Research Square

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4’ Fluoro Uridine is a Broadly Active Inhibitor of Aedes Mosquito- Transmitted Flavivirus Replication

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Research Article

Keywords: flavivirus, dengue, yellow fever, Zika, inhibitor, RNA polymerase

Posted Date: November 27th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11815606/v1

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Additional Declarations: Competing interest reported. The authors declare that they have no competing financial interests; however, SFM serves as an editorial board member of Virology Journal.

Abstract

There are currently no approved inhibitors active against any of the mosquito-transmitted flaviviruses, despite this group containing globally important human pathogens. Here we show inhibition of dengue 1–4, Zika and yellow fever viruses with the nucleoside analog 4'-fluoro uridine, using a quantitative reverse transcriptase PCR viral replication assay system. 4'-fluoro uridine has previously been shown to have strong antiviral activity against a large number of other positive and negative sense RNA viruses, including respiratory syncytial virus, SARS CoV-2, Influenza A viruses, several types of arena and bunyaviruses, as well as chikungunya virus, Venezuelan equine encephalitis virus, Sindbis virus, human parainfluenza virus and vesicular stomatitis virus. All flaviviruses tested showed dose-responsive replication inhibition from 4'-fluoro uridine treatment, with tissue culture IC50s ranging from 0.42 to 8.76 μM. This range of sensitivity does not appear to correlate with any sequence differences in the highly conserved NS5 viral polymerase and may be due to structural differences in other viral genes such as the NS3 helicase or the viral RNA genome itself that are part of the viral replication complex.

Introduction

4'-fluoro uridine (4'-FlU) is a fluorinated nucleoside analog that is converted to the triphosphate through cellular kinase pathways and can be incorporated by viral RNA polymerases. It was originally developed and patented in 2015 against hepatitis C virus but was ultimately not advanced further due to the development of alternate inhibitors. More recently, 4'-FlU has been shown to have strong antiviral activity against a large number of other positive and negative sense RNA viruses, including respiratory syncytial virus and SARS CoV-2 [1], Influenza A viruses [2, 3], several types of arena and bunyaviruses [4–6] as well as chikungunya virus [7], Venezuelan equine encephalitis virus, Sindbis virus, human parainfluenza virus and vesicular stomatitis virus [8]. A recent review of antivirals under development characterizes 4'-FlU as "remarkably broad spectrum" [9]. Multiple *in vivo* studies have shown good oral bioavailability of 4'-FlU with low toxicity in non-human models including mice, ferrets, guinea pigs and African green monkeys [1–7]. Importantly, delayed *in vivo* treatment has been documented as curative in several model systems [4, 5]. The 3' position of 4'-FlU is an unaltered hydroxyl group, meaning that the mechanism of action is distinct from nucleoside mimics that cause chain termination by preventing the addition of further nucleotide bases to the growing RNA chain [10]. Instead, 4'-FlU appears to become incorporated into the growing RNA chain and functions by causing steric hindrance in the polymerized RNA chain, resulting in either immediate or delayed polymerase stalling, depending on the sequence [1, 2]. In this way, 4'-FlU may not be a direct inhibitor of the viral polymerase, but rather an inhibitor of the structural flexibility of the RNA replication transcript, with important consequences for the development of viral resistance.

One group of positive sense RNA viruses that has not been well-investigated as targets for 4'-FlU are the *Aedes* mosquito-transmitted flaviviruses. These viruses include important human pathogens such as the four distinct serotypes of dengue virus (DENV 1–4), yellow fever virus (YFV) and Zika virus (ZIKV), each of which have caused both historic and recent large outbreaks and have no clinically approved inhibitors [11, 12]. A recent publication has described inhibitory activity of 4'-FlU against a single DENV serotype,

indicating that these viruses may be sensitive to 4'-FlU [8]. Here we show the broad *in vitro* inhibitory activity of 4'-FlU against DENV 1–4, YFV, and ZIKV using a quantitative reverse transcriptase PCR (qRT-PCR) replication assay system, directly measuring viral RNA levels in infected cells and discuss variability in viral sensitivity and implications for the potential mechanism of action.

Materials and methods

Compound storage and handling

4'-C-fluoro-uridine (4'-FlU) was purchased (Cayman Chemical Inc., Ann Arbor, MI) as a solid. Single-use aliquots were weighed out and stored at the recommended temperature of -20°C. When needed aliquots were thawed and resuspended in serum-free media for same day assay use and used within six hours of preparation.

