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Anti-Zika Activity of a Library of Synthetic Carbohydrate Receptors

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ABSTRACT

Zika virus (ZIKV), a mosquito-borne flavivirus, is a global health concern because of its association with severe neurological disorders. Currently, there are no antiviral therapies that have been specifically approved to treat ZIKV, and there is an urgent need to develop effective anti-ZIKV agents. Here, we report anti-ZIKV activity of 16 synthetic carbohydrate receptors (SCRs) that inhibit ZIKV infection in Vero and HeLa cells. Using a ZIKV Reporter Virus Particle-based infection assay, our data demonstrates these SCRs are highly potent with IC_{50}'s as low as 0.16 μM and negligible toxicity at several fold higher concentrations. Time-of-addition studies showed that these SCRs inhibit the early stages of the virus infection, which is consistent with the proposed mode of action, where the SCRs likely inhibit binding between the virus and cell-surface glycans, thereby preventing viral entry into the cells, and, as such, this study demonstrates a potential new strategy against ZIKV.

INTRODUCTION

The *Flaviviridae* (FLVs), which is a family of viruses that includes Zika (ZIKV), West Nile, hepatitis C, dengue, yellow fever, and Japanese encephalitis, are an emerging global health threat.[1] ZIKV[2] was first isolated[3] in the Zika forest of Uganda in 1947 from a febrile sentinel rhesus monkey. Although it is mainly transmitted to humans by mosquitoes of *Aedes* genus,[4] sexual,[5] maternal-to-fetal,[6] blood transfusion, and other modes of transmission have also been reported.[4] ZIKV was detected in Asia in the 1980s and then outbreaks were reported in Micronesia and French Polynesia in 2007 and 2013, respectively.[2c] Since its arrival to Brazil in 2014, infecting millions of people, it has rapidly spread throughout the Americas causing an expanding pandemic.[7] ZIKV infection can cause symptoms such as fever, rash, muscle pain, headache, retro-orbital pain, joint pain, and conjunctivitis, but it is asymptomatic in most cases.[8] Recent studies, however, have shown that ZIKV is also linked to severe neurological disorders such as microcephaly[9] or other severe brain malformations in fetuses and newborn babies, and Guillan-Barré syndrome[10] in adults. Further studies revealed that ZIKV also causes severe eye diseases and blindness in newborns, and conjunctivitis and uveitis in adults.[11] Because of the severity of these symptoms, the World Health Organization declared ZIKV a global health emergency of international concern in February 2016.[12] Although great strides have been made since 2016 in the search for drugs for the treatment of ZIKV, there is to date no vaccine or antiviral therapy approved specifically to treat ZIKV.[13] Rather, treatment is focused currently on relieving symptoms with analgesics and antipyretics.[14] Thus, there is an urgent need to develop novel agents with anti-ZIKV activity that can prevent or mitigate infection.

To this end, significant recent efforts have been devoted to testing libraries of compounds and repurposing of drugs already approved towards viral targets or cellular targets.[15] Drugs such

as BCX4430,[16] brequinar,[17] gemcitabine,[18] sofosbuvir,[19] and finasteride[17] inhibit ZIKV replication by targeting RNA-dependent RNA polymerase. Other classes of agents such as viral protease inhibitors,[20] virucidal agents,[21] antimalarials,[22] antibiotics,[22c,23] immunomodulators,[24] immunosuppressants,[22c] fusion inhibitors,[17,18,22b,25] antiparasitic,[22c] proteasome inhibitors,[22c] antidepressant,[22c] cyclin-dependent kinase inhibitor,[25] apoptosis-related drugs[18,23a,25] and hypolipidemic drugs[23b] also possess anti-ZIKV activity. Although several compounds have advanced to phase I clinical trials,[26], without an approved compound to treat the infection, there still remains a pressing need to explore molecules that inhibit ZIKV using alternate, less conventional strategies.

An important part of the ZIKV lifecycle – and one that is not widely targeted by antiviral therapies – is the binding of proteins on the viral envelope to cell-surface glycans.[27] After making contact with host cell surface, FLVs enter the host cell through clathrin-mediated endocytosis involving conformational changes of envelope proteins, resulting in membrane fusion and release of the viral genome. A promising therapeutic strategy involves disrupting this process with compounds that can mimic or, alternatively, bind the glycans of the host or of the virus.[28] In FLVs this docking process involves cellular receptors like glycosaminoglycans (GAGs),[29] neolactotetraosylceramide,[30] Gas6-AXL tyrosine kinase receptor complex,[31] and the dendritic cell-specific intercellular adhesion molecule-grabbing non-integrin (DC-SIGN),[32] a carbohydrate-binding lectin abundant in immature dendritic cells that interacts with the highly mannosylated *N*-linked glycan on the FLV envelope protein. Natural and synthetic compounds that inhibit this process by mimicking or targeting glycans of host cells or of viruses have been investigated therapeutically. For example, the highly mannosylated *N*-glycans of the human immunodeficiency virus (HIV) have a crucial role in transmission of the pathogen into the target cells, and also act as

