Published in final edited form as:

Antiviral Res. 2017 November ; 147: 124–130. doi:10.1016/j.antiviral.2017.10.014.

Antiviral activity of *N*-(4-hydroxyphenyl) retinamide (4-HPR) against Zika virus

Jared D. Pitts¹, Pi-Chun Li², Melissanne de Wispelaere¹, and Priscilla L. Yang^{1,*}

¹Department of Microbiology and Immunobiology, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA

²Department of Cancer Biology, Dana-Farber Cancer Institute, 360 Longwood Avenue, Boston, MA 02215, USA

Abstract

The rapid spread of Zika virus (ZIKV) in recent years has highlighted the severe diseases associated with ZIKV infection, such as Guillain-Barré syndrome in adults and microcephaly in newborns; yet no vaccines or antivirals currently exist to prevent or treat ZIKV infection. We and others have previously identified *N*-(4-hydroxyphenyl) retinamide (fenretinide or 4-HPR) as an antiviral compound that inhibits dengue virus 2 (DV2) and other flaviviruses by limiting the steady-state accumulation of viral RNA. Here we show that 4-HPR potently inhibits ZIKV in mammalian cell culture and significantly reduces both serum viremia and brain viral burden in a murine model of ZIKV infection. Consistent with previous observations with dengue virus, this antiviral activity is associated with a significant reduction in the steady-state abundance of viral genomic RNA. We show this reduction is due to a major decrease in the rate of viral RNA synthesis, though not via direct inhibition of the activity of the viral replicase. These results establish 4-HPR's mode of action against DV and ZIKV and, taken with previous clinical trials that established 4-HPR's safety and tolerability, illustrate the potential utility of 4-HPR as an agent for treatment of ZIKV infection.

Keywords

Zika virus; fenretinide; *N*-(4-hydroxyphenyl) retinamide; 4-HPR; dengue virus; antiviral

1. Introduction

Zika virus (ZIKV) and the closely related dengue virus (DV), West Nile Virus (WNV), Japanese encephalitis virus (JEV), and yellow fever virus (YFV) are mosquito-borne RNA viruses of the genus flavivirus in the *Flaviviridae* family. The *Flaviviridae* family contains

*To whom correspondence should be addressed: Priscilla_yang@hms.harvard.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Competing financial interests

The authors have no competing financial interests to declare.

several important human pathogens, but the medical importance of Zika virus was not fully appreciated until the recent epidemic in the Americas. It is estimated that 80% of individuals infected with ZIKV are asymptomatic, while most symptomatic infections result in a mild fever with associated arthralgia, rash, headache, and conjunctivitis. Recently, the rapid spread of ZIKV has revealed more serious diseases associated with infection, most notably Guillain-Barré syndrome in adults and congenital malformations (*e.g.*, microcephaly) in infants born to infected mothers. The severe disease sequelae of ZIKV infection highlight the urgent need for effective counter-measures; however, no antivirals or vaccines against ZIKV are currently approved. Unlike most flaviviruses, which are known to cause acute infections that are cleared by the host immune response, ZIKV's persistence in body fluids (*e.g.*, semen, saliva, serum, and breast milk) (Paz-Bailey et al., 2017; Sotelo et al., 2017) for weeks to months after the onset of symptoms and its long-term infection of tissues such as the testis (Govero et al., 2016; Ma et al., 2016) suggest a need and opportunity for pharmacological interventions that can help to eliminate the virus from immune-privileged sites, limit damage to tissues and organs where ZIKV persists, and/or limit non-classical modes of ZIKV transmission (*e.g.*, sexual or breastfeeding).

The synthetic retinoid, *N*-(4-hydroxyphenyl)-retinamide (4-HPR) has been extensively tested in humans for treatment or prevention of a variety of cancers (Moore et al., 2010; et al., 2011; Schneider et al., 2009), macular degeneration (Mata et al., 2013), and Stargardt disease (Marmor et al., 2008). Studies of 4-HPR in children with neuroblastoma (Villablanca et al., 2011) indicate that the compound can be dosed at high levels with minimal adverse reactions (*e.g.*, rash, nausea, elevated transaminase levels) that were reversible after cessation of drug treatment. Additionally, a long-term, five year phase III clinical trial investigating the use of 4-HPR as a chemopreventative agent against recurrent breast cancer showed a significant reduction in the recurrence of cancer in premenopausal women (Veronesi et al., 1999). These clinical trials speak to the safety and tolerability profiles of 4-HPR, even when administered over long periods of time.

Recently, the synthetic retinoid *N*-(4-hydroxyphenyl)-retinamide (4-HPR) has been shown to be an inhibitor of dengue virus and closely related flaviviruses (Carocci et al., 2015; Fraser et al., 2014) including most recently against Zika virus (Wang et al., 2017). Against dengue virus, 4-HPR's antiviral activity is associated with reduced steady-state accumulation of viral RNA. This may be due to effects on viral RNA synthesis and/or viral RNA stability but is not notably associated with a reduction in translation of viral RNA (Carocci et al., 2015). 4-HPR's polypharmacology has posed serious challenges in elucidation of the relevant antiviral target(s) and mechanism(s) of action. Although 4-HPR's anti-cancer activities are linked to the production of ROS and inhibition of ceramide synthesis, neither of these pharmacological activities is required for the antiviral effect (Carocci et al., 2015; Fraser et al., 2014). The inhibition of NS5 and importin α/β (Fraser et al., 2014) and the activation of the PERK arm of the unfolded protein response (Fraser et al., 2016) have both been recently proposed as potential mechanisms underlying 4-HPR's inhibition of DV; however, the exact relationship between these activities and flavivirus replication (*i.e.*, correlation versus causality) remains unclear.

