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Erythrosin B is a potent and broad-spectrum orthosteric inhibitor of the flavivirus NS2B-NS3 protease

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Abstract

Many flaviviruses, such as Zika virus (ZIKV), Dengue virus (DENV1-4) and yellow fever virus (YFV), are significant human pathogens. Infection with ZIKV, an emerging mosquito-borne flavivirus, is associated with increased risk of microcephaly in newborns and Guillain-Barré syndrome and other complications in adults. Currently, specific therapy does not exist for any flavivirus infections. In this study, we found that erythrosin B, an FDA-approved food additive, is a potent inhibitor for flaviviruses, including ZIKV and DENV2. Erythrosin B was found to inhibit the DENV2 and ZIKV NS2B-NS3 proteases with IC₅₀ in low micromolar range, *via* a non-competitive mechanism. Erythrosin B can significantly reduce titers of representative flaviviruses, DENV2, ZIKV, YFV, JEV, and WNV, with micromolar potency and with excellent cytotoxicity profile. Erythrosin B can also inhibit ZIKV replication in ZIKV-relevant human placental and neural progenitor cells. As a pregnancy category B food additive, erythrosin B may represent a promising and easily developed therapy for management of infections by ZIKV and other flaviviruses.

Keywords

Flavivirus; Zika virus; Dengue virus; Antiviral; Protease inhibitor; Erythrosin B

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1. Introduction

The genus *Flavivirus* contains more than 70 viruses, many of which cause serious human diseases. The four serotypes of Dengue virus (DENV1-4), yellow fever virus (YFV), West Nile virus (WNV), Zika virus (ZIKV), St. Louis encephalitis virus (SLEV), Japanese encephalitis virus (JEV), Powassan virus (POWV), and tick-borne encephalitis virus (TBEV) are categorized as global emerging pathogens. Due to global travel, ZIKV outbreaks recently occurred worldwide, and was imported to many new territories including the UK, Canada, and the US (Attar, 2016; Bogoch et al., 2016; Chen, 2016; Korhonen et al., 2016). ZIKV transmission has been further increased by transfer between humans through sexual activities and blood transfusions (Foy et al., 2011; Musso et al., 2014, 2015; Patino-Barbosa et al., 2015). Importantly, ZIKV infection increases the risk of microcephaly in babies born to infected mothers, and may cause Guillain-Barré syndrome and other complications in adults (Calvet et al., 2016; Martinez et al., 2016; Rodriguez-Morales, 2016; Thomas et al., 2016). Published data in mice and humans have demonstrated that ZIKV infects the central nervous system during fetal development, including (but not limited to) regions like the cortex, thalamus, and hypothalamus, as well as neurogenic regions including the subventricular and subgranular zones, with fetal infection occurring through placenta cells (Cugola et al., 2016; Lancaster et al., 2013; Tabata et al., 2016; Tang et al., 2016). Infection with ZIKV results in a phenotypic and genetically measured infection in stem cell and neural progenitor cells with cortical thickness, cell apoptosis, and most critically, a phenotype similar to microcephaly (Cugola et al., 2016; Lancaster et al., 2013; Li et al., 2016; Tang et al., 2016).

The flavivirus genomic RNA is single-stranded with positive polarity. The viral genome is approximately 11 kb in length, composed of a 5' untranslated region (UTR), a single long open reading frame (ORF), and a 3' UTR. The single ORF encodes a large precursor polyprotein (PP) that requires post-translational processing by host and viral proteases (Chambers et al., 1990). These processes lead to mature viral proteins, including three structural proteins (capsid, pre-membrane or membrane, and envelope), and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Among the viral proteins, NS3 is the second largest protein encoded by flavivirus, and has multi-enzymatic functions, including protease, RNA triphosphatase, nucleoside triphosphatase, and helicase activities (Brecher et al., 2013; Lim et al., 2013; Luo et al., 2015; Sampath and Padmanabhan, 2009). The viral protease is encoded by the N-terminal region (~180 amino acids (aa)) of the NS3 protein. The viral NS2B protein is a membrane-associated protein, with a central hydrophobic core region (~40 aa) as an essential co-factor for the NS3 protease function. As an essential enzyme, the flavivirus NS2B-NS3 protease is highly conserved (Brecher et al., 2013, 2017; Chambers et al., 1991; Falgout et al., 1991).

In a recent study, we developed a split luciferase complementation (SLC)-based high-throughput screening assay to identify inhibitors blocking the essential interactions between the DENV2 protease NS3 and its co-factor NS2B (Li et al., 2017a). Pilot screening of the NCATS Chemical Genomic Center (NCGC) Pharmaceutical Collection compound library resulted in a few priority "hits" as potent orthosteric inhibitors capable of abolishing NS2B-NS3 interactions (Li et al., 2017a).