a shield to protect the virus from the host immune response.[33] To this end, lectins such as microvirin and cyanovirin interact with the densely mannosylated gp120 of HIV envelope and inhibit viral transmission.[34] However, because of their high molecular weight and peptidic nature, further development of these lectin-based therapeutic agents was unsuccessful. Alternatively, small molecule-based carbohydrate-binding agents can also disrupt the viral docking process. For example, the antibiotics pradimicin A, benanomicin A, and their analogues that bind terminal D-mannopyranosides exhibit antiviral activity in cell culture with 50% effective concentration against HIV-1 in the micromolar concentration range.[35] Similarly, 1,3,5-triazines bind gp120 of the HIV envelope and inhibit HIV-1.[36] Aminopyrrolic synthetic carbohydrate receptors (SCRs) – synthetic molecules that are designed to form supramolecular complexes with carbohydrates – bind gp120 and inhibit HIV-1 infection at micromolar concentrations.[37] With respect to FLVs, bovine lactoferrin, an antimicrobial protein, and basic peptides derived from antimicrobial chemokines, CXCL9 and CXCL12γ, show anti-FLV activity by binding GAGs.[38] In addition, high mannose-based dendrimers achieve anti-FLV activity by competing with the high mannose glycans of the viral envelope protein that interact with DC-SIGN.[39] Similarly, iminosugar-based α-glucosidase inhibitors that permanently modify the viral glycan structure in cytoplasm have also been developed.[40] There are still no reports, however, on SCRs whose anti-FLV activity derives from binding of glycans on the viral envelope protein or disrupting interactions between host-cell glycans and glycan binding proteins on the viral envelope, and pursuing this strategy could lead to new lead compounds with potent anti-FLV activity.

In an effort to explore molecules with just such a mode of action, our group has developed recently a series of small molecule SCRs that preferentially bind mannosides and glucosides. The binding of 10 of these SCRs with a series of monosaccharides was studied by ^{1}H NMR in

chloroform[41b] and dichloromethane[41a], and their association constants (K_as) towards a series of biologically-relevant monosaccharides were reported, with selectivities as high as 103:1 β-Man:β-Gal. This preference for binding mannosides is driven by cooperative binding modes that arise from the flexible and multivalent structures of the SCRs. As association between glycan binding proteins on the envelope of ZIKV and glycans on the cell surface is an important part of viral entry into the host cell, we envisioned that the SCRs could disrupt this process, so we assessed the anti-ZIKV activity of these SCRs, and the results of those studies are described herein. While our study clearly demonstrates the potential of SCRs to inhibit ZIKV, further studies are needed to define the precise mechanism of action.

RESULTS AND DISCUSSION

Here, we report the ability of the components of a library containing 16 novel, small molecule SCRs to mitigate ZIKV infection in Vero and HeLa cells using a ZIKV reporter virus-based infection assay. The C-prM-E (Capsid-preMembrane-Envelope) gene construct of ZIKV is used to generate Reporter Virus Particles (RVPs) that package a GFP reporter expressing WNV replicon. These RVPs infect cells in a manner identical to native ZIKV, with the advantage of providing a rapid GFP readout in a 96 well format.[42] Results of cell viability/cell toxicity, inhibition of ZIKV infection, the IC_{50} values of these compounds, and some mechanistic insights based on time of compound addition are presented herein. Structure-activity relationships and correlations between mannose-binding of the SCRs and anti-ZIKV activity are discussed.

Synthesis of carbohydrate receptors. The 16 SCRs studied here (Figure 1) were based upon the structure of **3** – a mannose-selective SCR[41b] – with systematic structural alterations designed to explore relationships between molecular design and anti-ZIKV activity. Compounds **4**–**11**

maintain the biphenyl core and vary the heterocycle. Dimeric SCRs **15** and **16** were designed to investigate the role of multivalency on binding carbohydrate guests. Compounds **1**–**2** and **12**–**14** are intermediates in the syntheses of the receptors and were assayed to investigate the importance of the heterocyclic ring and the linker on anti-ZIKV activity. All of these compounds were synthesized from common intermediate **1**[41b]. We have reported the synthesis of compounds **2**–**11**, **13**, and **15** previously,[41a] and the preparation of compounds **12**, **14**, and **16** is described in the Supporting Information. The strategy used to prepare these compounds is modular, scalable, and amenable to formulating large libraries of similar molecules that can be readily synthesized to maximize antiviral activity or to understand relationships between molecular structure and viral inhibition.

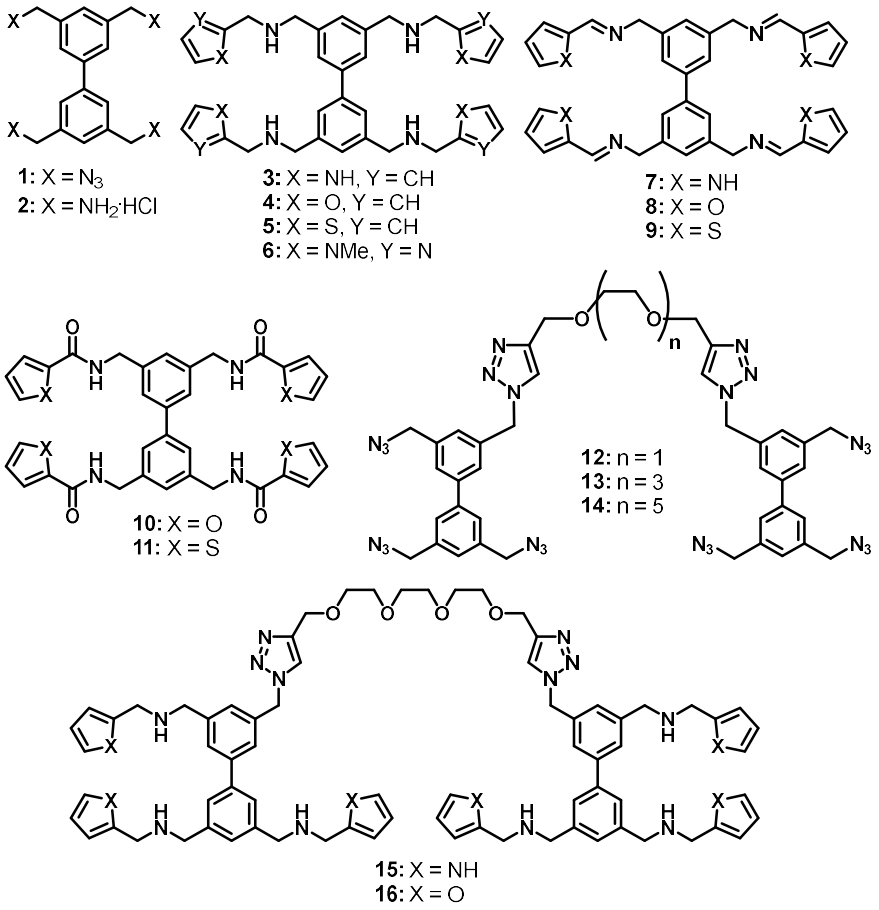

Figure 1. Synthetic Carbohydrate Receptors (SCRs) screened for activity against ZIKV.

