-8**. Changing the -O-linkage at 2-position to an -NH- in **8** (IC₅₀: 400 nM) resulted in a 2-fold activity reduction. Changing the central pyrazine ring in **8** to a pyridine in **6** (IC₅₀: 790 nM) further reduced the potency. As compared with **7** (IC₅₀: 530 nM) with a *N*-methyl secondary amine or **4** (IC₅₀: 1.1 μ M) with an amide at the 5-position, the primary amine in **9** is more favored. Changing the furan-3-yl group in **9** to a pyrazol-4-yl in **5** (IC₅₀: 710 nM) or a fused pyrrole ring in **3** (IC₅₀: 1.1 μ M) also decrease the inhibitory activity.

Compounds **3-9** also inhibited DENV serotype-2, -3, and West Nile protease (DV2pro, DV3pro and WVpro)^{15, 16} with IC₅₀ values of 120-1340 nM (Table 2). However, **9** exhibited negligible activities against several human serine-, cysteine-, aspartic- and metallo-proteases (Table S2). These results show **9** is a broadly active inhibitor of Flavivirus proteases with a high selectivity.

We determined X-ray structures of DV2pro in complex with compounds **5**, **8** and **9** at 2.7-3.0 Å. Statistics for diffraction data and structure refinement are shown in Table S3. The three structures are very similar to each other, with each asymmetric unit containing two inhibitor-bound proteins (Fig. 1a–c, Fig. S6 and S7). Similar to the apo-protein¹⁶, the DV2pro-inhibitor complexes adopt an open conformation, with NS2B binding partially to NS3 (Fig. S8a). The inhibitor-bound NS3 does not deviate significantly from the apo- or substrate-bound protein, except that the residues 152-164 are disordered (with no observed electron density) upon inhibitor binding. In contrast, the U-shaped peptide segment is well organized in both the apo- and substrate-bound NS3 (Fig. S8b/c). In the latter case, residues 152-164

constitute part of the S1 and S2 pockets of the active site and have interactions with the substrate¹⁵. Inhibitor binding pushes the loops 71-75 and 117-122 outwards by ~1.3 Å and 3 Å (Fig. S8d). All of these movements remodel the surface of NS3 and create a deep, L-shaped pocket (Figures 1d and S8e) that accommodates the inhibitors. The compounds are allosteric inhibitors, which do not occupy the substrate binding site (Fig. 1e).

Mechanistically, these inhibitors bind to and stabilize DV2pro in the open conformation, which prevents NS2B from folding into the active site as well as the binding of the substrate.

The inhibitor-protein interactions are illustrated in Figures 1d/f and S9. The central pyrazine ring of **9** is located at the junction of the L-shaped pocket. The furanylphenyl group is deeply inserted into the pocket with favorable hydrophobic interactions. The 2- and 5-substituents occupy a deep surface groove, having mostly hydrophobic interactions. The positively charged -NH₂ of **9** has hydrogen-bond and electrostatic interactions with Asp75, one of the protease catalytic triad.

High structural and sequence similarities between DV2pro and ZVpro, particularly for the inhibitor-interacting residues (Fig. S10a/b), suggest compound **9** binds to ZVpro similarly. Enzyme kinetics studies showed that **9** is a non-competitive inhibitor of DV2pro (Fig. S10c), consistent with its X-ray structure. Similar enzyme kinetics results support **9** is also an allosteric inhibitor of ZVpro (Fig. S10d).

