Mechanism through Which Retrocyclin Targets Flavivirus Multiplication

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ABSTRACT Currently, there are no approved drugs for the treatment of flavivirus infection. Accordingly, we tested the inhibitory effects of the novel defensin retrocyclin-101 (RC-101) against flavivirus infection and investigated the mechanism underlying the potential inhibitory effects. First, RC-101 robustly inhibited both Japanese encephalitis virus (JEV) and Zika virus (ZIKV) infections. RC-101 exerted inhibitory effects on the entry and replication stages. Results also indicated that the nonstructural protein NS2B-NS3 serine protease might serve as a potential viral target. Furthermore, RC-101 inhibited protease activity at the micromolar level. We also demonstrated that with respect to the glycoprotein E protein of flavivirus, the DE loop of domain III (DIII), which is the receptor-binding domain of the E protein, might serve as another viral target of RC-101. Moreover, a JEV DE mutant exhibited resistance to RC-101, which was associated with deceased binding affinity of RC-101 to DIII. These findings provide a basis for the development of RC-101 as a potential candidate for the treatment of flavivirus infection.

IMPORTANCE Retrocyclin is an artifically humanized circular defensin peptide, containing 18 residues, previously reported to possess broad antimicrobial activity. In this study, we found that retrocyclin-101 inhibited flavivirus (ZIKV and JEV) infections. Retrocyclin-101 inhibited NS2B-NS3 serine protease activity, suggesting that the catalytic triad of the protease is the target. Moreover, retrocyclin-101 bound to the DE loop of the E protein of flavivirus, which prevented its entry.

KEYWORDS retrocyclin-101, flavivirus, antiviral effect, NS2B-NS3 protease, DE loop

Flaviviruses are taxonomically classified in the genus Flavivirus and family Flaviviridae. These viruses include more than 70 different pathogens and are transmitted mostly by arthropods. Emerging and reemerging flaviviruses, such as Zika virus (ZIKV), Japanese encephalitis virus (JEV), dengue virus (DENV), West Nile virus (WNV), and yellow fever virus, cause public health problems worldwide (1). Flaviviruses contain an approximately 11-kb positive-stranded RNA genome that encodes three structural proteins, including the capsid (C), membrane (premembrane [prM] and membrane [M]), and envelope (E), as well as seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (2). The envelope glycoprotein (E) is responsible for receptor binding and membrane fusion and thus plays essential roles in virus entry. E proteins exist as homodimers on the surface of the virus. Among the three domains of the E protein, domain I (DI) connects the DII and DIII domains, and DII contains fusion polypeptides that facilitate membrane fusion, whereas DIII has been proposed to act as the receptor binding region (3–5). It has been reported that several key residues, such as the glycosylation site N154 and the DE loop (T363SSAN367), are responsible for receptor binding (6, 7), whereas H144 and H319

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are thought to play critical roles in DI and DIII interactions (8). Moreover, Q258, located in DI, and T410, located in the stem, are indispensable for low-pH-triggered conformational changes, in which the stem region undergoes zippering along with DI, thus leading to the postfusion conformation and membrane fusion (9–11). As it envelops the surface of the virion, the E protein is the natural target for antibodies and the design of entry inhibitors to prevent receptor binding and membrane fusion (4, 9, 12, 13). Likewise, viral proteases such as NS2B-NS3 protease-helicase and the NS5 RNA-dependent RNA polymerase represent attractive drug targets in an attempt to identify replication inhibitors (14, 15).

Retrocyclin (RC) is an artificially humanized \( \theta \)-defensin that has been reported to possess broad antimicrobial activity (16–21). RC-101 has the sequence GICRCICGKGICRCICGR and is an analogue of RC-1 (GICRCICGRGICRCICGR). It contains 18 residues, including three disulfide bonds and four positively charged residues (Fig. 1A and B), which confers high binding affinity to glycosylated proteins, such as HIV gp120 (22), influenza virus hemagglutinin (23), and herpes simplex virus 1/2 (HSV-1/2) glycoprotein (24), thus preventing virus entry. Additionally, some viral proteases with negatively charged surfaces might serve as targets for RC-1 (20).