Anti-ZIKV activity of SCRs. To determine the anti-ZIKV activity of the 16 SCRs, Vero cells were pre-incubated with the compounds for 30 min at room temperature followed by infection with ZIKV GFP RVPs (Figure 2 and Supplementary Figure S10A). Vero cells were chosen because they are highly permissive to infection by FLVs. The number of GFP-positive cells, which is a measure of virus infection, was determined 72 h post-infection. Compounds were screened for anti-ZIKV activity at 100 µM concentration, and those compounds that showed activity were further assayed in dose response curves. Figure 2A shows a representative image of ZIKV RVP infection in the presence of different concentrations of **15**. As depicted in the fluorescent microscopy image of a single well of a 96 well plate, the number of GFP-positive cells increases with increasing dilution of **15**, with the control (complete absence of **15**) showing

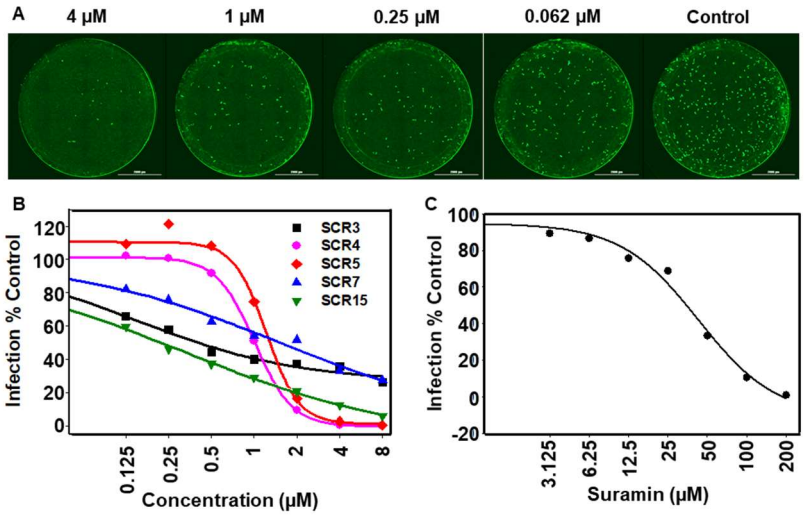

Figure 2. Anti-ZIKV activity of SCRs in Vero cells and comparison with Suramin. (A) Representative images of individual wells of a 96 well plate showing inhibition of ZIKV infection in the presence of SCR **15**. ZIKV infection is represented by the number of GFP positive cells. Control represents ZIKV infection in the absence of any SCR. (B) Inhibition curves of ZIKV infection in the presence of indicated SCRs. (C) Inhibition curves of ZIKV infection in the presence of Suramin.

Table 1. Inhibitory activity of SCRs against ZIKV infection in Vero cells

SCR	IC_{50} (μM)	TC_{50} (μM)	K_a (M^{-1}) with α-Man[a]	K_a (M^{-1}) with β-Man[a]
1	>100	b	b	b
2	>100	b	b	b
3	0.36 ± 0.15	34.15	1.4×10^3	3.6×10^4
4	1.13 ± 0.23	5.43	1.1×10^2	5.4×10^1
5	1.31 ± 0.13	4.12	1.4×10^2	c
6	>100	b	4.4×10^3	5.9×10^2
7	1.36 ± 0.27	46.11	3.0×10^3	2.4×10^3
8	>100	b	2.0×10^2	3.7×10^1
9	>100	b	c	c
10	Not soluble	b	b	b
11	>100	b	b	b
12	>100	b	b	b
13	>100	b	b	b
14	>100	b	b	b
15	0.16 ± 0.05	36.2	2.6×10^3	1.7×10^3
16	12.37 ± 2.99	51.52	b	b
Suramin	44.02 ± 4.19	>200	b	b

a) Association constant (K_a) between octyloxy pyranosides and SCRs from NMR titrations in CD_2Cl_2 at 298 K.[41a] b) Not determined. c) No detectable binding. IC_{50}: 50% inhibitory concentration; TC_{50}: 50% toxic concentration.

maximum infection. This same assay was then used to measure ZIKV infection in the presence of all 16 SCRs. Six of the 16 SCR receptors showed some level of activity against ZIKV infection in Vero cells, with **15** being the most potent (Table 1). Suramin, an FDA-approved drug for the treatment of trypanosomiasis, has recently been shown to have activity against several viruses, including ZIKV, by inhibiting different aspects of the virus life cycle including, attachment, fusion, and reverse transcription.[43] As Suramin has been reported to interfere with ZIKV attachment, we

conducted the same assay using Suramin for comparison. As demonstrated in Figure 2C, Suramin showed a dose dependent inhibition of ZIKV infection, although with significantly lower potency than the screened SCRs. These findings establish the anti-ZIKV activity of this class of molecules. Data for selected compounds are shown in Figure 2 (data for all compounds are provided in Supplementary Figure S10), and these data indicate that receptor **15** shows the best infection control followed by its monomeric counterpart, **3**.

As Vero cells are derived from African green monkeys, we also tested the anti-ZIKV activity of SCRs in cells of human origin. HeLa cells were recently used by Barrows *et al.*[15a] to screen for ZIKV inhibitors and by Chan *et al.*[44] to study ZIKV pathogenesis and transmission. To this end, we determined IC_{50} values for the six SCRs that were the strongest inhibitors of ZIKV infection in Vero cells using the same RVP assay (Supplementary Figure 11A). In HeLa, SCR **15** remains the most potent, with a similar IC_{50} value (Table 2). This result confirms that the anti-ZIKV activity of the SCRs is maintained against human cell lines.