In this study, we explored additional hit compounds that were not characterized by previous study. Among these uncharacterized compounds, erythrosin B (EB), an FDA-approved food additive, was also found to act as a potent inhibitor for the flavivirus NS2B-NS3 protease. Erythrosin B moderately inhibited the interactions between the DENV2 NS2B and NS3 with IC_{50-SLC} of 15 μM , and significantly inhibited the DENV2 and ZIKV protease activities with IC_{50-pro} in low micromolar range *via* a non-competitive mechanism (IC_{50} : half maximal inhibitory concentration, defined as the compound concentration required to inhibit protease activity (IC_{50-pro}) or SLC (IC_{50-SLC}) by 50%). Results from cell-based assays indicated that EB significantly reduced titers of DENV2, ZIKV, WNV, YFV, and JEV with EC_{50} in low micromolar potency (EC_{50} : half maximal effective concentration, defined as the compound concentration required to reduce virus titer by 50%). In contrast, significantly higher concentrations of EB (> 150 μM) will be required to cause death of human cells, including human placental epithelial cells and neural progenitor cells relevant to ZIKV pathogenesis. As an FDA pregnancy category B food additive, EB may represent a promising and easily developed therapy for management of infections by DENV, ZIKV and other flaviviruses.

2. Materials and methods

2.1. Compounds

Erythrosin B was procured from Sigma-Aldrich.

2.2. Cloning, expression and purification

All clones were generated as described previously (Li et al., 2017a). The MBP-NS3 mutants were generated using the Strategene Quick-Change II mutagenesis strategy with primer pairs shown in Supplemental Table S1. All proteins were expressed in *Escherichia coli* strain Rosetta 2(DE3) (EMD Biosciences) and purified through a nickel-nitrilotriacetic acid (NTA) column (Qiagen) or Glutathione sepharose 4B (GE HealthCare), followed by a gel filtration 16/60 Superdex 200 column (GE HealthCare).

2.3. Protease inhibition assay

The DENV2 NS3-MBP or the ZIKV GST-NS3 fusion protein (50 nM) was mixed with erythrosin B at various concentrations or with DMSO control in reaction buffer (20 mM Tris pH 8.0, 100 mM NaCl, 5% Glycerol, and 0.05% CHAPS) and incubated at 4 °C for 30 min. Then the DENV2 or ZIKV His-NS2B was added at 1 μM final concentration to the EB-NS3 mixture of DENV2 or ZIKV, respectively. The peptide substrate Abz-RRRSAG-nTyr (NeoBiolab) were added to the mixture at a concentration of 50 μM , respectively, and substrate cleavage was monitored over time at 37 °C in a BioTek Flx800 at excitation/emission wavelengths of 360 nm/420 nm. The rate of increase in RFU over time was calculated in the linear range and normalized as fluorescence defined as $RFU_{final}-RFU_0$.

For all bar graphs and dose-responsive curves, all data were presented as means and standard deviations of experimental data in triplicate, or as specified. Sigmoidal function within the Origin Suite6.0 (Origin Lab, Wellesley Hills, MA) was used for dose-responsive curve fittings, unless otherwise specified.

2.4. Cytotoxicity assay

Cytotoxicity for erythrosin B was measured by a MTT cell proliferation assay using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide method (ATCC), as described previously (Chen et al., 2013a, 2013b). Briefly, approximately 1×10^5 cells in 100 μ l of media were seeded into 60 wells of a 96 well plate, while the remaining wells held media. Plates were held at RT for 1 h and then incubated for 20–24 h. The media was removed, and 100 μ l of media containing decreasing concentrations of antiviral compound in 1% DMSO were added to the wells. All determinations were performed in triplicate. After 48 h incubation at 37 °C, the MTT assay was performed according to manufacturer's protocol. A microtiter plate reader (Ely808, BioTek Instruments, Inc.) with a 570 nm filter (MTT) was used to record absorbance. After adjusting the absorbance for background and comparing to untreated controls, the cytotoxic concentration CC_{50} was calculated using a sigmoidal nonlinear regression function to fit the dose–response curve using the ORIGIN Suite6.0 (Origin Lab, Wellesley Hills, MA).

