Material and Methods

Cells

293T, BHK-21, Vero, and C6/36 cell lines were purchased from ATCC (American type culture collection) and maintained as instructed by the manufacturer (Cat# CRL-11268, CCL-10, CCL-81, and CCL-1660). Cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% Penicillin and streptomycin, 1% non-essential amino acids and 10% fetal bovine serum (Invitrogen). The immortalized BBB cell line hCMEC/D3 (Millipore #SCC066) was maintained in EndoGRO™-MV Complete Media Kit (Millipore # SCME004) supplemented with 1 ng/mL FGF-2 (Millipore #GF003). Cell lines were tested negative for mycoplasma contamination using a homemade PCR detection kit. Primary HUVECs were purchased from ATCC (PCS-100-010, Lot# 62245802, passage #2) or Cell Applications (200K-05, Donor #1 & #2, passages 2 and 3 respectively). Primary HSaVECs and HAoECs were from PromoCell (C-12231 and C-12271, passage 3, 5, and 6.) and HCoAECs were purchased from Lonza (CC-2585, passage 3 and 6 respectively) All ECs were maintained under standard cell culture conditions in endothelial growth medium (EGM-2MV) from Lonza as we have published[1]. The purity of ECs was validated by the presence of CD31/PECAM1 on cell surface (Online Figure 2A).

Viruses

Zika virus strains/isolates MR766 (Rhesus/1947/Uganda), IbH 30656 (Human/1968/Nigeria), PRVABC59 (Human/2015/Puerto Rico), and FLR (Human/2015/Colombia) were initially obtained from Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Manassas, VA) and subsequently propagated in C6/36 cells. Approximately 2 x 10^6

cells were seeded 24 hours prior to infection in a T75 cell culture flask. When the cells had reached ~60-80% confluency they were infected with virus at a multiplicity of infection (MOI) of 0.01. The cells were incubated for 7 days or until they began to die from the cytopathic effect caused by the virus. After propagation, the virus containing media was collected and centrifuged at 1500g for 5 minutes to remove extracellular debris. The supernatant was then passed through a 0.22 μm filter syringe and aliquoted before it was frozen at -80 °C for long term storage. The infectious titers of stock virus were determined by plaque assays and summarized in Online Table I. The production of DENV-2 strain Thailand 16681 has been previously published[2]. Experiments involving infectious virus were approved by SRI Biosafety Committee and conducted in a Biosafety level 2 laboratory.

Antibodies and Reagents

The pan-flavivirus anti-E protein murine monoclonal antibody D1-4G2-4-15 was produced from hybridoma cells purchased from ATCC (HB-112) and purified as instructed by the manufacturer. The Alexa Fluor 568 goat-anti-mouse and Alexa Fluor 488 goat-anti-rabbit secondary antibodies were purchased from molecular probes (Invitrogen cat # A11019 and #A-11008). Goat anti-human AXL polyclonal antibody (AF-154), mouse anti-human AXL antibody (MAB 154), and CD31/PECAM-1 antibody (BBA7) were purchased from R&D systems. Polyclonal ZO-1 antibody (Cat No. 61-7300) was from ThermoFisher Scientific. Cabozantinib (XL-184, VEGFR2/Met/Ret/Kit/FLT//AXL inhibitor), Sorafenib (Raf kinases and tyrosine kinases inhibitor), Sunitinib malate (VEGFR/PDGFRβ/ KIT/ FLT3/RET/CSF-1R inhibitor), and R428 (Selective Axl inhibitor) were purchased from APExBIo Technology (Houston, TX). Notably, Cabozantinib potently inhibits several RTKs including AXL phosphorylation[3]. R428

has been well characterized in its ability to inhibit AXL phosphorylation and downstream signaling[4]. pWZL-Neo-Myr-Flag-AXL[5] and the AXL kinase dead AXL (K567R)[6] were obtained from Addgene. Mutating the K567 residue of AXL is known to destroy an ATP-binding site and to inhibit Axl phosphorylation[6-8].

ZIKV plaque assay

Vero cells were plated the day before infection into 12 well plates at 5 x 10^5 cells/well. On the day of the experiment, serial dilutions of virus were made in 0.4 ml media. Five different 10-fold dilutions of purified virus were spread onto monolayers of Vero cells at 37°C for 1 h to initiate binding to cells. , then medium containing virus particles was replaced with 1 ml overlay containing a 1:1 mixture of 1% SeaPlaqueTM agarose(Lonza) and 2X Modified Eagle Medium (Gibco) with 10% (vol/vol) FBS. Cells were maintained at 37°C in 5% CO_2 .On day 5, cells were stained with 1 ml secondary overlay containing a 1:1 mixture of SeaPlaqueTM agarose (Lonza) and 2X Modified Eagle Medium and 0.33% neutral red (Sigma) . Cells were incubated at 37°C and plaques were counted after 48 hours. Resultant plaques were photographed using a Canon scanner. For each ZIKV strain, averages of 10 plaques from two plates were analyzed using the ImageJ software (NIH). The size of a plaque was determined by comparing the measured area of a plaque to that of a well in the 12-well (380 mm^2) using the formula: Size of a plaque = $AREA_{plaque}/AREA_{well} \times 380mm^2$.