Table 2. Inhibitory activity against ZIKV infection and toxicity of select SCRs in HeLa cells.

SCR	IC_{50} (μM)	TC_{50} (μM)
3	0.45 ±0.06	2.43
4	0.35 ± 0.09	2.51
5	0.56 ± 0.08	2.47
7	3.06 ± 0.59	17.59
15	0.24 ± 0.02	2.48
16	1.37 ± 0.18	2.22

IC_{50}: 50% inhibitory concentration; TC_{50}: 50% toxic concentration.

Cytotoxicity and cell viability study. For a compound to have therapeutic potential it should have a high efficacy with minimum toxicity. To this end, the cytotoxic activity of the screened

compounds was assessed in Vero cells. For this, Vero cells were incubated with different concentrations of the SCRs for 72 h (Table 1 and Supplementary Figure S10B). Cell viability was determined by measuring intracellular ATP levels using Cell Titer Glo assay. Cell viability curves were fit using Sigma plot and 50% toxic concentration (TC_{50}) of each compound was determined. Changes in cell morphology were also assessed via microscopy. As demonstrated in Table 1, the TC_{50} values for all compounds that showed anti-ZIKV activity were several fold higher than their anti-ZIKV IC_{50}, suggesting the potential for therapeutic exploration. For the most potent ZIKV inhibitor, **15**, the TC_{50} of 36.2 μM was >220-fold higher than the IC_{50} of 0.16 μM. For comparison, Suramin, which also demonstrated anti-ZIKV activity, had an IC_{50} that was much higher than the SCRs. Cytotoxicity was also assessed in a cell line of human origin-HeLa cells for the most active subset of SCRs. As demonstrated in Table 2 and Supplementary Figure S11B, the TC_{50} values of the SCRs tested were several fold higher than the IC_{50} value.

Time-of-addition study. To gain insight into the mechanism via which the synthetic carbohydrate receptors inhibit ZIKV infection in the Vero cells, time-of-addition experiments were carried out. The compounds were added to the Vero cells either 30 min prior to infection or at 4 h or 24 h post-infection. Plates were incubated for 72 h and degree of infection was determined by the number of GFP+ cells. Suramin was also studied for comparison. As seen in Figure 3, the SCRs were most effective when added –30 min (prior to infection), and were less effective at 4 h and least effective at 24 h post-infection. These results were similar to those obtained with Suramin, a known inhibitor of virus attachment and infection,[43] suggesting that – similar to Suramin – the SCRs act upon the virus by inhibiting early stages in the virus life cycle, most likely by preventing virus attachment and/or viral entry. These data are consistent, although not conclusive, with a proposed mechanism of activity, where the SCRs operate on the virus by binding glycans involved with viral docking.

While these studies provide mechanistic insights regarding SCR mediated inhibition of ZIKV infection, inhibition of replicating ZIKV by the SCR supports the idea that the compounds are active in targeting multiple round virus replication as well. Further experiments will be needed before a mechanism of inhibition is conclusively determined.

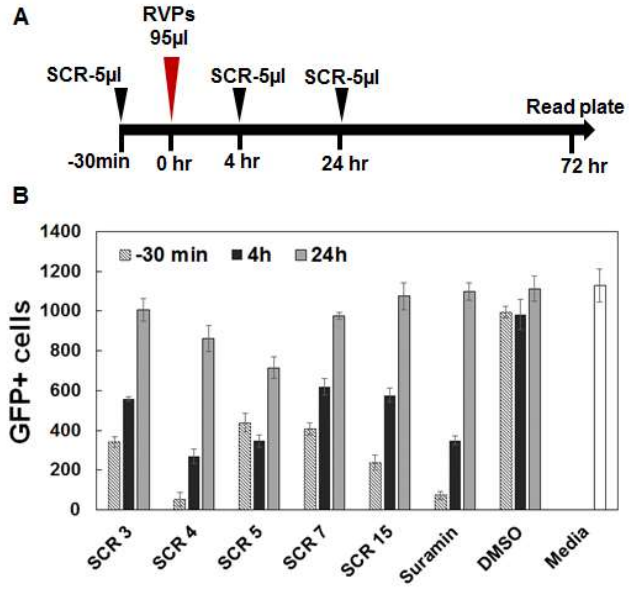

Figure 3. Time of compound addition studies and inhibition of ZIKV infection with the five most potent SCRs. (A) Schematic of time-of-addition experiments. Cells were treated with SCRs either 30 min prior to infection or 4 h and 24 h post ZIKV RVP infection. Plates were read 72 h post infection. (B) Vero cells were treated with the indicated compounds or DMSO control as indicated in part (A) above. The number of GFP positive cells was determined 72 h post infection.

Inhibition of infectious virus with SCRs. As RVPs are only capable of initiating a single round of infection, we next tested the anti-ZIKV activity of the most potent SCR, **15**, using infectious Zika virus isolate PRVABC-59[45] as well as Suramin and DMSO as controls. Vero cells were pre-incubated with the compounds or DMSO followed by infection with a predetermined amount of ZIKV based on titration data (Supplementary Figure S12)**.** Cells were then fixed and stained for

ZIKV protein expression using the anti-FLV group antigen antibody 4G2. As shown in Figure 4, there is excellent inhibition of infectious ZIKV with **15** and to a lesser extent with Suramin (Figure 4B). As expected, there was no inhibition seen with the DMSO control (Figure 4A). Moreover, fluorescent microscopy analysis showed characteristic peri-nuclear staining pattern for ZIKV Envelope in DMSO treated but not **15** (8 μM) or Suramin (200 μM) treated cells. This suggests that the SCRs are not only capable of inhibiting RVPs but also infectious virus in multiple round infection assays.

