

# A Nuclear Transport Inhibitor That Modulates the Unfolded Protein Response and Provides In Vivo Protection Against Lethal Dengue virus Infection

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**Background.** Dengue virus (DENV) is estimated to cause 390 million infections each year, but there is no licensed vaccine or therapeutic currently available.

**Methods.** We describe a novel, high-throughput screen to identify compounds inhibiting the interaction between DENV nonstructural protein 5 and host nuclear transport proteins. We document the antiviral properties of a lead compound against all 4 serotypes of DENV, antibody-dependent enhanced (ADE) infection, and ex vivo and in vivo DENV infections. In addition, we use quantitative reverse-transcription polymerase chain reaction to examine cellular effects upon compound addition.

**Results.** We identify *N*-(4-hydroxyphenyl) retinamide (4-HPR) as effective in protecting against DENV-1–4 and DENV-1 ADE infections, with 50% effective concentrations in the low micromolar range. 4-HPR but not the closely related *N*-(4-methoxyphenyl) retinamide (4-MPR) could reduce viral RNA levels and titers when applied to an established infection. 4-HPR but not 4-MPR was found to specifically upregulate the protein kinase R-like endoplasmic reticulum kinase arm of the unfolded protein response. Strikingly, 4-HPR but not 4-MPR restricted infection in peripheral blood mononuclear cells and in a lethal ADE-infection mouse model.

**Conclusions.** 4-HPR is a novel antiviral that modulates the unfolded protein response, effective against DENV1–4 at concentrations achievable in the plasma in a clinical setting, and provides protection in a lethal mouse model.

**Keywords.** dengue virus; antiviral; nuclear transport; unfolded protein response.

Dengue virus (DENV), the causative agent of dengue fever, potentially lethal dengue hemorrhagic fever, and dengue shock syndrome, is a reemerging arthropod-borne virus threatening >40% of the world's population [1]. DENV is an enveloped, positive-sense,

single-stranded RNA virus, classified within the flavivirus genus alongside other significant pathogens, including West Nile virus. Despite the global burden of DENV, there is no licensed vaccine or antiviral therapeutic available. Eliciting a neutralizing antibody response against all 4 serotypes of circulating DENV (DENV-1–4) by vaccination has proven highly problematic, with only 30% efficacy reported to date [2]. The search for efficacious antivirals against DENV [3] has largely focused on compounds that target key enzymatic activities of viral proteins, the RNA-dependent RNA polymerase (RdRp) and protease, emulating approaches used for human immunodeficiency virus [4, 5] and hepatitis C virus [6]. So far, there has been limited progress for DENV, with toxicity and lack of efficacy in clinical trials hampering the development

Received 24 March 2014; accepted 26 May 2014; electronically published 5 June 2014.

Presented in part: Lorne Infection and Immunity Conference, Lorne, Australia, February 2014.

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The Journal of Infectious Diseases® 2014;210:1780–91

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DOI: 10.1093/infdis/jiu319

of compounds such as the nucleoside analogues NITD-008 and balapiravir [7, 8]. Compounds directed at alternative targets include the  $\alpha$ -glucosidase I/II inhibitor, celgosivir, currently under clinical trial [9, 10], but there is clearly an urgent need for the identification of new targets to enable development of truly efficacious anti-DENV agents.

Previously, we showed that DENV nonstructural protein 5 (NS5; RdRp) traffics into and out of the nucleus during infection and that NS5 nuclear accumulation is significantly reduced when association of NS5 with the host cell nuclear import proteins, importin  $\alpha$  (IMP- $\alpha$ ) and IMP- $\beta$ 1, is blocked, either through mutation of key NS5 nuclear localization signal (NLS) residues [11, 12] or by small-molecule inhibitors, such as ivermectin [13, 14]. In either case, production of infectious DENV is significantly attenuated, highlighting the potential of targeting NS5 nuclear import as an antiviral strategy.

Here, we build on this work by screening for small-molecule inhibitors targeting the interface between NS5 and IMP- $\alpha$ / $\beta$ 1 [13, 14] and identifying the synthetic retinoid *N*-(4-hydroxyphenyl) retinamide (4-HPR). We demonstrate its usefulness in protecting against DENV-1–4 in cell culture, antibody-dependent enhanced (ADE) infection (in which subneutralizing antibodies enhance infection, possibly contributing to severe dengue [15]), as well as *ex vivo* infection (in peripheral blood mononuclear cells [PBMCs]). Intriguingly, we find that 4-HPR leads to specific activation of the unfolded protein response (UPR), culminating in rapid elimination of viral RNA from infected cells. Most importantly, we show that 4-HPR can protect against DENV ADE infection in a lethal mouse model. Specific induction of the UPR thus appears to promote an anti-DENV environment, with 4-HPR a promising new antiviral in this context.

## MATERIALS AND METHODS

### Cell Lines and Virus Strains

Cell lines used include Vero, Huh-7, BHK-21, THP-1, and C6/36. DENV-1–4 strains were described previously [16]. See the [Supplementary Materials](#) for cell culture conditions and GenBank accession numbers.

### Antibodies

Mouse anti-E antibody (4G2) was produced in hybridoma cells. Humanized 4G2 immunoglobulin G (IgG) [17], human anti-NS3 IgG (3F8), and anti-NS5 IgG (5M1) [18, 19] and human anti-NS5 [13] (immunofluorescence experiments) have been described. Rabbit anti- $\beta$ -actin and mouse anti-ceramide antibodies were from Sigma and Enzo Life Sciences, respectively.

### Therapeutic Compounds

4-HPR, arotinoid acid (AA), 13-*cis* retinoic acid (13-*cRA*), *N*-(4-methoxyphenyl) retinamide (4-MPR), and all-*trans*-retinoic

acid (ATRA) were purchased from Sigma, with additional 4-HPR purchased from Tocris Bioscience ([Supplementary Materials](#)).

### AlphaScreen

AlphaScreen binding assays were performed as previously described ([Supplementary Materials](#)) [13, 14, 20], using recombinant His<sub>6</sub>-NS5 [13, 14], IMP- $\alpha$ , biotinylated IMP- $\beta$  [14], and biotinylated IMP- $\alpha$  lacking the IMP- $\beta$ -binding domain (residues 51–529; IMP- $\alpha$  $\Delta$ IBB [21]).

### Virus Infection

Antiviral experiments were performed using Vero or Huh-7 cells infected with DENV-2 at a multiplicity of infection (MOI) of 1 or 0.3, in the absence or presence of the therapeutic compounds indicated above. The virus load in the culture medium was measured 24 hours (in Vero cells) or 48 hours (in Huh-7 cells) after infection, by quantitative reverse-transcription polymerase chain reaction (qRT-PCR; Vero) or plaque assay (Huh-7). ADE infection in THP-1 and PBMCs was achieved as previously described [17], with slight modifications ([Supplementary Materials](#)).

