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A screen of FDA-approved drugs for inhibitors of Zika virus infection

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Summary

Currently there are no approved vaccines or specific therapies to prevent or treat Zika virus (ZIKV) infection. We interrogated a library of FDA-approved drugs for their ability to block

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Author Contributions

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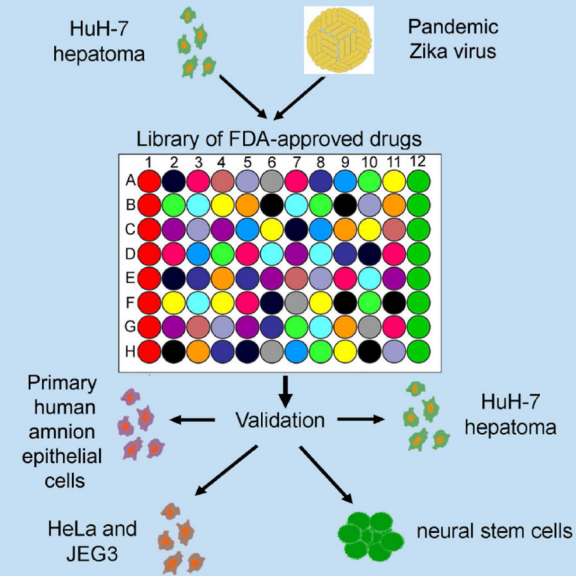
infection of human HuH-7 cells by a newly isolated ZIKV strain (ZIKV MEX_I_7). More than 20 out of 774 tested compounds decreased ZIKV infection in our *in vitro* screening assay. Selected compounds were further validated for inhibition of ZIKV infection in human cervical, placental and neural stem cell lines, as well as primary human amnion cells. Established anti-flaviviral drugs (e.g., bortezomib and mycophenolic acid) and others that had no previously known anti-viral activity (e.g., daptomycin) were identified as inhibitors of ZIKV infection. Several drugs reduced ZIKV infection across multiple cell types. This study identifies drugs that could be tested in clinical studies of ZIKV infection and provides a resource of small molecules to study ZIKV pathogenesis.

Graphical Abstract



Introduction

Arthropod-borne flaviviruses, particularly those transmitted by *Aedes* mosquitoes, pose significant threats to global health. Zika virus (ZIKV) was isolated in 1947 in the Ziika Forest in Uganda from a sentinel *Rhesus* macaque and subsequently found in *Aedes africanus* mosquitoes (Dick et al., 1952). ZIKV remained a footnote among neglected tropical diseases due to the mild disease described from a limited number of cases. That changed in 2007 when ZIKV emerged in a series of outbreaks across the Pacific (Duffy et al., 2009; Dupont-Rouzeyrol et al., 2015; Roth et al., 2014; Tognarelli et al., 2016), and changed dramatically in 2014 and 2015 when Zika exploded into the Americas causing a large and expanding pandemic (Fauci and Morens, 2016; Lednicky et al., 2016). One of the first countries to be affected in the Americas was Brazil, where ZIKV has caused an estimated 1.5 million infections (Weaver et al., 2016). In the last year diseases caused by/associated with ZIKV infection have evolved to become a more pressing flaviviral threat (Weaver et al., 2016). Since ZIKV can be transmitted by *Aedes* species mosquitoes, which are widely distributed in tropical and temperate regions of the world (Weaver et al., 2016), it is likely that ZIKV has become pandemic.

Most human infections are transmitted by mosquito, although ZIKV can spread directly from person to person through sexual contact and also vertically from mother to fetus (Petersen et al., 2016). This sets ZIKV apart from other pathogenic flaviviruses and creates significant and unexpected public health concerns. ZIKV infection is usually asymptomatic, and most symptomatic infections are mild and resemble those observed with dengue: rash, fever, arthralgia, conjunctivitis, myalgia, headache, and retro-orbital pain (Petersen et al., 2016). There have been reports of hematospermia and symptoms resembling prostatitis following infection (Foy et al., 2011), and viral RNA and infectious virus have been detected in the semen of men weeks after clearing of acute symptoms (Mansuy et al., 2016). Most symptomatic ZIKV infections are self-limited and resolve in less than a week; however, there are documented cases of severe acute ZIKV infection in patients with other underlying conditions (Arzuza-Ortega et al., 2016). Moreover Zika has been strongly associated with neurological sequelae, most commonly Guillain-Barré Syndrome, but also meningo-encephalitis and myelitis (Fontes et al., 2016; Petersen et al., 2016). The most dreaded complications of infection occur in pregnancy and include severe fetal abnormalities and death. An association between microcephaly and ZIKV infection was first noted in Brazil when a 20-fold increase in microcephaly was reported from 2014 to 2015, and today compelling geographic and epidemiological evidence indicate a causal relationship between ZIKV and birth defects (Fauci and Morens, 2016; Kleber de Oliveira et al., 2016; Rasmussen et al., 2016). This conclusion is supported by finding ZIKV RNA and infectious virus in placental and fetal tissues (Brasil et al., 2016; Mlakar et al., 2016), and very recently by animal model studies that demonstrate fetal infection and neurological damage in fetuses of experimentally infected pregnant mice (Cugola et al., 2016; Miner et al., 2016). The neurological complications and the severe repercussions of ZIKV infection on the fetus have led the World Health Organization to declare a global health emergency and compel the scientific community to find solutions to the Zika threat.

The first line of defense against Zika is preventing mosquito bites using repellents or pesticides (Benelli, 2015). Unfortunately these methods failed to contain recent arbovirus epidemics. There is no approved vaccine or specific therapy to prevent or treat ZIKV infection and physicians are restricted to administering supportive care. Indeed, there is nothing that physicians can do to prevent fetal damage from ZIKV infection during pregnancy or to prevent severe Zika outcomes in individuals with underlying medical conditions.

To address the immediate need for anti-ZIKV therapy we interrogated a library of FDA-approved drugs for the ability to block ZIKV infection. Our work identified more than 20 therapeutics that decreased ZIKV infection in HuH-7 cells. Among these were drugs previously shown to have anti-flaviviral activity and some that had no previously reported anti-viral activity. We further showed that multiple drugs reduced ZIKV infection in human cell lines derived from the female genital tract, placenta, primary neural stem cells, and primary amniotic epithelial cells. This study identifies drugs that could be immediately tested by clinical experts for ZIKV infection during pregnancy or in the context of severe ZIKV.

Results

Isolation of a pandemic ZIKV from mosquitos

In order to work with a pandemic ZIKV we isolated ZIKV MEX_I_7 from *Ae aegypti* mosquitos in Tapachula, Chiapas, Mexico (Guerbois et al., submitted), re-sequenced the majority of the genome by massively parallel sequencing, and compared its open reading frame sequence with other publically available ZIKV sequences (Figure S1). Phylogenetic analysis identified the established African and Asian lineages for ZIKV (Faye et al., 2014), and placed ZIKV MEX_I_7 within the Asian lineage, segregating closest to Guatemalan isolates from the American pandemic.

