Discovery of Zika Virus NS2B/NS3 Inhibitors That Prevent Mice from Life-Threatening Infection and Brain Damage

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ABSTRACT: Zika virus (ZIKV) infection, which initially was endemic only in Africa and Asia, is rapidly spreading throughout Europe, Oceania, and the Americas. Although there have been enormous efforts, there is still no approved drug to treat ZIKV infection. Herein, we report the synthesis and biological evaluation of agents with noncompetitive mechanism of the ZIKV NS2B/NS3 protease inhibition through the binding to an allosteric site. Compounds 1 and 2 showed potent activity in both enzymatic and cellular assays. Derivative 1 efficiently reduced the ZIKV protein synthesis and the RNA replication and prevented the mice from life-threatening infection and the brain damage caused by ZIKV infection in a ZIKV mouse model.

KEYWORDS: Zika, NS2B/NS3 protease, mouse model, modeling, synthesis

Zika virus (ZIKV) is one of the biggest threats to global health. The WHO declared ZIKV outbreak Public Health Emergency of international concern since 2007 mainly because of the rapid spread and the sexual and vertical human-to-human transmission.¹ ZIKV is a mosquito-borne small enveloped positive single-stranded RNA virus in the Flavivirus genus and in the Flaviviridae family that is phylogenetically related to West Nile and Dengue viruses.²⁻⁴ ZIKV is transmitted to human by a vector bite during the day, primarily through the infected female Aedes aegypti, but the virus has been isolated from multiple Aedes species that are probably also involved in the transmission of ZIKV infection.¹ The symptoms are generally mild. Nevertheless, ZIKV virus infection during pregnancy can cause infants with microcephaly or other inborn malformations, known as congenital ZIKV syndrome.⁵ The infection is also related with fetal loss and preterm birth.⁶ Moreover, an increasing number of neurological complications such as Guillain–Barré syndrome, meningoencephalitis, and myelitis have recently been reported in ZIKV-infected adults.⁷ The ZIKV infection was also a cause of orchitis and long-term male subfertility.⁸ Thus, far, there is still no specific drug or vaccine for the treatment of ZIKV infection, with only a few candidates advanced into clinical trials.⁹ The treatment therapies just aim to mitigate the infection symptoms. Therefore, developing new agents for prophylaxis and postinfection therapy remains an urgent challenge.

The ZIKV genome encodes a precursor protein that is processed by proteases into three structural proteins, capsid (C), premembrane/membrane (prM), and envelope glycoprotein (E), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).¹⁰ The NS3 is a large multifunctional protein with two functionally distinct domains: a 176-residue N-terminal domain with protease activity and a 444-residue C-terminal domain with helicase, nucleoside 5' transferase (NTPase), and S' terminal RNA triphosphatase (RTPase) activities.¹¹ The NS2B polypeptide cofactor plays a key role for NS3 catalysis.¹² The infection symptoms are generally mild. Nevertheless, ZIKV virus infection during pregnancy can cause infants with microcephaly or other inborn malformations, known as congenital ZIKV syndrome. The infection is also related with fetal loss and preterm birth. Moreover, an increasing number of neurological complications such as Guillain–Barré syndrome, meningoencephalitis, and myelitis have recently been reported in ZIKV-infected adults. The ZIKV infection was also a cause of orchitis and long-term male subfertility. Thus, far, there is still no specific drug or vaccine for the treatment of ZIKV infection, with only a few candidates advanced into clinical trials. The treatment therapies just aim to mitigate the infection symptoms. Therefore, developing new agents for prophylaxis and postinfection therapy remains an urgent challenge.
to Ala (153) and Lys to Asp (139), from the DENV to ZIKV NS2B/NS3 exosite sequence (Figure 1S, Supporting Information). In preliminary docking studies, a previously reported DENV NS2B/NS3 allostERIC inhibitor, 1, and 2 (Table 1).17

Table 1. Antiviral Data vs ZIKV in HepG2 Cellsα

| Compd | R₁ | R₂ | X        | Survival % ± SD | Viability % ± SD |
|-------|----|----|----------|-----------------|-----------------|
| 1     | COOMe | Cl  | C=O      | 47 ± 8          | 100 ± 14        |
| 2     | COOEt | Br  | C=O      | 66 ± 12         | 100 ± 4         |
| 3     | COOEt | H   | C=O      | 0               | 100 ± 2         |
| 4     | COOEt | H   | CH₂      | 0               | 100 ± 5         |
| 5     | COOMe | Br  | C=O      | 22 ± 4          | 100 ± 7         |
| 6     | COOMe | Br  | CH₂      | 2 ± 0.01        | 100 ± 6         |
| 7     | COOEt | Br  | CH₂      | 20 ± 5          | 100 ± 7         |
| 8     | Ph    | Br  | C=O      | 23 ± 8          | 100 ± 5         |
| 9     | COOMe | Cl  | CH₂      | 23 ± 1          | 100 ± 3         |
| 10    | COOMe | MeO | C=O      | 49 ± 6          | 100 ± 6         |
| 11    | COOEt | MeO | C=O      | 47 ± 4          | 100 ± 4         |
| 12    | COOEt | MeO | CH₂      | 28 ± 2          | 100 ± 1         |
| 13    | COOEt | OH  | C=O      | 0               | 94 ± 4          |
| 14    | COOMe | Cl  | CH₂      | 16 ± 1          | 100 ± 5         |
| Ribavirin |      |     |          | 82 ± 6         | 100 ± 1         |