In this study, we tested the inhibitory effect of RC-101 against flavivirus infection. As flaviviruses possess only one conserved N-linked glycan on the E protein (25), whether RC-101 exerted the inhibitory effect against flavivirus entry by targeting the glycan chain was tested in this study. Meanwhile, we determined that RC-101 could also inhibit flavivirus replication by blocking the NS2B-NS3 serine protease.

RESULTS

RC-101 inhibits ZIKV infection. To test the inhibitory effect of RC-101 against ZIKV infection, two strains were used to determine the 50% inhibitory concentration (IC\(_{50}\)) of RC-101. Notably, the ZIKV PRVABC 59 strain, belonging to the Asian lineage ZIKV strains, contains one N-linked glycosylation site (N-X-S/T) at residue N154 of E, which is conserved among the flaviviruses, whereas the stocks of the African lineage MR766 may or may not lack the E glycosylation motif due to their extensive passaging (26–31). To this end, an MR766 strain lacking the N-glycosylation motif (GenBank accession no. MK105975.1) was used in this study. The cytotoxicity of RC-101 was initially tested on Vero cells, which showed a marginal response even at 100 \( \mu \)M (Fig. 1C). An immunofluorescence antibody (IFA) staining plaque assay for the antiviral effect of RC-101 against ZIKV PRVABC 59 showed a dose-dependent inhibition, with an IC\(_{50}\) of 7.033 \( \mu \)M (Fig. 1D to F). Similarly, RC-101 inhibited ZIKV MR766 infection, with an IC\(_{50}\) of 15.58 \( \mu \)M (Fig. 1G to I). To verify the result, an additional cell line, the U251 glioma cell line, was used in the plaque assay. As shown in Fig. 1J, RC-101 robustly inhibited PRVABC 59 virus production; few plaques were found when 100 \( \mu \)M peptide was included, and an approximately 4- to 5-log unit reduction was found in the 12.5 \( \mu \)M treatment group. Similarly, RC-101 robustly inhibited MR766 virus production, with a reduction of approximately 7 log units when 100 \( \mu \)M peptide was used and a reduction of approximately 1 log unit when 12.5 \( \mu \)M RC-101 was used (Fig. 1K). To validate the comparison results, the replication kinetics of both strains were evaluated. As shown in Fig. 1L, both strains had similar growth curves, with an accumulation of infectious virions that reached the highest titer at 72 h postinfection.

RC-101 inhibits ZIKV infection at both the entry and replication steps. To test whether RC-101 blocked the entry step or the replication step, a time-of-addition experiment was performed (Fig. 2A). As shown in Fig. 2B and C, no suppression of viral titers was observed in the pretreatment or the virucidal treatment groups, indicating that RC-101 does not inhibit ZIKV infection—either by blocking the cellular receptors that prevent virus binding or by inactivating the virus directly. However, RC-101 exerted significant inhibitory effects when its addition was synchronized with the virus via coadministration. Moreover, RC-101 inhibited MR766 strain infection when it was added 1 h postinfection. These results suggested that viral entry and replication are the stages at which RC-101 shows inhibitory activity.
To confirm the inhibitory effect on viral replication, we investigated the effects of RC-101 on the ZIKV replicon (32, 33). As shown in Fig. 3, RC-101 showed little effect on the initial translation of replicon RNA (Fig. 3A), whereas an appreciable reduction in the luciferase signal was observed at 48 h postelectroporation (Fig. 3B). This confirmed that RC-101 has an inhibitory effect on the ZIKV replication state.