### Immunofluorescence

Huh-7 cells were infected with DENV-2 at an MOI of 10 and then fixed 24 hours after infection, before staining and imaging ([Supplementary Materials](#)).

### Quantitative PCR (qPCR) of Cellular Targets

Huh-7 cells that were infected with DENV-2 (MOI, 1) and/or treated with drugs were lysed in Trizol at 1–36 hours after infection/drug addition. Complementary DNA was generated with polydT primers (cellular transcripts) or with random primers (viral RNA), using Improm II (Promega) according to the manufacturer's instructions. RNA levels in infected and/or drug treated cells were determined relative to RNA levels of the gene encoding actin and to RNA levels in untreated cells, using the  $2^{-T_{\Delta\Delta C}}$  method ([Supplementary Materials](#)) [22].

### In Vivo Infections

AG129 mice [23] were infected as described previously [9]. 4-HPR was administered orally (200  $\mu$ L at 20 mg/kg) once or twice daily for 5 days from the time of infection. All work was performed in accordance with Singapore National guidelines under protocol 2012/SHS/713, approved by SingHealth Animal Care and Use Committee, Singapore General Hospital/NUS.

## RESULTS

### Identification of 4-HPR as an Anti-DENV Agent

Trafficking of DENV NS5 into the nucleus, dependent on high-affinity interaction with IMP- $\alpha$ / $\beta$ 1, is important for efficient DENV replication [11–14, 24]. To build on the capability of

our established AlphaScreen platform [14, 20], we performed a robotized, high-throughput screen of the Library of Pharmacologically Active Compounds (Sigma), to identify inhibitors that block the association of His-tagged DENV-2 NS5 with biotin-labeled IMP- $\alpha$ / $\beta$ 1. Twenty-nine compounds that reduced the AlphaScreen signal generated by NS5-IMP- $\alpha$ / $\beta$ 1 binding by >20% were counter-screened against a biotinylated hexahistidine peptide (B-His) to remove false-positive compounds that interfered with AlphaScreen chemistry (Figure 1A). Of 3 compounds that did not inhibit B-His by  $\geq$ 10%, 4-HPR was selected for further study, in part because of its documented safety, particularly at high doses in children [25–27], the age group at greatest risk of death due to severe dengue [28].

4-HPR interfered with association of NS5 and IMP- $\alpha$ / $\beta$ 1, as well as NS5 and IMP- $\alpha$ IBB (a truncated form of IMP- $\alpha$  lacking the autoinhibitory IMP- $\beta$ -binding domain [21]), with 50% inhibition of binding occurring at similar half maximal inhibitory concentration (IC<sub>50</sub>) values of 1–1.5  $\mu$ M in 3 independent experiments (Figure 1B), while NS5 binding to IMP- $\beta$ 1 alone was largely unaffected. Together, these data suggest that 4-HPR prevents NS5 from binding to the NLS binding site on IMP- $\alpha$  by targeting NS5 and/or IMP- $\alpha$ .

To investigate the structure/activity relationship for 4-HPR, a synthetic retinoic acid analogue, we tested the ability of ATRA (the parent compound of 4-HPR) and 4-MPR (a closely related analogue of 4-HPR; Figure 1C) to inhibit association of NS5 with IMP- $\alpha$ / $\beta$ 1 by AlphaScreen. In contrast to 4-HPR, neither ATRA nor 4-MPR were able to disrupt association of these proteins, demonstrating the specificity of the interaction (Figure 1D).

We next tested the antiviral activity of 4-HPR by infecting Vero cells with DENV-2 in the presence of 10  $\mu$ M 4-HPR. Viral RNA copies in the culture medium were determined by qRT-PCR 24 hours after infection, as an indicator of virus titer [29]. 4-HPR had potent antiviral activity in this context, causing a 2-log reduction in viral RNA copies 24 hours after infection ( $P < .0001$ ; Figure 1E). The results were not attributable to toxicity, with an XTT assay indicating a 50% cytotoxicity concentration (CC<sub>50</sub>) of 13.5  $\mu$ M (Supplementary Figure 1A). Neither ATRA nor 4-MPR, nor 2 other 4-HPR analogues, AA and 13-cRA, had any effect on DENV-2 infection (Figure 1E), correlating with their inability to inhibit IMP- $\alpha$ / $\beta$ 1-NS5 interaction. Thus, the antiviral activity of 4-HPR is not a general property of retinoids, which primarily signal through retinoic acid receptors (RARs) to modulate cell differentiation and development [30]. The high specificity of the inhibitory activity of 4-HPR is highlighted by 4-MPR, which differs from 4-HPR by a single methyl group and lacks inhibitory activity toward IMP- $\alpha$ / $\beta$ 1-NS5 interaction, as well as antiviral activity toward DENV-2. Since 4-MPR itself is the major metabolite of 4-HPR [31, 32], this suggests the antiviral activity is likely to be elicited by 4-HPR itself, rather than by this byproduct.