Immunofluorescence assay

Cells were plated on gelatin-coated glass coverslips the day before infection into 24 well plates at 5 x 10^4 cells/well. The cells were washed for 5 minutes 3 times with 1X phosphate

buffered saline. After wash, cells were fixed in 4% paraformadelhyde for 15 minutes at room temperature and then permeabilized with 0.2% triton-X 100 for 10 minutes. The ZIKV envelope protein was detected using a 1:300 dilution of the 4G2 primary antibody isolated and 1:500 of the Alexa Fluor 568 goat-anti-mouse antibody. Images were captured by a Zeiss LSM 700 laser scanning confocal microscope.

Real-Time PCR

Quantification of RNA was conducted using iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad) with an in-house developed protocol on a Step One Real-Time PCR system (Applied Biosystems). The copy number of Zika RNA was calculated by comparing to a standard curve obtained with serial dilutions of a full-length Zika genome encoding plasmid. Primers for qPCR were as follows: ZIKV MR766 forward: 5′- CTTGAAATCCGGTTTGAGGA-3′ and reverse: 5′-CTTTCCTGGGCCTTATCTCC-3′; ZIKV IbH 30656 forward: 5′-CACAAAGGGAGGTCCTGGTC-3′ and reverse: 5′- CACAGCAAAGTGTCACACGG-3; ZIKV PRVABC59 and ZIKV FLR forward: 5′- CACTGTGAGAGGTGCCAAGA-3′ and reverse: 5′- TTTGTGTTCAGACCCAACCA-3′; human GAPDH RNA forward: 5′-GAATTTGCCATGGGTGGAAT-3′ and reverse: 5′-ATGTCCAGCCTCAGAACTTC-3′.

Flow cytometry

$4x10^5$ cells were trypsinized and resuspended in PBS. Control mouse IgG or mouse anti-human AXL antibody (MAB 154) was added to the cells at 1:100 and then incubated on ice for 30 minutes. After three washes in PBS, cells were incubated with Goat-anti-Mouse-FITC antibody (1:100) for 30 minutes on ice. Expression of AXL was quantified by BD FACSCalibur (BD Biosciences). Data was analyzed by flowing software.

Transendothelial Electric Resistance Measurement

2000 HUVECs cells were seeded on a 24-well transwell plate (Corning, 6.5-mm membrane diameter, 0.4-μm pore size). Cells that were resuspended in 0.2 ml of medium were added to the upper chamber, and 0.5 ml of medium was added to the lower chamber. After reaching full confluence, cells were infected with ZIKV (MOI 1). Transendothelial electric resistance (TEER) was measured daily for 2 days using an epithelial voltohmmeter (EVOM, World Precision Instruments, Sarasota, FL). A blank well with no cells was maintained, and the average resistance from the blank was subtracted from the sample. Resistance (R) per unit area was calculated as $(R_{sample} - R_{blank}) \times 0.33$ cm^2. The data represent means of two independent experiments ± SD, each done in duplicate.

Time-of-addition assays

ZIKV was added to HUVECs at 4°C and incubated for 2 hrs. Unbound virus was washed off with cold media, and the cells were shifted to 37°C (set as 0 hr time point) to initiate synchronous infection. At the indicated time points, R428 or DMSO was added into the media and incubated for two hours prior to removal (exception is t= -2hr where inhibitors were added back after removal of the virus and incubated for additional two hours prior to removal). Infected cells were incubated at 37° for an additional 24 hours prior to quantification of ZIKV RNA by RT-qPCR. Inhibition was calculated as 100 – % infection relative to infections containing no inhibitors.

Immunoblotting

Cells were grown in 6-well plates and lysates were prepared with RIPA buffer (50 mM Tris-HCl [pH 7.4]; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; protease inhibitor cocktail (Sigma); 1 mM sodium orthovanadate), and insoluble material was precipitated by brief centrifugation. Protein concentration of lysates was determined by BCA protein assay (Thermo Scientific). Lysates containing equal amounts of protein were loaded onto 4-20% SDS-PAGE gels and transferred to a nitrocellulose membrane (LI-COR, Lincoln, NE), blocked with 10% milk for 1 h, and incubated with the primary antibody overnight at 4 °C. Membranes were blocked with Odyssey Blocking buffer (LI-COR, Lincoln, NE), followed by incubation with primary antibodies at 1:1000 dilutions. Membranes were washed three times with 1X PBS containing 0.05% Tween20 (v/v), incubated with IRDye secondary antibodies (LI-COR, Lincoln, NE) for 1 h, and washed again to remove unbound antibody. Odyssey CLx (LI-COR Biosystems, Lincoln, NE) was used to detect bound antibody complexes.