**RC-101 inhibits NS2B-NS3 serine protease activity.** To investigate the potential viral target of RC-101, we tested the inhibitory effect of RC-101 on ZIKV NS2B-NS3 protease activity. It has been reported that RC-1, which possesses the same residues as RC-101 except for one lysine (K) instead of arginine (R) in RC-101, might dock at the NS2B-NS3 interface and thus inhibit DENV-2 replication by...
interfering with the activity of the NS2B-NS3 serine protease (20). Considering the sequence and structural conservation of Flavivirus NS proteins, we reasoned that RC-101 might have a similar effect on the ZIKV NS2B-NS3 protease. To test this hypothesis, we first produced NS2B-NS3 pro in Escherichia coli as a single-chain peptide (20, 34, 35). Protease activity was assessed using a fluorogenic peptide as a substrate at 37°C for 30 min. As shown in Fig. 4A, the Michaelis-Menten constant ($K_{m}$) value was 11.77 μM, indicating that the enzyme kinetic assay was robust and suitable to investigate the inhibitory effect. As shown in Fig. 4B, RC-101 effectively inhibited NS2B-NS3 protease activity, with an IC$_{50}$ of 7.20 μM, indicating that this protease serves as a viral target of RC-101.

Inhibition of the protease activity of NS3 by RC-101 was further supported by the detection of the unprocessed polyprotein precursor (PP) and NS3 in the infected cells (36). As shown in Fig. 4C to D, the expression of JEV NS3 (~70 kDa) was inhibited in a dose-dependent manner by RC-101. Notably, the unprocessed polyprotein precursor (>180 kDa) was present in the low-RC-101-concentration

**FIG 2** Time-of-addition analysis of the antiviral activity of the RC-101. (A) Schematic illustration of the time-of-addition experiment. For virucidal treatment, ZIKV (MOI of 2.5) was incubated with RC-101 (40 μM) at 37°C for 1 h, and the mixture was diluted 25-fold to infect Vero cells for 1 h. For pretreatment (pre), Vero cells were incubated with RC-101 (40 μM) for 1 h (from −1 to 0 h) and then infected with ZIKV (MOI of 0.1) for 1 h (from 0 to 1 h), co-administration treatment. Vero cells were incubated with a mixture of RC-101 (40 μM) and ZIKV (MOI of 0.1) for 1 h (0 to 1 h). Posttreatment, Vero cells were infected with ZIKV (MOI of 0.1) for 1 h and then incubated with RC-101 (40 μM) for an additional 47 h (PRVABC 59) and 71 h (MR766), respectively. (B and C) Time-of-addition analysis of the antiviral effect of RC-101 against PRVABC 59 (B) and MR766 (C). The inhibitory effect of the drugs in each group was determined by plaque assays. Data are presented as the mean ± SD from 5 to 8 independent experiments. LOD, limit of detection. *, P < 0.05; ***, P < 0.001.
groups (0.78125 and 3.125 μM), and the level of the polyprotein precursor at 3.125 μM was significantly higher than that at 0.78125 μM, indicating that the protease activity of NS3 was inhibited at these RC-101 concentrations. The presence of the polyprotein precursor decreased in the high-RC-101-concentration groups (12.5 and 50 μM), since the viral infection was robustly blocked in these groups (Fig. 4C and E). Based on both the in vitro enzyme kinetic assays and the experiments in infected cells, it was concluded that RC-101 inhibits flavivirus NS2B-NS3 serine protease activity.

RC-101 inhibits flavivirus entry by targeting the DE loop of E glycoprotein. As RC-101 was found to inhibit ZIKV infection at both the entry and replication stages (Fig. 2), we further investigated the mechanism underlying the inhibitory effect on the entry stage. As previously mentioned, RC has been reported to inhibit different types of enveloped viruses by binding to the negatively charged glycan chains on the surface of the glycoprotein, thus blocking virus entry (22–24). However, flaviviruses contain only one glycosylation motif on the E glycoprotein, but this the number is not absolutely conserved, as DENV has two glycosylation motifs, whereas some African lineage ZIKV strains have no glycan chain on the surface (26–31, 37–39). As shown in Fig. 1, RC-101 exerted similar inhibitory effects on both the ZIKV Asian strain PRVABC 59 (one glycan) and the African strain MR766 (no glycan), suggesting that glycans might not be the target of RC-101. As RC-101 could block ZIKV infection at the entry stage (Fig. 2), we further investigated its effect on the E protein.