A screen for anti-ZIKV activity among FDA-approved drugs identifies several candidates for drug repurposing

Flaviviruses require a large number of human host factors and many of these are targets of approved drugs. An expeditious path to identify candidate anti-ZIKV therapeutics is to repurpose previously approved drugs. To this end we screened a library of 774 FDA-approved therapeutics for anti-ZIKV activity (Figure 1A). Human HuH-7 cell monolayers were treated with each drug in the library, a positive control anti-flaviviral compound (NITD008) (Yin et al., 2009) or vehicle (DMSO) for 1 hour before infection with ZIKV MEX_I_7 (see Methods). The following day, cells were fixed and the viral envelope protein was labeled for immunofluorescent detection. Automated imaging and analysis quantified cell number and the percentage of cells that were productively infected. This assay design should effectively identify drugs that inhibit viral entry, translation or RNA synthesis, but may not identify drugs that target late stages of infection such as viral egress. In negative control wells 48.30% ± 4.63% of cells were infected compared to less than 1% of those treated with NITD008 (Figure 1B). The Z' factor, a quantitative metric that assesses screen robustness, for the population of the 44 negative and 44 positive controls in the initial screen was 0.7, a value interpreted as evidence of an excellent assay (Zhang et al., 1999). We independently performed a replicate screen using the same library and controls. The distribution of the infection rates for the replicate screens was similar, yielding a correlation coefficient of 0.86 (Figure 1C). The full data sets for the screens are in Table S1.

Most drugs showed little to no effect on either ZIKV infection rate or number of cells detected (Figure 1D). The population mean infection rate was 49.18% (95% CI: 48.32%-50.05%), which is similar to the mean of the negative controls. Forty five drugs significantly inhibited ZIKV infection (Z-score </= −3.00) in both replicates of the screen. Some of these also reduced cell number. The median cell count for the population in replicate 1 was 3,085, which was similar to the average cell count for the DMSO control wells (2866 ± 275). Replicate two performed similarly (Table S1). We concluded that our screen was able to differentiate candidate antiviral compounds from the distribution of the population.

Of the 45 drugs that reduced virus infection, we selected 30 based on their efficacy in our system and considerations of their clinical use (these 30 are indicated by red circles in Figure 1D; highlighted in Table S1). The percent infection within the 30 selected hits ranged

from uninfected to 28.07% and cell number ranged from 3 to 3,905 cells, with a median 1,868 cells. In general there were fewer cells in wells treated with these 30 drugs compared to the average of the negative control. Therefore, toxicity by candidate antivirals may impact results at the concentration tested (10 μM). Nevertheless, hits including daptomycin, mycophenolate mofetil and sertraline reduced viral infection without affecting cell count (Figure 1E). Using a low passage, pandemic-associated ZIKV virus isolate, this screen systematically evaluated FDA-approved therapeutics to identify ZIKV infection inhibitors. Furthermore, the results show that drugs that are approved for use during pregnancy are among those that block ZIKV MEX_I_7 in our assay.

Follow up and validation of selected anti-ZIKV drugs

We selected the aforementioned 30 drugs (red circles, Figure 1D) for follow up analysis based primarily on their efficacy in reducing ZIKV infection in primary screens. Each drug was used to pre-treat HuH-7 cells at concentrations of 0.001 μM, 0.01 μM, 0.1 μM, 1 μM and 10 μM, for one hour prior to infection with ZIKV. For comparison we used NITD008 and vehicle as positive and negative controls, respectively. After 24 hours of infection cells were fixed, stained for viral antigen, and analyzed for percent infection and cell number. As expected most drugs displayed clear antiviral activity at 10 μM concentrations (Figure S2), in agreement with the primary screen data. Six drugs, which reduced percent infection values by less than half or only eliminated cells were excluded from further testing, leaving a list of 24 drugs with validated anti-ZIKV activity (Table S1).

The reduction of infection rate was highly correlated with reduction of cell numbers for multiple drugs, indicating that these compounds target processes essential for both cell survival/proliferation and ZIKV replication. This profile was exemplified by the antineoplastic proteasome inhibitor bortezomib (Figure 2). This was expected since HuH-7 cells are derived from a human hepatoma and are susceptible to antineoplastic drugs. Several drugs reduced infection rates without large effects on cell number. Among the most potent of these were ivermectin, mycophenolic acid (MPA), and daptomycin (Figure 2). The latter two drugs showed EC_{50} values of between 0.1 and 1.0 μM while the EC_{50} for ivermectin was between 1 and 10 μM. Of note, bortezomib (Choy et al., 2015), ivermectin (Mastrangelo et al., 2012; Wagstaff et al., 2012) and MPA (Diamond et al., 2002) have been shown to inhibit replication of flaviviruses, whereas daptomycin has not been previously associated with antiviral activity. Other notable drugs that reduced infection without strongly reducing cell numbers were sertraline-HCl, pyrimethamine, palonosetron-HCl, and cyclosporine A (Figure S2). Only the latter has been previously documented to inhibit flavivirus infection (Qing et al., 2009). Given their anti-ZIKV activity and their clinical profile the drugs listed on Table 1 could be considered for treatment of high risk Zika patients.

Flaviviruses require hundreds of pro-viral host factors for efficient replication (Krishnan et al., 2008; Le Sommer et al., 2012; Sessions et al., 2009) and it is possible that some of the anti-ZIKV drugs target these factors. We explored this idea by comparing the gene targets of the 24 drugs to human gene products identified in RNAi screens for YFV and DENV host factors in HuH-7 cells (Le Sommer et al., 2012) (Barrows et al., manuscript in preparation).

Interestingly, 4 out of the 24 validated drug targets have been identified previously as host genes affecting YFV or DENV propagation respectively in these RNAi screens (Table S2).

Drugs inhibit ZIKV MEX_I_7 infectivity in human cell lines derived from genital, placental and neural tissues

Given the evidence for sexual transmission, possibly through infected semen coming in contact with vaginal mucosa or cervix, and the clear evidence of placental infection (Mlakar et al., 2016; Noronha et al., 2016) we also studied established cell lines derived from the genital tract and trophoblasts. Both HeLa cells, which were derived from a cervical adenocarcinoma, and JEG3 cells, which were derived from a placental choriocarcinoma, were readily infected with ZIKV MEX_I_7 (Figure 3). We selected eight drugs for follow-up testing in HeLa and JEG3 cells: ivermectin, daptomycin, MPA, sertraline, pyrimethamine, cyclosporine A, azathioprine, and mefloquine. In HeLa cells all drugs tested reduced virus infection rate at 10 μM concentrations (Figure S3A). 1 μM MPA completely inhibited infection in HeLa cells as did 10 μM ivermectin (Figure 3A and C). Daptomycin was not as potent of an inhibitor in HeLa compared to HuH-7 but still reduced infection significantly. In JEG3 cells four out of eight drugs tested showed anti-viral effect and MPA was the most potent (Figure 3B and C, and Figure S3B), reducing infection at 1 and 10 μM. An unusually strong reduction of JEG3 cell count after treatment of 10μM ivermectin was observed in some experiments, while treating HeLa cells with 10μM ivermectin did not show the same impact on cell count. These experiments show that the inhibitory capacity of several FDA-approved drugs was not strictly cell line specific and importantly could be seen in cells types that may be relevant to ZIKV infection.