In the present study, we show that 4-HPR inhibits ZIKV in mammalian cell culture and reduces both viremia and brain viral burden in a murine model. This antiviral activity is correlated with a significant decrease in the abundance of ZIKV RNA. We further show that the addition of 4-HPR to virus-infected cells leads to a rapid reduction in the rate of viral RNA synthesis, which is not due to direct inhibition of the viral replicase. Together with past studies demonstrating the safety and tolerability of 4-HPR, this work establishes a foundation for pursuit of 4-HPR as an antiviral against ZIKV.

2. Materials and Methods

2.1. Compounds

N-(4-hydroxyphenyl) retinamide (4-HPR, fenretinide) was purchased from A Chem Tek and LC labs. N-(4-methoxyphenyl) retinamide (4-MPR) was purchased from Sigma. 4-HPR and 4-MPR were suspended in DMSO, aliquoted into small 10 mM stock solutions, and stored at -20°C . Actinomycin D (Sigma) was diluted to 10 mg/mL in acetone and stored at -20°C .

2.2. Viruses and cells

All work using viral pathogens and recombinant DNA was done in accordance with protocols approved by the Harvard Medical School Committee on Microbiological Safety. Zika virus strains MR-766 (Genbank accession no. [KX377335](#)) and PF13-251013-18 (Genbank accession no. [KX369547](#)) and dengue virus type 2 strain New Guinea C (NGC) (Genbank accession no. [KM204118](#)) were propagated by infecting a monolayer of C6/36 cells supplemented with 2% fetal bovine serum (FBS) at an MOI of 0.1 and harvesting supernatants at ~72 hours post infection (Zika) or ~96 hours post infection (dengue). Vero, Huh7, and SK-N-SH cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS at 37°C and 5% CO_2 . BHK-21 cells were grown in Minimum Eagle medium (α -MEM) supplemented with 5% FBS at 37°C and 5% CO_2 . C6/36 cells were maintained in L-15 medium supplemented with 10% FBS at 27°C .

2.3. Virus infections and plaque forming assay (PFA)

Experiments for all *in vitro* and *in cellulo* studies of 4-HPR antiviral activity were conducted in triplicate unless otherwise indicated. To initiate infections for IC_{90} determination, 5×10^4 (Vero, Huh7, BHK) or 1×10^5 (SK-N-SH, C6/36) cells were seeded into each well of a 24-well plate and incubated at 37°C overnight. Virus was diluted into Earle's balanced salt solution (EBSS), and cells were infected at a multiplicity of infection (MOI) of 1 in 100 μL virus solution and incubated at 37°C with rocking every 15 minutes. Unabsorbed virus was washed away with 1 mL of 1XPBS, after which 1 mL of the appropriate medium containing 2% FBS and appropriate concentration of 4-HPR was added to the cells. Cells were incubated at 37°C and 5% CO_2 for 24 hours, and supernatant was harvested, briefly centrifuged, and stored at -80°C until analysis of viral yield by plaque formation assay (PFA). To establish concentrations at which robust antiviral activity occurs, we determined IC_{90} values, defined as the concentration of compound needed to reduce single-cycle viral yield by 1 $\log_{10}$ unit under these condition. IC_{90} values were determined for each cell type using non-linear regression analysis with GraphPad Prism software.

To analyze steady-state RNA levels during infection, cells were seeded as above, and virus was diluted in EBSS to allow infection at an MOI of 10 for 1 hour at 37°C with rocking every 15 minutes. Unabsorbed virus was washed away with two washes of 1XPBS, and then DMEM containing 2% FBS and DMSO or 4-HPR (final concentration 5 µM) was added. At various time points post-infection, culture supernatants were removed, cells were suspended in 350 µL RLT buffer (Qiagen RNeasy Kit), and RNA was purified according to the manufacturer's instructions. Viral RNA was quantified by RT-qPCR and compared to abundance of GAPDH mRNA.

For PFA, Vero cells were seeded at 7.5×10^4 cells per well of a 24-well plate and incubated at 37°C with 5% CO₂ for 16–20 hours. Infectious virus supernatants were diluted in a series of 10-fold dilutions into EBSS and 100 µL of each dilution was added to the cell monolayer and incubated at 37°C for 1 hour with gentle rocking every 15 minutes. Unabsorbed virus was removed with a 1 mL 1XPBS wash, and cells were overlaid with 1 mL of DMEM supplemented with 2% carboxymethylcellulose (CMC), 2 % FBS, and penicillin/streptomycin. Plates were incubated at 37°C with 5% CO₂ for 3 days, and the cells fixed, and stained with crystal violet.

2.4. Monitoring viral RNA synthesis with 4-thiouridine

Experiments for all *in vitro* and *in cellulo* studies of 4-HPR antiviral activity were conducted in triplicate unless otherwise indicated. This method was adapted from previously published protocols (Duffy and Simon, 2016; Dölken, 2013) which we optimized to monitor flavivirus RNA production. 2.4×10^6 Vero cells in DMEM with 10% FBS were seeded in a T75 flask and incubated for 12–16 hours at 37°C with 5% CO₂. Cells were infected with dengue strain NGC at an MOI of 10 in EBSS for 1 hour at 37°C with gentle rocking every 15 minutes after which time unabsorbed virus was removed, and DMEM containing 10% FBS was added to the flask. At 15 h post-infection, actinomycin D (final concentration 10 µg/mL) and 4-HPR (final concentration 10 µM) or DMSO were added, followed by 4-thiouridine (4sU) (final concentration 500 µM) at 16h post-infection. At four time points taken every 2h post-addition of 4sU, culture supernatants were removed, and cells were washed once with 1×PBS and then lysed by the addition of 4 mL Trizol reagent (Thermo Fisher). Cell lysates were spiked with 4sU-containing nanoluciferase RNA as a labelling and enrichment control and stored at –80°C until further processing. RNA was purified, biotinylated with MTSEA-biotin (Biotium), and 4sU-containing RNA was isolated by streptavidin beads (GE Healthcare) as described in papers by Duffy et al. (Duffy and Simon, 2016; Duffy et al., 2015). RNA from total, unbound (previously synthesized), and bound (newly synthesized) samples were then quantified by RT-qPCR.