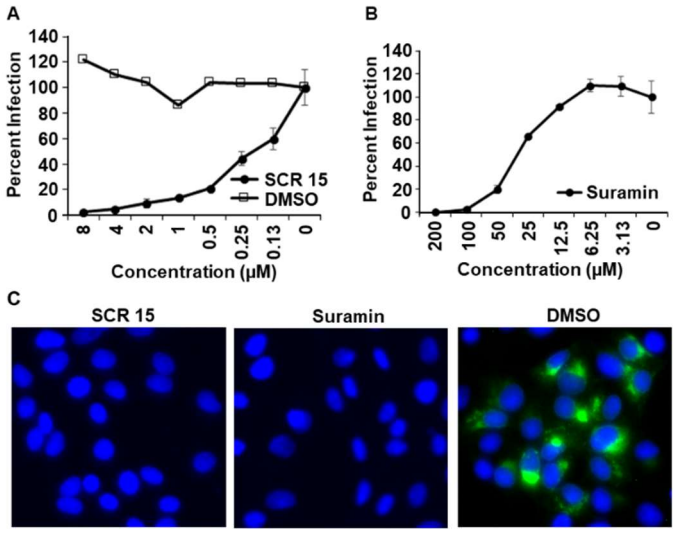

Figure 4. Anti-ZIKV activity of SCR **15** and Suramin against infectious ZIKV. Vero cells were treated with (A) SCR **15**, DMSO control, or (B) Suramin for 30 min at room temperature followed by infection with ZIKV. Cells were fixed and stained for ZIKV protein expression using the 4G2 antibody. Images of wells were acquired and number of GFP positive cells quantified. Data are mean ±SD of triplicate observations. Data from one representative experiment are shown. (C) Representative fluorescent microscopy images of cells treated with SCR **15** (8 μM) or Suramin (200 μM) or DMSO followed by ZIKV infection and staining. Blue=DAPI; Green=ZIKV Env.

SCRs do not affect the N154 glycosylation site of ZIKV Env. Based on our preliminary data we anticipate that the SCRs could likely be binding to *N*-mannosylated regions of Zika E protein. One such glycosylation site, N154, has been shown to be important for ZIKV cell surface binding and infection[46]. To understand whether this site was involved in the antiviral activity of SCRs we generated the N154Q mutant and analyzed inhibition mediated by SCR **15**. Interestingly, the N154Q mutant was inhibited with both SCR **15** and Suramin, similar to WT RVPs (Figure 5A and B). This suggests that glycosylation sites other than N154Q may be important for ZIKV attachment in Vero cells, or that **15** may disrupt other virus carbohydrate interactions. These results are strikingly similar to Suramin, our control compound, suggesting that the mechanism of inhibition by SCRs may be similar to other entry inhibitors like Suramin. In support of this, it was shown for a related flavivirus, WNV that the presence of a single *N*-linked glycosylation sites on the prM or E protein was sufficient for virus tropism under certain conditions and in certain cell types.[47]

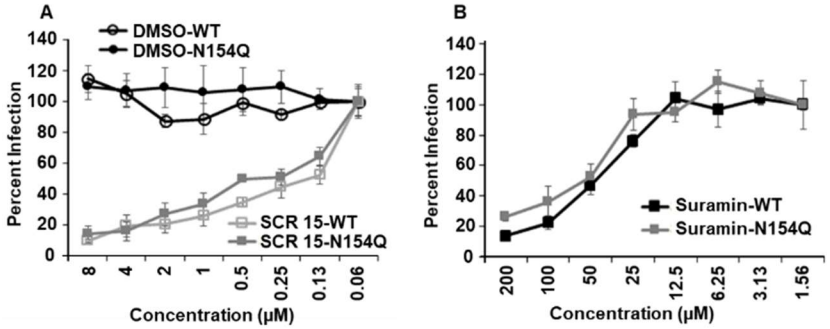

Figure 5. Abolishing the ZIKV Env glycosylation site N154 does not affect the inhibition mediated by SCRs. Vero cells were treated with (A) SCR **15**, DMSO control, or (B) Suramin for 30 min at room temperature followed by infection with WT or ZIKV-N154Q mutant RVPs. Images of wells were acquired 72h post infection and number of GFP positive cells quantified. Data are mean ±SD of triplicate observations. Data from one representative experiment is shown.

Structure-activity analysis. In Vero cells, the best inhibitory activity corresponded to SCR **15** and its monomer **3**, respectively, indicating that the pyrrolic heterocycles and secondary amine groups are important for anti-ZIKV activity. Further, the improved activity of SCR **15** compared to **3** (approximately double) shows the importance of multivalency for antiviral activity: **15** has approximately double the number of aminopyrrolic groups compared to **3**. The synthetic intermediates did not show activity against ZIKV as anticipated, confirming the necessity of both the biaryl core and the pendant π-electron rich heterocycles. Receptors **4**, **5**, and **7**, which lack either a secondary amine group or a pyrrole ring, are less potent in Vero cells, although **4** is more potent than **3** in HeLa cells. However, furan-based multivalent receptor **16** shows activity far lower than that of its monomer **4**, the reasons for which are not well understood. Imine- and amide-based receptors **8**–**11** were not effective against ZIKV. These data indicate that both the aminopyrrolic groups and secondary amine linkers contribute to high ZIKV inhibition.