The dramatic effect of Zika on fetal neural development (Petersen et al. 2016) and strong evidence that neural progenitor or stem cells and neurons can be infected by ZIKV (Garcez et al., 2016; Hanners et al., 2016; Qian et al., 2016; Tang et al., 2016) led us to investigate the effect of several drugs on ZIKV MEX_I_7 infection of a human fetal brain-derived neural stem cell (hNSC) cell line (K048), which was obtained without genetic modifications (Svendsen et al., 1998). These were infected much less efficiently by ZIKV MEX_I_7 than HuH-7, HeLa or JEG3 cells, with infection rates of ~8% using an MOI of 3 (Figure 4). We tested the effects of selected drugs at 1 and 10 μM on infection. Cells were pre-treated with drugs and then infected for 96 hours before analysis by flow cytometry for viral antigen positivity. At concentrations of 1 μM, only NITD008 and MPA reduced rates of virus infection of hNSCs (Figure 4A). Unexpectedly, cyclosporine A enhanced infection at 1 μM compared to the DMSO control. In contrast, at 10 μM concentrations NITD008, MPA, cyclosporine A and ivermectin each significantly blocked ZIKV MEX_I_7 infection (Figure 4B). It should be noted, however, that at 10 μM concentrations, ivermectin, mefloquine, and to a lesser extent cyclosporine A, altered cellular light scattering features, which suggests cell toxicity, and reduced the number of cells that could be confidently analyzed by flow cytometry (Figure 4C and D, and Figure S4). We additionally tested a ZIKV strain from the African lineage (ZIKV DAK_41525)(Ladner et al., 2016) on hNSCs with a drug panel including sertraline and bortezomib. At 1 μM concentrations MPA, ivermectin and bortezomib all exerted antiviral effects and only the latter exhibited moderate toxicity (Figure 4 E and F).

Drugs inhibit ZIKV MEX_I_7 infectivity in primary human amnion epithelial cells

Primary human amnion epithelial cells (HAEC) form the lining of the amniotic sac. These cells were efficiently infected *in vitro* by ZIKV MEX_I_7 and thus represent a primary cell line amenable to our screening model. We tested ivermectin, daptomycin, MPA, sertraline, pyrimethamine, cyclosporine A, azathioprine, and mefloquine, using a concentration range with a higher maximum (16 μM). At 16 μM none of these drugs caused substantial reduction of cell numbers (Figure S5A). In contrast, we observed moderate inhibition of ZIKV MEX_I_7 infectivity by daptomycin and strong inhibition by ivermectin, sertraline and mefloquine at 16 μM (Figure 5A and 5B). MPA inhibited ZIKV MEX_I_7 infection at 1.6 μM (Figure 5B). We tested these drugs on HAECs from another donor and observed similar antiviral effects for MPA, ivermectin, daptomycin, mefloquine, and sertraline (data not shown). These results confirmed that potential anti-ZIKV therapeutics are effective in primary human fetal cells. These data extend the range of the anti-ZIKV activity to cells believed to be attacked by the virus *in vivo* and suggest that combinations of well tolerated drugs should be considered for clinical use to optimize effectiveness in different organs and cell types.

Discussion

Here we identified FDA-approved drugs that can inhibit ZIKV infection in several human cells, including those of genitourinary and neural origin (Table 2) and suggest that these drugs be carefully considered for expedited trials among Zika patients. It is important to point out that given their safety profiles many of these drugs have been used during pregnancy for other indications, both in the U.S. as well as globally. Some are FDA category B, meaning that “animal reproduction studies have failed to demonstrate a risk to the fetus and there are no adequate and well-controlled studies in pregnant women OR Animal studies have shown an adverse effect, but adequate and well-controlled studies in pregnant women have failed to demonstrate a risk to the fetus in any trimester”. Even those that are category C or D (risk not ruled out or positive evidence of risk, respectively) can be used in pregnancy when potential benefit outweighs the risk, which is likely in the case of Zika. For example, sertraline (Zoloft®) is one of the better studied and most used anti-depressants in pregnancy even though it is category C. We should also keep in mind that the use of these medications to treat Zika infection during pregnancy may require shorter courses and may involve gestational age windows that are different, and therefore better safety profile, than what is used for the currently accepted indications. In addition, many of these drugs have been shown to cross the placenta (e.g. mefloquine, sertraline), allowing the opportunity to treat not only the mother but also the fetus. Furthermore, where data support lack of negative drug-drug interactions, clinical studies could test combinations of two or more of these to achieve maximal efficacy.

It is critically important to note that many of the drugs shown to have anti-ZIKV activity could have untoward effects, particularly in the context of pregnancy, and therefore their use should be only in the hands of clinical experts, preferably under research protocols. The use of these drugs in a clinical setting will obviously rely on the best diagnostic evidence, and every effort should be made to use the most sensitive and specific tests to optimize the

accuracy of a Zika diagnosis. Like any treatment in pregnancy, the risks of the treatment and the potential for overtreatment have to be weighed against the risk of no treatment, in this case a devastating neurodevelopmental adverse outcome.

Several of the drugs have been previously shown to have antiviral activity. For example MPA has been demonstrated to inhibit DENV (Diamond et al., 2002; Kang et al., 2014; Ng et al., 2007; Takhampunya et al., 2006) and our observation that it also inhibited ZIKV provides evidence that our screen successfully identified established flavivirus inhibitors. Ivermectin had previously been shown to inhibit Venezuelan equine encephalitis virus (Lundberg et al., 2013), chikungunya virus (Varghese et al., 2016), and several flaviviruses (Mastrangelo et al., 2012) (Lundberg et al., 2013). Daptomycin, however, had not been previously shown to have antiviral activity. Daptomycin is a lipopeptide antibiotic that inserts into cell membranes rich in phosphatidylglycerol (PG) (Baltz, 2009), and this suggests an effect on PG-rich late endosomal membranes, which are critical for viral entry (Zaitseva et al., 2010). The identification of daptomycin as potentially useful to treat Zika patients highlights the power of unbiased screens. Therefore, while we wholeheartedly agree with Ekins et al in calling for an open drug discovery for ZIKV anti-virals, we do not favor the priority these authors give to screening known antivirals (Ekins et al., 2016).

The hits reported herein are FDA approved therapeutics for a variety of diseases, and the pharmacokinetics have been reported for some of these drugs. For example, the maximum plasma concentrations (C_{max}) for high dose daptomycin were reported to exceed 180 mg/mL and the duration to achieve C_{max} (t_{max}) was within the first 30 minutes of intravenous delivery (Benvenuto et al., 2006). In addition, C_{max} for MPA was between 24.2 and 47.2 mg/L an hour after drug delivery (Bullingham et al., 1998). In each case, the effective experimental concentrations reported here were below the C_{max} reported in the literature and t_{max} was achieved within an hour of drug delivery. Ivermectin inhibited the African lineage ZIKV infection at 1 μM in our hNSC model. The ivermectin C_{max} has been reported to be 260ng/mL (Guzzo et al., 2002), so the effective concentration we report is 4× higher than the observed C_{max}. Mefloquine inhibited ZIKV infection at 10 μM. The mefloquine C_{max} has been reported as 1872 ng/L (Karbwang and White, 1990). Therefore the concentration for mefloquine that resulted in anti-viral activity *in vitro* was approximately 2.2X higher than the potential C_{max}. For ivermectin and mefloquine, t_{max} was less than 5 or 24 hours, respectively (Guzzo et al., 2002; Karbwang and White, 1990). Although it is difficult to extrapolate from *in vitro* models to *in vivo* activity, it is promising that our preclinical testing discovered anti-ZIKV activity for several drugs within a range reasonably achievable in humans.

