Identification of Estrogen Receptor Modulators as Inhibitors of Flavivirus Infection

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ABSTRACT Flaviviruses such as Zika virus (ZIKV), dengue virus (DENV), and West Nile virus (WNV) are major global pathogens for which safe and effective antiviral therapies are not currently available. To identify antiviral small molecules with well-characterized safety and bioavailability profiles, we screened a library of 2,907 approved drugs and pharmacologically active compounds for inhibitors of ZIKV infection using a high-throughput cell-based immunofluorescence assay. Interestingly, estrogen receptor modulators raloxifene hydrochloride and quineestrol were among 15 compounds that significantly inhibited ZIKV infection in repeat screens. Subsequent validation studies revealed that these drugs effectively inhibit ZIKV, DENV, and WNV (Kunjin strain) infection at low micromolar concentrations with minimal cytotoxicity in Huh-7.5 hepatoma cells and HTR-8 placental trophoblast cells. Since these cells lack detectable expression of estrogen receptors- α and - β (ER- α and ER- β) and similar antiviral effects were observed in the context of subgenomic DENV and ZIKV replicons, these compounds appear to inhibit viral RNA replication in a manner that is independent of their known effects on estrogen receptor signaling. Taken together, quineestrol, raloxifene hydrochloride, and structurally related analogues warrant further investigation as potential therapeutics for treatment of flavivirus infections.

KEYWORDS flavivirus, dengue virus, Zika virus, West Nile virus, high-throughput screening, antiviral, estrogen receptor, antiviral agents

Mosquito-borne flaviviruses such as Zika virus (ZIKV), dengue virus (DENV), and West Nile virus (WNV) are responsible for significant morbidity and mortality worldwide. For example, DENV, which is classified into four antigenically distinct serotypes (DENV-1 to DENV-4), is estimated to cause ~100 million symptomatic infections and ~25,000 deaths each year (1). In contrast, closely related ZIKV was thought to only cause mild febrile illness until recent large outbreaks in 2007 in the Pacific islands, in 2013 in French Polynesia, and in 2014 to 2016 in South America revealed the unexpected association of ZIKV with serious neurodevelopmental disorders in infants and Guillain-Barré syndrome in infected adults (2). Interestingly, while ZIKV is predominantly transmitted via mosquitoes, recent studies involving clinical samples and animal models have shown that it can also spread vertically from the pregnant mother to developing fetus and via sexual contact (3). In the developing fetus, ZIKV targets neural progenitor cells and limits their growth and induces apoptosis, providing a likely mechanism for ZIKV-induced congenital microcephaly and other neurodevelopmental

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disorders that are now collectively referred to as Zika congenital syndrome (ZCS) (2, 4–8).

The recent explosive outbreak of ZIKV and continual seasonal outbreaks of DENV and other related flaviviruses highlight the need for safe and effective preventative vaccines and therapeutic treatments to combat the spread and impact of these flaviviruses. While a vaccine for DENV is now available, due to its moderate efficacy and safety concerns related to sensitization of unexposed individuals to more severe disease, there remains an urgent need for improved vaccines that are ideally effective against multiple DENV serotypes and related flaviviruses, but do not exacerbate viral infection and disease severity due to antibody-dependent enhancement (ADE) of infection (9). Furthermore, although antiviral therapies would be of great value in reducing the impact and global health burden of flavivirus infections, no such antivirals are currently available.

Given the costly and time-consuming nature of development of novel antiviral drugs, drug repurposing has emerged as a popular approach to accelerate the identification of safe and effective antiviral therapeutics (10). This approach often involves screening of libraries of approved and well-characterized drugs for compounds that inhibit viral infection or associated pathogenesis, either directly via unanticipated inhibition of viral factors or processes or indirectly through perturbation of host factors or pathways that are required for the viral replication cycle. Given that the safety, bioavailability, half-life, and, in most instances, biological targets of these drugs have already been well-characterized, any effective antiviral drugs identified via drug-repurposing screens could potentially be rapidly repositioned as antiviral therapeutics. In the context of flavivirus infection, several recent screening studies have reported the identification of promising antiviral compounds among libraries of approved and biologically active compounds, including known inhibitors of flaviviral infection, such as mycophenolic acid (MPA), and unexpected inhibitors of viral replication, such as the antihelminthic drug niclosamide (11–14).

Here, we screened a library of 2,907 approved drugs and pharmacologically active compounds for inhibitors of ZIKV infection in Huh-7.5 hepatoma cells. These screens revealed 15 inhibitors of ZIKV infection, including previously identified antivirals such as the nucleoside analogue thioguanine and novel antivirals such as the estrogen receptor (ER) modulators raloxifene hydrochloride and quinestrol. Validation studies revealed that these ER modulators similarly inhibit ZIKV, DENV-2, and WNV (Kunjin strain) infection at low micromolar concentrations with minimal cytotoxicity. Mechanistically, these antiviral effects appeared not to involve the major estrogen receptors, ER- α and ER- β , and could predominantly be attributable to inhibition of viral RNA translation and/or replication. Taken together, our study identifies ER modulators as inhibitors of flaviviral infections and supports their further exploration and improvement of their antiviral activity by medicinal chemistry as strategies to develop effective therapeutics to treat flavivirus infections.

RESULTS

Identification of candidate antiviral drugs via a high-throughput screen of approved drugs and pharmacologically active compounds. To identify novel inhibitors of ZIKV infection, we developed a high-throughput cell-based assay of viral infection using an immunofluorescence readout in a 96-well plate format. Specifically, compounds from a library comprised of 2,907 approved drugs and pharmacologically active compounds and vehicle (DMSO) controls were dispensed into 96-well black-walled imaging plates prior to plating of Huh-7.5 hepatoma cells to achieve a compound concentration of 10 μ M. Huh-7.5 cells were chosen for the screen as their gene expression profile and growth properties have been characterized in numerous studies and they are susceptible to infection with a range of flaviviruses, including ZIKV (15, 16). Following plating, cells were then cultured for 24 h prior to infection with ZIKV (strain PRVABC59; multiplicity of infection [MOI] $\sim$ 3.6) and then cultured for a further 24 h prior to fixation, immunofluorescent labeling using an antibody against the viral