2.5. Viral titer reduction assays

A viral titer reduction assay was used to determine the effect of erythrosin B on selected flaviviruses, including DENV2 (New Guinea-C), ZIKV (PRVABC59), WNV (NY99), JEV (17D), and YFV (YF-Vax) using A549 cells (American Type Culture Collection) as described previously (Chen et al., 2013a, 2013b; Li et al., 2017a). Briefly, approximately 2×10^5 human A549 cells in 500 μ l of media were seeded into each well of a 24 well plate. At 24 h after seeding, dilutions at 2X the desired concentration of erythrosin B were made in 1% DMSO media and 250 μ l was added to wells in triplicate. Immediately following, 250 μ l of media containing viruses at a concentration to yield a MOI 0.1 PFU/cell was added to the wells. After 48 h incubation at 37 °C, culture media were collected and stored at –80 °C for later quantification using a plaque assay. For the plaque assay, Vero cell monolayers in 6-well plates were seeded 3–4 days prior to infection to achieve a confluent monolayer. Depending on virus type, three to eight 10-fold serially dilutions of the harvested samples were made. To inoculate, 100 μ l of the dilution was inoculated into each of 2 wells, rocked gently to distribute virus, and incubated for 1 h at 37 °C. Cells were then overlaid with a nutrient medium containing 0.6% oxoid agar. The agar was allowed to solidify and the plates were then incubated at 37 °C. A second overlay containing 2% neutral red was added after the plaques began to appear on day 2, and then incubated overnight. Plaques were counted daily for 1–3 days until no significant increase was seen. The effective concentration EC_{50} was determined by sigmoidal nonlinear regression fitting of the dose–response curve using the ORIGIN software package.

Human primary placental epithelial cells (HPECs) derived from the inner surface of the amnion were purchased from Cell Applications, Inc., and cultured according to manufacturer's manual. Human hNPC, derived from iPSC generated using the STEMCCA Cre-Excisable Constitutive Polycistronic (OKSM) lentivirus, was purchased from EMD Millipore, and cultured according to manufacturer's manual.

The antiviral efficacy experiments with HPECs and HNPCs were carried out as described (Chen et al., 2013a, 2013b; Li et al., 2017a), with a ZIKV MOI of 2.

All cells were tested as free of Mycoplasma contamination.

2.6. Immunofluorescence assay

ZIKV-infected cells treated with DMSO or erythrosin B were grown in 96-well black imaging plates (Corning). At 48-hr post-infection, growth medium was removed. The cells were washed once with PBS and fixed on ice in 100% pre-chilled (-20°C) methanol for 15 min. The fixed cells were incubated for 1 h with blocking and permeabilisation buffer containing 0.5% Triton X-100, 0.2 $\mu\text{g/ml}$ EDTA and 1% BSA in PBS. The cells were then treated with a mouse monoclonal pan anti-E antibody 4G2 (ATCC) overnight and washed three times with PBST buffer (1x PBS with 0.2% Tween 20). The cells were then incubated with the DyLight[®] 488 goat anti-mouse IgG (ImmunoReagents, Inc.) for 1 h in blocking buffer, after which the cells were washed three times with PBST. Nuclear staining dye Hoechst was added and incubated for 5 min. Fluorescence images were recorded under a fluorescence microscope equipped with an Olympus DP71 imaging system.

2.7. Quantitative qRT-PCR

50 μl of cell supernatant samples were extracted on Applied Biosystems MagMAX Express-96 Deep Well Magnetic Particle Processor. TaqMan gene expression qRT-PCR assays were performed with 5 μl of the extracted RNA using the TaqMan One-step RT-PCR Master Mix Reagents Kit (PE Biosystems) on Applied Biosystems 7500 Real-time PCR System. TaqMan primers for ZIKV were CCGCTGCCCCA ACACAAG and CCACTAAYGTTCTTTTGCAGACAT with ZIKV probe Cy5-AGCCTACCT/TAO/TGACAAGCAGTCAGACACTCAA-IAbRQSp. Samples were analyzed by relative quantification using the $2^{-\Delta\Delta\text{Ct}}$ (“delta-delta Ct”) compared with the endogenous control.

2.8. Protein thermal shift assay

The protein thermal-shift assay (PTSA) was conducted using an Applied Biosystem 7500 Fast Real-Time PCR System (ThermoFisher Scientific) from 25 to 80°C . The DENV2 His-MBP-NS3 or each mutant (final concentration of 2.5 μM in 1x PBS) was mixed with erythrosin B to attain a 4.8 μM final concentration in 1.6% DMSO in the MicroAmp[®] Fast Optical 96-Well Reaction Plate (ThermoFisher Scientific). Thermal denaturation was monitored using SYPRO Orange (Life Technologies) according to manufacturer's manual. The denaturation of the proteins was monitored by following the increase of the fluorescence emitted by the probe that binds exposed hydrophobic regions of the denatured protein. The melting temperature (T_m) was calculated as the mid-log of the transition phase from the native to the denatured protein, using a derivative model in the Protein Thermal Shift[™] Software v1.0 (ThermoFisher Scientific). The reference unfolding temperature of proteins in 1.6% DMSO ($T_{m\text{-DMSO}}$) was subtracted from the values in the presence of each compounds ($T_{m\text{-comp}}$) to obtain thermal shifts, $T_m = T_{m\text{-comp}} - T_{m\text{-DMSO}}$. Compounds were considered to be binders when $T_m > 0.5^{\circ}\text{C}$.