It should also be noted that the information derived from our screen can shed light on the biology of ZIKV, and re-analysis of our data will likely lead to identification of pathways critically important to the virus (Table 2). Indeed, the potent antiviral action of bortezomib (Figures 2 and 4), which had been noted for activity against DENV (Choy et al., 2015), indicates that proteasome action is essential for ZIKV. The role of iron metabolism in the ZIKV life cycle, highlighted by sensitivity to deferasirox (Figure S2), may be of interest for future exploration. Interestingly, we observed that YFV infection was sensitive to knockdown of the iron-sulfur containing protein ISCU, suggesting a shared requirement

between diverse flaviviruses (Le Sommer et al., 2012). ZIKV is also sensitive to perturbation of neurotransmitter signaling as evidenced by the inhibitory effects of sertraline and palonosetron (Figures 5 and S2). Here again, we observed consistency with our prior data that identified cellular genes HTRC3 and GRK2 as important YFV HDFs (Le Sommer et al., 2012). Although not the primary target of our screen, compounds that enhanced infection were also identified and these may provide insights into host pathways that restrict infection or those that when activated make cells most suitable for viral replication. Importantly, we note that desogestrel was one of the drugs that enhanced infection. Although other progestins in the chemical library did not cause similar effects, this observation may have implications for pregnant women and those on hormonal contraception.

Confronted with the large and growing Zika pandemic we have few options to intervene. Insecticide-based vector control methods (Araujo et al., 2015; Benelli, 2015) complemented by more sophisticated approaches, such as release of genetically modified (Moreira et al., 2009) or Wolbachia-infected mosquitos (Harris et al., 2012) have shown promise to reduce mosquito populations, but even if successful these campaigns take months if not years to implement. Furthermore, ZIKV sexual transmission presents a problem not dealt with by vector control alone. The situation in terms of treatment or prevention is not much better. While there are several approaches being considered there is no available vaccine for ZIKV and it may take years for final approval. The antiviral approach is equally limited: there are very few approved drugs to treat acute viral infections and there are no approved anti-flavivirals (Menendez-Arias and Richman, 2014). It is critical to pursue strategies, similar to those effective against human immunodeficiency and hepatitis C viruses, that hold promise for infections caused by the related DENV and WNV (e.g. direct acting antivirals) (Kok, 2016), however these anti-flaviviral compounds are in early phases of development and may take years to move into clinical use. The work presented here provides therapeutic possibilities to consider and test when confronted with ZIKV infection in pregnancy and in individuals with increased risk due to underlying medical conditions. Moreover, this work highlights how little we know about the molecular vulnerabilities of emerging pathogens, and the importance of correcting this deficit to be able to provide rapid and effective responses to future epidemics.

Experimental Procedures

Cell culture

Vero, HuH-7 and HeLa cells were maintained in DMEM supplemented with 10% FBS and penicillin and streptomycin (P/S). JEG3 cells were maintained in MEM supplemented with 10% FBS and P/S. C6/36 cell line was maintained in RPMI supplemented with 10% FBS and P/S. Human fetal brain neural stem cells (K048) were originally derived from the cortical region of a 9-week post mortem fetus, and grown as suspending neurosphere cultures. They were maintained in serum-free medium (DMEM/F12 3:1) supplemented with B27, 20 ng/ml fibroblast growth factor 2, 20 ng/ml epidermal growth factor (EGF), 10 ng/ml leukemia inhibitory factor and P/S (Wu et al., 2002). Primary human amnion epithelial cells (HAEC) were amplified in DMEM:F12 (1:1) supplemented with 10 mg/L EGF, 10% FBS and P/S.

Primary human amnion cell cultures

Fetal membrane tissues were obtained after placental delivery from women undergoing elective repeat cesarean section and uncomplicated pregnancies at term, not in labor at the John Sealy Hospital at UTMB, TX, USA. The IRB approval for discarded tissues was obtained prior to sample collection. Fetal membranes were dissected and the amnion layer was peeled from choriodecidua, washed in warm saline and small pieces (0.5 cm^2) were digested twice with trypsin (1 mg/mL) and collagenase (0.5 mg/mL) for 30 minutes at 37°C. The digestion buffer was inacti vated by DMEM complete media [(DMEM/F12 (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 15% FBS (Sigma-Aldrich) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin)] and the cells were collected by centrifugation. Cells were counted with a hemocytometer, and seeded in 10 cm culture plates with DMEM complete media, at 37°C in a humidified atmosphere containing 5% CO_2. The purity of the epithelial cells was greater than 95%, as determined by staining with cytokeratin antibodies (Pan-Cytokeratin, Abcam, Cambridge, MA, USA, #ab80826).

Virus Culture

ZIKV MEX_I_7/2015 (Accession number KX247632) was amplified once in Vero cells followed by three passages in C6/36 cells. Zika virus/A.africanus-tc/SEN/1984/41525-DAK (ZIKV DAK_41525) stocks were generated on Vero cells. Virus containing media was collected in RPMI supplemented with 5% FBS, P/S with 0.01M HEPES (pH 7.4). The virus containing material was cleared by centrifugation at 1000g for 20min at 4 °C and stored at −80°C.

Drug library screening

2×10^4 HuH-7 cells were seeded onto collagen (Sigma # C8919) coated ThermoNunc 96-well, black wall, optical bottom microwell plates (#165305) using DMEM (Gibco #11995-065) media supplemented with 10% FBS (OmegaSci #FB-12) and P/S (Gibco #15140-122). Cells were pretreated with experimental compound (Enzo #BML-2843-0100 SCREEN-WELL FDA-Approved Drug Library V2) or controls, diluted to a final concentration of 13.8 μM in DMEM, 5% FBS, P/S, 0.01M HEPES (Gibco #15630-080), and incubated 1hr at 37°C. Approximately 1 hour after addition of compounds, 55 μL of ZIKV MEX_1_7 diluted 1 in 125 using 5% FBS, P/S, 0.01M HEPES, DMEM was added to each well resulting in a 10 μM final concentration for each compound. The calculated virus of 8,718 FFU, or MOI 0.4, was added to each well. The infected cells were incubated 24–26 hours post infection. Infection was stopped by rinsing each well once with PBS with calcium and magnesium. (Stock # Corning # 21–030-CV) and fixing the cells with 4% paraformaldehyde (PFA). Cells were permeabilized using PBS with 0.1% TritonX100 and blocked using 1% Normal Goat Serum in PBS with 0.1% Tween20. Primary antibody, 4G2 (pan-flavivirus, anti-envelope antibody)(Henchal et al., 1982) was diluted in 1% normal goat serum in PBS with 0.1% Tween and incubated on cells overnight at 4°C. Secondary antibody, goat anti-mouse Alexa647, and the nuclear stain, Hoechst was applied the following day. Both antibody stocks and Hoechst were diluted 1:2000 in 1% Normal Goat Serum in PBS with 0.1% Tween20. One field per well was imaged using the 10× objective

on a PerkinElmer Opera Phenix High Content Screening System and the images were analyzed using the associated Harmony® Office Software.