envelope protein (4G2), and automated fluorescence microscopy and image analysis to quantify the percentage of infected cells (Fig. 1A and B). The extended period (24 h) of compound exposure prior to infection was chosen to enable identification of additional compounds that may take time to accumulate or alter cellular gene expression profiles, metabolism, or machinery to create an environment that is unfavorable to viral infection. The relatively short window of infection (24 h) and high multiplicity of infection were chosen to focus on inhibitors of early events in viral infection, such as entry, translation, establishment of viral replication complexes, and viral RNA replication. For a negative control, wells that were treated with DMSO vehicle alone (0.2% final concentration), $45.01\% \pm 4.40\%$ and $51.66\% \pm 3.93\%$ of cells were infected in the respective repeat screens. In this context, DMSO-treated controls were associated with an average cell count of $5,386.10 \pm 457.58$ cells in the imaged area of each well in the first replicate screen (Fig. 1B, Table S1). As a positive control for inhibition of infection, cells were treated with the polyether ionophore antibiotic nanchangmycin at a final concentration of $1 \mu\text{M}$, given a recent report that ZIKV infection is potentially inhibited by this drug (12). However, under the conditions of our screen this treatment resulted in unacceptable levels of cellular cytotoxicity and a $>50\%$ reduction in cell counts compared to vehicle controls (0.2% DMSO). Accordingly, robust Z' analysis was applied for each plate and robust Z-scores for individual treatments were determined, which uses the median and median absolute deviation (MAD) for calculations of screen robustness and drug effects (17). The average robust Z' factors for each plate in the two replicate screens were 0.87 ± 0.05 and 0.89 ± 0.05 , respectively, indicating excellent robustness of the screens and a good power to accurately identify compounds with significant effects. With regard to reproducibility of the screens, similar infection rates were observed for each compound in the replicate screens, resulting in an R^2 correlation coefficient of 0.72 (Fig. 1C). Similarly, analysis of robust Z-scores for each compound across the replicate screens revealed an R^2 correlation coefficient of 0.66 (Fig. S1), indicating acceptable reproducibility of the screens.

For our screens, hits were defined as having a robust Z-score of ≤ -2.0 , an infection rate of $\leq 50\%$, relative to that of the mean of DMSO controls, and a cell count of $\geq 50\%$ of that of the mean of DMSO controls, to exclude compounds that overtly affected cell viability. According to these criteria, the repeat screens identified 53 and 56 hits, respectively, with 15 inhibitors that were common to both screens (Table 1). Among these common hits were compounds that have previously been identified as inhibitors of flavivirus infection, such as thioguanine, a purine synthesis inhibitor, and imatinib, a tyrosine kinase inhibitor (11, 18). Additional hits included the antimalarials dihydroartemisinin and artesunate, the antihistamine azelastine HCl, and the estrogen receptor modulators quineestrol and raloxifene HCl (Table 1, Fig. 1B and C). Quineestrol and raloxifene hydrochloride (henceforth referred to as raloxifene) were chosen for further analysis given that little is known regarding the impact of these compounds on flavivirus infections and our demonstration that estrogen receptor modulators were highly represented among the hits identified in the individual repeat screens, with additional related compounds that met the hit criteria in one of the repeat screens, including estradiol, ethinyl estradiol, and estriol (Table S1).

Dose-dependent inhibition of ZIKV, DENV-2, and WNV (Kunjin) infection by quineestrol and raloxifene. We next sought to validate the antiviral activity of quineestrol and raloxifene against ZIKV and determine whether these compounds were similarly efficacious against related flaviviruses DENV-2 and WNV (Kunjin). For this we plated Huh-7.5 cells into black-walled imaging plates containing the inhibitors to achieve a range of concentrations from 0.1 to $20 \mu\text{M}$. Twenty-four hours later, cells were infected with ZIKV, DENV-2, or WNV (Kunjin) at a multiplicity of infection (MOI) of ~ 3.6 and fixed 24 h later, to mirror the conditions of the original screens. Immunofluorescent labeling of the viral E protein and automated imaging and analysis were then performed, prior to quantification of infection levels, relative to DMSO-treated controls (Fig. 2). This revealed comparable low micromolar antiviral efficacy of quineestrol against ZIKV, DENV-2, and WNV (Kunjin), with approximate 50% inhibitory concentration (IC_{50})

FIG 1 Identification of inhibitors of ZIKV infection among a library of FDA-approved and pharmacologically active compounds using an immunofluorescence-based assay. (A) Drug screening timeline and workflow. Huh-7.5 cells were plated into 96-well (Continued on next page)

TABLE 1 Inhibitors of ZIKV infection in Huh-7.5 cells identified via high-throughput screening

Drug name	Robust Z-score (rep 1)	Robust Z-score (rep 2)	% infected (rep 1)	% infected (rep 2)
Quinestrol	−8.960543891	−10.40147721	3.54	19.83
Raloxifene	−5.594885708	−6.664829682	13.02	16.98
Citrinin	−6.458823564	−8.277304205	22.06	19.13
Chlorhexidine dihydrochloride	−9.529448426	−13.20775169	8.09	11.57
Tioguanine	−12.91379595	−11.24947726	4.83	11.57
Chlorindanol	−5.29865036	−6.151139657	16.77	18.86
Erythritol	−6.462380225	−7.869080309	20.95	23.09
Imatinib base	−4.07701258	−5.876354389	20.94	24.46
Enrofloxacin	−4.10359615	−4.224948397	5.42	18.89
Deoxysappanone B trimethyl ether	−4.028366822	−3.388602238	5.82	23.99
Azelastine hydrochloride	−9.859143907	−8.477809972	17.89	23.69
Arteminol	−7.181930945	−3.669083741	12.12	15.32
Dequalinium chloride	−7.542871498	−10.03461574	7.62	6.02
Deacetylgedunin	−6.798053157	−7.180822605	11.24	18.48
Artesunate	−4.975058417	−5.45911868	20.1	26

values of 11.4 μM , 15.3 μM , and 10.1 μM , respectively. Similarly, raloxifene displayed similar but slightly more potent antiviral efficacy against ZIKV, DENV-2, and WNV (Kunjin), with approximate IC_{50} values of 7.4 μM , 9.3 μM , and 7.7 μM , respectively.

Given that infection of placental trophoblasts appears to be an important aspect of the ability of ZIKV to infect the placenta and spread to the developing fetus in ZIKV-infected pregnant mothers (19, 20), we also investigated the antiviral activity of quinestrol and raloxifene in HTR8/SVneo cells derived from human first-trimester trophoblasts. This revealed similar low micromolar antiviral activity of quinestrol against ZIKV, DENV-2, and WNV (Kunjin) (Fig. S2A). Consistent with the results seen in Huh-7.5 cells, the antiviral activity of raloxifene was slightly stronger than that of quinestrol, with approximate IC_{50} values of 4.5 μM for each of ZIKV, DENV-2, and WNV (Kunjin) (Fig. S2B).

While the above immunofluorescence-based experiments demonstrated dose-dependent antiviral effects of quinestrol and raloxifene toward these flaviviruses, it was not clear whether the observed effects could be attributed to changes in viral entry, RNA replication, and/or infectious virus production. To explore these possibilities further, Huh-7.5 cells were infected with DENV-2 (MOI ~ 0.05), washed extensively, and returned to culture for 48 h in the presence of a range of concentrations of quinestrol and raloxifene prior to quantitation of intracellular DENV-2 RNA and virus infectivity in cell culture supernatants (Fig. 3). This revealed dose-dependent reductions in viral RNA levels in response to both quinestrol and raloxifene treatment (Fig. 3A). These effects were closely mirrored by reductions in the production of infectious virus (Fig. 3B). Together, these results imply that quinestrol and raloxifene inhibit early events in DENV-2 infection and/or viral RNA replication but do not noticeably exert additional inhibitory effects on infectious virus particle production.