2.5. RT-qPCR

Purified RNA was first quantified by UV absorbance at 260 nm, and 0.25 – 1 µg of total RNA was used as template for cDNA synthesis using the iScript cDNA synthesis kit (Bio-rad) following the manufacturer's instructions. cDNA was treated with RNaseH (NEB) for 20 minutes at 37°C and then mixed with iQ SYBR Green Supermix (Bio-Rad) using primers ZIKVPRFOR1 - 5'-GCAAACCTGGTTGGAATCAA-3' and ZIKVPRREV1- 5'-GAGCTTCCCAAAAGCCAAG -3' for Zika virus and DV2CapF-5'-

AATATGCTGAAACGCGAGAGA-3' and DV2CapR-5'-GGGATTGTTAGGAAACGAAGG-3' for dengue virus. Several different control RNAs were used for these studies, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for analysis from Vero cells, hypoxanthine guanine phosphoribosyl transferase (HPRT) for mouse studies, and NanoLuciferase (NanoLuc) for spike in controls in the 4-thiouridine (4sU) experiments. Control primers used were GAPDHF -5'-GAGTCAACGGATTTGGTCTGT-3' and GAPDHRev -5'-TTGATTTGGAGGGATCTCG-3', msHPRTFW -5'-CTGGTGAAAAGGACCTCTCG-3' and msHPRTREV -5'-TGAAGTACTCATTATAGTCAAGGGCA - 3', NanoLucF -5'-ATGGTCTTCACACTCGAAGA-3' and NanoLucRev -5'-TTACGCCAGAATGCGTTCGC-3'. Reactions were run using a 2-step protocol with a 5 minute activation step at 95°C followed by 40 alternating cycles of a 15 second denaturing step at 95°C and 30 second annealing and extension step at 60°C with fluorescence measurements taken at the end of each cycle with a CFX96 real-time PCR detection system (Bio-Rad).

2.6. CRC purification and *in vitro* ZIKV RNA replication

Crude replication complexes (CRC) of dengue and Zika viruses were purified from T150 flasks of Vero cells infected at MOI of 1 at 2 days post-infection. Infected cells were harvested by trypsinization and centrifuged at 800×*g* for 10 minutes, washed once with 1XPBS and again centrifuged at 800×*g* for 10 minutes. The cell pellet was resuspended in hypotonic buffer (10 mM Tris pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, and Roche protease inhibitor cocktail), and the cell suspension disrupted with a Parr nitrogen cell disruption bomb 4369 pressurized at 500 psi for 15 minutes on ice. The suspension was then centrifuged at 1200×*g* for 10 minutes, and the supernatant collected and again centrifuged at 10,000×*g* for 10 minutes. The supernatant was then collected and centrifuged in a Beckman Coulter ultracentrifuge using a SW60Ti rotor at 69,000×*g* for 75 minutes. The pellet was suspended in hypotonic buffer containing 15% glycerol then aliquoted and flash frozen in liquid nitrogen prior to storage at -80°C. Total protein concentration was determined by Bradford assay, and the presence of NS5 and NS3 proteins was verified by western blot.

To assess *in vitro* RNA replication by Zika CRCs, 20 ng of CRC was added to reaction buffer (20 mM Tris-HCL pH 7.5, 5 mM KCl, 10 mM MgCl₂, 0.5 mM MnCl₂, 5 mM DTT, 1.25 mM NTP, 40 µg/mL actinomycin D, 1U RNasin). For dengue CRC, the reaction buffer was the same except with 4 mM MnCl₂ and no MgCl₂. 4-HPR, 4-MPR, dUTP (Trilink), or DMSO were added to a total DMSO percentage of 1%. 40 µCi of P³²-UTP was then added and samples were incubated at 37°C for 90 minutes. The reaction was transferred to ice, 560 µL of AVL buffer (QiaAmp Viral RNA Mini kit) was added to each sample, and the RNA was purified according to the manufacturer's protocols. Purified RNA was added to 6× RNA sample buffer and boiled at 95°C for 5 minutes, then run on a 10% urea-TBE gel (Bio-Rad) at 100 V for ~12–16 hours. The gel was fixed and exposed to a phosphor screen for 24 hours before imaging with a Typhoon FLA 9500. Densities of bands were quantified using ImageJ (Schneider, C.A., 2012).

2.7. 4-HPR in ZIKV mouse models of infection

All animal protocols used for this study were reviewed and approved by the Institutional Animal Care and Use Committee of Harvard University. The AG129 murine model of ZIKV was conducted primarily to assess whether 4-HPR could inhibit virus replication *in vivo*. Thus our primary outcomes were to determine if reductions in viral burden in the blood as well as relevant organs (*i.e.*, brain) could be observed. These experiments were designed to obtain basic measures of 4-HPR's antiviral efficacy when delivered prophylactically to justify further testing of the compound (*e.g.* effects on pathogenesis, post-exposure treatment, etc.). ZIKV infection kinetics were initially monitored in 6–8 week old AG129 mice of both sexes. While the infection kinetics changed by varying the inoculum, no differences were noted between male and female mice. Mouse experiments with 4-HPR were conducted in 3 separate trials with aged-matched 6–8 week old male mice. Groups of 10 AG129 mice (deficient in interferon alpha/beta/gamma receptors) were infected with 1×10^3 PFU Zika virus strain PF13-251013-18 via the intraperitoneal (IP) route. The IP route was chosen for this study due to unavailability of the Lym-X-Sorb vehicle used for the oral delivery in previous reports (Carocci et al., 2015). IP formulations were based upon published studies (Vratilova et al., 2004) to achieve drug exposures in serum equivalent to levels observed via oral delivery. Treatments of vehicle alone or 4-HPR were dosed at 60 mg/kg and delivered by intraperitoneal injection every 12 hours from 24 hours pre-infection to 84 hours post-infection. The vehicle used for 4-HPR delivery consisted of 5% ethanol/5% cremophor-EL (Millipore)/90% saline. Mice were monitored daily for weight and morbidity for up to 7 days post-infection, at which point the mice were sacrificed and organs harvested, flash frozen on dry ice, and stored at -80°C until processed. Blood was collected on days 2, 3, and 4 post-infection by the retro-orbital route, and the serum separated and harvested after centrifugation in a microvette blood collection tube (Sarstedt). Serum viremia was assessed by viral plaque formation assay.