Compound Validation Studies

Essentially the same protocol as in the primary screens was used with the exception that the compounds were serially diluted to achieve final concentrations of 10 μM, 1 μM, 0.1 μM, 0.01 μM, and 0.001 μM. For HeLa and JEG3 infections, twice as much virus (MOI of 0.8) was used compared to HuH-7 infections. HuH-7, HeLa and JEG3 validation experiments were performed twice with at least three replicates per condition. hNSC experiments were performed at least once with at least three replicates per condition. For hNSC experiments, cells were infected at an MOI of 3 for 48 or 96 hours with ZIKV DAK_41525 or ZIKV MEX_I_7, respectively. Cells were then harvested, fixed in 1X PBS buffer containing 4% PFA and incubated with the primary antibody anti-envelope 4G2 diluted 1:1000 in permeabilizing / blocking solution (1XPBS, 0.1% saponin and 1% bovine serum albumin) overnight. After two washes with the permeabilizing / blocking solution, cells were incubated for 1 hour with goat anti-mouse Alexa 488 and analyzed in a Guava® easyCyte flow cytometer. Collected data were analyzed with the FlowJo v.10 software. The criteria for determining whether cells were analyzed were based on light scattering features for the negative controls.

Drug validation using HAECs

Two experiments (A & B, respectively) were performed using cells from two different donors. In both experiments, 1.5×10^4 HAEC cells were seeded in DMEM: F12, 5% FBS, P/S, EGF onto collagen coated microwell plates. All subsequent chemical or viral dilutions were performed using the same media. Compounds were serially diluted such that cells were pretreated for 1 hour at 37°C with experimental compounds or controls, diluted to a final concentration between 0.00288 and 28.8 μM or between 0.00138 and 13.8 μM for experiments A & B, respectively. Approximately 1 hour after addition of compounds, 55 μL of ZIKV MEX_1_7 diluted 1 in 62.5 was added to each well resulting in a final concentration between 0.0016 and 16 μM or between 0.001 and 10 μM for experiments A & B, respectively. The calculated virus of 1.8×10^4 FFU, or MOI 1.2, was added to each well for both experiments. The infected cells were incubated 24–26 hours post infection. Infection was stopped by rinsing each well once with PBS with calcium and magnesium and fixing the cells with 4% PFA. The cells were stained consistent with HuH-7 HeLa and JEG3 experiments. Nine fields per well were imaged using a 10× objective and analyzed as described for the other experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- 774 FDA-approved drugs screened for anti-Zika virus activity in a human hepatoma cell line
- Over 20 compounds showed anti-Zika virus activity
- Selected compounds validated in human neural stem cells and primary amnion cells

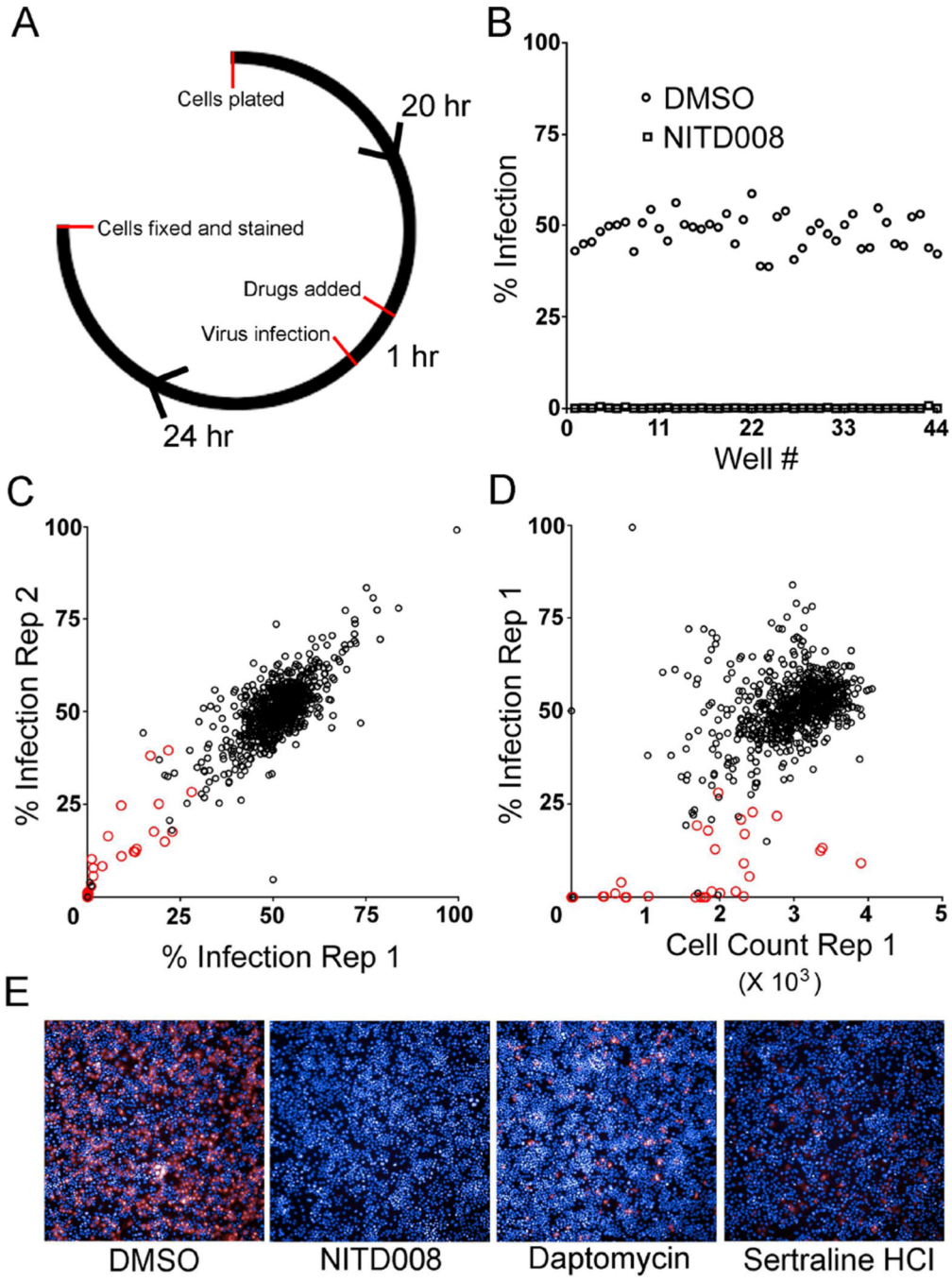

Figure 1. A screen for inhibitors of ZIKV infection among FDA approved drugs

(A) Screen timeline. HuH-7 cells were plated 20 hours prior to treatment with drugs for one hour. Cells were then infected with ZIKV MEX_I_7 (see Figure S1) for 24 hours before fixation and staining. Final concentration of drugs was 10 μM. (B) Screen controls. Rates of infection are shown for all positive (NITD008) and negative (DMSO) controls across screening plates. (C) Screen reproducibility. Points represent the % infection for each drug in each replicate of the screen. Points colored red were selected for validation studies. (D) Infection rate versus cell count. Cell count and corresponding % infection values for each

drug are plotted for replicate screen 1. (E) Representative images showing viral antigen (red) and nuclei (blue) for controls and selected drugs. Images were acquired using a 10X objective. Data for the screen are presented in Table S1.