Anti-ZIKV activity of **9** was evaluated in U87 glioma cells^{23,24}. The passage-3 stock of ZIKV FLR strain²⁵ was used for clinical relevance. Upon infection with ZIKV-FLR, U87 cells were incubated with **9** for 48 h. Newly generated ZIKV viruses in the media were determined quantitatively. Compound **9** significantly reduced ZIKV RNA in a dose-dependent manner (Fig. 2a). Because ~1/10⁴ of RNAs represent infectious viruses²⁵, an end-point dilution assay was used to determine viral titers more accurately. Half-log (0.32×) serial dilutions of the media were added to Vero cells in quadruplicate. Upon incubation for 7 days, ZIKV infection in each sample was determined with cytopathic effects. TCID₅₀ (tissue culture infective dose) was calculated based on the highest dilution in which 50% of the quadruplicate samples were infected with ZIKV. As shown in Fig. 2b for a representative experiment, compound **9** reduced infectious ZIKV viruses by 68% at 300 nM, 90% at 600 nM, 97% at 1.2 μM, 99% at 2.5 μM, and 99.7% at 5 μM. Multiple experiments showed that EC₆₈ of **9** was 300 or 600 nM. Compound **9** exhibited similar antiviral activities against ZIKV HN16 strain²⁶ (Fig. 2c). **9** also showed significant activity against DENV-2 (strain K0049)²⁷, inhibiting viral replication in Vero cells by 97% at 5 μM. These results demonstrate compound **9** has potent cellular antiviral activity against Zika and dengue viruses. Moreover, cellular antiviral activities of compounds **1-9** are generally correlated with their biochemical activities against ZVpro (Table 1). Compound treatment also dose-dependently inhibited the viral proteins capsid, NS3 and NS5 in infected Vero cells (Fig. S11). These results support ZVpro is the cellular target of these compounds.

In vivo anti-ZIKV activity of **9** was evaluated in C57BL/6 mice with both interferon-α/β and -γ receptor genes knocked out²⁸. We found that intraperitoneal (ip) injection of 100 TCID₅₀ of ZIKV-FLR caused rapid viral replication and death of the mice in ~10 days. Started 1h before inoculation of ZIKV, ip treatment with **9** (15 mg/kg/12h) for 24h (when ZIKV

replication is in a rapidly growing phase) reduced ZIKV RNA copies in both plasma and brains of the mice by 96% and 98% (Fig. 2d). Treatment at 30 and 20 mg/kg/day for 3 days significantly prolonged the survival of ZIKV-infected mice, with the average values for the control, 20 and 30 mg/kg groups (N=12) being 11.7, 13.7 and 15.1 days (Fig. 2e). These results show that **9** can effectively inhibit ZIKV replication in vivo.

In summary, compound **9** is a broadly active inhibitor of Flavivirus proteases and exhibits significant cellular and in vivo activities against Zika virus. In addition, X-ray studies reveal that it binds to an allosteric pocket of NS3 and provide, for the first time, a druggable pocket of the Flavivirus protease, as contrasted to the shallow active site recognizing polar and positively charged Arg or Lys of the substrate. Rational inhibitor development based on the pharmacological leads and structural platform could lead to compounds with improved potency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

DENV	dengue virus
DV2pro	dengue serotype 2 NS2B-NS3 protease
DV3pro	dengue serotype 3 NS2B-NS3 protease
ip	intraperitoneal
TCID₅₀	tissue culture infective dose
WVpro	West Nile NS2B-NS3 protease
ZIKV	Zika virus
ZVpro	Zika NS2B-NS3 protease

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Figure 1.

X-ray structure of the DV2pro-9 complex. (A) The overall structure of DV2pro in complex with compound 9 (ball and stick model with C atoms in brown). NS3 is shown in green and NS2B in purple; (B) The $2F_o - F_c$ electron density map of DV2pro-9 at the inhibitor binding site (contoured at 1σ) and (C) the $F_o - F_c$ omit map (at 3σ); (D) Compound 9 is located in an L-shaped, deep pocket of NS3 (shown as an electrostatic surface) that is mostly hydrophobic; (E) Aligned structures of DV2pro-9 with the substrate-bound DV3pro (PDB: 3U1I, NS3 in cyan and NS2B in yellow), showing 9 occupies a different binding site from the substrate (tube model with C atoms in black); (F) Interactions between compound 9 and DV2pro.

Figure 2.