#### 4-HPR Restricts Production of Infectious DENV-1–4 and ADE-Mediated Infection in Cell Culture

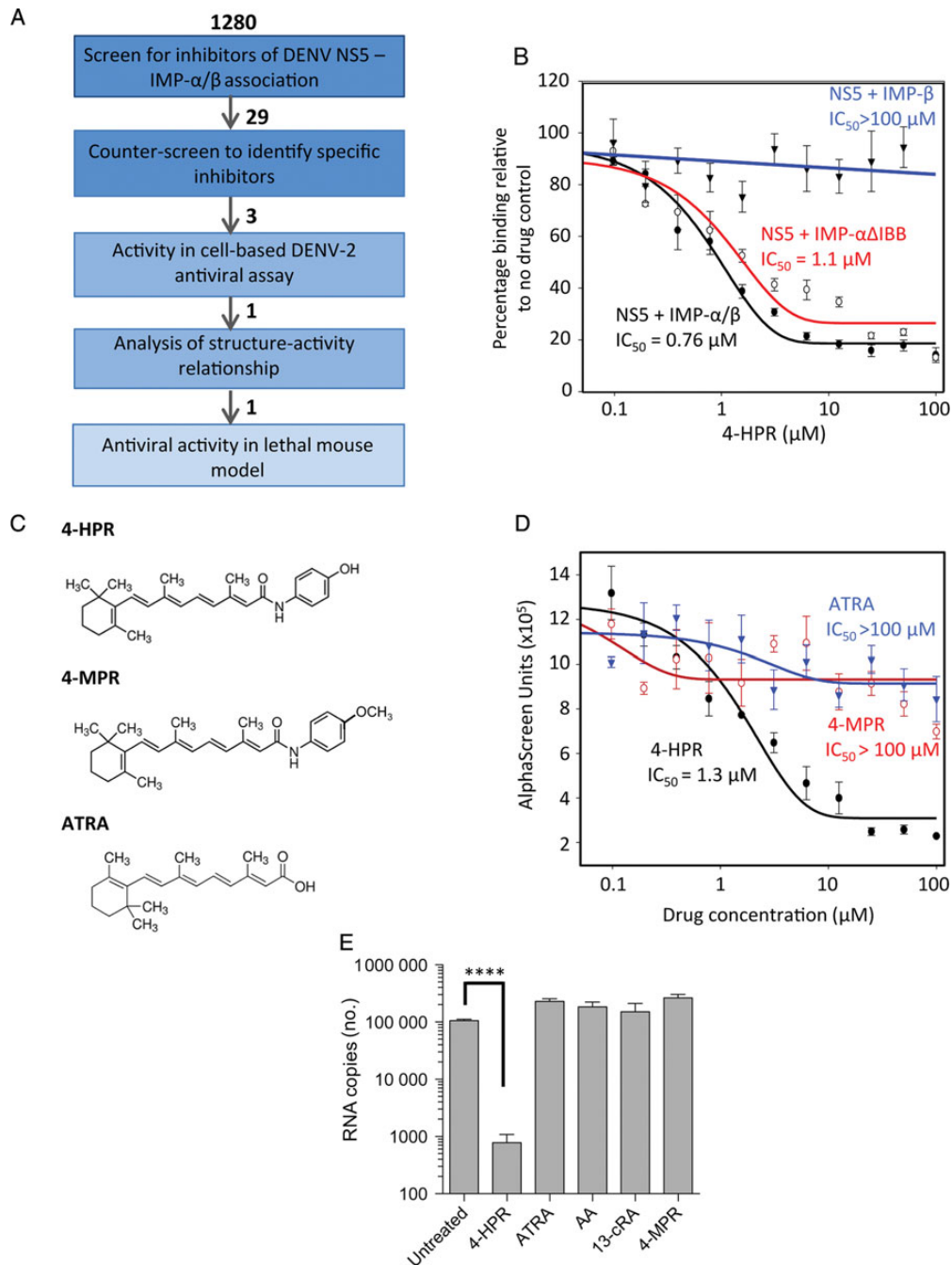
To test the efficacy of 4-HPR in inhibiting different serotypes of DENV, Huh-7 cells were infected with DENV-1–4 in the presence of increasing concentrations of 4-HPR, with virus titers determined by plaque assay at 48 hours after infection. Strikingly, all 4 DENV serotypes were restricted with similar 50% effective concentrations (EC<sub>50</sub>; 2.6, 2.1, 1.4, and 2.1  $\mu$ M for DENV-1, 2, 3 and 4, respectively, from 2 independent experiments; Figure 2A), strongly implying that 4-HPR targets a component/pathway common to all DENV serotypes. In addition, 4-HPR showed activity against the related flavivirus, West Nile virus (Kunjin strain [WNV<sub>KUN</sub>]; Supplementary Figure 1B).

To further characterize the antiviral activity of 4-HPR, we used a model for DENV ADE infection, whereby subneutralizing antibodies increase DENV uptake into monocytes via Fc receptors, mimicking events hypothesized to occur in patients infected with a second DENV serotype [23]. Subneutralizing concentrations of the anti-DENV-2 E antibody, 4G2 [17], were incubated with DENV-1 for 1 hour prior to addition of the virus/antibody complex to THP-1 monocytes in the presence of 4-HPR. Virus titers were determined by plaque assay 48 hours later. The average EC<sub>50</sub> from 2 independent experiments was 0.78  $\mu$ M (Figure 2B), with a >2-log reduction in infectious virus at 7.5  $\mu$ M 4-HPR, demonstrating that ADE-DENV infection is also susceptible to 4-HPR treatment.

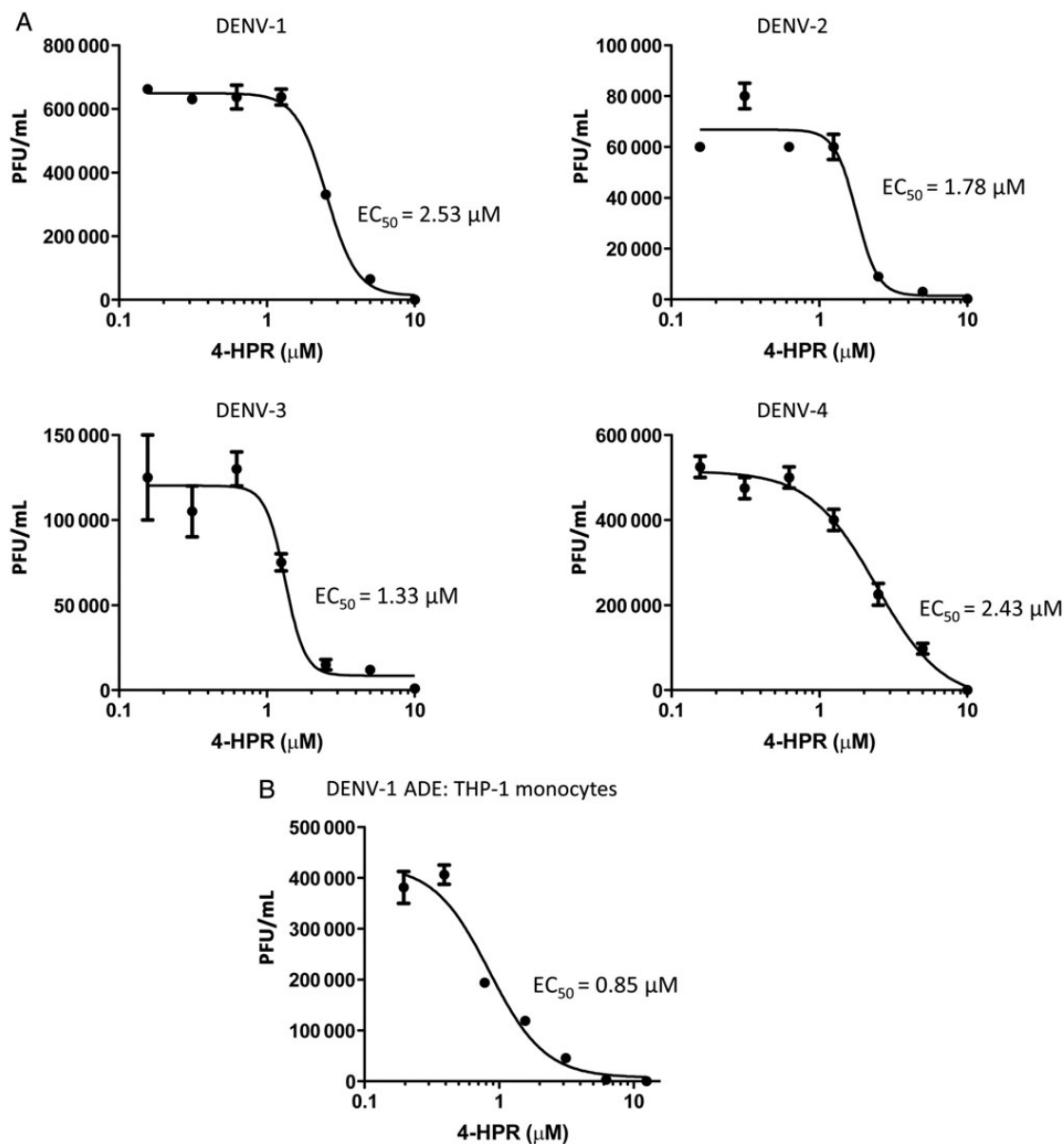
#### 4-HPR Effectively Reduces DENV-2 RNA Replication When Added 12 Hours After Infection