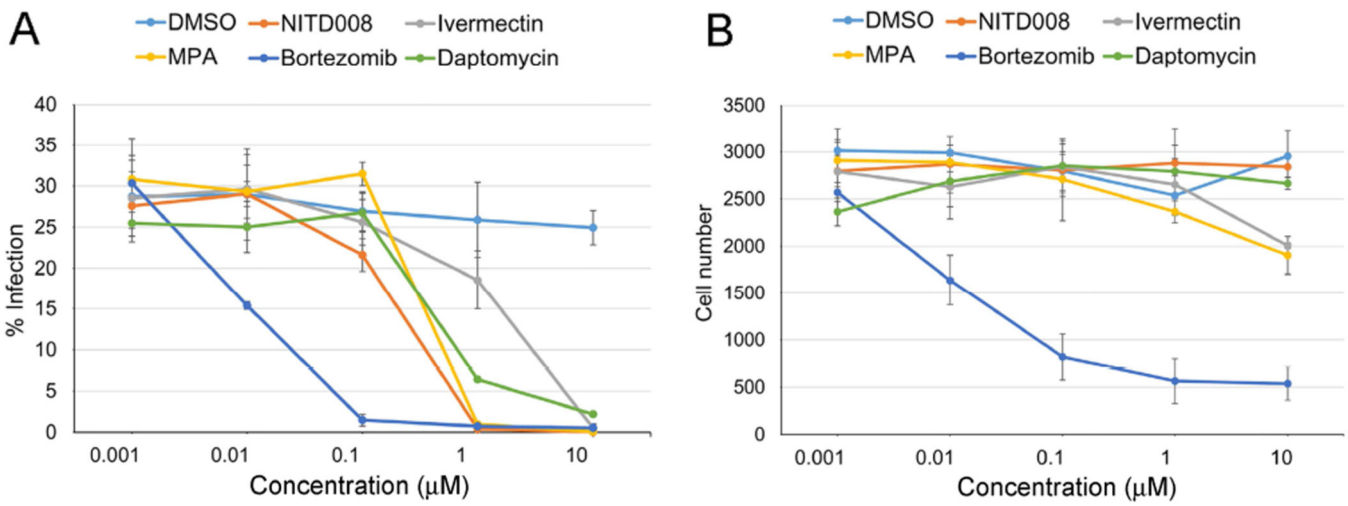

Figure 2. Validation of selected drugs in ZIKV-infected HuH-7 cells

(A) Infection rates as a function of drug concentration are shown. (B) Cell numbers for the indicated drug treatments are shown. Data for all 30 drugs tested in follow up experiments are in Figure S2. Data are represented as mean +/− standard deviation.

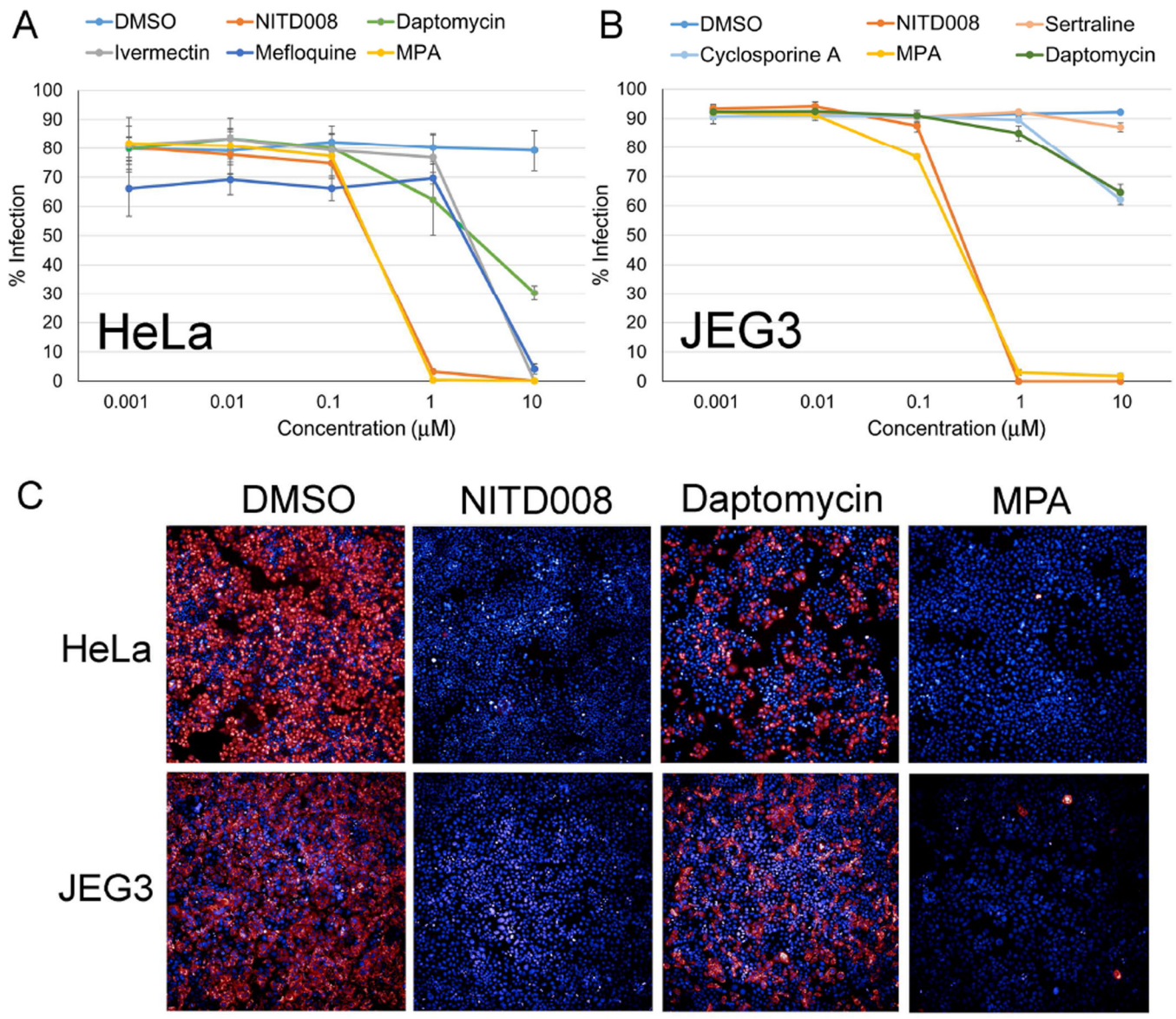

Figure 3. Evaluation of selected anti-viral drugs in ZIKV-infected human cell lines of cervical and trophoblast origin

(A) Infection rates as a function of drug concentration are shown for HeLa cells. (B) Infection rates as a function of drug concentration are shown for JEG3 cells. Data are represented as mean +/− standard deviation. (C) Representative immunofluorescence images showing virus antigen (red) and nuclei (blue) are shown for the 10 μM concentration for each drug indicated. Data for all 8 drugs tested in follow up experiments are in Figure S3.