Cellular and in vivo antiviral activities of compound **9**. (A-C) Treatment of U87 cells with **9** caused dose-dependent reduction of (A) RNA copies of ZIKV (FLR strain), (B) infectious ZIKV (FLR strain), and (C) infectious ZIKV (HN16 strain); (D) Treatment with compound **9** significantly reduced ZIKV RNA in plasma (left) and brains (right) of ZIKV-infected mice; (E) Treatment with compound **9** significantly prolonged the survival of ZIKV-infected mice.

Scheme 1.

General synthesis for compounds 2-9.^a

^a*Reagents and conditions:* (i) *N*-Iodosuccinimide, DMSO; (ii) NaNO₂, H₂SO₄(Conc.); (iii) *N*-Boc-piperidin-4-ylmethanol, PPh₃, diisopropyl azodicarboxylate, THF; (iv) R⁵-boronic acid, Pd(PPh₃)₄, Na₂CO₃, 1,4-dioxane-H₂O, 80 °C; (v) R⁶-boronic acid, Pd(PPh₃)₄, Na₂CO₃, 1,4-dioxane-H₂O, 110 °C; (vi) 4M HCl, CH₂Cl₂, 0 °C; (vii) (*N*-Boc-piperidin-4-yl)methylamine, K₂CO₃, DMF, 100 °C.

Table 1.Structures and activity of compounds **1-9**.

For **1-5, 7, 9**: X = O, Y = N
 For **6**: X = NH, Y = CH
 For **8**: X = NH, Y = N

Cpd	R ⁵	R ⁶	linked-ZVpro IC ₅₀ (μM)	ZIKV-FLR EC ₆₈ (μM)
1	4-Br-Ph	4-Br-Ph	21.7 ± 1.5	>10 ^a
2	4- <i>t</i> -Bu-Ph	4- <i>t</i> -Bu-Ph	3.14 ± 0.09	5.0
3	4-(NH ₂ CH ₂)-Ph	Indol-5-yl	1.12 ± 0.07	2.50
4	4-(NH ₂ CO)-Ph	4-(furan-3-yl)-Ph	1.05 ± 0.07	2.50
5	4-(NH ₂ CH ₂)-Ph	4-(pyrazol-4-yl)-Ph	0.71 ± 0.07	2.50
6	4-(NH ₂ CH ₂)-Ph	4-(furan-3-yl)-Ph	0.79 ± 0.03	1.20
7	4-(MeNHCH ₂)-Ph	4-(furan-3-yl)-Ph	0.53 ± 0.06	1.20
8	4-(NH ₂ CH ₂)-Ph	4-(furan-3-yl)-Ph	0.40 ± 0.05	1.20
9	4-(NH ₂ CH ₂)-Ph	4-(furan-3-yl)-Ph	0.20 ± 0.01	0.30-0.60

^aEC₆₈ cannot be determined because **1** showed cytotoxicity at >10 μM. All other compounds had no significant cytotoxicity.

Table 2.IC₅₀ (μM) of **3-9** against Flavivirus proteases.

	linked-ZVpro	DV2pro	DV3pro	WVpro
3	1.12 ± 0.07	0.64 ± 0.03	0.54 ± 0.01	0.93 ± 0.03
4	1.05 ± 0.07	0.98 ± 0.02	0.93 ± 0.02	1.34 ± 0.01
5	0.71 ± 0.07	0.21 ± 0.04	0.20 ± 0.07	0.12 ± 0.02
6	0.79 ± 0.03	0.86 ± 0.01	0.86 ± 0.02	1.27 ± 0.03
7	0.53 ± 0.06	0.73 ± 0.02	0.53 ± 0.01	0.87 ± 0.02
8	0.40 ± 0.05	0.29 ± 0.06	0.21 ± 0.07	0.51 ± 0.04
9	0.20 ± 0.01	0.59 ± 0.02	0.52 ± 0.06	0.78 ± 0.02