To determine the viral burden in organs, the frozen samples were directly homogenized in 1 mL of DMEM (unsupplemented) using a TissueLyser LT (Qiagen) following the manufacturer's instructions. After homogenization 100 μL of homogenate was added to 350 μL LBP buffer, and RNA extraction was performed using a Nucleospin RNA plus kit (Clontech) following the manufacturer's protocols. Viral RNA was quantified by RT-qPCR using the Ct method normalized to mouse HPRT RNA (hypoxanthine guanine phosphoribosyl transferase), a housekeeping RNA to standardize total cellular RNA.

3. Results

3.1. ZIKV infection is inhibited by 4-HPR *in cellulo*

In addition to possessing antiviral activity against all 4 serotypes of dengue virus (Fraser et al., 2014), 4-HPR has also been shown to inhibit several other flaviviruses, such as Kunjin (West Nile virus) and Modoc virus, as well as hepatitis C virus (Family *Flaviviridae*, genus *hepacivirus*) (Carocci et al., 2015). We assessed 4-HPR's activity against both African and Asian strains of ZIKV by infecting Vero cells in the presence of varying concentrations of 4-HPR and harvesting culture supernatants 24 hours later to quantify single-cycle viral yield. We observed significant inhibition of both MR-766 (African) and PF13-251013-18 (Asian),

as reflected by a greater than one-log decrease in infectious virus production with as little as 1 to 5 μM 4-HPR (Fig. 1A–B). 4-HPR appeared to be a more potent inhibitor of the PF13-251013-18 strain at lower concentrations; however, ZIKV replication was completely suppressed with no virus detected for either strain at concentrations of 20 μM . Due to its close relation to the virus currently circulating in the Americas, strain PF13-251013-18 was used in the remainder of this study.

We quantified 4-HPR's antiviral potency against ZIKV PF13-251013-18 in additional mammalian and insect cell lines, including the human neuroblastoma cell line SK-N-SH and *Aedes albopictus* cell line C6/36, by measuring IC_{90} values, defined as the concentration of 4-HPR required to reduce single-cycle viral yield by 1 $\log_{10}$ unit. While some inhibition of ZIKV by 4-HPR was observed in C6/36, we were unable to achieve 90% inhibition at concentrations that did not affect cell viability (Fig. 1C and data not shown). In contrast, 4-HPR inhibited ZIKV PF12-251013-18 in all of the mammalian cell lines tested with IC_{90} values near 1 μM , approximately twenty-fold lower than 4-HPR's CC_{50} (Fig. 1D–E, and data not shown). The consistency of 4-HPR's antiviral potency against ZIKV PF130251013-18 across mammalian cell lines is in agreement with our previous observation that 4-HPR's antiviral activity is likely mediated by a host factor conserved in its importance in the replication of diverse members of the *Flaviviridae* in mammalian cells.

3.2. 4-HPR reduces ZIKV viremia and viral burden *in vivo*

To investigate whether 4-HPR's anti-ZIKV activity observed *in cellulo* translates to an *in vivo* model, we utilized a murine model of ZIKV infection to assess 4-HPR's antiviral activity as reflected by serum viremia. We inoculated AG129 mice, which lack type I and II interferon receptors, with 1×10^3 plaque-forming units (PFU) of ZIKV strain PF13-251013-18 by intraperitoneal injection and established that viremia peaks at 3 days post-infection, wanes starting on day 4, and is undetectable by plaque formation assay by day 5 post-infection (Supp. Fig. A.1). To assess antiviral activity of 4-HPR in this model, we prophylactically dosed mice ($n = 10$) with either 4-HPR at 60 mg kg^{-1} or vehicle twice daily starting 24 hours prior to ZIKV infection (Fig. 2A). Continuing 4-HPR administration through 3.5 days post-infection, we observed a statistically significant reduction of approximately 1 $\log_{10}$ in ZIKV viremia for 4-HPR treated mice compared to vehicle controls at all time points tested (days 2, 3, 4 post-infection) (Fig. 2B). Since virus only becomes consistently detectable in brain tissue on days 4–5 post-infection (data not shown), we monitored the mice until day 7 post-infection, at which time we observed a nearly 50% reduction in viral RNA in the brain (Supp. Fig. A.2), demonstrating a significant reduction in viral burden in this tissue and indicating that 4-HPR may limit viral dissemination to and/or inhibit replication in this organ. Together, these observations establish 4-HPR as a potent inhibitor of ZIKV *in vivo*.