In our previously published work, we constructed a series of JEV variants with mutations in the receptor-binding motif or in amino acids critical for membrane fusion on the E protein (6). Considering the relative conservation of the sequence and structure of flavivirus E proteins, we used the constructed JEV variants to investigate the potential target of RC-101. Among the selected variants, the N154A and DE mutants (T363SSAN367 to A363AAAA367) impaired receptor binding by the virus, H144A and H319A abrogated the interaction between DI and DIII, and Q258A and T410A resulted in failure of the E protein to refold to form its postfusion conformation (6). Notably, these six tested sites were conserved between JEV and ZIKV (Fig. 5).

First, the antiviral effect of RC-101 against JEV was investigated. As shown in Fig. 6A to C, RC-101 dose-dependently inhibited JEV infection in BHK-21 cells, with an IC50 of 10.67 μM. Furthermore, the viral titer reduction assay confirmed that RC-101 robustly inhibited JEV infection in both BHK-21 and U251 cells (Fig. 6D).

The investigation was conducted using coadministration (Fig. 7A). As shown in Fig. 6B and C, RC-101 at 50 μM, corresponding to the approximate IC98 against ZIKV (Fig. 1), robustly inhibited JEV infection, which made the prM band hardly detectable.
and the viral titers decreased by approximately 3 log units. Similarly, RC-101 inhibited infections by viruses harboring N154A and H144A, suggesting that neither N154 nor H144 is the target of RC-101. Of note, the outcome indicating that abolishing the glycosylation motif (N154A) resulted in retained sensitivity to RC-101 was in line with the notion that differences in the number of glycan chains in different strains have little effect on RC-101 inhibition (Fig. 1). This further confirmed that RC-101 has a unique antiflavivirus mechanism, which is unlike the effects on other enveloped viruses. Notably, as shown in Fig. 7B and C, the Q258A mutant likely had increased sensitivity to RC-101, whereas H319A resulted in resistance to RC-101 at the protein level and in the low-multiplication of infection (MOI) assay. Among the six tested mutants, the DE mutant and T410A showed robust resistance to RC-101 in all assays, indicating that these two mutants do confer resistance and might serve as the viral glycoprotein target(s) of RC-101. As T410 is located in the stem region of the E protein, buried by the compacted E dimer and hardly accessible in the prefusion conformation, the DE mutant was selected for further investigation of the binding affinity to RC-101.

DE loop mutant decreases binding affinity to RC-101. To test the possibility that the DE loop is the target of RC-101, and to test whether the DE mutant would disrupt the binding of RC-101 to DIII, the binding affinities of the wild-type (WT) and the DE mutant DIII to RC-101 were examined by biolayer interferometry. The interactions between DIII and RC-101 were calculated using a 1:1 binding model at three different concentrations (Fig. 8). The results showed that RC-101 bound to WT DIII with a kinetic
association ($K_a$) of 1.46 × 10^4 M$^{-1}$ s$^{-1}$, kinetic dissociation ($K_d$) of 1.18 × 10^{-4} s$^{-1}$, and an equilibrium dissociation constant ($K_D$) of 8.10 × 10^{-9} M, indicating that RC-101 has high affinity for DIII. The binding affinity of RC-101 to the DE mutant was decreased by 1 order of magnitude, to a $K_D$ of 2.37 × 10^{-8} M, which suggested that the DE loop might be the binding site of RC-101 and that the DE mutant would disrupt this interaction.

**FIG 5** The potential viral target of RC-101 on flavivirus E protein. Shown is the side view of monomer prefusion Japanese encephalitis virus (JEV) E protein ectodomain conformation (cyan; PDB no. 3P54) in alignment with the full-length Zika virus (ZIKV) E protein (gray; PDB no. 5IRE). The potential targets tested in this study were enlarged and highlighted by color.