Next, we sought to determine whether the antiviral effects of quinestrol and raloxifene were attributable to cytotoxic and/or antiproliferative effects. For this, Huh-7.5 cells and HTR8/SVneo cells were plated as above into 96-well plates containing a range of inhibitor concentrations. Cells were then cultured for 48 h prior to analysis of cell viability/proliferation using a commercial resazurin-based cell viability assay (Fig. 4). This revealed that cell viability/proliferation was only appreciably inhibited at the

FIG 1 Legend (Continued)

imaging plates containing compounds to achieve a final drug concentration of 10 μM . Cells were then cultured for 24 h and infected with ZIKV (PRVABC59, MOI ~ 3.6). Cells were then cultured for a further 24 h, fixed, labeled by anti-E indirect immunofluorescence (green), counterstained with DAPI (blue and red in upper and lower micrographs, respectively), and analyzed by automated fluorescence microscopy. (B) Graphical representation of ZIKV infection rates and cell numbers for each compound in the first screen replicate. (C) Screen reproducibility. Infection rates for each of the replicate screens were plotted against one another and the R^2 correlation was determined. For panels B and C, red spheres represent compounds that met the hit selection criteria (see Materials and Methods), while the data points for raloxifene HCl and quinestrol are also indicated (boxes and lines).

FIG 2 Dose-response analysis of the antiviral effects of quinnestrol and raloxifene. Huh-7.5 cells were treated with quinnestrol or raloxifene at a range of concentrations, as indicated, for 24 h prior to infection with ZIKV (A), DENV-2 (B), or WNV-KUNV (C) at an MOI of ~ 3.6 . Cells were then cultured for 24 h and processed for immunofluorescence and automated imaging analysis to determine infection rates, relative to DMSO-treated (carrier) controls. For quinnestrol, IC_{50} values were 11.4 μM , 15.3 μM , and 10.1 μM against ZIKV, DENV-2, and WNV-KUNV, respectively. For raloxifene, IC_{50} values were 7.4 μM , 9.3 μM , and 7.7 μM against ZIKV, DENV-2, and WNV-KUNV, respectively. Data are means $\pm$ standard deviations (SD) ($n = 3$ or 4), representative of at least two similar repeat experiments. Fitted curves represent best fits for IC_{50} calculations.

highest drug concentrations (40 to 50 μM) in both cell types, with trends indicating that HTR8/SVneo cells are most sensitive to high concentrations of quinnestrol. While we were not able to determine 50% cytotoxic concentration (CC_{50}) values from these data for all conditions (i.e., $CC_{50} > 50 \mu M$), the CC_{50} of quinnestrol in HTR8/SVneo cells was 36.3 μM , while the CC_{50} of raloxifene in Huh-7.5 cells was 38.7 μM . Similar effects were observed when an alternative ATP quantitation-based commercial cell viability assay was employed (Fig. S3), with quinnestrol displaying CC_{50} values of 31.5 μM and 49.7 μM in HTR8/SVneo and Huh-7.5 cells, respectively. Taken together these results indicate that the estrogen receptor modulators quinnestrol and raloxifene inhibit ZIKV, DENV-2, and WNV (Kunjin) infection at low micromolar concentrations in both Huh-7.5 and HTR8/SVneo cell lines in the absence of overt cellular cytotoxicity.

The antiviral activities of quinnestrol and raloxifene are independent of cellular estrogen receptor expression. Both quinnestrol and raloxifene target estrogen receptors. Quinnestrol is the 3-cyclopentyl ether of ethinyl estradiol. Following ingestion and

FIG 3 Dose-response analysis of the antiviral effects of quinnestrol and raloxifene on DENV-2 RNA and infectious virus production. Huh-7.5 cells were infected with DENV-2 (MOI ~0.05) for 2 h, washed twice with PBS, and returned to culture for 48 h in the presence of quinnestrol (left panels) or raloxifene (right panels) at the indicated concentrations, after which samples were collected for analysis. (A) Intracellular DENV-2 RNA levels by qRT-PCR (normalized to the housekeeping gene RPLPO and expressed as a percentage of DMSO-control values). (B) Infectious virus levels in cell culture supernatants (determined by focus-forming assays). Data are means $\pm$ SD ($n = 3$).

gastrointestinal absorption, it accumulates in adipose tissue and is slowly released, contributing to its long half-life. It is then readily metabolized to ethinyl estriol, a synthetic derivative of the natural estrogen estradiol that acts as an estrogen receptor (ER) agonist. It is commonly used in hormone replacement therapy and treatment of the symptoms of menopause and, less commonly, in the treatment of breast and prostate cancers. In contrast, raloxifene is a selective estrogen receptor modulator (SERM) that acts as an ER agonist in the cardiovascular system, bone, and liver, but acts as an ER antagonist in breast tissue and the endometrium. It is used in the prevention and treatment of osteoporosis and to reduce the risk of breast cancer development in at-risk individuals. Accordingly, we queried whether the antiviral activities of quinnestrol and raloxifene could be attributed to their actions upon ER- α and/or ER- β signaling and we used Western blotting to assess ER- α and ER- β expression in Huh-7.5 and HTR8/SVneo cell lines. This revealed that ER- α protein was not detectable in either Huh-7.5 or HTR8/SVneo cell lines, whereas an ~60 kDa band corresponding to ER- α was readily detected in lysates prepared from the ER- α -positive MCF-7 breast cancer cell line (21) (Fig. 5A). Given controversy surrounding the specificities of various commercially available antibodies that are reported to target ER- β and related conflicting reports surrounding expression of ER- β in various cell lines (22, 23), we assessed ER- β expression in Huh-7.5 cells and HTR8/SVneo cells using a validated ER- β -specific monoclonal antibody (22), and as a positive control we used a stable LNCaP cell line that heterologously expresses ER- β in response to doxycycline (23). As was seen for ER- α , we found that ER- β protein was not detectable in either Huh-7.5 or HTR8/SVneo cell lines, despite ready detection of ER- β in the doxycycline-induced positive-control cell line (Fig. 5B). While it is possible that these cell types express very low levels of ER- α/β that are undetectable by Western blotting, our analysis suggests that quinnestrol- and raloxifene-mediated inhibition of flavivirus infection is not dependent upon expression of ER- α or ER- β and instead involves an “off-target” mechanism of action.

FIG 4 Dose-response analysis of the impact of quinnestrol and raloxifene on cell viability. Huh-7.5 cells (A) or HTR-8 cells (B) were treated with quinnestrol or raloxifene at the indicated concentrations (0.5 to 50 μ M) for 48 h prior to analysis of viability using a resazurin-based fluorescent cell viability assay. Data are means $\pm$ SD ($n = 4$), representative of similar repeat experiments.