2.9. Western blot

At 48 h post infection with an MOI of 0.1, ZIKV-infected A549 cells treated with DMSO or erythrosin B were washed twice with PBS buffer and manually scraped out of the 6-well

plates. Cells were spun down and supernatant was removed. 30 µl of complete protease inhibitor cocktail in PBS was added to the cells, followed by addition of 30 µl of SDS-PAGE loading buffer. The mixtures were boiled at 95 °C for 10 min, followed by centrifugation at 15,000 rpm for 10 min. Sample was analyzed using 12% SDS-PAGE. The primary antibodies used were anti-ZIKV NS3 (GTX133309, GeneTex, Inc.) and anti-GAPDH (CB1001, EMD Millipore). Western blots were quantified using the Bio-Rad Gel Doc EZ system and Image Lab™ software #1709690 (Bio-Rad).

2.10. Modeling

The 3D structure for erythrosin B was downloaded in SDF format from the ZINC15 library (Sterling and Irwin, 2015). The NS2B-bound crystal structures of the Dengue NS3 protease (PDB ID: 3U1I (Noble et al., 2012)) and of the Zika NS3 protease (PDB ID: 5LC0 (Lei et al., 2016)) were used as docking targets to predict the possible bound conformations of these compounds.

An Induced Fit Docking (IFD) protocol of Schrödinger Small-Molecule Drug Discovery Suite (Schrödinger, 2016) was employed in this docking study. Crystal structures of DENV3 (PDB Code: 3U1I) and ZIKV (PDB Code: 5LC0) were used as docking templates. Only the NS3 protease was kept from each structure and prepared with Protein Prepared Wizard. During this step, hydrogens were added, crystal waters were removed, partial charges were assigned using the OPLS-2005 force field, and protonation states were assigned. The 3D conformations of ligands were created using the Schrödinger Maestro and prepared with LigPrep. During this step, a single, low-energy, 3D structure of each ligand was produced. The Induced Fit Docking protocol was run from Schrödinger Maestro using the Induced Fit Docking panel. On NS3 chains of DENV3 and ZIKV, the center of box for docking was chosen on the centroid of a set of selected residues within the 2B53 pocket. The box size was set to 26 Å on each side. Selected side chains (R24, K26, and T59 of DENV3; M1026, S1060, and Y1023 of ZIKV) were temporarily removed (the equivalent of being temporarily mutated to alanine) to create more space during the initial IFD process and were restored after the docking step. The IFD procedure used reduced van der Waals radii scaling (0.7 for nonpolar receptor atoms and 0.5 for non-polar ligand atoms) for a flexible docking. For each pose generated from initial Glide docking, a Prime structure prediction was then used to accommodate the ligand by reorienting nearby side chains. These residues and the ligand were then minimized. During this process, the receptor structures were optimized. Finally, each ligand was re-docked into the structures within 30 kcal/mol, and within the top 20 structures using Glide XP mode. As a result, multiple docking poses were generated and ranked according to the IFD scores. The poses with lower values of IFD scores are more favorable for binding. The receptor-ligand complexes structures were imported into Schrödinger Maestro for visualization and analysis of binding site interactions.

3. Results

3.1. Erythrosin B abolishes interactions between NS2B and NS3 of DENV2

Previously, we developed an SLC-based NS2B-NS3 interaction assay by generating GST-CLuc398-NS3 and His-NLuc416-NS2B₄₉₋₆₆ fusion proteins in which the C-terminal

fragment (CLuc398) of firefly luciferase (FLuc, aa 398–550) was fused between the GST-tag and viral NS3, and the N-terminal fragment (NLuc416) of FLuc (aa 1–416) was fused between the His-tag and viral NS2B peptide 49–66 (Li et al., 2017a). Interactions between the NS2B peptide 49–66 and NS3 result in complementation of NLuc and CLuc to form a complete active luciferase, leading to significant increase of luminescence when given luciferin substrate (Li et al., 2017a). Using this SLC assay, we found that EB moderately inhibited the interactions between NS2B₄₉₋₆₆ and NS3 with IC_{50-SLC} of 15 μ M (Fig. 1A).

3.2. Inhibition of the DENV2 and ZIKV protease activities by EB

To establish an *in vitro* protease assay, we first generated His-NS2B and NS3 of DENV2 and ZIKV fused to maltose-binding protein (MBP) fusion proteins (Li et al., 2017a). Unlike NS3 alone, which forms inclusion bodies and requires *in vitro* refolding, the DENV2 MBP-NS3 can be expressed and purified as soluble proteins. In our previous study (Li et al., 2017a), we found that the MBP-NS3 and the refolded NS3 are equally active when trans-activated by His-NS2B.

We performed a protease inhibition assay using a FRET substrate Abz-RRRR↓SAG-nTyr (NeoBiolab), where the arrow indicates the cleavage site (Li et al., 2017a). The RRRRSAG represents an authentic substrate within the DENV2 capsid protein (Bera et al., 2007). Our data showed that EB effectively inhibited the DENV2 NS2B-NS3 protease activity with IC_{50-pro-DENV} of 1.9 μ M (Fig. 1B).