Statistical analysis

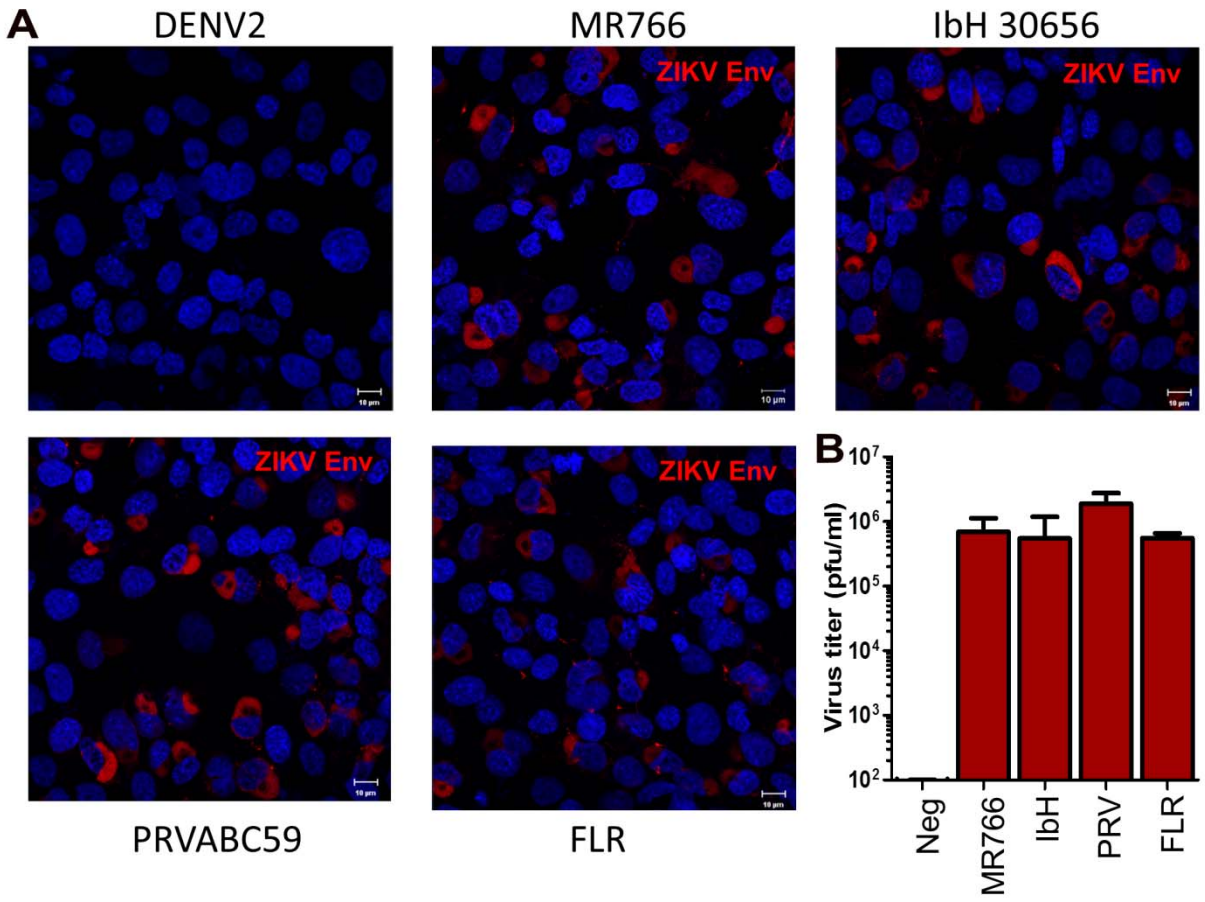
All experiments were independently performed at least twice times as indicated in the Figure legends. Except were specified, bar graphs were plotted to show mean ± standard deviation (SD). Statistical analyses were performed using Prism 6. A p value of <0.05 in the Wilcoxon and Kruskal-Wallis tests was considered statistically significant.