Quinnestrol and raloxifene do not markedly alter viral protein localization. To investigate whether the antiviral effects of raloxifene coincided with changes in the subcellular localization and/or the appearance of viral replication organelles and putative sites of virus particle production, Huh-7.5 cells were simultaneously infected with DENV2-NS1-FLAG and treated with 7.5 μ M raloxifene or DMSO alone for 48 h prior to fixation, immunofluorescent labeling, and confocal fluorescence microscopy (Fig. 6). This DENV-2 derivative, which features a FLAG epitope insertion within the viral nonstructural protein 1 (NS1) (24), enabled immunofluorescent labeling of viral NS1 protein, double-stranded RNA (dsRNA), and either E or capsid proteins in the same samples. While raloxifene treatment was predictably associated with a marked reduction in the overall level of infection in treated cell populations (not shown), there was no appreciable impact of raloxifene on viral E, capsid, NS1, or dsRNA localization. Similarly, the strong colocalization of NS1 and dsRNA at putative viral replication sites and the infrequent colocalization of dsRNA with capsid and E protein at putative viral assembly sites were not significantly altered by raloxifene treatment. Consistent with this, we did not observe any appreciable effects of quinnestrol treatment on viral protein localization or colocalization under the same infection and treatment conditions (not shown). Taken together, the antiviral activity of these drugs against DENV-2 does not appear to be attributable to overt effects on viral protein localization in infected cells.

Quinnestrol and raloxifene inhibit viral RNA replication. Next, we performed time-of-addition experiments to determine whether the antiviral effects of raloxifene and quinnestrol were strongest when the drugs were added before, during, or after the infection period (Fig. 7A, D, and G). For these experiments a fluorescent reporter virus, DENV2-NS1-mScarlet (24), was used to enable simple live cell imaging by fluorescence microscopy and quantification of infection-associated fluorescence using a multimode plate reader. Interestingly, comparable inhibition of viral infection levels was observed regardless of whether drugs were applied to cells before (and after), during (and after),

FIG 5 Western blot analysis of ER- α and ER- β protein expression in Huh-7.5 and HTR-8/SVneo cells. Whole-cell lysates were prepared from the indicated cell lines and subjected to SDS-PAGE and Western blotting using antibodies against ER- α (A) and ER- β (B). Lysates from MCF-7 cells were used as a positive control for ER- α (A), while lysates from LNCaP cells heterologously expressing ER- β were used as a positive control for expression of ER- β (B). Actin (β -actin) served as a loading control.

or only after the infection period (Fig. 7B, C, E, F, H to I). Accordingly, it is possible that these drugs disrupt an aspect of the virus replication cycle that is common to all of the treatment conditions, such as viral RNA replication. This is consistent with our earlier demonstration that treatment of Huh-7.5 cells with these drugs immediately following infection with wild-type DENV-2 resulted in dose-dependent reductions in viral RNA levels and commensurate reductions in infectious virus production (Fig. 3).

To further explore whether raloxifene and quinestrol impact viral RNA replication and/or viral translation, we employed luciferase-encoding DENV-2 and ZIKV sub-

FIG 6 Raloxifene does not appreciably alter the localization or appearance of DENV-2 viral RNA replication or assembly sites. Huh-7.5 cells were simultaneously infected with DENV2-NS1-FLAG (MOI ~ 0.1) and treated with raloxifene ($7.5 \mu\text{M}$) or DMSO as a carrier control. At 48 h postinfection, cells were fixed and processed for indirect immunofluorescent labeling of combinations of (A) NS1 (green), dsRNA (red), and E (cyan) or (B) NS1 (green), dsRNA (red), and capsid (cyan). DAPI (gray in merged images) was used to stain nuclear DNA. Scale bars in merged images are $10 \mu\text{m}$ and $5 \mu\text{m}$ for main images and insets, respectively.

genomic replicons in transient viral replication assays. For this, Huh-7.5 cells stably expressing firefly luciferase were treated with drugs ($5 \mu\text{M}$) or DMSO carrier for 2 h prior to transfection with *in vitro*-transcribed RNA for DENV-2 or ZIKV subgenomic replicons and culture for 24 to 72 h in the presence of drug or DMSO, as indicated (Fig. 8A). To

FIG 7 Time-of-addition analysis of the antiviral effects of raloxifene and quinestrol against DENV-2. Huh-7.5 cells were treated with raloxifene (5 or 10 μ M), quinestrol (5 or 10 μ M), or DMSO (control) and infected with DENV2-NS1-mScarlet (MOI $\sim$ 0.01) for 3 h, according to the timelines depicted in panels A, D, and G. Cells were then labeled with Hoechst 33342 and imaged by live cell imaging, as depicted in micrographs shown in panels B, E, and H. Plates were then processed for quantification of NS1-mScarlet-associated fluorescence using a multimode plate reader. Graphs (C, F, and I) show infection-associated fluorescence levels, expressed as a percentage the DMSO-treated controls. Data are means $\pm$ SD ($n = 4$), representative of similar repeat experiments. Asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) indicate statistically significant differences compared to DMSO-treated controls, as determined by unpaired Student's t tests.

gauge levels of *Renilla* luciferase activity that were associated with transfected “input” RNA and enable assessment of the impact of these drugs on viral RNA translation, replication-defective “GND” or “GAA” replicons were also employed. Although there were no observable effects of drug treatments on cell appearance in these experiments, to account for any impact of drug treatment on cell viability and/or proliferation, viral replication-associated *Renilla* luciferase levels were normalized to cellular firefly luciferase levels. As shown in Fig. 8B, compared to controls, normalized DENV-2 RNA replication levels in raloxifene-treated cells were $\sim$ 10-fold lower across the first 48 h posttransfection and $\sim$ 6-fold lower at 72 h posttransfection. Strikingly, at 24 h posttransfection, when virally encoded *Renilla* luciferase activity is comparable for wild type and replication-defective GND replicons, raloxifene treatment was associated with a marked $\sim$ 16-fold reduction in GND-associated luciferase activity compared to DMSO-treated GND controls. This suggests a substantial impact of raloxifene on viral poly-protein translation. Consistent with this, raloxifene treatment resulted in a greater than 10-fold reduction in virally encoded luciferase levels for both replication-competent and replication-defective GAA ZIKV subgenomic replicons (Fig. 8C).

In contrast, quinestrol treatment was associated with a moderate $\sim$ 2 to 4-fold inhibition of DENV-2 subgenomic replicon-encoded luciferase activities and no appreciable changes in virally encoded luciferase activities for the replication-defective

FIG 8 Inhibition of DENV-2 and ZIKV viral RNA replication in response to raloxifene and quinnestrol treatment. (A) Timeline depicting treatment of Huh-7.5+FLuc cells with raloxifene (RALOX) or quinnestrol (QUIN) at 5 μ M for 2 h prior to and 24 to 72 h following transfection with *in vitro*-transcribed viral RNA for *Renilla* luciferase-encoding subgenomic replicons (SGR). For cells transfected with sgDV.R2A replicon RNA (B and D) or sgZV.R2A replicon RNA (C and E), samples were harvested at 3, 24, 48, and 72 h posttransfection, as indicated, and normalized luciferase activities (RLuc/FLuc) were determined and expressed as a percentage of average values for each group at 3-h time points. Data are means $\pm$ SD ($n = 4$), representative of similar repeat experiments.