3.3. 4-HPR inhibits viral RNA synthesis *in cellulo*

Replication of the flavivirus (+)-RNA genome is a membrane-associated process catalyzed by a “replicase” complex that includes the viral NS5 and NS3 proteins which provide the viral RNA-dependent RNA polymerase (RdRp) and helicase activities, respectively. The other viral non-structural proteins are also essential for RNA replication but have

biochemical functions that are not as clearly defined. In addition, both biochemical studies and loss-of-function screens have implicated multiple host factors that co-localize with the membrane-bound replication complex and that appear to be required for viral RNA replication, although their functions and mechanisms of recruitment and retention in the replicase remain active areas of study (Heaton et al., 2010; Taguwa et al., 2015). We have previously shown that 4-HPR-mediated inhibition of dengue, West Nile, Modoc, and hepatitis C viruses is correlated with a reduction in the abundance of both viral genomic RNA and double-stranded RNA, which is an obligate intermediate in the replication of these viruses (Carocci et al., 2015). To confirm that 4-HPR's anti-ZIKV activity has a similar mode of action, we monitored its effect on ZIKV genomic RNA abundance and found that 4-HPR treatment prevents accumulation of ZIKV RNA (Fig. 3A–B).

To investigate whether 4-HPR acts by directly inhibiting the viral RdRp in the replication complex, we isolated crude replication complexes (CRCs) from ZIKV- and DV2-infected Vero cells (Costello et al., 2016; Lindenbach and Rice, 1999) and measured CRC-associated RdRp activity by monitoring incorporation of ^{32}P -UTP into viral RNA in the absence of NTPs (control) or in presence of dCTP (control), 4-HPR, or *N*-(4-methoxyphenyl) retinamide (4-MPR), a breakdown product of 4-HPR that lacks antiviral activity (Fraser et al., 2014). Whereas replicase-catalyzed incorporation of ^{32}P -UTP into viral RNA was significantly reduced by dUTP or the absence of NTPs (Fig. 3C), we observed no effect of 4-HPR or 4-MPR on the RdRp activity of ZIKV and DV2 CRCs (Fig. 3C), even at concentrations at which over 99% of viral replication is inhibited in cell culture (Fig. 1B, D, E).

These results provide strong evidence that 4-HPR's antiviral activity is not due to direct inhibition of the viral RdRp or to biochemical disruption of active CRC; however, these results could not exclude the possibility that the compound inhibits viral RNA synthesis in the infected host cell by an indirect mechanism. To test this hypothesis explicitly, we examined 4-HPR's effect on viral RNA synthesis. DV2 was used instead of ZIKV as the experimental model due to increased efficiency of metabolic labelling of RNA in pilot studies. Cells infected with DV2 were treated with 4-HPR or DMSO and labeled with the nucleoside analog 4-thiouridine (4sU) starting at 16 hours post-infection in the presence of actinomycin D to inhibit cellular transcription (Duffy et al., 2015; Dölken et al., 2008). 4sU-labeled RNA was then biotinylated and purified from total RNA by affinity chromatography (Fig. 4A). Quantification of labelled RNA at pulse times of two to eight hours (corresponding to 18–24 hours post-infection) showed that 4-HPR significantly reduces synthesis of new viral RNA (Fig. 4B–C), resulting in at least a four-fold reduction in newly synthesized viral RNA over the full 8 hour pulse period. Taken together, these results demonstrate that 4-HPR reduces steady-state RNA replication by inhibiting viral RNA synthesis and that this effect is most likely due to an effect on a host target important for replicase assembly, stability, or activity in the infected host cell since 4-HPR does not directly affect viral replicase activity.

4. Discussion

During the peak of the Zika pandemic in the Americas, hundreds to thousands of people were infected each week. Currently, there are no approved antivirals for treating individuals infected with dengue, Zika, or other pathogenic flaviviruses. The persistence of Zika virus for weeks or months after the loss of viremia (Musso et al., 2015) suggests an even greater need and also greater opportunity to utilize antivirals against this flavivirus. In the current study, we have demonstrated that the known drug 4-HPR is a potent inhibitor of Zika virus in multiple cell lines and that prophylactic delivery of the drug results in significant reductions in viremia and brain viral burden in AG129-infected mice. The antiviral activity of 4-HPR in this *in vivo* model suggests that 4-HPR could be a useful drug to protect travelers from Zika infection when given prior to potential risk of exposure. In addition, our observation that 4-HPR reduces viral RNA synthesis *in cellulo* even when inhibitor treatment is delayed until 16 hours post-infection (Fig. 4B–C) suggests that 4-HPR can inhibit viral RNA replication during an established infection and should prompt more extensive studies to determine 4-HPR's utility in reducing viral replication when administered post-exposure. Such studies would inform whether 4-HPR may be useful in treating individuals in pandemic regions who present with clinical symptoms of infection or in accelerating elimination of Zika virus from testis and other sites where viral RNA persists long after the elimination of viremia (Oliveira Souto et al., 2016; Paz-Bailey et al., 2017). This would be particularly impactful in both reducing transmission by infected individuals who have become asymptomatic yet continue to shed virus in semen, vaginal secretions, saliva, etc. (Duggal et al., 2017; D'Ortenzio et al., 2016; Musso et al., 2017, 2015) as well as possibly limiting the reproductive damage which results from persistent replication in reproductive tissues (*e.g.*, testis) (Govero et al., 2016; Ma et al., 2016).

4-HPR has been proposed as a candidate for drug repurposing due to its demonstrated activity against multiple flaviviruses and the hepatitis C virus HCV along with its well-established safety and tolerability. A better understanding of the target(s) and mechanism(s) underlying 4-HPR's antiviral activity is clearly important for establishing the conditions under which it may be effectively deployed as an antiviral agent. The effect of 4-HPR on dengue virus was first reported in an ALPHAscreen that identified it as an inhibitor of the interaction between dengue NS5 and importin α/β (Fraser et al., 2014). Since the potency of inhibition by ALPHAscreen correlated well with antiviral potency in cell culture, this suggested a potential mechanism for 4-HPR's antiviral activity. In a more recent study, Fraser and colleagues (Fraser et al., 2016) presented evidence that 4-HPR activates the PERK arm of the Unfolded Protein Response (UPR), leading to phosphorylation of eIF2 α and likely global shutdown of translation. Similarly, inhibition of the protein phosphatase 1 regulatory subunit GADD34 with guanabenz was shown to prevent the dephosphorylation of eIF2 α (Tsaytler et al., 2011) associated with reduced dengue virus replication, although it remains unclear that this effect of 4-HPR is required for (rather than associated with) antiviral activity. Notably, the observations that dengue virus translation is not inhibited by 4-HPR (Carocci et al., 2015) and that dengue virus infection alone can activate phosphorylation of eIF2 α (Peña and Harris, 2011; Umareddy et al., 2007) suggest that additional activities of 4-HPR are required for inhibition of these viruses.