To gain further insight into the mode of action of 4-HPR, infected Huh-7 cells were treated by delayed addition of 4-HPR, inactive analogue 4-MPR, or the previously described adenosine nucleoside inhibitor, NITD-008 [8], 12 hours after infection. Intracellular RNA levels were monitored by qPCR from 12 to 60 hours after infection. In the untreated DENV infection control and in infected cells treated with 7.5  $\mu$ M 4-MPR, viral RNA levels increased steadily until 60 hours after infection (Figure 3A). In contrast, addition of 7.5  $\mu$ M 4-HPR to Huh-7 cells at 12 hours after infection resulted in significantly reduced viral RNA levels relative to levels in the infection control, with a 1-log reduction observed within 12 hours (24 hours after infection;  $P < .05$ ). Interestingly, the amount of viral RNA present did not increase between 24 hours and 60 hours after infection. This result was comparable to the reduced RNA levels in infected cells treated with NITD-008, implying that 4-HPR acts at the RNA replication stage of infection rather than at virus entry, assembly, or egress. However, 4-HPR did not inhibit RdRp activity in vitro (not shown), implying another mode of action.

Parallel analysis of virus titers revealed consistent results, where delayed addition of 4-HPR resulted in significantly reduced virus levels throughout the 60 hours of infection, relative to levels in the untreated and 4-MPR controls ( $P < .001$ ;



**Figure 1.** Identification of *N*-(4-hydroxyphenyl) retinamide (4-HPR), a potent anti-dengue virus (DENV) agent. *A*, Schematic showing the strategy for identifying 4-HPR from a roboticized drug screen, beginning with the Library of Pharmacologically Active Compounds (Sigma). *B*, AlphaScreen technology was used to examine the binding of 30 nM His<sub>6</sub>-NS5 to 10 nM importin  $\alpha/\beta$ 1 (IMP- $\alpha/\beta$ 1; IMP- $\alpha/\beta$ 1 heterodimer containing biotinylated IMP- $\beta$ 1), 3 nM IMP- $\alpha\Delta\text{IBB}$  (biotinylated IMP- $\alpha$  with the autoinhibitory IMP- $\beta$ -binding domain removed), or 3 nM biotinylated IMP- $\beta$ 1, in the presence of increasing concentrations of 4-HPR. Data are the mean  $\pm$  standard deviation of triplicate wells from a single typical experiment. Average half maximal inhibitory concentrations from 3 independent experiments are indicated in the text. *C*, Structures of hit compound 4-HPR, its analogue *N*-(4-methoxyphenyl) retinamide (4-MPR), and the parent compound all-*trans*-retinoic acid (ATRA). *D*, Analysis is similar to that in panel *B*, except NS5 and IMP- $\alpha/\beta$ 1 binding were compared in the presence of increasing concentrations of 4-HPR, 4-MPR, or ATRA. *E*, Vero cells were infected with DENV-2 (multiplicity of infection, 1) in the presence of drug diluent (untreated) or the indicated compounds (10  $\mu\text{M}$ ) for 2 hours, followed by removal of the virus/drug inoculum, and addition of fresh 2% fetal calf serum maintenance medium. At 24 hours after infection, culture medium was collected, and the viral RNA extracted using the QIAamp viral RNA mini kit (Qiagen). The absolute number of RNA copies in each sample was determined by Taqman One Step reverse-transcription polymerase chain reaction (Applied Biosystems) as an estimate of virus titer, by extrapolation from a standard curve generated from *in vitro*-transcribed DENV-2 RNA. Data are the mean  $\pm$  standard error of the mean for 3 independent experiments. Statistical analysis (by the Student *t* test) was performed using GraphPad Prism software. \*\*\*\**P* < .0001.  $IC_{50}$ , half maximal inhibitory concentration.