Orthosteric inhibitors targeting the NS2B-NS3 binding interface are likely to be broad-spectrum inhibitors for flaviviruses (Li et al., 2017a, 2017b). To this end, we expressed and purified the protease components of an additional flavivirus, ZIKV, for which therapeutics are urgently needed. The GST-tagged NS3 and Flag-tagged His-NS2B of ZIKV were generated as described previously (Li et al., 2017a). Using the same Abz-RRRR↓SAG-nTyr FRET substrate, we showed that EB has a slightly higher inhibition potency (IC_{50-pro-ZIKV}: 1.7 μ M) towards the ZIKV NS2B-NS3 protease activity, compared to that of DENV2 (Fig. 1C). The results indicated that EB is a potential broad-spectrum inhibitor of flavivirus proteases.

3.3. Cytotoxicity of EB in cell culture

To evaluate cytotoxicity, we measured the viability of A549 cells in the presence of EB using the MTT cell viability assay as we described previously (Brecher et al., 2015; Li et al., 2017a) (Fig. 2A). EB did not show any cytotoxicity even at very high concentrations, indicating that the CC₅₀ for EB in A549 cells is more than 150 μ M, the highest concentration tested (CC₅₀: half maximal cytotoxic concentration, defined as the compound concentration at which 50% of cells remain viable) (Fig. 2B). Our results indicate that the antiviral effect of EB has excellent cytotoxicity profile in addition to its antiviral effect.

3.4. Broad-spectrum inhibition of flaviviruses

We next performed a viral plaque reduction assay to evaluate the antiviral efficacy of EB. We first infected human lung carcinoma A549 cells with DENV2 in the presence of a concentration series of EB or a DMSO control, and measured plaque-forming viral titer at

48 h post-infection. The results indicated that EB is a potent inhibitor of DENV2 with EC₅₀ of 1.2 μ M (Fig. 2B).

Because inhibitors targeting the NS2B-NS3 interaction site are potentially broad-spectrum flavivirus inhibitors, we next investigated the antiviral efficacy of EB against a panel of flaviviruses (Fig. 2C–F; Table 1). Our data indicate that EB is a very potent inhibitor for all tested flaviviruses, namely ZIKV, WNV, YFV, and JEV and can reduce the viral titers in a dose-dependent manner. The selectivity indices, defined as CC₅₀/EC₅₀, range from 125 to 428 for these viruses (Table 1). The results indicate that EB has good therapeutic window for these viruses.

3.5. Inhibition of RNA synthesis and protein expression of ZIKV

In addition to the viral plaque reduction assay, we also chose ZIKV to evaluate the inhibitions of viral RNA synthesis and viral protein expression by EB. As shown (Fig. 3A), the ZIKV RNA copy number was also significantly reduced in a dose-dependent manner by EB. Moreover, using a pan flavivirus anti-E antibody 4G2, an immunofluorescence assay (IFA) assay indicated that EB also greatly reduced ZIKV antigen expression in a dose-dependent manner (Fig. 3B).

We further used Western blots (WB) to investigate whether EB could inhibit viral NS3 protein expression. The expression of ZIKV NS3 (~70 KDa) was significantly inhibited by EB in a dose-dependent manner (Fig. 3C).

Overall, these experiments confirmed that EB inhibits viral infectivity, viral RNA replication, and viral protein expression, and is a broad-spectrum antiviral for flaviviruses.

3.6. Inhibition of ZIKV in cells relevant to ZIKV pathogenesis

To investigate whether EB is effective in human primary cells demonstrated to be relevant to ZIKV pathogenesis (Garcez et al., 2016; Qian et al., 2016; Tabata et al., 2016; Tang et al., 2016; Zhang et al., 2016), we investigated inhibition of ZIKV by EB in human placental epithelial cells (HPECs) that derived from the inner surface of the amnion, and in human induced pluripotent stem cell (iPSC)-derived neural progenitor cell (HNPC) (Chambers et al., 2009). Our results showed that EB effectively inhibited ZIKV in HPEC and HNPC in dose-dependent manners (Fig. 3D and E). EB had an EC₅₀ value (0.6 μ M) in HPEC, similar to that in the A549 cells (Table 1). However, it was slightly less effective in inhibition of ZIKV in HNPC than in A549, with an EC₅₀ of 1.39 μ M (Table 1). Moreover, results from IFA and qRT-PCR indicated that viral protein expression and RNA replication were also drastically decreased (Fig. 3, F to I). Overall, these experiments demonstrate that EB is an effective antiviral in placental and neural progenitor cells relevant to ZIKV infection.