The hypothesis that 4-HPR's antiviral activity is mediated by a host factor is supported by prior studies, including our work showing that 4-HPR inhibits multiple flaviviruses and hepatitis C virus (Carocci et al., 2015). Our observation that 4-HPR inhibits ZIKV across multiple mammalian cell lines while exhibiting significantly lower antiviral potency in the mosquito cell line C6/36 (Fig. 1C-E) further suggests that 4-HPR acts via a mammalian host target that may be only partially conserved in the insect host. Efforts directed towards elucidation of this target and of the molecular mechanism underlying 4-HPR's antiviral activity provide a unique opportunity to advance our understanding of how host factors regulate replication of the flavivirus genome. In addition, studies of 4-HPR's antiviral target and mechanism may be critical for developing 4-HPR as an antiviral agent since this knowledge would enable the development of small molecules selective for 4-HPR's antiviral target and lacking activity against 4-HPR's other known targets (*e.g.*, mTor, RBPs, RAR β). On-going and future work in these areas may be especially valuable due to 4-HPR's broad-spectrum activity against the flaviviruses and the hepacivirus HCV.

Our demonstration that 4-HPR significantly and rapidly reduces RNA synthesis (Fig. 4C) for the first time demonstrates the compound's direct effect on a specific biochemical process in the viral life cycle. Our data indicate that 4-HPR reduces RNA synthesis (Fig. 4C) without direct inhibition of the viral polymerase or chemical destabilization of membrane-associated replication complexes (Fig. 3C). Rather, 4-HPR appears to act via an indirect mechanism mediated by a host factor. While further studies are needed to examine potential mechanisms of action, we note 4-HPR's well-established safety and tolerability may enable its use as a broad-spectrum agent against other known and potentially emerging flaviviruses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Suján Shresta for supplying breeders for the AG129 mice, Dominique Burri for critical reading of the manuscript, Didier Musso and Nathalie Pardigon for Zika virus strain PF13-251013-18, Congressman Michael Capuano and his staff (MA) for assistance with import of Zika virus, and the Medicinal Chemistry Core of the Dana-Farber Cancer Institute for assistance with compound acquisition and curation. This study was funded by NIH grants R01AI095499 (Yang) and U19AI109740 (Whelan). J.D.P was partially supported by NIH 3T32AI007245-33S1 (Whelan).

References

- Carocci M, Hinshaw SM, Rodgers MA, Villareal VA, Burri DJ, Pilankatta R, Maharaj NP, Gack MU, Stavale EJ, Warfield KL, Yang PL. The bioactive lipid 4-hydroxyphenyl retinamide inhibits flavivirus replication. *Antimicrob. Agents Chemother.* 2015; 59:85–95. [PubMed: 25313218]
- Costello DA, Villareal VA, Yang PL. Desmosterol Increases Lipid Bilayer Fluidity during Hepatitis C Virus Infection. *ACS Infect Dis.* 2016; 2:852–862. [PubMed: 27933788]
- Duffy EE, Rutenberg-Schoenberg M, Stark CD, Kitchen RR, Gerstein MB, Simon MD. Tracking Distinct RNA Populations Using Efficient and Reversible Covalent Chemistry. *Mol. Cell.* 2015; 59:858–66. [PubMed: 26340425]
- Duffy EE, Simon MD. Enriching s(4) U-RNA Using Methane Thiosulfonate (MTS) Chemistry. *Curr Protoc Chem Biol.* 2016; 8:234–250. [PubMed: 27925666]