Online Table I. Infectious titers of ZIKV stocks used in the study

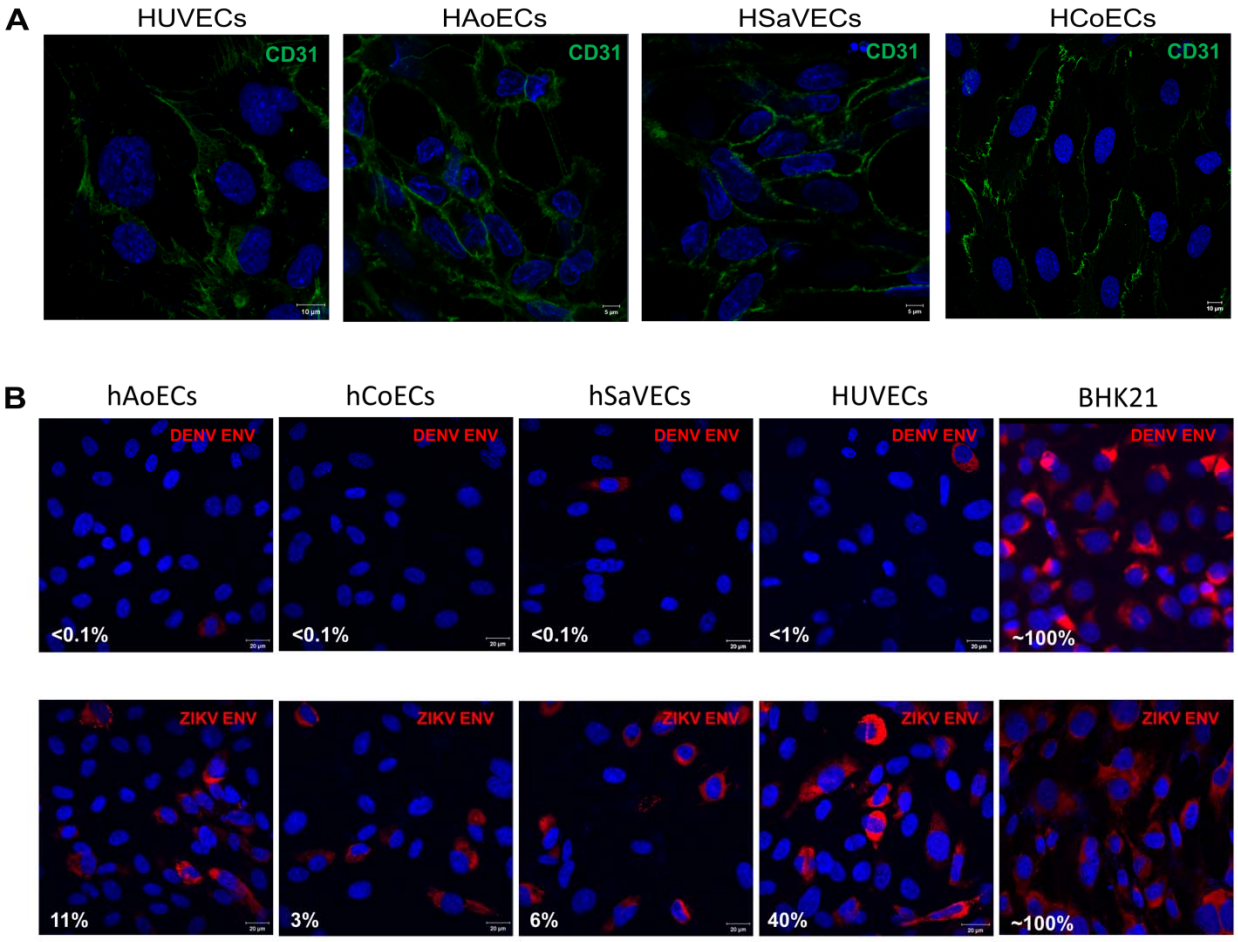
ZIKV Strain/Isolate	Viral Titer(pfu/ml)
PRVABC59	8.6×10^6
MR766	3.6×10^6
IbH30656	2.1×10^6
FLR	2.0×10^6