DENV-2 subgenomic replicon (Fig. 8D), indicating that DENV-2 polyprotein translation is not inhibited by quinnestrol. While ZIKV subgenomic replicon-encoded luciferase activities were also moderately reduced by quinnestrol treatment, moderate quinnestrol-mediated reductions in luciferase activity were also observed for the replication-defective GAA mutant replicon (Fig. 8E). In this context, we also explored the possibility that quinnestrol impacts directly upon ZIKV NS5 RNA-dependent RNA polymerase (RdRp) activity by using a fluorescence-based assay of viral RNA replication and recombinant ZIKV NS5. This analysis revealed no significant impairment of RdRp activity at 10 or 100 μ M (Fig. S4).

To further examine the apparent impact of raloxifene on viral RNA translation, we next investigated replication-defective subgenomic replicon-encoded *Renilla* luciferase

FIG 9 Inhibition of viral RNA translation by raloxifene. (A) Timeline depicting cotransfection of Huh-7.5 cells with the indicated replication-defective subgenomic replicon RNA (sgDV.R2A [GND] or sgZV.R2A [GAA]) and FLuc mRNA and either immediate harvest of samples (4 h) or culture for 8, 16, or 24 h with medium containing DMSO (0.05% [vol/vol]), cycloheximide (CHX, 25 μ g/ml) or raloxifene (RALOX, 5 μ M) prior to sample collection and analysis by dual-luciferase assay. (B) Quantification of sgDV.R2A (GND)-encoded RLuc activity (left panel) and FLuc activity (right panel), expressed as a percentage of average 4-h values. (C) Quantification of sgZV.R2A (GAA)-encoded RLuc activity (left panel) and FLuc activity (right panel), expressed as a percentage of average 4-h values. Data are means $\pm$ SD ($n = 4$).

activity following short-term treatment with raloxifene or cycloheximide, a well-characterized inhibitor of eukaryotic translational elongation. To simultaneously examine the effects of these drugs on nonviral protein translation, the viral subgenomic replicon RNA was cotransfected with a 5'-capped firefly luciferase reporter mRNA. Following transfection, cells were treated with raloxifene (5 μ M) or cycloheximide (25 μ g/ml) prior to determination of *Renilla* luciferase (RLuc) and firefly luciferase (FLuc) activities at 8, 16, and 24 h (Fig. 9A). Unexpectedly, FLuc activity was comparably inhibited, up to approximately 13-fold, by both raloxifene and cycloheximide under these conditions (Fig. 9B and C, right panels). In contrast, virally encoded RLuc activity was markedly reduced by raloxifene treatment, up to approximately 75-fold, while cycloheximide treatment moderately inhibited virally encoded RLuc activity, up to approximately 5-fold (Fig. 9B and C, left panels). Taken together, these results indicate that raloxifene treatment strongly impairs DENV-2 and ZIKV RNA replication in a manner that may be attributable to inhibition of viral polyprotein translation and/or reduced stability of viral RNA. In contrast, quinestrol treatment only modestly inhibits DENV-2 and ZIKV RNA replication and/or translation, although the mechanism(s) involved remain unclear.

Next, we investigated whether hepatitis C virus (HCV) RNA replication is also sensitive to raloxifene and quinestrol, given its genetic relationship with flaviviruses as a member of the *Flaviviridae* family and previous reports that SERMs inhibit multiple

aspects of the HCV replication cycle (25–27). For this, dose-response experiments were performed for raloxifene and quinestrol using Huh-7.5 cells harboring a NanoLuc luciferase-tagged HCV subgenomic replicon. As shown, raloxifene and quinestrol treatments inhibited HCV RNA replication at low micromolar concentrations, with IC_{50} values of approximately 5.4 μ M and 7.4 μ M, respectively (Fig. S5A and B). For these experiments, the NS5A inhibitor velpatasvir was used as a positive control (Fig. S5C). As expected, this treatment resulted in potent inhibition of HCV RNA replication, with an IC_{50} of ~41 pM.

DISCUSSION

In this study, we developed and performed high-throughput screens to identify candidate antiviral drugs against ZIKV among a library of approved drugs and well-characterized pharmacologically active compounds. This led to the identification of 15 compounds that reproducibly inhibited ZIKV infection at 10 μ M in the absence of overt cytotoxicity. Several similar recent high-throughput screening studies have been performed using libraries of FDA-approved drugs to identify safe and effective anti-ZIKV therapies among drugs that have already been approved for treatment of unrelated conditions and can therefore be considered for expedited trials for treatment of ZIKV-infected individuals (11–14). While our screens identified several hits that were also identified in similar previous screens, including the purine analogue thioguanine (11) and the Bcr-Abl tyrosine kinase inhibitor imatinib (28), to our knowledge our screens are the first to identify estrogen receptor modulators as candidate antivirals for treatment of ZIKV infection.

Raloxifene is an archetypal SERM that was originally approved for use in the treatment and prevention of postmenopausal osteoporosis and, later, to reduce the risk of breast cancer development in at-risk individuals. It acts as an ER agonist in bone, cardiovascular tissue, and the liver, and as an ER antagonist in breast tissue and the endometrium. It is also classified as a cationic amphiphilic drug (CAD), i.e., a broad range of compounds that feature a hydrophobic aromatic ring or ring system and a side chain that is hydrophilic and contains an ionizable amine functional group (29). CADs include other SERMs, such as clomiphene and toremifene, and certain antimalarials, antidepressants, antipsychotic drugs, antiarrhythmic drugs, and cholesterol-lowering drugs (29). Interestingly, many CADs display antiviral activities against a range of viruses, including Ebola virus (EBOV), DENV, ZIKV, HCV, Middle East respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus (SARS-CoV). In many instances, these antiviral effects have been attributed to the pH-dependent accumulation of CADs in late endosomes/lysosomes (LE/Lys) and subsequent enlargement of these vesicles and disruption of their involvement in the replication cycles of these viruses (25, 26, 29–34).

While many of the studies of the antiviral activity of CADs have focused upon inhibition of viral fusion (31, 32, 35, 36), the strongest antiviral effects of raloxifene in our studies involved the use of ZIKV and DENV-2 subgenomic replicons. This suggests that impairment of virus entry/fusion is not the major antiviral mechanism of raloxifene against these viruses, and instead disruption of viral RNA replication/translation predominates. We hypothesize that the antiviral impact of raloxifene on flavivirus RNA replication/translation may be attributable to its alteration of endosomal cholesterol content, trafficking, and/or biogenesis, in a similar manner to U18666A, another CAD which inhibits lysosomal cholesterol export via targeting Niemann-Pick C1 (NPC1) (37) and inhibits DENV RNA replication and HCV RNA replication via perturbation of cholesterol recruitment to viral replication organelles (38, 39). While previous studies have shown that raloxifene and other SERMs disrupt cholesterol trafficking in macrophages and other cell types (29, 40), it will be important to investigate whether the antiviral effects of raloxifene against flaviviruses that we have observed are attributable to perturbation of LE/Lys cholesterol content and trafficking. Of note, while the manuscript was in preparation, Tohma et al. reported that the SERMs cyclofenil, clomiphene, and tamoxifen also inhibit DENV and ZIKV replication and, in particular,

infectious virus production (41). While our study indicates that raloxifene predominantly disrupts viral RNA replication/translation, consistent with our findings, Tohma et al. also found that the antiviral effects of SERMs appeared to be independent of ER signaling.