- Duggal NK, Ritter JM, Pestorius SE, Zaki SR, Davis BS, Chang G-JJ, Bowen RA, Brault AC. Frequent Zika Virus Sexual Transmission and Prolonged Viral RNA Shedding in an Immunodeficient Mouse Model. *Cell Rep.* 2017; 18:1751–1760. [PubMed: 28199846]
- Dölken L. High resolution gene expression profiling of RNA synthesis, processing, and decay by metabolic labeling of newly transcribed RNA using 4-thiouridine. *Methods Mol. Biol.* 2013; 1064:91–100. [PubMed: 23996251]
- Dölken L, Ruzsics Z, Rädle B, Friedel CC, Zimmer R, Mages J, Hoffmann R, Dickinson P, Forster T, Ghazal P, Koszinowski UH. High-resolution gene expression profiling for simultaneous kinetic parameter analysis of RNA synthesis and decay. *RNA.* 2008; 14:1959–72. [PubMed: 18658122]
- D’Ortenzio E, Matheron S, Yazdanpanah Y, Lamballerie X, de Hubert B, Piorkowski G, Maquart M, Descamps D, Damond F, Leparç-Goffart I. Evidence of Sexual Transmission of Zika Virus. *N. Engl. J. Med.* 2016; 374:2195–8. [PubMed: 27074370]
- Fraser JE, Wang C, Chan KW, Vasudevan SG, Jans DA. Novel dengue virus inhibitor 4-HPR activates ATF4 independent of protein kinase R-like Endoplasmic Reticulum Kinase and elevates levels of eIF2 α phosphorylation in virus infected cells. *Antiviral Res.* 2016; 130:1–6. [PubMed: 26965420]
- Fraser JE, Watanabe S, Wang C, Chan WKK, Maher B, Lopez-Denman A, Hick C, Wagstaff KM, Mackenzie JM, Sexton PM, Vasudevan SG, Jans DA. A nuclear transport inhibitor that modulates the unfolded protein response and provides in vivo protection against lethal dengue virus infection. *J. Infect. Dis.* 2014; 210:1780–91. [PubMed: 24903662]
- Govero J, Esakky P, Scheaffer SM, Fernandez E, Drury A, Platt DJ, Gorman MJ, Richner JM, Caine EA, Salazar V, Moley KH, Diamond MS. Zika virus infection damages the testes in mice. *Nature.* 2016; 540:438–442. [PubMed: 27798603]
- Heaton NS, Perera R, Berger KL, Khadka S, Lacount DJ, Kuhn RJ, Randall G. Dengue virus nonstructural protein 3 redistributes fatty acid synthase to sites of viral replication and increases cellular fatty acid synthesis. *Proc. Natl. Acad. Sci. U.S.A.* 2010; 107:17345–50. [PubMed: 20855599]
- Lindenbach BD, Rice CM. Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function. *J. Virol.* 1999; 73:4611–21. [PubMed: 10233920]
- Ma W, Li S, Ma S, Jia L, Zhang F, Zhang Y, Zhang J, Wong G, Zhang S, Lu X, Liu M, Yan J, Li W, Qin C, Han D, Qin C, Wang N, Li X, Gao GF. Zika Virus Causes Testis Damage and Leads to Male Infertility in Mice. *Cell.* 2016; 167:1511–1524.e10. [PubMed: 27884405]
- Marmor MF, Jain A, Moshfeghi D. Total rod ERG suppression with high dose compassionate Fenretinide usage. *Doc Ophthalmol.* 2008; 117:257–61. [PubMed: 18523815]
- Mata NL, Lichter JB, Vogel R, Han Y, Bui TV, Singerman LJ. Investigation of oral fenretinide for treatment of geographic atrophy in age-related macular degeneration. *Retina (Philadelphia, Pa.).* 2013; 33:498–507.
- Moore MM, Stockler M, Lim R, Mok TS, Millward M, Boyer MJ. A phase II study of fenretinide in patients with hormone refractory prostate cancer: a trial of the Cancer Therapeutics Research Group. *Cancer Chemother. Pharmacol.* 2010; 66:845–50. [PubMed: 20082080]
- Musso D, Richard V, Teissier A, Stone M, Lanteri MC, Latoni G, Alsina J, Reik R, Busch MP. Detection of Zika virus RNA in semen of asymptomatic blood donors. *Clin. Microbiol. Infect.* 2017
- Musso D, Roche C, Robin E, Nhan T, Teissier A, Cao-Lormeau V-MM. Potential sexual transmission of Zika virus. *Emerging Infect. Dis.* 2015; 21:359–61. [PubMed: 25625872]
- Oliveira Souto I, Alejo-Cancho I, Gascón Brustenga J, Peiró Mestres A, Muñoz Gutiérrez J, Martínez Yoldi MJ. Persistence of Zika virus in semen 93 days after the onset of symptoms. *Enferm. Infecc. Microbiol. Clin.* 2016
- Paz-Bailey G, Rosenberg ES, Doyle K, Munoz-Jordan J, Santiago GA, Klein L, Perez-Padilla J, Medina FA, Waterman SH, Gubern CG, Alvarado LI, Sharp TM. Persistence of Zika Virus in Body Fluids - Preliminary Report. *N. Engl. J. Med.* 2017
- Peña J, Harris E. Dengue virus modulates the unfolded protein response in a time-dependent manner. *J. Biol. Chem.* 2011; 286:14226–36. [PubMed: 21385877]
- Rao RD, Cobleigh MA, Gray R, Graham ML, Norton L, Martino S, Budd GT, Ingle JN, Wood WC. Phase III double-blind, placebo-controlled, prospective randomized trial of adjuvant tamoxifen vs.

tamoxifen and fenretinide in postmenopausal women with positive receptors (EB193): an intergroup trial coordinated by the Eastern Cooperative Oncology Group. *Med. Oncol.* 2011; 28(Suppl 1):S39–47. [PubMed: 20878269]

Schneider BJ, Worden FP, Gadgeel SM, Parchment RE, Hodges CM, Zwiebel J, Dunn RL, Wozniak AJ, Kraut MJ, Kalemkerian GP. Phase II trial of fenretinide (NSC 374551) in patients with recurrent small cell lung cancer. *Invest New Drugs.* 2009; 27:571–8. [PubMed: 19225720]

Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods.* 2012; 7:671–675.

Sotelo JRR, Sotelo AB, Sotelo FBJ, Doi AMM, Pinho JRRR, Oliveira R, de C, Bezerra AMPSM, Deutsch AD, Villas-Boas LS, Felix AC, Romano CM, Machado CM, Mendes-Correa MCJC, Santana RAFA, Menezes FG, Mangueira CLPL. Persistence of Zika Virus in Breast Milk after Infection in Late Stage of Pregnancy. *Emerging Infect. Dis.* 2017; 23:856–857.