There appears to be a correlation between anti-ZIKV binding and the previously determined binding affinities of the SCRs for mannosides and glucosides (Table 1).[41a] While SCRs **3**, **6**, and **15** are the strongest carbohydrate binders, SCR **15** exhibits the best inhibitory activity. This result suggests that carbohydrate binding may play a role in the anti-ZIKV activity of the SCRs, but stronger inhibition of **15** may suggest that the effects of multivalency are magnified in the dense cellular environment compared to in solution. SCR **6**, which also binds α-mannosides strongly in solution,[41a] but does not show any anti-Zika activity, further suggests the importance of pyrroles in cellular environments and that other glycans, besides mannosides, may be involved in viral entry. Other cell-surface glycans, such as GAGs, which are densely decorated with *N*-acetyl glucosamines have a role in ZIKV infection and may also be involved in the anti-ZIKV activity of these compounds, so these studies are inconclusive with respect to the mechanism of

inhibition, and clearly indicate that further research is needed to confirm the origin of anti-ZIKV activity.

CONCLUSION

In conclusion, we have synthesized and evaluated the anti-ZIKV activity of 16 SCRs, a class of compounds whose anti-ZIKV activity has not been previously reported. Both **3** and **15** are active at sub-micromolar concentrations, which is comparable to the best anti-ZIKV agents known, and significantly more potent than Suramin. The TC_{50} values are significantly greater than the IC_{50} values, suggesting that these compounds merit further therapeutic exploration. Based on the importance of pyrrolic heterocycles, secondary amine groups, and multivalency on the potency of SCRs, we propose that the anti-ZIKV activity can be enhanced by increasing multivalency by incorporating more pyrrolic heterocycles and secondary amine groups in future inhibitors. Time-of-addition studies imply a mode of action whereby the SCRs inhibit attachment of the virus to the host cell. Structure-activity analysis suggests that anti-Zika activity may correlate to glycan binding ability, and further studies are needed to confirm the mode of inhibition. These results confirm that SCRs have the potential to become powerful therapeutic agents in the battle against ZIKV, and they may act by a mechanism that has not yet been explored widely despite its therapeutic potential. Given the proposed mode of action of these SCRs, involving disrupting glycan-protein binding on the cell surface, it is worth evaluating SCRs as probes for studying virus-host interactions.

EXPERIMENTAL SECTION

Synthetic procedures

General. All solvents, reagents and starting materials were purchased from commercial sources and used without further purification unless otherwise noted. All solvents were dried using a JC Meyer solvent purification system. Aqueous solutions were prepared from nanopure water from a Milli-Q plus system, with a resistivity over 18 MΩ cm^{-1}. Chromatography purifications were performed using silica gel (60 Å, 70-230 mesh). Thin-layer chromatography (TLC) was carried out using aluminum sheets precoated with silica gel 60 (EMD 40-60 mm, 230-400 mesh with 254 nm dye). TLC plates were visualized by UV-light and using charring solution (prepared by dropwise addition of conc•H_2SO_4 (5 mL) to a solution of $H_3PMo_{12}O_{40}$ (1 g) and $Ce(SO_4)_2$ (2 g) in water (95 mL)), alkaline $KMnO_4$ solution (prepared by dissolving $KMnO_4$ (2 g) and $NaHCO_3$ (4 g) in water (100 mL)), and heat as developing agents. All reactions were carried out under an inert atmosphere of Ar using standard Schlenk techniques unless otherwise noted. Reaction flasks were dried in an oven at 100 °C for 12 h. Compounds **1**[41b], **2–11**[41a], **13**[41a], **15**[41a], 1,2-bis(prop-2-yn-1-yloxy)ethane[48], and 3,6,9,12,15,18-hexaoxaicosa-1,19-diyne[48] were synthesized according to published literature procedures. Deuterated solvents were purchased from Cambridge Isotope Laboratories Inc. and used as received. NMR spectra were obtained on a Bruker AVANCE 300 MHz spectrometer. All chemical shifts are reported in δ units (ppm) using the solvent residual signal as an internal standard. The following abbreviations are used for signal multiplicities: s, singlet; br s, broad singlet, d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets. High-resolution electrospray ionization mass spectra were obtained on Agilent Q-TOF system. The purity data of all the compounds screened for anti-Zika activity were determined by the quantitative nuclear magnetic resonance (qNMR) method and were found to be >95% pure, except for compound **7**, which could only be purified to 93%.

Synthesis of 1,2-bis((1-((3',5,5'-tris(azidomethyl)-[1,1'-biphenyl]-3-yl)methyl)-1*H*-1,2,3-triazol-4-yl)methoxy)ethane, 12. 1,2-bis(prop-2-yn-1-yloxy)ethane (200 mg, 1.5 mmol) and **1** (2.7 g, 7.2 mmol) were dissolved in 135 mL anhydrous DMF. 15 mL H_2O was added, followed by sodium ascorbate (1.2 g, 6.0 mmol), $CuSO_4$ (49 mg, 0.30 mmol), and bathocuproinedisulfonic acid disodium salt (200 mg, 0.38 mmol). The mixture was stirred at room temperature under Ar for 24 h. The reaction mixture was concentrated under reduced pressure, triturated with $CHCl_3$, passed through a silica column, and eluted with $CHCl_3$ to remove $4N_3$. Then the column was flushed with 10% MeOH/$CHCl_3$, and the fractions were concentrated to give the crude, which was further purified by column chromatography (SiO_2, 1 to 1.5% MeOH in $CHCl_3$) to provide **12** (310 mg, 24%) as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.59 (s, 2H), 7.51 (s, 2H), 7.48 (s, 2H), 7.45 (s, 4H), 7.29 (s, 2H), 7.23 (s, 2H), 5.58 (s, 4H), 4.65 (s, 4H), 4.44 (s, 8H), 4.42 (s, 4H), 3.68 (s, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 145.08, 141.76, 141.09, 137.38, 137.06, 136.16, 127.22, 127.18, 127.01, 126.84, 126.76, 122.74, 69.75, 64.65, 54.41, 54.28, 53.80; HRMS (ESI): m/z calcd for $C_{40}H_{39}N_{24}O_2$ $[M+H]^+$: 887.3682, found 887.3688.