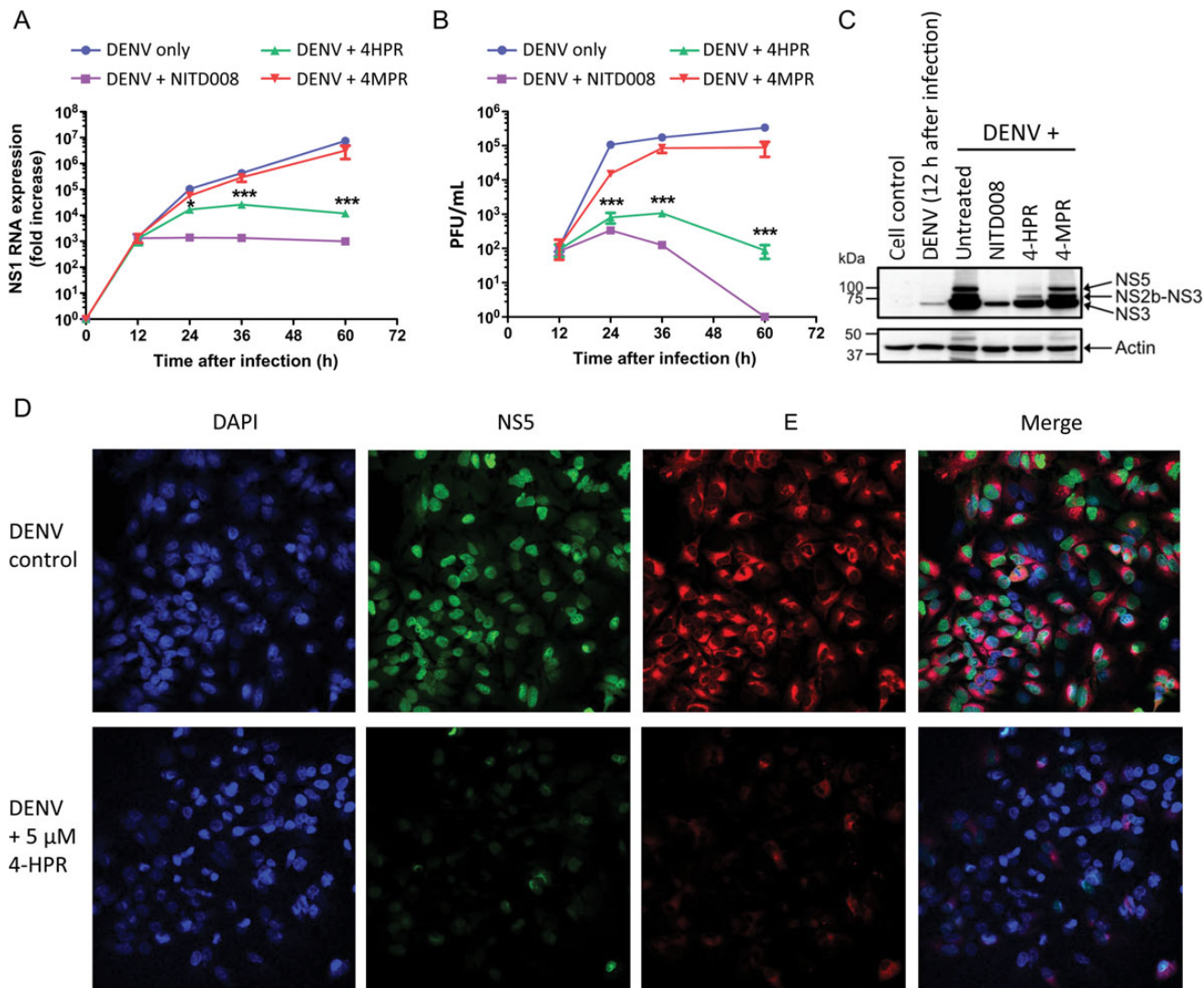


**Figure 2.** *N*-(4-hydroxyphenyl) retinamide (4-HPR) restricts production of infectious dengue virus serotypes 1–4 (DENV-1–4) and antibody-dependent enhanced (ADE)–infection in cell culture. *A*, Huh-7 cells were infected with DENV-1–4 (multiplicity of infection [MOI], 0.3) for 1 hour in the presence of the indicated concentrations of 4-HPR. Virus/drug inoculums were removed, and fresh 2% fetal calf serum maintenance medium was added, which contained the same indicated concentration of 4-HPR. Culture medium was collected 48 hours after infection, and viral titers were determined by plaque assay, represented as plaque-forming units (PFU) per milliliter. *B*, Antibody dependent enhanced (ADE) infection was performed as described in panel *A*, except DENV-1 (MOI, 10) was first incubated with subneutralizing concentrations of a monoclonal antibody directed toward E protein (4G2), prior to addition of immune complexes to THP-1 cells in the presence of 4-HPR. Data are the mean  $\pm$  standard deviation for duplicate wells from a single assay, representative of 2 independent experiments. Average 50% effective concentrations ( $EC_{50}$ ) from the 2 experiments are indicated in the text.

Figure 3*B*). Maximal reduction was reached at 60 hours after infection, when the virus titer was 3-logs lower in 4-HPR treated cells, compared with the DENV only control, similar to values for the NITD-008-treated cells. This finding suggests that the compound may be efficacious against an established infection, but this will require examination in an *in vivo* system. As

expected, NS5 and NS3 protein levels were also markedly lower at 36 hours after infection, following 4-HPR treatment but not 4-MPR treatment, relative to levels for the DENV only control (Figure 3*C*).

To observe the inhibitory effect of 4-HPR on NS5 nuclear accumulation, Huh-7 cells were infected with DENV-1–4 in the



**Figure 3.** *N*-(4-hydroxyphenyl) retinamide (4-HPR) effectively reduces dengue virus serotype 2 (DENV-2) RNA replication when added 12 hours after infection. *A–C*, Huh-7 cells were infected with DENV-2 (multiplicity of infection [MOI], 1) prior to addition of drug diluent dimethyl sulfoxide (DMSO; DENV only), 7.5 μM 4-HPR, 7.5 μM 4-MPR, or 10 μM NITD-008, 12 hours after infection. Cells were lysed at the indicated times, and RNA was extracted by Trizol and analyzed by quantitative polymerase chain reaction, with fold change in expression of NS1 calculated relative to that for the untreated control (*A*). Concurrently, culture medium was collected, and viral titers were determined by plaque assay (*B*). Alternatively, cells were lysed at 36 hours after infection (except for the infection control, which was lysed at 12 hours after infection, as indicated) by addition of reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis loading dye, and Western analysis was performed using antibodies directed toward NS3, NS5, and actin (loading control; *C*). Data are the mean ± standard deviation of duplicate wells from 2 independent experiments (*A* and *B*) or are representative of 2 independent experiments (*C*). Statistical analysis (by the Student *t* test) was performed using GraphPad Prism software. \**P* < .05 and \*\*\**P* < .001. *D*, Huh-7 cells were infected with DENV-2 (MOI, 10) for 2 hours in the presence of DMSO (DENV control) or 5 μM 4-HPR as indicated, then replaced with fresh 2% fetal calf serum maintenance medium containing the indicated compound. Cells were fixed and stained 24 hours after infection with monoclonal antibodies directed toward E protein, NS5, or were stained with DAPI to visualize the cell nucleus. Images were captured by confocal laser scanning microscopy, using a Nikon C1 invert microscope. Images are representative of a single typical experiment.

absence/presence of 4-HPR. Cells were fixed and stained 24 hours later using a cross-reactive monoclonal antibody for NS5 [13] or E protein (a secondary marker for infection [17]). In DENV-2-infected control cells, NS5 could be detected in virtually all cells in a given field, which also stained positive

for cytoplasmic E protein, and was localized almost exclusively to the nucleus, consistent with previous reports (Figure 3*D*) [12, 13]. Upon treatment with 5 μM 4-HPR, very few cells stained positive for infection, with low to undetectable levels of NS5 or E protein in most cells, consistent with the markedly reduced

levels of NS5 protein detected by Western blot analysis of 4-HPR-treated cells (Figure 3C). This strong inhibition of infection meant that it was not possible to quantify the extent to which 4-HPR inhibited NS5 nuclear accumulation, with similar results in cells infected with DENV-1, 3, and 4 (data not shown).

#### 4-HPR Specifically Activates the Protein Kinase R-like Endoplasmic Reticulum Kinase (PERK) Pathway

4-HPR has been previously reported to inhibit dihydroceramide desaturase (DEGS-1), an enzyme that catalyses the final step in de novo ceramide synthesis [33]. However, DEGS-1 inhibition is unlikely to be the basis of protection against DENV infection by 4-HPR, as 2 other distinct ceramide synthesis inhibitors, C<sub>8</sub>-cyclopropenylceramide (DEGS-1 competitive inhibitor [34]) and myriocin (inhibitor of serine palmitoyltransferase, which catalyses the first step in sphingolipid biosynthesis [35]), did not exhibit antiviral activity toward DENV-2, despite clearly reducing cellular ceramide levels (Supplementary Figure 2).