Cell Lines

Vero CCL-81 (ATCC) were grown in Eagle's Minimum Essential Medium (EMEM) (American Type Culture Collection, Manassas, VA) supplemented with 10% (v/v) heat-inactivated, fetal bovine serum (HI-FBS), 2mM GlutaMax, 100 $U{\cdot}mL^{-1}$ penicillin G, 100 $\mu g{\cdot}mL^{-1}$ streptomycin, and 0.25 $\mu g{\cdot}mL^{-1}$ amphotericin B at 37°C, 5% (v/v) CO_2 with relative humidity. LLC-MK2 CCL-7 (ATCC) were grown in Dulbecco's Modified Eagle Medium (DMEM) (American Type Culture Collection, Manassas, VA) supplemented with 10% (v/v) heat-inactivated, fetal bovine serum (HI-FBS), 2mM GlutaMax, 100 $U{\cdot}mL^{-1}$ penicillin G, 100 $\mu g{\cdot}mL^{-1}$ streptomycin, and 0.25 $\mu g{\cdot}\ mL^{-1}$ amphotericin B at 37°C, 5% (v/v) CO_2 with relative humidity. All reagents were purchased from Thermo Fisher Scientific, Waltham, MA, unless noted otherwise.

Virus Stocks

DENV1 strain HI-1(GenBank: KM204119.1), DENV2 strain New Guinea-2 (GenBank: MK506264.1), DENV3 strain H-87 (GenBank: KU050695.1), DENV4 strain H-42 and ZIKV strain MR766 (GenBank: MW143022.1) were provided by Robert B. Tesh from the University of Texas Medical Branch Galveston through the World Reference Center for Emerging Viruses and Arboviruses. ZIKV strain PRVABC59 (GenBank: KU501215.1) was provided by the Centers for Disease Control and Prevention, Arbovirus Branch. YFV-17D construct (GenBank: JX949181.1), also known as clone pACNR-17D, was provided by Charles Rice at the Rockefeller Institute [13]. Virus stocks were propagated and titered for concentration by plaque-assay in both LLC-MK2 and Vero cell lines.

Plaque assay

To determine titer for each virus, plaque assays were conducted as previously described [14, 15]. Briefly, a monolayer of cells was plated to be 100% confluent in 24–48 hours. A serial dilution of virus was made in serum-free medium, plated on cells in technical replicates, and allowed to infect cells for one hour at 37°C with 5% (v/v) CO_2 and relative humidity. After one hour, inoculum was removed and cells overlaid with 1.2% (v/v) microcrystalline cellulose (AVICEL, FMC Biopolymer, Philadelphia, PA) and EMEM 2X supplemented with 10% (v/v) heat-inactivated, fetal bovine serum, 2mM GlutaMax, 100 $U \cdot mL^{-1}$ penicillin G, 100 $\mu g \cdot mL^{-1}$ streptomycin, and 0.25 $\mu g \cdot mL^{-1}$ amphotericin B. Infected cells were incubated for 2–3 days at 37°C with 5% (v/v) CO_2 and relative humidity. Overlay of complete media and microcrystalline cellulose was removed, and cells were fixed in Formalde-Fresh Solution. Virus was stained using anti-DENV, human IgG monoclonal antibodies, previously described [16], followed by secondary anti-human IgG (H + L) horse-radish peroxidase conjugated antibodies (Pierce, Rockford, IL). Plaque forming units were developed using 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St Louis, MO).

Inhibition assay

Vero cells were plated 24 hours ahead of time, approximately 6.85 x 10^4 cells per well in 24 well plates. Uniformity of monolayers and confluency were assessed the day of assay performance for multiplicity of infection (MOI) calculations. 4'-FlU was resuspended in serum-free EMEM and measured using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific). Absorbance values and extinction coefficient (9,660 $M \cdot cm^{-1}$) were used to calculate exact concentration. A stock concentration was further prepared from the resuspension and filtered through 0.2μM EZFlow PES syringe filter (Foxx Life Sciences, Londonderry, NH) to sterilize before application with cell culture. Stock compound was mixed with complete EMEM and then virus added at an MOI of 0.01 or 0.001 to achieve the final 4'-FlU compound concentrations desired (0, 0.1, 1, 10 and 100μM). Mixtures were added to cells immediately with compound concentrations tested in triplicate. Treated cells were incubated at 37°C with 5% CO_2. DENV 1–4 and YFV-17D were used at MOI of 0.01 and incubated less than 72 hours. ZIKV PRVABC59 at MOI of 0.01 and ZIKV MR766 at MOI of 0.001 were incubated less than 48 hours. Cell lysate was collected for analysis according to RNeasy Mini kit (Qiagen, Hilden, Germany) recommendations and stored at -80°C until ready for further processing.