References

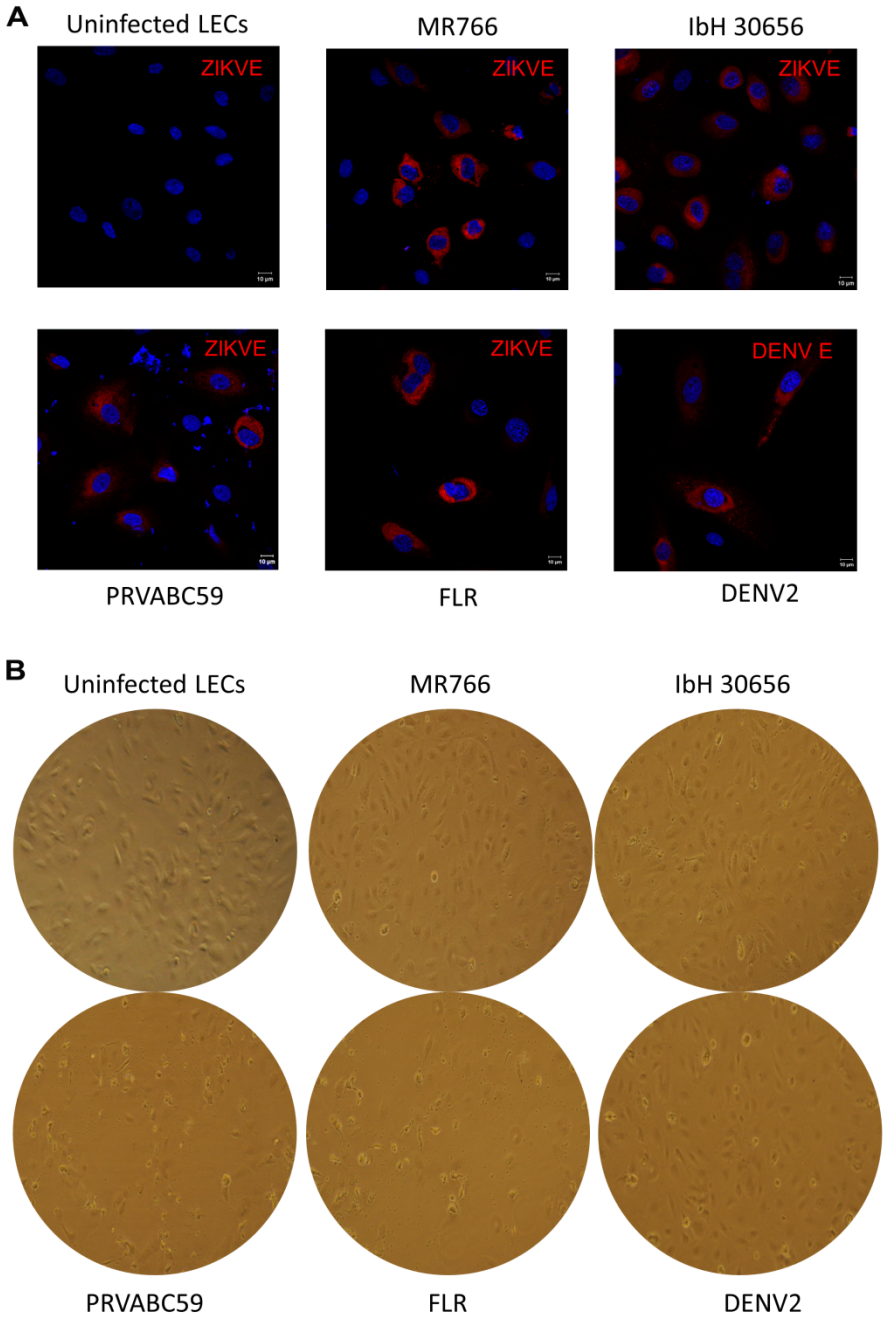
1. Lohman AW, Leskov IL, Butcher JT, Johnstone SR, Stokes TA, Begandt D, DeLalio LJ, Best AK, Penuela S, Leitinger N, Ravichandran KS, Stokes KY, Isakson BE. Pannexin 1 channels regulate leukocyte emigration through the venous endothelium during acute inflammation. *Nat Commun*. 2015;6:7965
2. McCormick KD, Liu S, Jacobs JL, Marques ET, Jr., Sluis-Cremer N, Wang T. Development of a robust cytopathic effect-based high-throughput screening assay to identify novel inhibitors of dengue virus. *Antimicrob Agents Chemother*. 2012;56:3399-3401
3. Zhou L, Liu XD, Sun M, Zhang X, German P, Bai S, Ding Z, Tannir N, Wood CG, Matin SF, Karam JA, Tamboli P, Sircar K, Rao P, Rankin EB, Laird DA, Hoang AG, Walker CL, Giaccia AJ, Jonasch E. Targeting met and axl overcomes resistance to sunitinib therapy in renal cell carcinoma. *Oncogene*. 2016;35:2687-2697
4. Holland SJ, Pan A, Franci C, Hu Y, Chang B, Li W, Duan M, Torneros A, Yu J, Heckrodt TJ, Zhang J, Ding P, Apatira A, Chua J, Brandt R, Pine P, Goff D, Singh R, Payan DG, Hitoshi Y. R428, a selective small molecule inhibitor of axl kinase, blocks tumor spread and prolongs survival in models of metastatic breast cancer. *Cancer Res*. 2010;70:1544-1554
5. Boehm JS, Zhao JJ, Yao J, Kim SY, Firestein R, Dunn IF, Sjostrom SK, Garraway LA, Weremowicz S, Richardson AL, Greulich H, Stewart CJ, Mulvey LA, Shen RR, Ambrogio L, Hirozane-Kishikawa T, Hill DE, Vidal M, Meyerson M, Grenier JK, Hinkle G, Root DE, Roberts TM, Lander ES, Polyak K, Hahn WC. Integrative genomic approaches identify ikbke as a breast cancer oncogene. *Cell*. 2007;129:1065-1079
6. Meyer AS, Zweemer AJ, Lauffenburger DA. The axl receptor is a sensor of ligand spatial heterogeneity. *Cell Syst*. 2015;1:25-36
7. Braunger J, Schleithoff L, Schulz AS, Kessler H, Lammers R, Ullrich A, Bartram CR, Janssen JW. Intracellular signaling of the ufo/axl receptor tyrosine kinase is mediated mainly by a multi-substrate docking-site. *Oncogene*. 1997;14:2619-2631
8. Shimojima M, Ikeda Y, Kawaoka Y. The mechanism of axl-mediated ebola virus infection. *J Infect Dis*. 2007;196 Suppl 2:S259-263