4-HPR also induces cell stress responses, including the UPR [36], a pathway located within the endoplasmic reticulum and controlled by the gateway proteins PERK, activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1), each of which regulates a distinct outcome for the cell by modulating transcription of various target genes (Figure 4A). Typically, the UPR is activated when the endoplasmic reticulum becomes overloaded with misfolded proteins, directing the cell to stall protein translation to allow restoration of homeostasis [37]. Intriguingly, DENV infection activates all 3 arms of the UPR pathway by 24 hours after infection [17, 38].

To examine the effect of 4-HPR on the UPR, Huh-7 cells were treated with 7.5  $\mu$ M 4-HPR or 4-MPR in the presence or absence of DENV infection. Cells were lysed at various times up to 36 hours after addition, and qPCR analysis was performed to assess the level of activation of each pathway. Transcripts representing growth arrest DNA damage-inducible protein 34 (GADD34) and CCAAT/enhancer-binding protein-homologous protein (CHOP) were evaluated for the PERK pathway [17], X-box-binding protein1 (XBP1) was evaluated for the ATF6 pathway [17], and p58 inhibitor of protein kinase R (p58<sup>IPK</sup>) was evaluated for the IRE1 pathway [39].

Following infection with DENV alone, transcription of GADD34 and CHOP peaked 36 hours after infection, increasing by 35- and 23-fold, respectively, while XBP1 and p58<sup>IPK</sup> transcript levels also increased with infection, peaking 36 hours after infection at approximately 3-fold, relative to values for the untreated control (Figure 4B) [38]. The potent UPR activator thapsigargin [40], used as a control, induced rapid and massive upregulation of all transcripts tested. Addition of 4-HPR to infected cells caused levels of PERK pathway transcripts to increase earlier and to a much higher extent than DENV alone, with a 12-fold increase in CHOP and GADD34 transcript

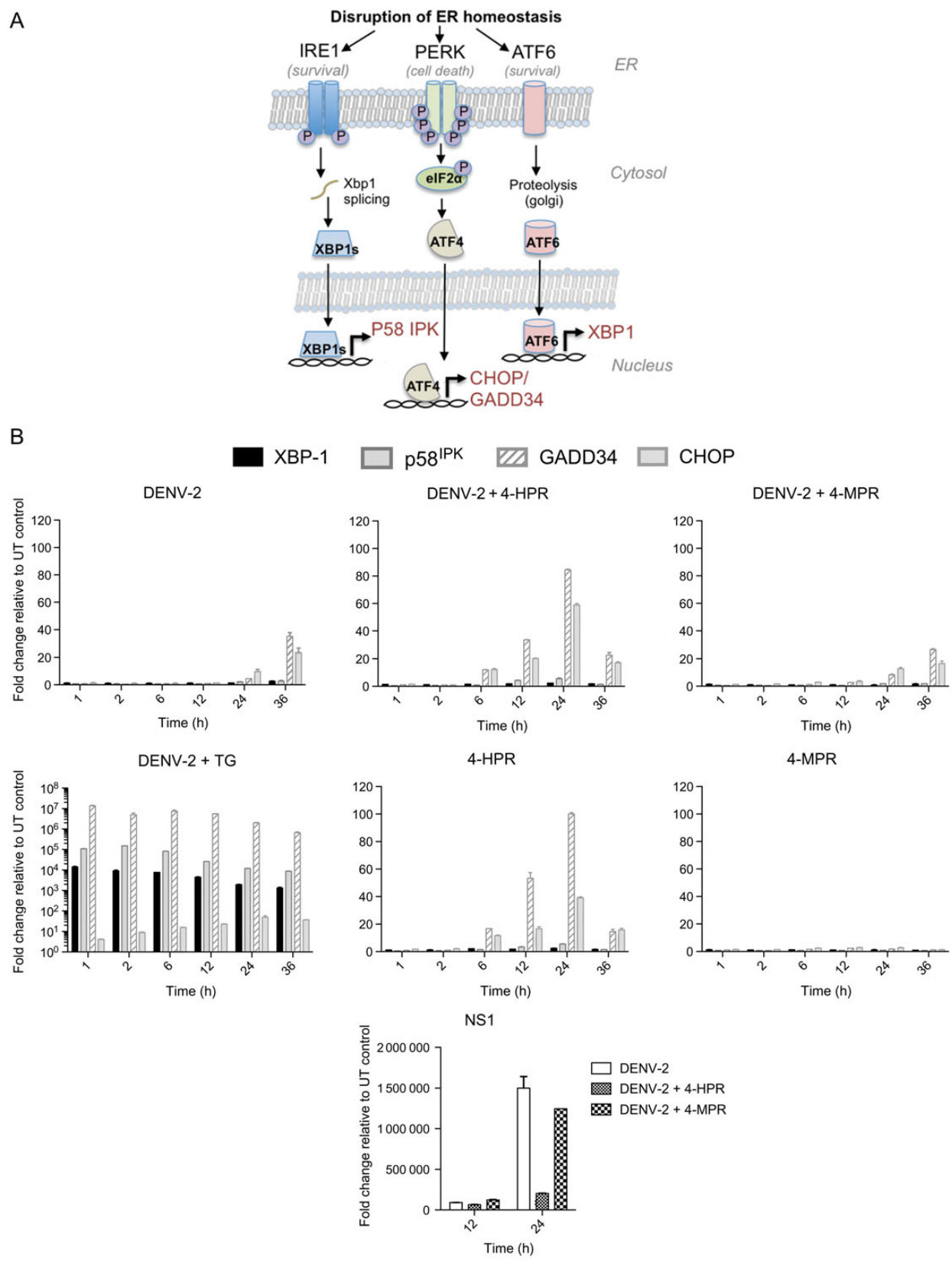
levels at 6 hours after infection, which peaked at 24 hours after infection, with 84- and 59-fold increases, respectively, relative to transcripts from the untreated control. 4-HPR was also found to activate the IRE1 and ATF6 pathways, but to a much lower extent, inducing p58<sup>IPK</sup> transcript levels by approximately 5-fold, and XBP1 transcript levels by approximately 2-fold, at 24 hours after addition. Addition of 4-MPR to DENV-2-infected cells resulted in a similar transcript profile to that for DENV-2 alone, consistent with 4-MPR not displaying any antiviral activity. Assessment of NS1 transcript levels as a measure of viral replication in the DENV-2 control, 4-HPR-treated, and 4-MPR-treated samples showed RNA levels to be substantially decreased in the 4-HPR-treated cells only. Interestingly, induction of the PERK pathway by 4-HPR was also observed in the absence of DENV-2 infection, suggesting the effect is not dependent on the presence of virus (Figure 4B).