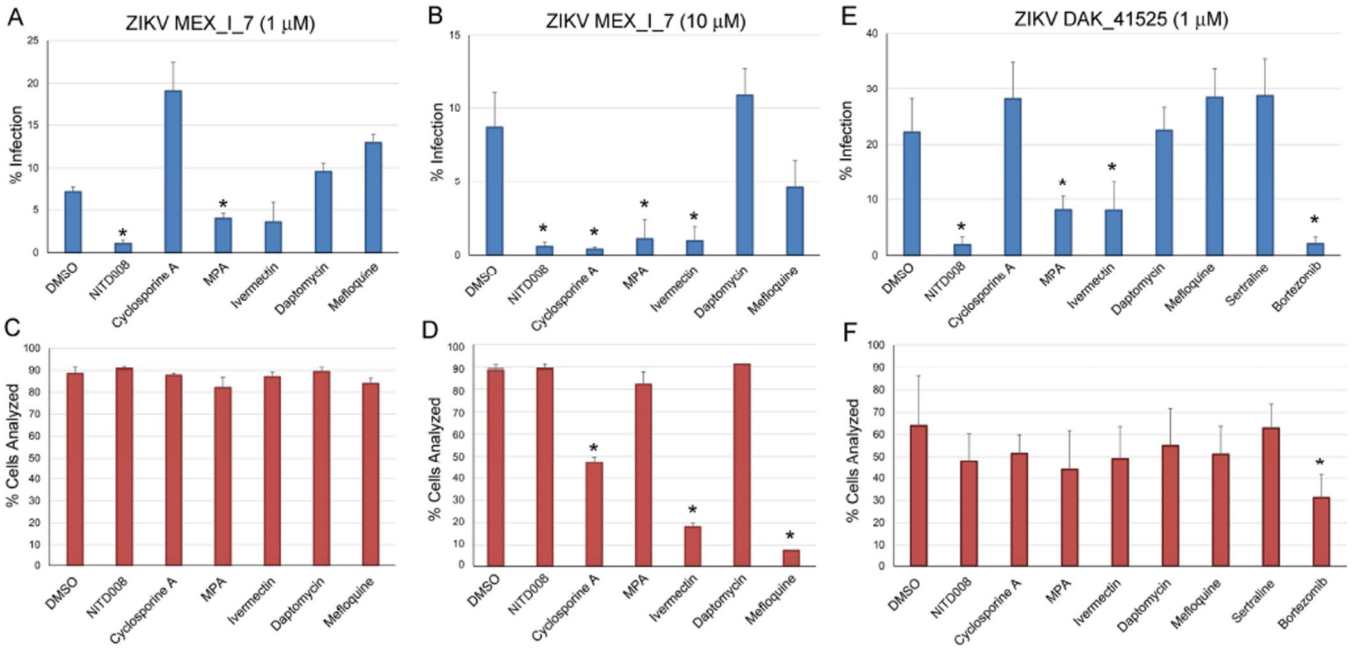

Figure 4. Effects of selected drug on infection of human neural stem cells (hNSC)

Rates of hNSC infection by ZIKV MEX_I_7 are shown for treatment conditions of 1 μM (A) and 10 μM (B). The % of cells that were analyzed for virus infection is shown for 1 μM (C) and 10 μM (D) conditions for ZIKV MEX_I_7. (E) Infection by ZIKV DAK_41525 under conditions of 1 μM drug treatment. Data are represented as mean +/− standard deviation (F) The % of cells that were analyzed for ZIKV DAK_41525. See Figure S4 for light scattering and fluorescence intensity data associated with the ZIKV MEX_I_7 infection. Asterisks indicate statistically significant reductions of virus infection or cells analyzed at a P value of <0.05 as calculated by unpaired t-test.

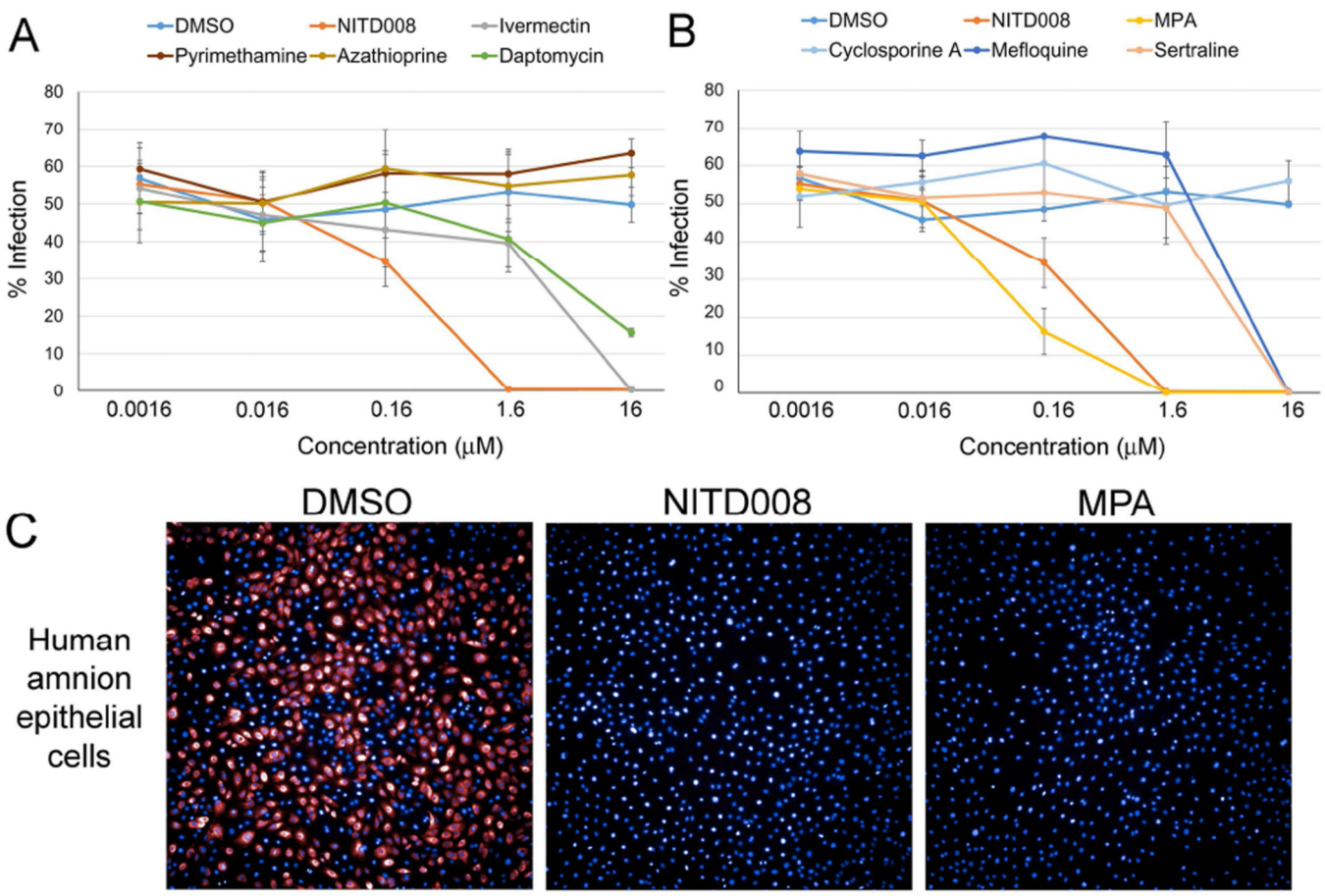

Figure 5. Preclinical evaluation of select therapeutics repurposed to target ZIKV infection of human amnion epithelial cells (HAECs)

Rates of HAEC infection by ZIKV MEX_I_7 are shown for the indicated drugs at concentrations between 0.0016 and 16 μM. Vehicle (DMSO) and positive control (NITD008) from the same experiment are shown in panels (A) and (B). Infection rates for ivermectin, pyrimethamine, azathioprine, and daptomycin are shown in (A), while MPA, cyclosporine A, mefloquine, and sertraline are shown in (B). Data are represented as mean +/− standard deviation Representative micrographs for vehicle (DMSO), positive control (NITD008) and MPA are shown in (C). Virus antigen (red) and nuclei (blue) are shown for the 16 μM concentration for each drug indicated. Effects on cell number for aforementioned conditions is shown in Figure S5.