3.7. Non-competitive inhibition of the DENV2 and ZIKV NS2B-NS3 proteases

We next performed enzymatic kinetic experiments on the DENV2 His-NS2B/MBP-NS3 and the ZIKV His-NS2B/GST-NS3 protease heterocomplexes in the presence or absence of EB, to determine whether EB inhibited the viral protease by means of non-competitive orthosteric mechanism (Fig. 4A and B). Compared to the DMSO control, EB greatly

reduced the $V_{\max}$ values for the DENV2 and ZIKV hetero protease complexes, whereas the K_m values did not change. The results, including a lowered $V_{\max}$ but similar K_m values for the EB-treated samples, suggest a non-competitive inhibition mechanism according to classical Michaelis-Menten enzyme kinetics. These results are consistent with the proposed mechanism by which EB inhibits the viral protease activity by orthosterically abolishing the binding of NS2B to NS3, but does not compete with the protein substrate at the active site.

3.8. Modeling of binding of erythrosin B to flavivirus NS3 proteases

To further explore the potential mode of action, we docked EB to the crystal structures of the ligand-bound NS3 proteases of DENV3 (PDB ID: 3U1I) (Noble et al., 2012) and ZIKV (PDB ID: 5LC0) (Lei et al., 2016), after excluding the cofactor NS2B peptides during the protein preparation for molecular docking (Fig. 4, C to E).

As shown in Fig. 4C using the DENV3 NS3 as a representative model, EB was well docked into the NS3 pockets holding the NS2B residues at positions 51 and 53 (termed as 2B51 and 2B53 pockets), respectively, on both of DENV3 and ZIKV. EB interacts with F46 and H60 through the π - π stacking and interacts with K33 *via* the π cation (Fig. 4D). The isobenzofuran ring of EB is seated nicely in a cage formed by the NS3 residues Y23, I25, F46, L58 and H60. The cage normally holds residue 53 of the co-factor NS2B in the NS2B-NS3 heterocomplex (Fig. 4C). One of the hydroxyl groups of EB forms hydrogen bond with N61 and the other one forms hydrogen bond with Q27 and V57. It also interacts with K26 and K33 through additional hydrogen bonds. The docking pose shown in Fig. 4E demonstrates that EB binds at the 2B51 and 2B53 pockets on NS3 protease of ZIKV in a similar manner. Same π - π stacking interaction forms with F1046. One of the hydroxyl groups of EB forms hydrogen bond with S1060, while the other one forms hydrogen bond with M1026 and R1028. An additional halogen bond is formed in between iodine atom and T1081.

3.9. Determination of residues essential for binding of erythrosin B to the DENV2 MBP-NS3 protease

In order to investigate whether EB binds to the NS3 protein, we performed a protein thermal shift assay (PTSA). Our data showed that EB treatment led to an increase of 1.2 °C in T_m for purified DENV2 MBP-NS3 protein, compared to the DMSO control (Fig. 5A). The data suggest that EB binds to the NS3 protein, resulting in stabilization of the NS3 conformation that leads to a T_m increase (Fig. 5A).

To further investigate the interactions between EB and the viral NS3 protein, we generated six mutants of the DENV2 MBP-NS3 protein based on the docked complex structure (Fig. 4D). We used PTSA to evaluate whether these mutants retain the binding capacity to EB. Our results indicated that alanine mutations of residues Y23, I25, F46, L58 and H60 completely abolished the binding of EB to the mutant proteins, as reflected by loss of T_m increase upon EB binding (Fig. 5A). In addition, Q28A mutation led to reduced T_m increase (0.8 °C *vs* 1.2 °C), compared to that of WT.

The PTSA results are consistent with our docking model, in which the residues Y23, I25, F46, L58 and H60 form a cage to accommodate the isobenzofuran ring of EB, whereas Q28

is located on the edge of the binding pocket for EB and does not have productive interactions with the bound EB (Fig. 4D). Therefore, mutations of the cage-forming residues lead to the loss of binding of these mutants to EB, whereas Q28A mutation only results in moderate impact in T_m change upon EB binding compared to WT. These results indicated that these residues (Y23, I25, F46, L58, and H60) are key residues in interactions with EB, and further confirmed the non-competitive nature of mode of inhibition.

Furthermore, we evaluated the protease activities of these mutants. Our results showed that mutations of the cage-forming residues (Y23, I25, F46, L58, and H60) substantially reduced the protease activities (Fig. 5B). In contrast, Q28A only moderately reduced the protease activity. The results are consistent with the structural model of the NS2B-NS3 protease (Fig. 4C) and that of PTSA. The residues Y23, I25, F46, L58, and H60 form a cage to hold the essential residue at position 53 of the co-factor NS2B. Our results suggest that these residues are essential for binding of NS3 to its co-factor NS2B. Mutation of these residues will greatly reduce the protease activity.

Overall, our results suggest that EB binds into the NS3 pocket normally holding the essential residues at positions 51 and 53 of the cofactor NS2B. Mutations of the cage-forming residues not only greatly reduced the binding of EB to NS3, but also significantly diminished the protease activity.