αCompounds were assayed at 10 μM. 1: IC₅₀ = 13.7 ± 2.7 μM; 2: IC₅₀ = 15.8 ± 0.9 μM. Experiments were performed in triplicate. 
βRibavirin was at 100 μM.

highlighted a consistent binding mode with the two studied NS2B/NS3 structures. Notably, 1 and 2 did not show any interactions with the 2 mutated residues. Therefore, we synthesized a small compound library and evaluated it in ZIKV-infected HepG2 cells (Table 1). Compounds 1–14 were synthesized according to Scheme 1 by Friedel–Crafts reaction of an appropriate indole with 3,5-dimethoxy-4-hydroxybenzoyl chloride or 3,5-dimethoxybenzoyl chloride in the presence of anhydrous aluminum chloride by heating at reflux overnight in dichloroethane or, alternatively, by microwave irradiation in a closed vessel at 250 W and 100 °C for 6 min. Methylenec compounds were obtained by reduction of the carbonyl group with triethylsilane in trifluoroacetic acid by stirring for 48 h at 25 °C. The experimental procedures are reported in the Supporting Information.

All compounds, except 13, were nontoxic at 10 μM in HepG2 (liver) cells. The best ranking by activity/survival vs ZIKV virus in percent of mock was 2 (66%), 10 (49%), 1 (47%), and 11 (47%). Ribavirin protected over 80% of HepG2 cells in the presence of ZIKA-U at a concentration of 100 μM. The SAR summary for the inhibition of ZIKV in HepG2 cells by compounds 1–14 is shown in Chart 1S of the Supporting Information.

Analyses of the binding mode of 1 and 2 at the ZIKV NS2B/NS3 exosite highlighted (i) an H-bond of the 4-OH with Ala152 and hydrophobic contacts of (ii) the 3,5-dimethoxy groups with Trp148, Leu150, and Ile212, (iii) the indole ring with Leu141 and Leu214, and (iv) the ester moiety close to Ile188 (Figure 1). Compounds 1 and 2 formed a strong H-

bond with Ala152, also thanks to the presence of the carbonyl group. On the contrary, the 3,5-dimethoxyphenyl group of 14, deprived of the 4-OH group, lacked the interaction with Ala151 and adopted a different pose in the binding pocket (data not shown). Compounds 1, 2, and 14 as a negative control, were evaluated as inhibitors of the ZIKV NS2B/NS3 protease and in ZIKV-infected Hep7 cells (Table 2). The activity of recombinant ZIKV protease was measured through a FRET assay, using as substrate the Bz-nKRR-MCA peptide that generates a fluorescence signal when AMC is cleaved by

Table 2. Activity against ZIKV NS2/NS3 Protease

| Compd | ZIKV Protease IC₅₀ ± SD (μM) | Huh-7 cells EC₅₀ ± SD (μM) |
|-------|-----------------------------|----------------------------|
| 1     | 158 ± 25                    | 13.9 ± 1.1                 |
| 2     | 33 ± 7                      | 16.2 ± 0.6                 |
| 14    | 770 ± 210                   | nd                         |

αActivity of recombinant ZIKV protease measured through FRET assay. βAnti-ZIKV activity in Huh-7 infected cells line. γUnder the same assay conditions, 1 inhibited the DENV2 protease with IC₅₀ of 112 μM.

Figure 1. Proposed binding mode for 1 (cyan) and 2 (orange). Residues involved in interactions are reported as gray stick. H-bond is reported as a yellow dotted line. NS2B/NS3 is shown as a light blue surface, with Leu141 and Leu214, and (iv) the ester moiety close to 188 (Figure 1). Compounds 1 and 2 formed a strong H-bond with Ala152, also thanks to the presence of the carbonyl group. On the contrary, the 3,5-dimethoxyphenyl group of 14, deprived of the 4-OH group, lacked the interaction with Ala151 and adopted a different pose in the binding pocket (data not shown). Compounds 1, 2, and 14 as a negative control, were evaluated as inhibitors of the ZIKV NS2B/NS3 protease and in ZIKV-infected Hep7 cells (Table 2). The activity of recombinant ZIKV protease was measured through a FRET assay, using as substrate the Bz-nKRR-MCA peptide that generates a fluorescence signal when AMC is cleaved by

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the protease.\textsuperscript{18} Half maximal inhibitory concentration (IC\textsubscript{50}) was measured through activity assays, keeping the substrate (10 \( \mu\)M) and the protein (25 nM) in constant concentration, while using different concentrations of inhibitors.