Synthesis of 1,18-bis(1-((3',5,5'-tris(azidomethyl)-[1,1'-biphenyl]-3-yl)methyl)-1*H*-1,2,3-triazol-4-yl)-2,5,8,11,14,17-hexaoxaoctadecane, 14. 3,6,9,12,15,18-hexaoxaicosa-1,19-diyne (310 mg, 1.0 mmol) and **1** (1.87 g, 5.0 mmol) were dissolved in 90 mL anhydrous DMF. 10 mL H_2O was added, followed by sodium ascorbate (825 mg, 4.17 mmol), $CuSO_4$ (34 mg, 0.21 mmol) and bathocuproinedisulfonic acid disodium salt (140 mg, 0.26 mmol). The mixture was stirred at room temperature under Ar for 24 h. The reaction mixture was concentrated under reduced pressure, triturated with $CHCl_3$, passed through a silica column, and eluted with $CHCl_3$ to remove $4N_3$. Then the column was flushed with 10% MeOH/$CHCl_3$, and the fractions were concentrated to give the crude, which was further and purified by column chromatography (SiO_2, 1 to 3% MeOH

in $CHCl_3$) to provide **14** (425 mg, 40%) as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.62 (s, 2H), 7.51 (s, 2H), 7.48 (s, 2H), 7.46 (s, 4H), 7.29 (s, 2H), 7.23 (s, 2H), 5.60 (s, 4H), 4.66 (s, 4H), 4.44 (s, 8H), 4.42 (s, 4H), 3.71-3.54 (m, 20H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 141.76, 141.12, 137.37, 137.06, 136.22, 127.20, 127.17, 126.98, 126.82, 126.76, 122.81, 70.51, 70.47, 70.29, 69.82, 64.70, 54.42, 54.30, 53.81; HRMS (ESI): m/z calcd for $C_{48}H_{54}N_{24}O_6$ $[M+H]^+$: 1063.4731, found 1063.4737.

Synthesis of 16. PPh_3 (1.0 g, 3.9 mmol) was added to a stirring solution of **13** (500 mg, 0.51 mmol) in THF (30 mL) at room temperature and refluxed under Ar atmosphere for 1 h before the addition of furan-2-carbaldehyde (370 mg, 3.85 mmol) at room temperature. The reaction mixture was refluxed for an additional 48 h, cooled to room temperature, and concentrated under reduced pressure. The resulting residue was dissolved in MeOH (30 mL), and $NaBH_4$ (291 mg, 7.69 mmol) was added portion wise at room temperature. After stirring for 16 h, the reaction mixture was poured into ice, and the MeOH was evaporated. The residue was acidified with 3N HCl at room temperature and washed with CH_2Cl_2 (3 x 40 mL). The aqueous layer was basified with 3N NaOH and extracted with CH_2Cl_2 (3 x 40 mL). The combined organic layers were dried over to anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to provide **16** (610 mg, 92%) as a brown gum. ^{1}H NMR (700 MHz, CD_2Cl_2) δ = 7.54 (s, 2H), 7.51 (s, 2H), 7.45-7.38 (m, 6H), 7.36 (s, 6H), 7.28 (s, 2H), 7.22 (s, 2H), 6.36-6.26 (m, 6H), 6.23-6.11 (m, 6H), 5.52 (s, 4H), 4.63 (s, 4H), 3.90-3.72 (m, 24H), 3.68-3.45 (m, 12H); ^{13}C NMR (75 MHz, CD_2Cl_2) δ = 153.75, 153.61, 145.59, 142.18, 141.92, 141.87, 141.61, 140.80, 140.54, 135.20, 127.53, 127.49, 126.94, 125.84, 122.56, 110.16, 107.22, 107.14, 70.50, 70.47, 69.74, 64.70, 54.14, 52.81, 52.52, 45.58, 45.52; HRMS (ESI): m/z calcd for $C_{74}H_{83}N_{12}O_{10}$ $[M+H]^+$: 1299.6350, found 1299.6341.

Biological studies

Zika reporter virus particles. The codon optimized version of ZIKV C-prM-E construct has been described previously[42] and was synthesized using the complete ZIKV sequence available from the current outbreak in Americas (accession number KU312312.1)[49]. The C-prM-E variant lacking the E-glycosylation site N154Q was constructed by site directed mutagenesis using forward primer 5'-agcggcatgatcgtccaggacaccggccacgag-3' and reverse primer 5'-ctcgtggccggtgtcctggacgatcatgccgct-3' using the Quick Change II XL site directed mutagenesis kit (Stratagene). The entire C-PrM-E region was sequenced to verify the presence of the mutations and authenticity of insert. ZIKV RVPs were generated using the protocol described below. 293T cells stably expressing the Zika virus CprME (293T-CPrME-F6) and described previously[42] were transfected with the plasmid containing the sub-genomic GFP expressing replicon derived from lineage II strain of WNV[50]. For generation of N154Q RVPs, 293T cells were transfected with plasmids C-PrME-N154Q and sub-genomic GFP replicon at a ratio of 1:1. Transfections were performed using the Turbofect transfection reagent (ThermoFisher) strictly following the manufacturer's recommendations. The RVPs were harvested 48 h post-transfection, aliquoted, and stored for future use.

Titration of RVPs. Vero cells were plated in 96 well, clear bottom black plates at 5,000 cells per well. Serial 2-fold dilutions of RVPS were prepared in DMEM-10 medium and added to Vero cells starting with the highest dose of 50 µl/well. For each RVP dilution, infections were conducted in duplicates/triplicates and cells incubated with RVPs for 72 h. Thereafter, the plates were fixed with 4% formalin/PBS and images of whole wells acquired using the Cytation 5 imaging system (BioTek). The number of GFP+ cells were counted using the Gen5 imaging software which provides a read out of the number of GFP-positive cells per well. The optimal virus dose for infection experiments was then determined from the titration curves.