**FIG 6** RC-101 inhibits JEV infection. (A) Timeline of the assay. Cells were incubated with RC-101 at the indicated concentrations from 1 h preinfection and then infected with JEV AT31 at an MOI of 0.1 for 1 h. (B) BHK-21 cells infected with JEV were analyzed for prM expression using the IFA assay 24 h postinfection. Cells were imaged using an Operetta high-content imaging system (PerkinElmer). (C) Dose-response curve based on the IFA results. The percentages of infected and DAPI-positive cells were calculated using the Harmony 3.5 software in the Operetta high-content imaging system. (D) The inhibition effects were validated in both BHK-21 and U251 cells using the plaque assay. Data are presented as the mean ± SD from six independent experiments. LOD, limit of detection. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. 
Although RC has been reported to have inhibitory effects against different kinds of viruses with various antiviral mechanisms, few studies have investigated its effect on flaviviruses. In this study, we evaluated the antiviral effects of RC-101 against flaviviruses and elucidated the mechanism of action. As the analogue RC-1 has been reported to inhibit DENV NS2B-NS3 protease and viral replication, we first tested whether RC-101 could extend its antiviral spectrum to other flaviviruses. As a result, RC-101 was found to inhibit infections by different strains of ZIKV, as well as JEV. Furthermore, results suggest that the NS2B-NS3 protease might serve as one of the viral targets since RC-101 could block the serine protease activity of NS2B-NS3. The NS3 proteolytic domain forms a substrate-binding pocket with a catalytic triad, conserved in flaviviruses, of His-Asp-Ser (Fig. 9A). In an attempt to dock the analogue RC-2 (PDB no. 2LZI [GICRCICGRRICRCICGR]) (40) with ZIKV NS3 (PDB no. 5ZMS) (41), we found that glycine in RC-2 might interact with histidine (H1553) and serine (S1673) in the catalytic triad, and both of these residues are structurally conserved between ZIKV and JEV (Fig. 9B). RC-101 might thus inhibit NS2B-NS3 protease activity by competitively blocking the catalytic motif and thus preventing substrate binding. Meanwhile, as a cationic peptide, RC-101 might directly interact with the negatively charged NS2B and thus prevent the binding of NS2B and NS3 (20, 42).

**FIG 7** Sensitivity/resistance of the mutant viruses to RC-101. (A) Timeline of the assay. (B, top) JEV-infected BHK-21 cell lysates were analyzed by Western blotting at 24 h postinfection, and rabbit prM antiserum and the anti-GAPDH mouse monoclonal antibody were used as the primary antibodies (MOI of 0.1). (B, bottom) Quantification results of Western blotting are presented as the mean ± SD from 4 to 5 independent experiments. (C) The viral titers were tested by plaque assay using BHK-21 cells. Data are represented as the mean ± SD from 4 to 6 independent experiments. LOD, limit of detection. ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
As mentioned previously herein, RC has been extensively reported to inhibit enveloped viruses by targeting the negative glycan shield on the surface of the virus, thus blocking the initial entry of the virus into host cells (22–24). As the only glycan chain in the E protein of the ZIKV PRVABC 59 strain and JEV, the glycan linked to the N154YS glycosylation motif has been reported to interact with DC-SIGN, which is a candidate flavivirus receptor (43). Intriguingly, the N154A mutation had no impact on the sensitivity or resistance of JEV to RC-101. A possible explanation for this phenomenon is that RC-101 could easily bind with the dense glycan shield of gp120 and hemagglutinin (HA) of HIV and IAV, but in case of the flavivirus, RC-101 might pass through the unique glycan and interact with the E protein directly. The DE loop, which is the relatively higher tip of the E protein (Fig. 5), might serve as the viral target of RC-101. Although peptides derived from the DE loop were previously found to prevent JEV infection by interfering with virus attachment to BHK-21 cells (44), the DE loop is not the only or major receptor binding motif for JEV entry into different types of cells (6). Further studies should focus on whether RC-101 could inhibit flavivirus infection of different kinds of cells and whether the DE mutant confers resistance to RC-101 in other hosts and tissues.

Currently, there are no effective drugs approved for the treatment of flavivirus infection. Fortunately, several peptide inhibitors, derived from or targeting the E protein, have been used to successfully block flavivirus infection in vitro and in vivo (7, 9, 12, 45). As the flavivirus E protein has a highly conserved sequence and conformation, peptide inhibitors could be used for the treatment of emerging flavivirus infections or severe cases. In addition, peptide inhibitors have many advantages, such as high bio-compatibility, a low frequency of selecting resistant mutants, the ability to synergize with conventional drugs, and activity toward multidrug-resistant virus strains (46). The cyclic peptide RC-101, with a unique structure that provides long-lasting protection against viral infection (47, 48), is a potential candidate for the development of a successful drug to treat flaviviruses and other infectious diseases.