#### 4-HPR Provides Protection Against DENV Infection Ex Vivo and In Vivo

We next examined the antiviral activity of 4-HPR ex vivo, in human PBMCs, a major target of DENV infection [41]. PBMCs were prepared from a healthy donor buffy coat prior to ADE-mediated DENV-1 infection in the presence of increasing concentrations of 4-HPR or 4-MPR. Virus titers were determined by plaque assay 48 hours later, revealing an EC<sub>50</sub> for 4-HPR of 0.81  $\mu$ M (Figure 5A), with no reduction in titer observed for 4-MPR-treated PBMCs. Analysis of 4-HPR cytotoxicity in PMBCs indicated a CC<sub>50</sub> of >13  $\mu$ M, demonstrating that the antiviral effect is not due to cell death.

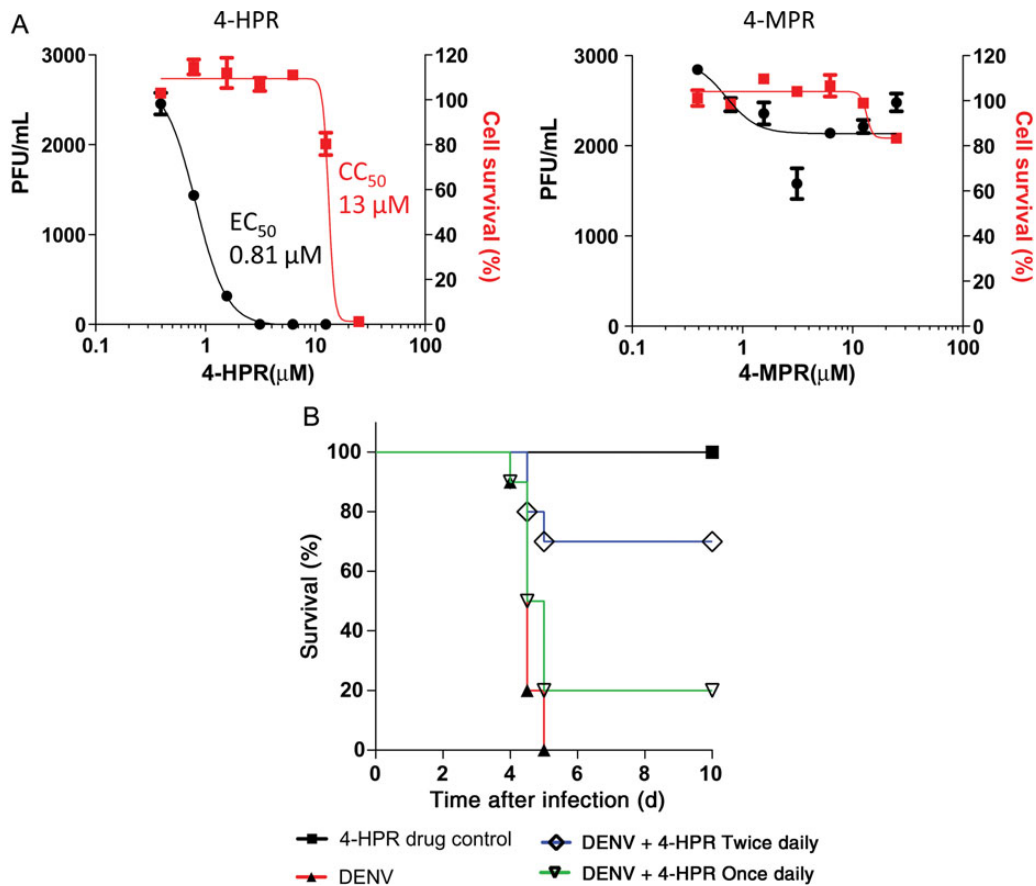
Mice have previously been used as an in vivo model for 4-HPR pharmacokinetics [42]. To examine the effect of 4-HPR on DENV infection in vivo, we used the previously described lethal mouse infection model, which uses a mouse adapted DENV-2 strain (S221) to infect Sv/129 mice deficient in type I/II interferon receptors (AG129) [23]. Viral infection is enhanced by intraperitoneal delivery of a subneutralizing concentration of 4G2 antibody 1 day prior to infection, leading to peak viremia 3 days after infection and death by day 5 [9]. Mice were treated with 20 mg/kg of 4-HPR, administered orally at the time of infection and then once daily or twice daily for 5 consecutive days. Consistent with previous reports, infected, untreated mice all died by day 5, without exception (Figure 5B) [9, 10]. Once-daily 4-HPR treatment was sufficient to protect 20% of mice, which recovered completely from the infectious challenge, while twice-daily treatment improved protection to an efficacious 70%.

Analysis of cytokines tumor necrosis factor  $\alpha$ , interleukin 6, interleukin 10, and interleukin 12 in DENV-infected mice all trended toward lower levels in the twice-daily treatment group in comparison to the virus control mice (Supplementary Figure 3), suggesting that 4-HPR reduces inflammatory cytokines associated with severe DENV disease [43].



**Figure 4.** Specific activation of the protein kinase R-like endoplasmic reticulum kinase (PERK) pathway by *N*-(4-hydroxyphenyl) retinamide (4-HPR) is concomitant with dengue virus (DENV) RNA reduction. *A*, Schematic representation of the unfolded protein response (UPR). Disruption of endoplasmic reticulum homeostasis by biochemical or physiologic stimuli activates the UPR, which may induce activation of the inositol-requiring enzyme 1 (IRE1), PERK, and/or activating transcription factor 6 (ATF6) pathway(s). Phospho-IRE1 can induce splicing of XBP1 transcripts, with the resulting XBP1 product





**Figure 5.** *N*-(4-hydroxyphenyl) retinamide (4-HPR) provides protection against dengue virus (DENV) infection ex vivo and in vivo. *A*, Peripheral blood mononuclear cells (PBMCs) were isolated from a healthy human donor by standard Ficoll-Paque (GE Healthcare) extraction. Prior to infection, DENV-1 (multiplicity of infection, 10) was incubated with monoclonal antibody 4G2, followed by addition of the immune complexes to peripheral blood mononuclear cells (PBMCs) in the presence of increasing concentrations of 4-HPR or *N*-(4-methoxyphenyl) retinamide. Culture medium was collected 48 hours after infection and viral titers determined by plaque assay (left  $\gamma$ -axis, circle symbols). PBMC viability was determined by addition of CellTiter Glo luminescent reagent (Promega) following identical drug treatment (but in the absence of virus; right hand  $\gamma$ -axis, square symbols). Cell survival is plotted relative to an untreated control. Data are the mean  $\pm$  standard deviation of duplicate (for the plaque assays) or triplicate (for the cell viability assays) wells from a representative assay. *B*, Sv/129 mice, deficient in type I and II interferon receptors (AG129), were injected with 10  $\mu$ g of 4G2 1 day prior to infection with mouse-adapted DENV-2 strain (S221) to promote virus uptake. Twenty-four hours later, mice were infected with  $2 \times 10^5$  plaque-forming units of S221, followed by immediate delivery of 4-HPR (or vehicle), administered orally (200  $\mu$ L at 20 mg/kg). 4-HPR treatment was continued once daily or twice daily for 5 days. Mice were scored for viability until day 10. Each group contained 10 mice, with the exception of the uninfected drug treatment control, which contained 2 mice.