Table 1

Candidate anti-ZIKV drugs and considerations for use in pregnancy.

Drug name	Pregnancy category[i]	Other considerations and notes
Auranofin	C	• Inform women of childbearing potential of the potential risk of therapy during pregnancy.
Azathioprine	D	•Contraindicated for use in pregnant women with Rheumatoid Arthritis
Bortezomib	D	• Women of reproductive potential should avoid becoming pregnant while on therapy.
Clofazimine	C	• Some animal studies have failed to reveal evidence of teratogenicity, but studies done at high doses have demonstrated fetotoxicity. There are no controlled data in human pregnancy.
Cyclosporine A	C	• Advise of the potential risks if used during pregnancy
Dactinomycin	D	
Daptomycin	B	
Deferasirox	C	
Digoxin	C	• Concentrations with anti-ZIKV activity may be toxic.
Fingolimod	C	• A pregnancy registry has been established to collect information about the effect of this drug during pregnancy.
Gemcitabine·HCl	D	
Ivermectin	C	
Mebendazole	C	• Inform of potential risk to fetus if taken during pregnancy, especially during first trimester.
Mefloquine·HCl	B	
Mercaptopurine Hydrate	D	
Methoxsalen	C	• Usually given in combination with ultraviolet (UV) radiation therapy.
Micafungin	C	
Mycophenolate Mofetil	D	• Boxed warning: Use during pregnancy is associated w/ increased risks of 1st trimester pregnancy loss and congenital malformations; counsel females of reproductive potential regarding pregnancy prevention and planning.
Mycophenolic Acid	D	• Boxed warning: Use during pregnancy is associated w/ increased risks of pregnancy loss and congenital malformations; counsel females of reproductive potential regarding pregnancy prevention and planning.
Palonosetron·HCl	B	• Drug interaction with SSRIs (Sertraline) causing serotonin syndrome
Pyrimethamine	C	• Women of reproductive potential should avoid becoming pregnant while on therapy.
Sertraline·HCl	C	• Boxed warning: Antidepressants increased the risk of suicidal thinking and behavior (suicidality) in children, adolescents, and young adults in short-term studies of major depressive disorder (MDD) and other psychiatric disorders. • Consider tapering dose during third trimester of pregnancy.
Sorafenib Tosylate	D	• Inform that the drug may cause birth defects or fetal loss during pregnancy; instruct both males and females to use effective birth control during treatment and for at least 2 weeks after stopping therapy. Instruct to notify physician if patient becomes pregnant while on therapy.
Thioguanine	D	• May cause fetal harm.

[i] FDA Pregnancy Categories are defined as follows (modified from www.drugs.com):

Category A: Adequate and well-controlled studies have failed to demonstrate a risk to the fetus in the first trimester of pregnancy (and there is no evidence of risk in later trimesters).

Category B: Animal reproduction studies have failed to demonstrate a risk to the fetus and there are no adequate and well-controlled studies in pregnant women.

Category C: Animal reproduction studies have shown an adverse effect on the fetus and there are no adequate and well-controlled studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks.
Category D: There is positive evidence of human fetal risk based on adverse reaction data from investigational or marketing experience or studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks.

It should be noted from that effective 30 June 2015 the FDA published the Content and Format of Labeling for Human Prescription Drug and Biological Products; Requirements for Pregnancy and Lactation Labeling, "*Pregnancy and Lactation Labeling Rule" (PLLR or final rule). The PLLR removes pregnancy letter categories from drug labels, however, these, categories remain useful as general guidelines for clinicians and investigators*.

Table 2

Summary of drug mechanisms and anti-ZIKV activity*

Drugs	Known mechanism	HuH-7	HeLa	JEG3	hNSC	HAEC	References
Auranofin	Inhibits thioredoxin reductase	Yes¶	N.D.	N.D.	N.D.	N.D.	(Madeira et al., 2012)
Azathioprine	Inhibits purine synthesis	Yes	Yes	No	N.D.	No	(Lennard, 1992)
Bortezomib	Proteasome inhibitor	Yes	N.D.	N.D.	Yes	N.D.	(Adams et al., 1999)
Clofazimine	Unknown (anti-microbial)	Yes	N.D.	N.D.	N.D.	N.D.	
Cyclosporine A	Cyclophilin inhibitor	Yes	No	No	Yes	No	(Qing et al., 2009)
Dactinomycin	Transcription inhibitor	Yes	N.D.	N.D.	N.D.	N.D.	(Sobell, 1985)
Daptomycin	Unknown (anti-microbial)	Yes	Yes	No	No	Yes	
Deferasirox	Chelator of intracellular iron	Yes	N.D.	N.D.	N.D.	N.D.	(Torti and Torti, 2013)
Digoxin	Na^+K^+ ATPase inhibitor; impacts calcium signaling	Yes	N.D.	N.D.	N.D.	N.D.	(Prassas and Diamandis, 2008)
Fingolimod	Sphingosine-1-phophate receptor modulator	Yes	N.D.	N.D.	N.D.	N.D.	(Spiegel and Milstien, 2011)
Gemcitabine HCl	Nucleoside analogue; blocks DNA replication	Yes	N.D.	N.D.	N.D.	N.D.	(Hertel et al., 1990)
Ivermectin	Unknown (anti parasitic); interferes with flaviviruses via inhibiting viral protein function	Yes	Yes	Yes	Yes	Yes	(Mastrangelo et al., 2012; Wagstaff et al., 2012)
Mebendazole	Unknown (anti helminthic)	Yes	N.D.	N.D.	N.D.	N.D.	
Mefloquine HCl	(Anti-parasitic) Disrupts autophagy, possibly disrupts lysosomal pH	Yes	Yes	Yes	No	Yes	(Geng et al., 2010; Poole and Ohkuma, 1981)
Mercaptopurine Hydrate	Inhibits purine synthesis	Yes	N.D.	N.D.	N.D.	N.D.	(Lennard, 1992)
Methoxsalen	DNA synthesis inhibitor	Yes	N.D.	N.D.	N.D.	N.D.	(Parrish et al., 1974)
Micafungin	Unknown (anti-fungal)	Yes	N.D.	N.D.	N.D.	N.D.	
Mycophenolate Mofetil	Prodrug of mycophenolic acid	Yes	N.D.	N.D.	N.D.	N.D.	

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Mycophenolic Acid	Inosine-5′-monophosphate dehydrogenase inhibitor	Yes	Yes	Yes	Yes	Yes	(Diamond et al., 2002)
Palonosetron HCl	5-hydroxytryptamine-3 receptor antagonist	Yes	N.D.	N.D.	N.D.	N.D.	(Navari, 2015)
Pyrimethamine	Dihydrofolate reductase antagonist	Yes	Yes	No	N.D.	No	(Schweitzer et al., 1990)
Sertraline HCl	Selective serotonin re-uptake inhibitor	Yes	No	No	No	Yes	(Koe et al., 1983)
Sorafenib Tosylate	Multi-target tyrosine kinase inhibitor	Yes	N.D.	N.D.	N.D.	N.D.	(Wilhelm et al., 2006)
Thioguanine	Inhibits purine synthesis	Yes	N.D.	N.D.	N.D.	N.D.	(Lennard, 1992)

*Validated at P = <0.05 with >50% inhibition of infection rate = Yes, Not validated = No. Not done = N.D.

¶Borderline effect.