While both raloxifene and quinestrol target estrogen receptors, the antiviral activity of quinestrol that we observed also appears to be independent of its effects on ER signaling. While quinestrol is not classified as a SERM or a CAD, it is possible that its high lipophilicity and accumulation in lipid storage vesicles may contribute toward its antiviral activity via perturbation of host cell lipid trafficking, given the intimate relationship between flaviviruses and host cell lipids (42). Further studies are required to dissect the antiviral mechanisms of quinestrol and structurally related estrogen receptor agonists. Additionally, while we have observed similar antiviral effects of quinestrol and raloxifene against ZIKV, DENV-2, and WNV/KUNV isolates, further studies are required to determine whether there are flavivirus-, DENV serotype-, and/or viral isolate-dependent differences in the antiviral activity of these drugs and whether the antiviral effects against *Flaviviridae* family viruses observed in this study extend to other unrelated viruses.

Taken together, our high-throughput screens for inhibitors of ZIKV infection among approved and pharmacologically active compounds identified 15 compounds from a broad variety of drug classes as candidate antiviral drugs that warrant further investigation. While some of these compounds have been identified in previous antiviral screening efforts (e.g., thioguanine and imatinib) and others are not suitable for oral or systemic administration and absorption (e.g., dequalinium chloride), we propose that the antiviral activities and mechanisms of action of estrogen receptor modulators raloxifene and quinestrol deserve further investigation in regard to the urgent and unmet need for safe and effective antiviral therapeutics to treat flavivirus infections. In particular, given the growing appreciation that SERMs and other CADs have broad spectrum antiviral activity against a range of viral pathogens (29, 43), raloxifene and existing and novel structurally related analogues may warrant investigation as candidate antiviral agents against pathogenic flaviviruses. Although the low micromolar antiviral activity of raloxifene described here is several hundred times higher than reported plasma concentrations in postmenopausal women following a single orally ingested 60 mg dose (~1 to 3 nM), the relatively strong safety profile of raloxifene and structurally related SERMs support the further exploration of these drugs as future antiviral therapeutics. We suggest that medicinal chemistry efforts to develop raloxifene analogues and/or derivatives with improved potency and safety profiles may yield safe and effective antiviral therapeutics that can be employed in treatment of flavivirus infections.

MATERIALS AND METHODS

Cell culture. Huh-7.5 cells (44) and Vero cells were generously provided by Charles Rice (Rockefeller University, New York, USA) and Jillian M. Carr (Flinders University, Adelaide, Australia), respectively. Both of these cell lines were cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies cat. no. 12430) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS), as described previously (45). C6/36 cells were also generously provided by Jillian M. Carr (Flinders University) and were cultured as described previously (24, 46). Huh-7.5+FLuc cells, which stably express firefly luciferase, were maintained in complete DMEM containing blasticidin (5 µg/ml) and have been described previously (45). Huh-7.5+SGR/5A-NLuc (E7) cells, which stably harbor a NanoLuc-encoding subgenomic HCV replicon have been described previously (47), and were cultured in complete DMEM containing blasticidin (5 µg/ml). The anti-E hybridoma cell line D1-4G2-4-15 (4G2) was purchased from ATCC and cultured in Hybri-Care medium (ATCC) supplemented with sodium bicarbonate and 10% FBS, as per the manufacturer's recommendations. LNCaP cells expressing ER-β in a doxycycline-inducible manner (LNCaP+ERβ) were generously provided by Jean Winter and Wayne Tilley (University of Adelaide, Adelaide, Australia) on behalf of Jason Carroll (University of Cambridge, Cambridge, UK) and were cultured in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FBS. FBS and cell culture plastic ware were purchased from Sigma-Aldrich. All other cell culture media and additives were purchased from Thermo Fisher Scientific.

Viruses and plasmids. ZIKV (PRVABC59; Puerto Rico, 2015) was originally obtained from ATCC. WNV (Kunjin strain NSW2011) was generously provided by Karla J. Helbig (La Trobe University, Melbourne, Australia). Plasmid pFK-DVs, which contains a full-length DENV-2 genome (strain 16681), and subgenomic

Renilla luciferase reporter-encoding replicon constructs for DENV-2 (pFK-sgDVs-R2A and pFK-sgDVs-R2A-GND) and ZIKV (pFK-synZIKV-sgR2A-H/PF/2013 and pFK-synZIKV-sgR2A-H/PF/2013-GAA) were generously provided by Ralf Bartenschlager (University of Heidelberg, Heidelberg, Germany) (48, 49). Plasmids pFK-DVs-NS1-FLAG and pFK-DVs-NS1-mScarlet and the derivative viruses were described recently (24). To initiate virus replication for the above cloned viral genome constructs, plasmids were first linearized with XbaI (DENV-2 constructs) or XhoI (ZIKV constructs) before use as the templates in *in vitro* transcription reactions using an mMessage mMachine SP6 transcription kit or mMessage mMachine T7 transcription kit (Thermo Fisher Scientific) for DENV-2 or ZIKV constructs, respectively. Following DNase treatment to digest plasmid template DNA, *in vitro*-transcribed viral RNA was purified using TRIpure (Bioline) and transfected into Huh-7.5 cells using DMRIE-C Reagent (Thermo Fisher Scientific), as described previously (45). Infectious viruses were amplified in C6/36 cells, clarified by centrifugation at $500 \times g$ for 10 min at 4°C, aliquoted, and stored at -80°C. Virus infectivity was determined as described below.

Plaque assays and focus-forming assays of viral infectivity. Virus infectivity was measured by plaque assays using Vero cells, as follows. Cells were seeded in 12-well trays at 2×10^5 cells/well and cultured overnight prior to infection with 0.5 ml of 10-fold serial dilutions of virus in normal medium. Following incubation for 1 h, virus was removed and replaced with 1 ml of 1.5% high viscosity sodium carboxymethylcellulose (Sigma-Aldrich) dissolved in serum-free DMEM. Cells were then returned to culture for 5 days prior to addition of 1 ml of 10% buffered formalin and fixation at 4°C overnight. Fixed cell monolayers were then washed 3 times with water, stained with 1% crystal violet solution for 20 min at room temperature, and washed extensively with water prior to enumeration of plaques and determination of the infectivity of original samples, expressed as plaque-forming units per milliliter (PFU/ml). Focus-forming assays were performed using Huh-7.5 cells and anti-E immunofluorescent staining, as described recently (24).