Compounds 1 and 2 showed IC\textsubscript{50} values of 158 \( \mu\)M and 33 \( \mu\)M, respectively. Compound 14 showed an IC\textsubscript{50} of 773 \( \mu\)M. It should be noted that, although we used very high concentrations of 14 (up to 500 \( \mu\)M), this compound did not completely inhibit the ZIKV protease (data not shown), thus it was removed from further analysis.

Enzyme kinetics was used to determine the mechanism of inhibition of the selected compounds, mainly 1 and 2. We used two inhibitor concentrations that flank the IC\textsubscript{50} value (150 \( \mu\)M and 250 \( \mu\)M for 1; 15 \( \mu\)M and 60 \( \mu\)M for 2) with different substrate concentrations, keeping ZIKV protease constant to 25 nM. This experiment was done in triplicate. For both compounds, the kinetic experiments showed noncompetitive inhibition, with \( K_i \) value of 280 \( \pm \) 30 \( \mu\)M for 1 (Figure 2) and about 48 \( \pm \) 6 \( \mu\)M for 2 (not shown). Microscale thermophoresis (MST) experiments were used to estimate the binding affinity of ZIKV protease to the two selected inhibitors 1 and 2. The purified ZIKV protein was fluorescently labeled using maleimide dye that binds to the sulfhydryl group and is titrated with di-[(aminooethyl)amino] ethane sulfonic acid (sulfonate) (data not shown).

The calculated \( V_{\text{max}} \), \( K_m \), and \( K_i \) parameters of 1 and 2 were predicted to have a good oral absorption according to both Lipinski’s rule of five\textsuperscript{19} and Veber’s rules\textsuperscript{20} (Table 1S, Supporting Information). The Pfizer 3/75 rule (\( tPSA < 75 \) and logP > 3)\textsuperscript{21} predicted that compound 2 would have higher likelihood of a preclinical toxicity event and experimental promiscuity than compound 1.

To investigate whether compound 1 exhibits anti-ZIKV activity, we treated the ZIKV-infected Huh-7 cells with various drug concentrations for 3 days. Western blotting and qRT-PCR were used to measure ZIKV protein synthesis and RNA replication, respectively. Compound 1 dose-dependently reduced ZIKV protein (Figure 3, top panel) and RNA (bottom panel) levels, with IC\textsubscript{50} value of 13.9 \( \pm \) 0.4 \( \mu\)M against ZIKV RNA replication. Moreover, 1 did not show cell toxicity at effective antiviral concentrations (data not shown).

6-Day-old ICR suckling mice were injected with 10\textsuperscript{4} PFU of ZIKV and compound 1 (1 mg/kg) by intraperitoneal injection at 1, 3, and 5 days post infection (dpi). Mice injected with heat-inactivated ZIKV (iZIKV) were used as mock control. The results of changes in viral titers (Figure 4), body weight, clinical scores, and survival rates (Figure 5S–7S, Supporting Information) revealed that compound 1 prevented the mice from life-threatening ZIKV infection.

ZIKV infection has been associated with severe neurological complications, with a remarkable increase in cases of Guillain–Barré syndrome and of microcephaly in newborns and fetuses.\textsuperscript{22–24} There is an unmet demand for safe drugs able to reach the main ZIKV targets by crossing the blood–brain and the placental barriers. The brain tissues were collected and subjected to hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) assay using anti-ZIKV NS1 antibody. The results revealed that compound 1 prevented the brain damage caused by ZIKV infection (Figure 5, top panel) and also significantly inhibited ZIKV replication in the brain tissue (Figure 5, bottom panel). To quantify monocyte infiltration, we performed an IHC assay using anti-Ly6C antibody, a monocytic marker. As presented in Figures 6 and 8S in the Supporting Information, monocyte infiltration caused by ZIKV was significantly reduced by 1, indicating the
potential of 1 against neurological disorders caused by ZIKV infection.

In conclusion, new ZIKV NS2B/NS3 protease inhibitors have been synthesized with potent activity in both enzymatic and cellular assays. The noncompetitive mechanism of the NS2B/NS3 inhibition through the binding to an allosteric site was confirmed by the enzyme kinetics experiments. Derivative 1 efficiently reduced the ZIKV protein synthesis and the RNA replication. As a proof of concept, compound 1 was evaluated in a mouse animal model. This compound was able to prevent the mice from life-threatening and the brain damage caused by ZIKV infection. These results pave the way to obtain new ZIKV drug candidates able to cross the blood–brain barrier to reach the neural cells.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.9b00405.

Additional information for experimental procedures of synthesis, computational studies, antiviral and cytotoxicity assay, NS2-NS3 protease inhibition, microscale thermophoresis, and animal model assay (PDF)

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A.C.: computational studies; M.P., M.N., G.L.R.: chemical synthesis; E.M., T.M., D.T.: NS2-NS3 protease inhibition and microscale thermophoresis; J.J.B., B.S. (temporarily at TUM),
J.N., F.S.: in vitro antiviral and cytotoxicity assay; C.-K.W., Y.-M.W., J.-C.L.: anti-ZIKV activity assay in animal model.

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Notes
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■ ABBREVIATIONS
ZIKV: Zika virus; DENV: Dengue virus; EDC: dichloroethane; ZIKV-U: 976 Uganda strain.

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