Inhibition of Zika infection using synthetic carbohydrate receptors. Vero and HeLa cells were obtained from ATCC, and cultured in DMEM supplemented with 10% FBS and Penicillin, streptomycin, and Glutamine. Cells were plated in 96 well, clear bottom black plates at 5,000 cells per well. Stock solutions of the compounds were made in DMSO at 10 mM concentration. Further dilutions of the compounds were made in cell culture media. Different compounds were added at the indicated concentrations in duplicates/triplicates and cells incubated with the compounds for 30 min at room temperature. Thereafter, a predetermined amount of Zika RVPs that yields up to 1000 GFP+ cells per well was added to the plates. Cells treated with the same amount (in µl) of DMSO as the input volume of the compounds and infected with Zika RVPs, were used as normalization control for determination of 100% infection. Plates were incubated for 72 h at 37 °C, after which images acquired using the Cytation5 imaging system (BioTek). The experiment was repeated 3 times and inhibition curves were generated for each experiment using the Sigma plot software and 50% inhibitory concentration (IC_{50}) value for each compound were determined.

Inhibition studies with infectious ZIKV, PRVABC59. The ZIKV isolate PRVABC59 derived from a human serum specimen from Puerto Rico in December 2015 was obtained from ATCC and propagated in Vero cells following the manufacturer's recommendations. The virus stocks were titrated in Vero cells using fluorescent microscopy. Briefly, Vero cells were infected with serial dilutions of the virus stocks and cells fixed with 4% formaldehyde/PBS 48 hr post infection. Subsequently cells were stained using 4G2 antibody (MAB10216, Millipore) followed by Alexa 488 conjugated secondary antibody (Invitrogen). Images for the whole wells were acquired on Cytation 5 imaging reader and number of GFP+ cells per well quantified using Gen5 Software. For subsequent experiments, a predetermined amount of virus that yields ~2000-3000 GFP+ cells per well was used.

For compound inhibition studies with infectious virus, Vero cells were plated in 96 well, clear bottom black plates at 7,500 cells per well. Cells were incubated with different concentrations of the SCRs for 30 min at room temperature as indicated above. Cells were then infected with a pre-determined amount of ZIKV PRVABC59 isolate that yields ~2000-3000 GFP+ cells per well determined from titration curves above. Thereafter, cells were fixed and number of infected cells determined via 4G2 antibody staining followed by Cytation5 imaging as above. The experiment was conducted in triplicate wells and the entire experiment was repeated.

Determination of cellular toxicity. Vero or HeLa cells were plated in 96 well clear bottom white plates at 5,000 cells per well. Different compounds were added at the indicated concentrations in duplicates, and the cells were incubated for 72 h at 37 °C. Cellular toxicity was measured using the CellTiter-Glo (Promega) luminescent viability assay that is based on quantitation of the ATP in cells, an indicator of metabolically active cells. Data was normalized to cells treated with DMSO as being 100% viable. Toxicity curves were generated using the Sigma plot software by fitting curves using Sigmoidal logistic 4 Parameter non-linear regression and TC_{50} concentrations determined for the compounds from the curves.

Time-of-addition experiments. ZIKV virus RVP inhibition assays in Vero cells were conducted as described above, with slight modification. Cells were infected with a pre-determined amount of ZIKV RVPs in a volume of 95 µl and the compounds were added either 30 min prior to infection or 4 h or 24 h post-infection in a volume of 5 µl. The plates were fixed 72 h post infection, and the number of GFP+ cells per well were determined using the Cytation5 imaging system.

ASSOCIATED CONTENT

Supporting Information. The following files are available free of charge. Spectral data of compounds **12**, **14** and **16**, detailed biochemical procedures and data (PDF) Molecular formula strings (CSV)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ZIKV, Zika virus; FLV, flavivirus; RVP, reporter virus particle; WHO, World Health Organization; RNA, ribonucleic acid; HIV, human immunodeficiency virus; SAR, structure-activity relationship; GAG, glycosylaminoglycan; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-grabbing non-integrin; C-prM-E, Capsid-preMembrane-Envelope; FDA, food and drug administration; PBS, Phosphate Buffered Saline; ATCC, American Type Culture Collection; DMEM, Dulbecco's Modified Eagle's Medium; FBS, Fetal Bovine Serum; GFP, Green Fluorescent Protein; ATP, Adenosine Triphosphate; WNV, West-Nile Virus; CUNY, City University of New York.

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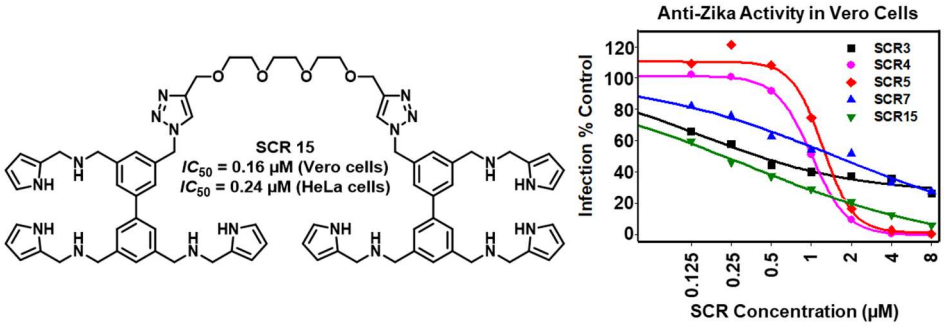
TOC Graphic


SCR 15
IC_{50} = 0.16 μM (Vero cells)
IC_{50} = 0.24 μM (HeLa cells)
Anti-Zika Activity in Vero Cells
Infection % Control
SCR3
SCR4
SCR5
SCR7
SCR15
120
100
80
60
40
20
0
0.125
0.25
0.5
1
2
4
8
SCR Concentration (μM)