4. Discussion

Since the outbreak of WNV in the New York City in 1999, the flavivirus protease enzyme has been an encouraging drug target. Recent study shows that inhibitors preventing the NS2B-NS3 interaction provide a promising approach for therapeutic development (Li et al., 2017a).

In this study, we identified erythrosin B as a candidate orthosteric inhibitor blocking interactions between flavivirus NS2B and NS3. Erythrosin B, also known as Red No. 3, erythrosine, or food additive E127, is an organoiodine compound primarily used in food coloring in many countries. In this study, we show for the first time that EB not only has novel anti-flaviviral activities but also has novel mechanism of action.

In this study, we showed that EB is a broad-spectrum inhibitor for the flavivirus NS2B-NS3 hetero-protease complexes. EB inhibited the viral protease activities of DENV2 and ZIKV with low micromolar efficacy *in vitro*. In cell culture, EB significantly inhibited the growth of representative flaviviruses including DENV2, ZIKV, WNV, YFV, and JEV with EC_{50} in low micromolar to high nanomolar range. IFA, qRT-PCR, and Western blot analyses indicated that not only was virus growth inhibited by EB, but also viral RNA synthesis and protein expression were drastically reduced by EB. Cytotoxicity analysis indicated that EB has large therapeutic window towards these flaviviruses. In addition, we showed a dose-dependent rescue from viral infection in all ZIKV-relevant cell types by EB. The findings establish the efficiency of EB to eliminate infection of ZIKV from human placental and progenitor cells, and provide a path forward to minimize the risk of fetal acquired microcephaly resulting from ZIKV infection of pregnant women (Lessler et al., 2016).

Furthermore, using biochemical binding assays, protease kinetics, mutagenesis, and docking experiments, we demonstrated that EB inhibited the viral protease *via* an orthosteric inhibition mechanism by interfering the interactions between viral protease components NS2B and NS3.

Erythrosin B has been used as a dye in foods, drugs and cosmetics since its approval by the US Department of Agriculture in 1907. Erythrosin B is a substance within the FDA pregnancy category B that has failed to demonstrate a risk to the fetus in animal reproduction studies, although there are no adequate and well-controlled studies in pregnant women. Our studies showed that erythrosin B is a potent ZIKV and DENV2 inhibitor with a novel mechanism by inhibition of interactions between viral protease components NS2B and NS3. As a category B substance, erythrosin B has low acute oral toxicity, and does not appear to cause reproductive or developmental toxicity ((ANS), 2011; Lin and Brusick, 1986; Tanaka, 2001). Evidence also suggests that it is unlikely to be genotoxic (Lin and Brusick, 1986). Based on these findings, erythrosin B was found to have a minimal effect in humans at a clinical oral dose of 200 mg daily over 14 days, and a no-adverse-effect-level (NOAEL) of 60 mg/kg body weight (bw)/day was derived for humans (SCCS, 2010). The LD₅₀ value for mouse (LD₅₀: lethal dose killing 50% of the test sample) was reported in the range from 2560 to 6800 mg/kg bw ((ANS), 2011). Erythrosin B was not found to adversely affect development of young animals at dose levels up to 500 mg/ kg bw/day ((ANS), 2011). Erythrosin B also does not adversely affect male fertility at dose levels up to 2000 mg/kg bw/day ((ANS), 2011). As an FDA pregnancy category B substance, erythrosin B is commonly used as cherry-pink food color in sweets such as some popsicles and candies, and even more widely used in cake-decorating gels. It is also frequently used as a dental plaque discoloring agent. Although there is no detailed report about the pharmacokinetics of EB, it was reported that the bioavailability of EB is low (Poulsen, 1993). Further experiment with enhanced drug formulation or efficient drug delivery systems (e.g. nanoparticles) may be required to improve the bioavailability of EB before its use as antivirals.

In a summary, our results demonstrate that flaviviral proteases can be targeted at an orthosteric site and provide a starting point for further development of orthosteric inhibitors; and we found a potent inhibitor, erythrosin B, which has excellent safety profile. Future *in vivo* efficacy and preclinical studies of erythrosin B will be essential to evaluate the pharmacokinetics, bioavailability, *in vivo* efficacy, dosage, and potential benefits and risks for patients infected with flaviviruses, including ZIKV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.antiviral.2017.12.018>.

Fig. 1. Inhibition of the NS2B-NS3 interactions and protease activity

(A) Dose-dependent inhibition of SLC upon binding of NLuc-NS2B₄₉₋₆₆ to GST-CLuc-NS3 by erythrosin B. N = 3. (B) Dose-response inhibitions of the DENV2 His-NS2B/His-MBP-NS3 protease activity by erythrosin B. N = 3. (C) Dose-response inhibitions of the ZIKV His-NS2B/GST-NS3 protease activity by erythrosin B. N = 3.