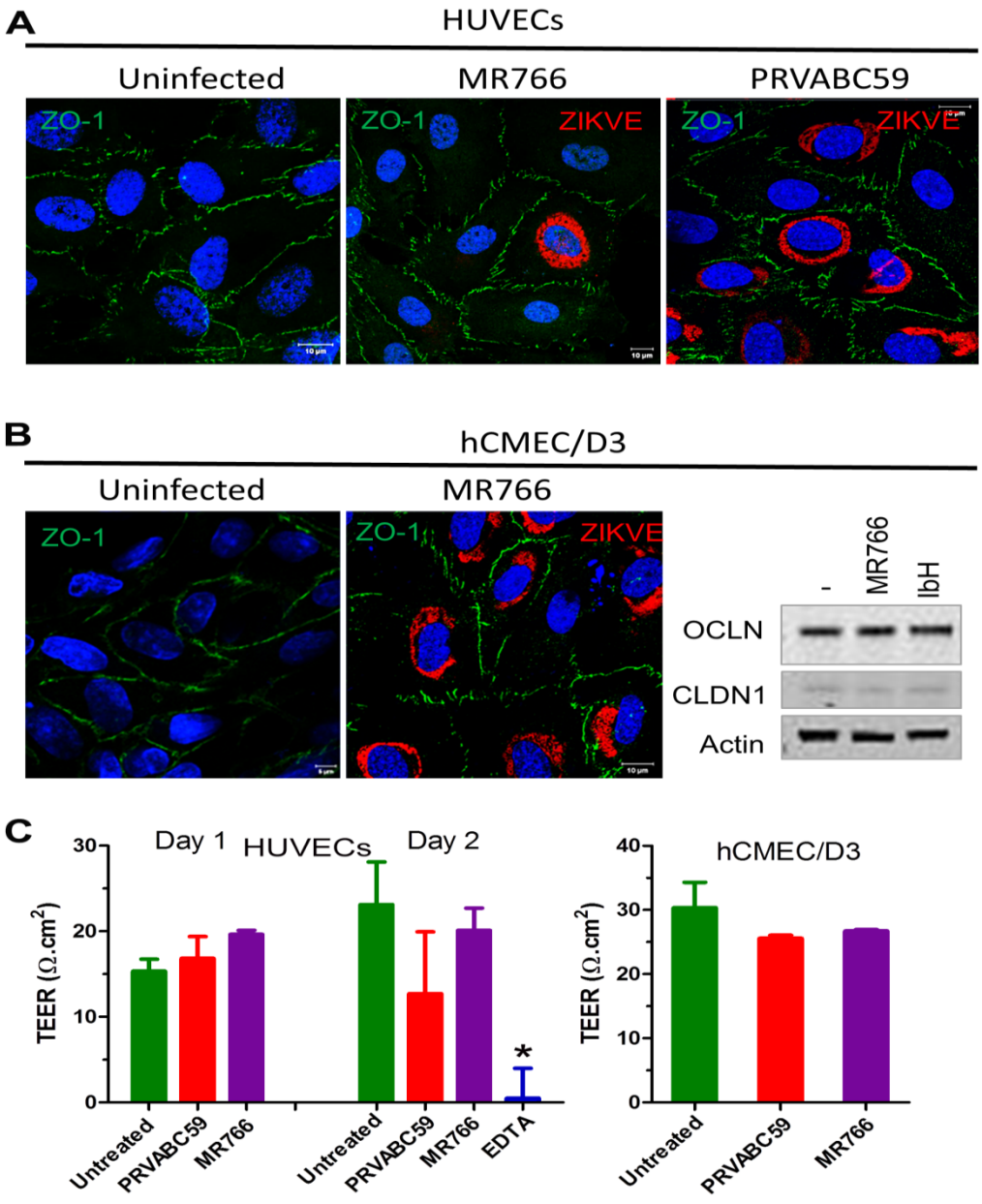
Online Figure I. Productive infection of hCMEC/D3 cells by ZIKV
(A) The human BBB cell line hCMEC/D3 were infected by indicated ZIKV strains/isolates at MOI of 1 for 48 hrs followed by immunostaining of ZIKV Env protein. (B) Infectious titers of supernatants collected at 48 hrs post-infection from cells in a. Data presented are averages of three independent experiments. N=3, Error bars, s.d.