**MATERIALS AND METHODS**

**Cells, viruses, and RC-101.** Vero, BHK-21, and U251 cells were maintained in Dulbecco’s modified Eagle’s medium and minimum essential medium containing 10% fetal bovine serum (FBS), respectively. The ZIKV PRVABC 59 strains were kindly provided by Jean K Lim (Icahn School of Medicine at Mount
The time-of-addition experiment was performed as previously described (51). Vero cells were infected and stained with 0.1% crystal violet for plaque visualization. For 1 h. Then, the supernatant was discarded, and the cells were overlaid with medium containing 1% prM antiserum, anti-JEV NS3 antibody (a gift from Bo Zhang, Wuhan Institute of Virology), and the anti-Operetta high-content imaging system (PerkinElmer), and the percentages of infected and DAPI-positive cells were evaluated by IFA assay and plaque assay.

Primary antibodies. Anti-ZIKV NS3 was a gift from Andres Merits, University of Tartu, Estonia, while the anti-GAPDH (anti-glyceraldehyde-3-phosphate dehydrogenase) mouse monoclonal antibody was purchased from AbClonal (AC033, Wuhan, China). The anti-JEV prM polyclonal antibody was prepared by expressing full-length prM in E. coli BL21 using a PET30a expression vector; purified protein was injected into rabbits to obtain the antiserum (6).

IFA assay. Cells were fixed with 4% paraformaldehyde, permeabilized using phosphate-buffered saline (PBS) containing 0.2% Triton X-100 for 15 min, and blocked with 3% fetal bovine serum (FBS; Gibco), followed by treatment with the primary antibody: anti-ZIKV NS3 or anti-JEV prM. After six rinses with PBS, the cells were stained with the secondary antibody, DyLight 488-labeled anti-rabbit IgG (KPL, Gaithersburg, MD, USA). Nuclei were then stained with DAPI (4',6-diamidino-2-phenylindole) according to the manufacturer’s instructions (Sigma-Aldrich, USA). Nine fields per well were imaged using an Operetta high-content imaging system (PerkinElmer), and the percentages of infected and DAPI-positive cells were calculated using the associated Harmony 3.5 software.

Western blotting. JEV-infected BHK-21 cells were lysed and subjected to 10% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane, which was blocked in 5% milk for 1 h. Then, the membrane was incubated with the primary antibodies: anti-ZIKV NS3 or anti-JEV prM. After a 1-h incubation at room temperature, the membrane was washed three times with TBS-T, before being incubated with a secondary antibody, DyLight 488-labeled anti-rabbit IgG (KPL, Gaithersburg, MD, USA). Nuclei were then stained with DAPI (4',6-diamidino-2-phenylindole) according to the manufacturer’s instructions (Sigma-Aldrich, USA). Nine fields per well were imaged using an Operetta high-content imaging system (PerkinElmer), and the percentages of infected and DAPI-positive cells were calculated using the associated Harmony 3.5 software.

To determine which stage of the ZIKV life cycle was inhibited by RC-101, a time-of-addition experiment was performed as previously described (51). Vero cells were incubated with ZIKV (MOI, 0.1) for 1 h before infection (~1 to 0 h), and the mixture was diluted 25-fold to infect Vero cells for 1 h. The inhibitory effect of RC-101 against ZIKV replication, BHK-21 cells were electroporated with the ZIKV replication (SZ-WIV001, GenBank accession no. KU963796) and then incubated with RC-101. Renilla luciferase activity in the cell lysates was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) (52).