Drug library screening and automated imaging. In our screen the Open Access Drugs library from Compounds Australia (Griffith University) was employed. This library is comprised of the MicroSource Spectrum FDA-approved drug library of 2,560 compounds and 347 additional pharmacologically active “in-house” compounds (2,907 compounds in total), dissolved in dimethyl sulfoxide (DMSO) to 5 mM. Compounds and controls (DMSO and Nanchangmycin [Selleck Chemicals] at 0.5 mM) were dispensed by Compounds Australia into 96-well black wall, clear bottom imaging plates (Greiner Bio-One CELLSTAR 96W microplates; cat. no. 655090) at 300 nL/well. Plates were then sealed and shipped at room temperature to the screening facility (CellScreen SA, Flinders University), where a BioTek EL406 Washer Dispenser was used to dispense 7,500 Huh-7.5 cells per well in 150 μ L of complete DMEM media (from a cell suspension of 5×10^4 cells/ml) to achieve a final drug concentration of 10 μ M. Cells were then returned to culture for 24 h prior to addition of 50 μ L of ZIKV PRVABC59 diluted in medium to achieve an MOI of ~ 3.6 . Following culture for a further 24 h, the medium was removed and cells were fixed with ice-cold methanol:acetone (1:1) at 4°C for 10 min. Following removal of the fixative and washing with phosphate-buffered saline (PBS), cell monolayers were blocked with 5% bovine serum albumin (BSA) in PBS (50 μ L/well) for 30 min at room temperature. The blocking solution was then removed and 40 μ L/well of anti-E hybridoma cell supernatant diluted 1:5 in PBS/1% BSA was added and incubated at 4°C overnight. Following a PBS wash step, cell monolayers were then incubated for ~ 2 h at 4°C with 40 μ L/well of Alexa Fluor-488-conjugated anti-mouse IgG (Life Technologies) diluted 1:200 in PBS/1% BSA. Cells were then washed, incubated with DAPI (4',6-diamidino-2-phenylindole dihydrochloride; 1 μ g/ml in PBS) (Sigma-Aldrich) for ~ 30 min at room temperature and washed again. Automated imaging was performed using an Operetta High Content Imaging and Analysis System (PerkinElmer). Briefly, for each well 4 separate fields were imaged for Alexa Fluor-488 and DAPI fluorescence using a 10 $\times$ objective. Image analysis was performed using Harmony and Columbus software (PerkinElmer), whereby infected cells were identified on the basis of Alexa Fluor-488 fluorescence intensity thresholds that were established using negative control (DMSO)- and compound-treated wells and cell segmentation that was performed on the basis of nuclear DAPI-associated fluorescence and cytoplasmic Alexa Fluor-488-associated fluorescence. As detailed in the Results, robust Z' analysis was performed for each plate and robust Z-scores for each compound treatment were calculated, based upon the median and median absolute deviation (MAD) for calculations of screen robustness and drug effects (17). Hits were defined as having a Z-score of ≤ -2.0 , a ZIKV infection rate of $\leq 50\%$ compared to DMSO-treated controls, and a cell count of $\geq 50\%$ of that of DMSO-treated controls.

Validation (dose-response) experiments were performed using the same experimental workflow, with the exception that cells were plated into 96-well imaging plates and allowed to adhere for 3 h prior to replacement of medium with medium containing compounds diluted to a range of different concentrations (0.1 μ M, 0.5 μ M, 1 μ M, 2.5 μ M, 5 μ M, 7.5 μ M, 10 μ M, 15 μ M, and 20 μ M) with DMSO at a fixed final concentration of 0.8% (vol/vol). Cells were infected 24 h later (MOI ~ 3.6), cultured for a further 24 h, and fixed and processed for immunofluorescence and analysis, as detailed above. IC₅₀ values for each compound were determined using variable slope (four parameters) least-squares fit analysis in Prism 8 (GraphPad Software).

Antibodies, chemicals, and compounds. Mouse anti-E monoclonal antibody (MAb) 4G2 was prepared from D1-4G2-4-15 hybridoma cells, cultured as described above. Anti-dsRNA mouse monoclonal antibody (MAb) 3G1 (IgM) hybridoma cell supernatant was generously provided by Roy Hall (University of Queensland, Brisbane, Australia). Mouse anti-capsid MAb 6F3.1 was generously provided by John Aaskov (Queensland University of Technology, Brisbane, Australia). Rabbit anti-FLAG MAb (D6W5B) was purchased from Cell Signaling Technology. Rabbit anti-ER- α MAb (60C) was purchased from Merck-Millipore. Mouse anti-ER- β MAb (PPZ0506) was purchased from R&D Systems. Mouse anti- β -actin

MAb (AC-74) was purchased from Sigma-Aldrich. Alexa Fluor 488-, 555-, and 647-conjugated secondary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Thermo Fisher Scientific.

Nanchangmycin and raloxifene HCl were purchased from Selleck Chemicals and dissolved in DMSO to 10 mM and 20 mM, respectively, aliquoted, and stored at -80°C . Quinestrol was purchased from Cayman Chemical, dissolved in DMSO to 20 mM, aliquoted, and stored at -80°C . Cycloheximide ready-made solution (100 mg/ml in DMSO) was purchased from Sigma-Aldrich and stored at 4°C . DAPI (4',6-diamidino-2-phenylindole dihydrochloride) and Hoechst (bisBenzimide H 33342 trihydrochloride) DNA dyes and doxycycline hydrochloride were purchased from Sigma-Aldrich, dissolved in sterile water to 1 mg/ml, and stored at 4°C .

Quantification of viral RNA by qRT-PCR. Total cellular RNA extraction and quantification of DENV-2 RNA via real-time quantitative PCR (qRT-PCR) was performed essentially as described (46). Briefly, following washing with PBS, RNA was extracted from near-confluent cells in 12-well trays using NucleoZOL (Macherey-Nagel), according to the manufacturer's instructions. DENV-2 RNA was quantified by qRT-PCR using Luna Universal one-step RT-qPCR kit (New England Biolabs) in 384-well plates, according to the manufacturer's instructions. Briefly, for each sample and primer pair, 10- μl reactions were prepared in technical duplicate, each containing 62.5 ng of isolated RNA (2.5 μl at 25 ng/ μl), 0.2 μl of each primer at 20 μM (0.4 μM final concentration; DENV-2 sense primer: 5'-ATC CTC CTA TGG TAC GCA CAA A-3'; DENV-2 antisense primer: 5'-CTC CAG TAT TAT TGA AGC TGC TAT CC-3'; RPLPO sense primer: 5'-AGA TGC AGC AGA TCC GCA T-3'; RPLPO antisense primer: 5'-GGA TGG CCT TGC GCA-3'), 1.6 μl nuclease-free water, and 0.5 μl Luna WarmStart RT enzyme mix. Reactions were performed using an Applied Biosystems QuantStudio 7 Flex real-time PCR system using the following program: 55°C for 10 min, 95°C for 1 min, and 40 cycles of the following: 95°C for 15 s, 60°C for 1 min. Melt curve analysis was performed using default settings of the instrument. DENV-2 RNA levels were expressed as a percentage of those of DMSO-treated controls, following normalization to RPLPO mRNA, using the threshold cycle ($\Delta\Delta\text{CT}$) method.