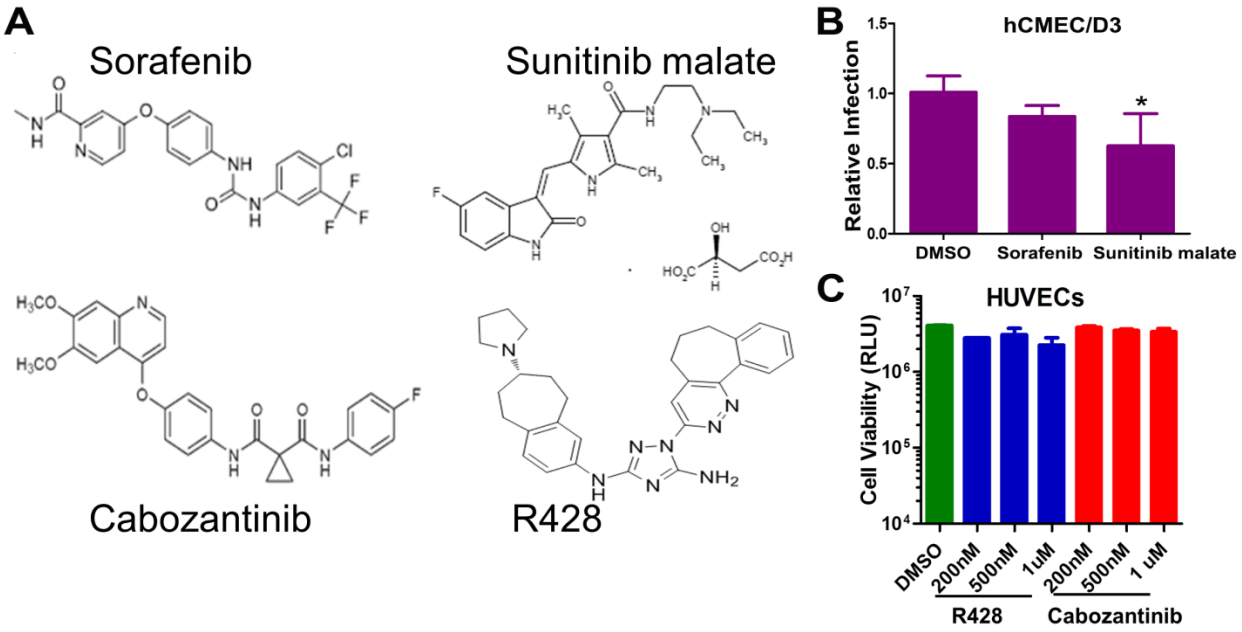
Online Figure II. Primary human ECs are much less permissive to DENV2 infection. (A) Expression of CD31/PECAM-1 on primary ECs. ECs were immunostained by anti-CD31 antibody (1:100) and visualized on cell surface (Green). Nearly all cells are positive for CD31. (B) Primary human ECs from the same donor were infected with DENV2 (top) or PRVABC59 at MOI of 1. Forty-eight hours post-infection, cells were immunostained for viral Env protein (red). The percentage of infection (shown at the lower left corner of each image) was calculated by enumerating the number of Env+ cells over the number of nuclei. BHK-21 cells were infected a positive control for infection.