## DISCUSSION

Antivirals efficacious against all serotypes of DENV are urgently needed following the continued increase in DENV infections worldwide. Here, we identify a novel antiviral agent, 4-HPR,

capable of blocking recognition of DENV NS5 by host nuclear import proteins, that is equally effective in protecting against DENV-1-4, ADE-mediated infection, including ex vivo (infected PBMCs) and in a lethal mouse model.

*Figure 4 continued.* (XBP1s) translocating to the nucleus to promote transcription of prosurvival genes, including p58<sup>IPK</sup>. Activation of PERK by phosphorylation induces phosphorylation of the translation initiation factor, eIF2 $\alpha$ , temporarily halting global translation, while also activating the transcription factor, ATF4. This leads to expression of proapoptotic genes, including those encoding CHOP and GADD34. Activation of ATF6 occurs by proteolytic cleavage, causing its relocalization to the nucleus and transcription of prosurvival genes, including XBP1. Levels of p58<sup>IPK</sup>, XBP1, and CHOP/GADD34 transcripts can be used as indicators of IRE1, ATF6, and PERK pathway activation, respectively. *B*, Huh-7 cells were infected without or with DENV-2 (multiplicity of infection, 1) in the presence of drug-diluent dimethyl sulfoxide (DENV-2 only), 500 nM thapsigargin (TG), 7.5  $\mu$ M 4-HPR, or *N*-(4-methoxyphenyl) retinamide. RNA was isolated from cells by Trizol extraction at the indicated times after infection/drug addition, and transcript levels determined by quantitative polymerase chain reaction, with fold change in expression of target genes calculated relative to the untreated (UT) control. Data are the mean  $\pm$  standard deviation of duplicate wells from a single assay, representative of 2 independent experiments.

We show that the antiviral activity of 4-HPR is highly specific, with structurally related compounds failing to demonstrate antiviral activity. Strikingly, modification of the hydroxyphenyl group of 4-HPR by a single methyl group (4-MPR) results in a complete loss of NS5:importin-binding inhibition, as well as antiviral activity. Furthermore, the parent compound of 4-HPR, ATRA, a pan-RAR agonist, does not inhibit DENV replication, consistent with the idea that the anti-DENV activity of 4-HPR is RAR independent [44]. Similarly, inhibition of DEGS-1, a key enzyme in ceramide synthesis [33], is unlikely to be the basis of 4-HPR antiviral action, since other ceramide synthesis inhibitors fail to impair DENV replication. In contrast, our results suggest an intriguing link between the antiviral action of 4-HPR and activation of the PERK arm of the UPR pathway.

4-HPR has been studied for its antitumor and chemopreventive properties, including in phase III clinical trials [45,46], with specific removal of cancerous cells dependent on induction of apoptosis by 4-HPR [36]. Here, we show for the first time that addition of 4-HPR to DENV-infected cells induces activation of the proapoptotic PERK arm of the UPR but does not result in cell death at concentrations that effectively remove the virus. DENV infection has previously been reported to activate all 3 arms of the UPR [17,38,47]. Interestingly, replication of DENV and WNV<sub>KUN</sub> is increased in PERK<sup>-/-</sup> mouse embryonic fibroblasts [47,48], while Salubrinal (which inhibits the GADD34-containing phosphatase complex, inducing translation attenuation) restricts DENV replication. Together with our findings here, this implies that activation of PERK may facilitate host responses against DENV and also WNV<sub>KUN</sub>, leading to a potent antiviral state. Since DENV disease pathology is in part due to an overactive inflammatory response [49], it is tempting to speculate that 4-HPR modulation of the UPR may lead to a rebalancing of cytokine levels to promote viral clearance. Consistent with this, cytokine levels in 4-HPR-treated mice are decreased overall relative to the infection control group. Future experiments in the mouse model will assess the role of cytokines in DENV pathology, in parallel with analysis of the stress pathways activated upon 4-HPR addition.

We identified 4-HPR as an inhibitor of DENV-2 NS5 association with the host nuclear transport proteins IMP- $\alpha$ / $\beta$ 1, and it is striking that the EC<sub>50</sub> values for 4-HPR in our cell culture and ex vivo infection experiments (range, 0.8–2.6  $\mu$ M) are essentially identical to the IC<sub>50</sub> for inhibition of NS5-IMP- $\alpha$ / $\beta$ 1 binding in vitro (1.2  $\mu$ M), consistent with the target for 4-HPR being the same. Because of the potent antiviral activity of 4-HPR, we were unable to show this directly and so cannot formally exclude the possibility that 4-HPR may interfere with the nuclear trafficking of another factor, such as one of the many transcription factors involved in UPR activation, or indeed other cellular targets, to modulate the cellular effects observed here.

Importantly, others have shown 4-HPR to be well tolerated in children, with plasma concentrations of up to approximately 21

$\mu$ M reported [50], well above the maximal EC<sub>50</sub> of 2.6  $\mu$ M determined here for DENV-1 in cell culture. This, coupled with its clear efficacy in our lethal mouse model, underline the great potential of 4-HPR as a promising anti-DENV agent, including as a therapeutic, showing activity when applied after infection. The focus of future work in this laboratory will thus be to progress characterization of the antiviral activity of 4-HPR toward a phase 1b, double-blind, placebo-controlled study to determine the efficacy of 4-HPR in DENV-infected patients.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

**Acknowledgments.** We thank Chandrakala Baswanacharya, for performing the polymerase assay; Steven Heaton, for assistance in establishing the robotic compound screen; Cassandra David, for cell culture assistance; Dr Sujan Shrestha (La Jolla Institute for Allergy and Immunology, CA), for providing the mouse-adapted strain of DENV-2 (S221); and Monash Micro Imaging Facility for use of their microscopes.

**Financial support.** This work was supported by the National Health and Medical Research Council Australia (project grants 606409 and APP59137, senior principal research fellowship APP1002486, and principal research fellowship APP1059015); the Victorian Infection and Immunity Network Industry Alliance's Collaborative, State Government of Victoria's Collaborative Networks Pilot Program (project grant), the Duke-NUS Signature Research Program, Singapore Ministry of Health; and the Singapore National Medical Research Council (NMRC/1315/2011).

**Potential conflicts of interest.** All authors: No potential conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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