To produce NS2B-GGGGSGGGG-NS3 protein, the ZIKV replication was used as the template, and the NS2B fragments were amplified by PCR using a forward (5’-TTAAGAACGAGGATCAGTTGCGATGACATGATGTTGAAGAAG-3’) and reverse (5’-CCACCATTTCCACCCCTTCTCCACCGTCGATCCTTCTCATGGGGGACC-3’) primer pair, and NS3 was also amplified using a forward (5’-GAGATCTGGTGAGTGATCGGCTGGGAATGGTGCTCTAATGGGA TGTCG-3’) and reverse (5’-CTCAGTGCTGCGTGTTGGGCTGTCGAGCTCTCTCACGACGCAAGGTGCGAAGG-3’) primer pair. The underlined letters represent restriction endonuclease sites, and the italic letters represent the GGGGSGGGG linker sequence (20). The PCR products were cloned into pET28a using an EnSpire multimode plate reader with the emission at 400 nm upon excitation at 350 nm. The kinetic parameter of NS2B-NS3pro was measured using an EnSpire multimode plate reader with the emission at 400 nm upon excitation at 350 nm. The kinetic parameter of NS2B-NS3pro was measured using an EnSpire multimode plate reader with the emission at 400 nm upon excitation at 350 nm. The kinetic parameter of NS2B-NS3pro was measured using an EnSpire multimode plate reader with the emission at 400 nm upon excitation at 350 nm. The kinetic parameter of NS2B-NS3pro was measured using an EnSpire multimode plate reader with the emission at 400 nm upon excitation at 350 nm. The kinetic parameter of NS2B-NS3pro was measured using an EnSpire multimode plate reader with the emission at 400 nm upon excitation at 350 nm. The kinetic parameter of NS2B-NS3pro was measured using an EnSpire multimode plate reader with the emission at 400 nm upon excitation at 350 nm. The kinetic parameter of NS2B-NS3pro was measured using an EnSpire multimode plate reader with the emission at 400 nm upon excitation at 350 nm. The kinetic parameter of NS2B-NS3pro was measured using an EnSpire multimode plate reader with the emission at 400 nm upon excitation at 350 nm. The kinetic parameter of NS2B-NS3pro was measured using an EnSpire multimode plate reader with the emission at 400 nm upon excitation at 350 nm. The kinetic parameter of NS2B-NS3pro was measured using an EnSpire multimode plate reader with the emission at 400 nm upon excitation at 350 nm. The kinetic parameter of NS2B-NS3pro was measured using an EnSpire multimode plate reader with the emission at 400 nm upon excitation at 350 nm. The kinetic parameter of NS2B-NS3pro was measured using an EnSpire multimode plate reader with the emission at 400 nm upon excitation at 350 nm.
WT DIII was expressed using E. coli BL21(DE3); the supernatant of the bacterial pellets was loaded onto a nickel column, and the bound protein was eluted with a gradient concentration of imidazole buffer. DE mutant DIII, expressed as inclusion bodies, was solubilized in 8 M urea (50 mM Tris-HCl, 100 mM NaCl, 1 mM dithiothreitol [DTT], 0.1% SDS, 8 M urea, pH 7.4). Refolding was carried out by titration dialysis at 4°C against refolding buffer (50 mM tris-HCl, 100 mM NaCl, 0.1% SDS, 1 mM l-(+)-arginine, 1 mM glutathione, 5% glycerol [pH 7.4]) until the concentration of urea was ≤ 2 M. Then, the supernatant was passed through a nickel column as described previously herein.

Binding affinity assay. Real-time binding assays between RC-101 and WT or the DE mutant DIII were performed using biolayer interferometry on an Octet QK system (Fortebio, USA) according to previously reported methods (7). Binding kinetics were calculated using the Octet QK software package, which fit the observation to a 1:1 model to calculate the association and dissociation rate constants. Binding affinities were calculated as the $K_d$ rate constant divided by the $K_a$ rate constant.

Docking of the NS2B-NS3/RC-2 complex. The crystal structures of RC-2 (PDB no. 2ZLI) and ZIKV NS3 (PDB no. 5ZMS) were used to build the complex using the ZDOCK 3.0.2 program (http://zdock.umassmed.edu) (54). The resulting model was represented by PyMOL (Fig. 9).

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