Confocal fluorescence microscopy and live cell imaging. For confocal fluorescence microscopy, Huh-7.5 cells were seeded into (no. 1.5H) μ -slide 8-well glass bottom chamber slides (Ibidi) that were precoated with 0.2% (wt/vol) gelatin at 10,000 cells/well and returned to culture overnight. Cells were then treated with medium containing raloxifene (7.5 μM), quinestrol (7.5 μM), or DMSO carrier (0.1% [vol/vol]), infected with DENV2-NS1-FLAG (MOI ~ 0.1) and returned to culture for 48 h. Cells were then fixed for 5 min at 4°C with ice-cold acetone:methanol (1:1), washed with PBS, and blocked with 5% BSA/PBS for 30 min. Cells were then incubated with primary antibody mixtures containing rabbit anti-FLAG (1 in 200) and either mouse anti-E hybridoma supernatant (1 in 5) or mouse anti-capsid hybridoma supernatant (1 in 5) diluted in PBS/1% BSA. Cells were then washed with PBS and incubated for 1 h at 4°C with Alexa Fluor 647-conjugated anti-mouse IgG (1 in 200) and Alexa Fluor 488-conjugated anti-rabbit IgG (1 in 200) diluted in 1% BSA/PBS. Following PBS wash steps, cells were then labeled by indirect immunofluorescence, as above, using anti-dsRNA hybridoma supernatant (1 in 5) followed by Alexa Fluor 555-conjugated anti-mouse IgM (1 in 200). Cells were then washed, labeled for 15 min with DAPI (1 $\mu\text{g}/\text{ml}$) diluted in PBS, and washed again with PBS prior to replacement with VECTASHIELD Antifade mounting medium (Vector Laboratories) and immediate imaging. Specificity of labeling was confirmed using mock-infected cells and infected cells that were labeled using irrelevant isotype control primary antibodies (BD Pharmingen) and omission of primary antibody as a control for anti-dsRNA labeling (not shown). Confocal imaging was performed using an Olympus FLUOVIEW FV3000 confocal microscope system using a $60\times$ NA 1.42 oil immersion objective and images were processed using NIS Elements AR v.3.22 (Nikon) and Photoshop 6.0 (Adobe) software.

For live cell imaging and plate reader-based quantitation of cellular fluorescence in live cells, Huh-7.5 cells were seeded into black-wall 96-well imaging plates in phenol red-free medium at 7,500 cells/well and cultured overnight prior to drug treatment and/or infection with DENV2-NS1-mScarlet, as appropriate. At the completion of the time course, cells were labeled with Hoechst 33342 (1 $\mu\text{g}/\text{ml}$) in phenol red-free medium for 30 min at 37°C . Cells were then washed once with normal medium and an optical adhesive film was applied to the plate before quantitation of mScarlet-associated fluorescence in each well using a PHERAstar FS multimode microplate reader (BMG Labtech) equipped with a 540/590 nm fluorescence intensity module using "well scanning" mode.

Cell viability assays. Cell viability assays were performed using PrestoBlue Cell Viability reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. Viability-associated fluorescence was quantified using a PHERAstar FS multimode microplate reader (BMG Labtech) and the 540/590 nm fluorescence intensity module. Alternatively, cells were cultured in 96-well white polystyrene cell culture plates (Costar cat. no. 3917), treated with drugs as appropriate, and cell viability was measured using a CellTiter-Glo Luminescent Cell Viability assay (Promega) as per the manufacturer's instructions using a GloMax-96 luminometer (Promega). Where possible, CC_{50} values for each compound were determined using variable slope (four parameters) least-squares fit analysis in Prism 8 (GraphPad Software).

Quantitative RdRp assays. To generate recombinant ZIKV RdRp, a pET24+ plasmid (NEB) containing the ZIKV RdRp coding sequence minus the N-terminal methyltransferase domain (nt 8,466 to 10,375 of strain MR766 [GenBank accession no. [LC002520](#)]) was first transformed into T7 Express competent *Escherichia coli* cells (NEB). ZIKV RdRp was overexpressed in *E. coli* by the addition of 0.5 mM isopropyl- β -thiogalactopyranoside, and overnight incubation (16°C , 200 rpm). RdRp purification was performed by chemically lysing *E. coli* pellets and then enriching the hexahistidine-tagged RdRp using Ni-NTA resin on a BioScale Mini Profinity IMAC Cartridge (Bio-Rad). Quantitative *in vitro* fluorescence-based assays of *de*

novo RdRp activity were performed using PicoGreen (Thermo Fisher Scientific) to detect formation of dsRNA from a poly(U) RNA template, as described previously (50, 51).

Immunoblotting and luciferase assays. Immunoblotting was performed essentially as described (52). Briefly, cell monolayers were washed with PBS and lysed in ice-cold NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris [pH 8.0]) containing protease inhibitor cocktail (Sigma-Aldrich). Samples were then homogenized using a 25-gauge needle/syringe and clarified by centrifugation ($10,000 \times g$, 5 min at 4°C). For each sample, ~50 µg of protein was separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane (GE Healthcare). Following blocking for 1 h at room temperature in TBS-Tween 20 (0.1%; TBS-T) containing 5% skim milk, membranes were incubated overnight in anti-ERα (1 in 500), anti-ERβ (1 in 500), or anti-β-actin (1 in 10,000) antibodies diluted in TBS-T containing 1% skim milk. Membranes were then washed in TBS-T (3 times for 10 min each) and incubated for 1 h at room temperature in HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibodies diluted in TBS-T containing 1% skim milk. Following extensive washing with TBS-T, membranes were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and imaged using a ChemiDoc MP imaging system (Bio-Rad).

Transient viral replication assays using subgenomic DENV-2 and ZIKV replicons were performed as follows. Huh-7.5+FLuc cells were treated with drugs or DMSO as indicated for 2 h prior to transfection with *in vitro*-transcribed RLuc-encoding replicon RNA using DMRIE-C (Thermo Fisher Scientific), according to the manufacturer's instructions. Following incubation for 3 h, transfection complexes were removed and cells were either lysed in Passive Lysis Buffer (Promega) or returned to culture for 24 h, 48 h, or 72 h in medium containing drug or DMSO, as appropriate, prior to lysis. Dual luciferase assays (Promega) were then performed according to the manufacturer's instructions using a GloMax-96 luminometer. *Renilla* luciferase (RLuc) values were normalized to firefly luciferase (FLuc) values and expressed as a percentage of average 3 h "input" values for each group. Assays of viral translation were performed as described above, with the exception that Huh-7.5 cells were cotransfected with equal amounts of RLuc-encoding replication-defective subgenomic replicon RNA and 5'-capped FLuc mRNA, prepared using the FLuc Control Template provided in the HiScribe T7 High Yield RNA Synthesis kit (New England Biolabs). Following collection of samples at 4, 8, 16, and 24 h posttransfection, dual-luciferase assays were performed and individual RLuc and FLuc values were expressed as a percentage of corresponding average 4 h input values. For HCV replicon experiments, Huh-7.5+SGR/5A-NLuc (E7) cells harboring a stable NanoLuc-encoding subgenomic replicon were treated with drugs at a range of concentrations and cultured for 48 h prior to lysis in Passive Lysis Buffer (Promega) and determination of luciferase activity using a Nano-Glo Luciferase Assay System (Promega), as per the manufacturer's instructions.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 7.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 1.6 MB.

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