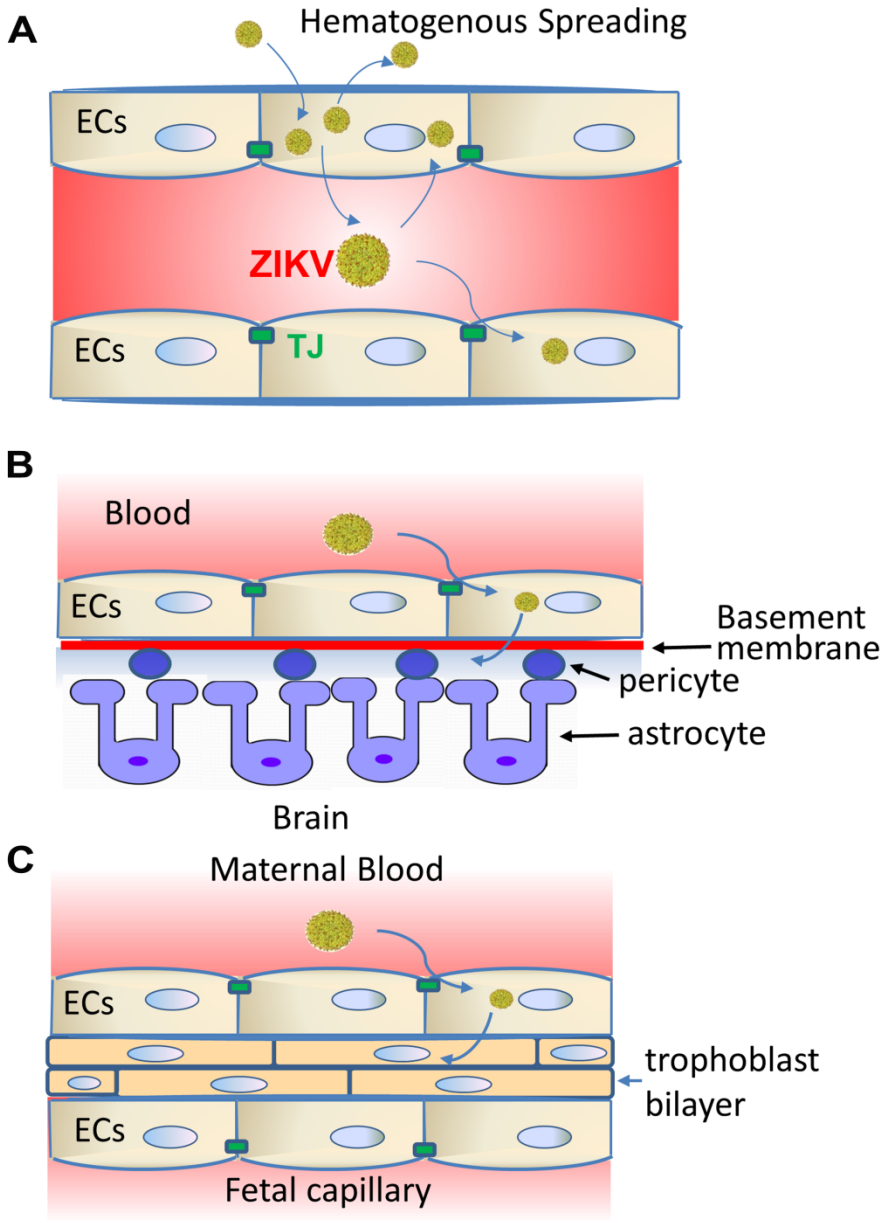
Online Figure III. Infection of lymphatic endothelial cells (LECs) by ZKIV. (A) LECs (passage 6) were infected by indicated ZIKV MR766 or PRV strain/isolate or DENV1 (Thailand 16681) at MOI of 1 for 48 hrs followed by immunostaining of ZIKV Env protein. (B) Representative phase-contrast images of infected cells at 48 hrs post-infection. Significant cell death was seen in PRV and FLR infected cells.



Online Figure IV. ZIKV infection does not directly disrupt tight junctions (TJs). Representative confocal images of ZIKV infected HUVEC cells from Fig. 1A (A) or hCMEC/D3 cells (B) at 48 hrs post-infection. Notably, only those viable cells were left on the glass cover slips for imaging. TJ protein ZO-1 (green) and ZIKV Env protein (red) were immunostained. Right inset within b is the western blotting image of cells by ZIKV MR766 or IbH strains after 48 hours post-infection. OCLN, occludin; CLDN1, claudin-1. (C) Transendothelial resistance (TEER) was measured on HUVECs or hCMEC/D3 (day 2 after PRV infection, MOI=0.1). (n=2, each done in duplicate; error bars, s.d.). As a positive control, EDTA (2 μM) was added to HUVECs at Day 2 for 40 minutes to disrupt TEER.



Online Figure V. AXL inhibitors. (A) Chemical structures of four compounds used in the study. (B) hCMEC/D3 cells were pre-treated with Sorafenib or Sunitinib malate (1 μM) for 1 hour followed by ZIKV infection (MOI 1) of 1 hour, after which a medium change occurred. Twenty-four hours post-infection cellular RNA was extracted for real-time RT-PCR quantification of ZIKV RNA. The obtained results were normalized against levels of GAPDH and the ZIKV RNA levels from DMSO-treated cells were set to 1. (n=2; error bars, s.d.; **P*<0.05.). (C) The cell viability of compound-treated HUVECs was quantified using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). The assay measures the cellular ATP levels using a luciferase based assay (in RLU). (n=3; error bars, s.d.)



Online Figure VI. Implications of ZIKV infection of ECs. (A) Productive of infection of vascular endothelium leads to shedding of virus to surroundings. (B) Productive infection of microvascular endothelial cells in the brain results in breaching the blood-brain barrier. (C) ZIKV virus carried from the infected mother feeds trophoblasts for potential breach of the placental-fetal barrier.