Fig. 2. Erythrosin B is a potent broad-spectrum inhibitor of representative flaviviruses
(A) Cell viability assay. A549 cells were incubated with various concentrations of erythrosin B and then assayed for viability at 48-hr post-incubation. N = 3. **(B–F)** Dose-dependent inhibition of representative flaviviruses DENV2 **(B)**, ZIKV **(C)**, WNV **(D)**, YFV **(E)**, JEV **(F)** by erythrosin B in A549 cells. Viral plaque reduction assay was used. N = 3.

Fig. 3. Inhibition of ZIKV in cells relevant to ZIKV

(A) qRT-PCR analysis of inhibition of viral RNA from ZIKV infected A549 cells by erythrosin B. (B) Immunofluorescence assay (IFA) of inhibition of viral protein expression by erythrosin B, using pan-flavivirus anti-E 4G2 antibody (green) (ATCC). Nuclei (blue) was stained in all IFA assays by the Hoechst stain solution. (C) WB analysis of dose response inhibition of ZIKV NS3 expression by erythrosin B (left panel). Right panel, NS3 expression normalized to the GAPDH loading control. ***, $p < .01$. Pre-seeded A549 cells in 6-well plate were treated with EB or DMSO control and infected with ZIKV with MOI of 0.1 as described previously (Li et al., 2017a). At 48 h post infection, cells were washed, harvested, and protected by protease inhibitor cocktail prior to lysis by SDS-PAGE loading buffer. Upon incubation at 95 °C for 10 min, sample was subjected to Western blot analysis with anti-ZIKV NS3 (GTX133309, GeneTex, Inc.) and anti-GAPDH (CB1001, EMD Millipore) as primary antibodies. (D,E) Viral plaque reduction assay for ZIKV-infected HPECs (D) and iPSC-derived hNPCs (E) by erythrosin B. ***, $p < .01$. (F,G) qRT-PCR analysis of inhibition of viral RNA of ZIKV-infected HPECs (F) and HNPCs (G) by erythrosin B. (H,I) IFA of inhibition of viral protein expression for ZIKV-infected HPECs (H) and HNPCs (I) by erythrosin B. For HPECs, erythrosin B (0.12 μ M) was used. For HNPCs, erythrosin B (1.67 μ M) was used.

Fig. 4. Inhibition of NS2B-NS3 protease complex via a non-competitive mechanism
 (A,B) Inhibition kinetic experiment of the DENV2 His-NS2B/His-MBP-NS3 (A) and the ZIKV His-NS2B/GST-NS3 (B) heterocomplexes by erythrosin B. Lineweaver–Burk plots of kinetics experimental data for inhibition of the His-NS2B/His-MBP-NS3 hetero protease complexes by erythrosin B. The DENV2 MBP-NS3 or ZIKV GST-NS3 (100 nM) was mixed with erythrosin B (3 μ M or various concentrations) for 30 min. The DENV2 His-NS2B or ZIKV His-NS2B (1 μ M) were added together with the Abz substrate at various concentrations (800 μ M–25 μ M in 2-fold dilutions). (C) Superimposition of bound EB (yellow) with the NS2B co-factor (cyan) of DENV3 (PDB: 3U1I). NS3 residues in contact with EB were in stick representation (grey). (D) Ribbon presentation of erythrosin B (green) docked into NS3pro of DENV3 (PDB: 3U1I). Key interaction residues are highlighted in stick presentation. Hydrogen bonds are shown in yellow dotted lines and π - π stacking is shown in blue line. (E) Ribbon presentation of erythrosin B (green) docked into NS3pro of ZIKV (PDB: 5LC0). The corresponding NS3pro β -strand hairpin loops on ZIKV are shown in green and grey. Key interaction residues are highlighted in stick presentation. Hydrogen bonds are also shown in yellow dotted lines and π - π stacking is shown in blue lines.

Fig. 5. Protein thermal shift assay (PTSA) and mutagenesis to determine essential residues of NS3 for binding of EB

(A) PTSA. Experimental details were described in the Method section. Experimental data were fitted and analyzed by using the Protein Thermal Shift™ Software v1.0 (ThermoFisher Scientific). $T_m = T_{m-EB} - T_{m-DMSO}$. N = 3. (B) Protease activity of each mutant NS3 protein relative to that of WT. All proteins were at 150 nM. N = 3. ***, $p < .01$, by one-way ANOVA.

Table 1

Cytotoxicity, antiviral efficacy, and selectivity index.

Cells	CC ₅₀ (μM)	Virus	EC ₅₀ (μM)	Selectivity index
A549	> 150	DENV2	1.2 ± 0.2	> 125
		ZIKV	0.62 ± 0.12	> 242
		WNV	0.66 ± 0.13	> 227
		YFV	0.57 ± 0.06	> 263
		JEV	0.35 ± 0.06	> 428
HPEC	> 150	ZIKV	0.60 ± 0.08	> 250
HNPC	> 150	ZIKV	1.39 ± 0.67	> 108