Taguwa S, Maringer K, Li X, Bernal-Rubio D, Rauch JN, Gestwicki JE, Andino R, Fernandez-Sesma A, Frydman J. Defining Hsp70 Subnetworks in Dengue Virus Replication Reveals Key Vulnerability in Flavivirus Infection. *Cell.* 2015; 163:1108–23. [PubMed: 26582131]

Tsaytler P, Harding HP, Ron D, Bertolotti A. Selective inhibition of a regulatory subunit of protein phosphatase 1 restores proteostasis. *Science.* 2011; 332:91–4. [PubMed: 21385720]

Umareddy I, Pluquet O, Wang QY, Vasudevan SG, Chevet E, Gu F. Dengue virus serotype infection specifies the activation of the unfolded protein response. *Viol. J.* 2007; 4:91. [PubMed: 17888185]

Veronesi U, Palo G, De Marubini E, Costa A, Formelli F, Mariani L, Decensi A, Camerini T, Turco MR, Del Mauro MG, Di Muraca MG, Vecchio M, Del Pinto C, D'Aiuto G, Boni C, Campa T, Magni A, Miceli R, Perloff M, Malone WF, Sporn MB. Randomized trial of fenretinide to prevent second breast malignancy in women with early breast cancer. *J. Natl. Cancer Inst.* 1999; 91:1847–56. [PubMed: 10547391]

Villablanca JG, London WB, Naranjo A, McGrady P, Ames MM, Reid JM, McGovern RM, Buhrow SA, Jackson H, Stranzinger E, Kitchen BJ, Sondel PM, Parisi MT, Shulkin B, Yanik GA, Cohn SL, Reynolds CP. Phase II study of oral capsular 4-hydroxyphenylretinamide (4-HPR/fenretinide) in pediatric patients with refractory or recurrent neuroblastoma: a report from the Children's Oncology Group. *Clin. Cancer Res.* 2011; 17:6858–66. [PubMed: 21908574]

Vratilova J, Frgala T, Maurer BJ, Patrick Reynolds C. Liquid chromatography method for quantifying N-(4-hydroxyphenyl)retinamide and N-(4-methoxyphenyl)retinamide in tissues. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2004; 808:125–30.

Wang C, Yang SNYN, Smith K, Forwood JK, Jans DA. Nuclear import inhibitor N-(4-hydroxyphenyl)retinamide targets Zika virus (ZIKV) nonstructural protein 5 to inhibit ZIKV infection. *Biochem. Biophys. Res. Commun.* 2017

Highlights

- Fenretinide (4-HPR) inhibits Zika virus in cell culture.
- 4-HPR decreases Zika virus serum viremia in murine model of disease.
- Metabolic RNA labelling experiments indicate that 4-HPR inhibits flavivirus RNA synthesis in cell culture.
- 4-HPR does not directly inhibit viral replicase activity *in vitro*.

Figure 1.

4-HPR inhibits Zika virus in cell culture. (A) Schematic of single-cycle infectivity used to determine 4-HPR IC₉₀ values against PF13-251013-18. (B) 4-HPR inhibits ZIKV from both the African (MR-766) and Asian (PF13-25103-18) strains as observed by dose-dependent reductions in single-cycle virus yield averaged from 3 independent experiments. The dotted line at 1×10^1 indicates the limit of detection in this experiment. (C–E) 4-HPR inhibits ZIKV strain PF13-251013-18 in multiple mammalian cell lines including Vero (kidney), Huh7 (liver), BHK (kidney), and SK-N-SH (neuronal) cells but has less potent antiviral activity in mosquito cell line C6/36 (mosquito larvae). Graphs are representative of one of three independent replicates. IC₉₀ values were determined for each cell line by averaging calculated values from 3 independent experiments. No IC₉₀ value was able to be determined for 4-HPR in C6/36 cells, as 90% inhibition of Zika virus was not obtained at the highest concentration evaluated (20 μ M).

Figure 2.

4-HPR inhibits ZIKV *in vivo*. **(A)** Schematic of ZIKV infection of AG129 mice and treatment of 60 mg/kg dose 4-HPR. Timing for blood collection and organ harvest are also noted. **(B)** ZIKV virus titers in sera of mice from each of 3 independent experiments on days 2, 3, and 4 post-infection were quantified by viral plaque formation assay. 4-HPR treated mice (black triangles) show a statistically significant decrease in serum viremia at each day tested when compared to vehicle treated mice (grey triangles). *p* values for 4-HPR- versus DMSO-treated mice were < 0.0001 on days 2 and 3 and 0.0001 on day 4 post-infection.

Figure 3.

4-HPR inhibits the accumulation of ZIKV RNA *in cellulo* but does not affect RdRp activity *in vitro*. (A) Schematic of time-course experiment monitoring steady-state abundance of viral RNA in ZIKV- or DV2-infected cells over time. (B) To assess 4-HPR's effect on steady-state accumulation of ZIKV RNA, we harvested total cellular RNA at various times after infection at MOI = 10, and quantified viral RNA by RT-qPCR relative to cellular GAPDH mRNA. Increasing viral RNA is observed at 12 h (ZIKV – black solid line) and 16 h (DV2 – black dashed line) post-infection for DMSO-treated controls, reflecting accumulation of newly synthesized viral RNA; however, this fails to occur in the presence of 5 μ M 4-HPR (red lines). (C) Upper panels show representative phosphor scans of *in vitro* RNA transcripts containing 32 P-UTP produced from CRCs purified from DV2- or ZIKV-infected Vero cells in the presence of DMSO, 4-HPR, 4-MPR, or dUTP. Lower panels show results of densitometry performed on phosphor scans using ImageJ software. The results of the average from 3 independent experiments for ZIKV and 2 experiments for DV2 are shown in the lower panels. Only the reactions lacking NTPs or containing dUTP show inhibition of RdRp activity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Figure 4.

4-HPR inhibits synthesis of dengue virus RNA. **(A)** Schematic of the workflow of incorporation, biotinylation, and purification of 4sU-containing RNA. **(B)** Schematic of experimental timing of the 4sU experiment with times of addition of 4sU, actinomycin D, and 4-HPR indicated. **(C)** Bound (nascent) DV2 RNA was quantified by RT-qPCR and normalized to a 4sU-labeled nanoluciferase RNA control that was spiked into each reaction. 10 μ M 4-HPR inhibits the synthesis of 4sU-containing RNA over time (squares with dashed line) when compared to DMSO control (circle with solid line). The graph is representative of one of three independent experiments.