RNA extraction

Cell lysate from inhibition assays was used as the measure of virus replication and inhibition for this study, as replication is dependent on host-cell function. RNeasy Mini Kit (Qiagen) was used to process cell lysates according to the manufacturer's protocol. RNA was double eluted in 45μL volumes of nuclease-free water. Supernatant from propagated virus stocks was extracted and used in conjunction with plaque-assay titers in order to create standard RNA stock aliquots, with known plaque-forming units, to generate standard curves for qRT-PCR quantification. Viral supernatant was processed by QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer's protocol and eluted in nuclease-free water.

qRT-PCR standard curves

Virus standards used to generate a curve and quantify samples were generated from tissue culture. Viral supernatants were assayed in cell monolayers to determine infectious titer, and the same supernatant was extracted for viral RNA (QIAamp Viral RNA, Qiagen) content. Serial dilutions of extract were evaluated for R^2 value and slope to create optimal curves for qRT-PCR application. The infectious particle titer calculated from plaque assays was then applied to viral RNA extraction volumes to determine infectious particle concentrations. We recognize that viral, genomic RNA and infectious particles are not identical, but reliably follow the same patterns under treatment conditions [14, 15].

qRT-PCR quantification and analyses iTaq Universal SYBR Green One-Step Kit (Bio-Rad Laboratories Inc., Hercules, CA) was used to amplify all viral RNA targets and qRT-PCR was conducted using a C1000 Touch Thermal Cycler chassis with CFX96 Real-Time System module (Bio-Rad Laboratories Inc., Hercules, CA). Universal DENV primer set was used for the amplification of DENV1-4 RNA. Briefly, primer (Integrated DNA Technologies, Coralville, IA) sequences include Forward-F10503 (5'-TTA GAG GAG ACC CCT CCC-3') and Reverse-R10599 (5'-TCT CCT CTA ACC TCT AGT CC-3') and target the 3'-UTR of dengue to create unique amplicon products for each serotype (94bp for DENV1, 97bp for DENV2, 95bp for DENV3, 102bp for DENV4) [17]. DENV qRT-PCR parameters include an initial reverse transcription (RT) at 50°C for 10min, initial inactivation of RT and activation of DNA dependent DNA polymerase at 95°C for 1min, followed by 45 total cycles - denaturation at 95°C for 10sec, annealing at 58°C for 15sec and extension at 60°C for 15sec with final melt curve application running from 65°C to 95°C in increments of 0.5°C for 5sec each.

YFV-17D primer sequences include Forward (5'-GAG GAG GAG TGG AAG GCA TTG GC-3') and Reverse (5'-CCA CCA CCA TCC ATT GCA GCC-3') targeting the NS5 region to create a 76 bp product. YFV-17D PCR parameters include an initial RT at 50°C for 10min, initial inactivation of RT and activation of polymerase at 95°C for 1min, followed by total of 45 cycles - denaturation at 95°C for 10sec and combined annealing and extension steps at 60°C for 30sec with final melt curve application running from 65°C to 95°C in increments of 0.5°C for 5sec each.

ZIKV primer sequences include a shared forward, Forward (5'-CTG CTG GCT GGG ACA CCC GC-3') paired with strain-specific reverse, Reverse-MR766 (5'-CGG CCA ACG CCA GAG TTC TGT GC-3') or Reverse-PRVABC59 (5'-GGC CAA TGC CAA GGC CC-3') targeting the NS5 region, creating a 99bp amplicon for strain MR766 and a 98bp amplicon for strain PRVABC59 [15]. ZIKV qRT-PCR parameters include an initial RT at 50°C for 10min, initial inactivation of RT and activation of polymerase at 95°C for 1min, followed by 40 total cycles - denaturation at 95°C for 10sec, annealing at 55°C for 15sec and extension at 60°C for 15sec with final melt curve application running from 65°C to 95°C in increments of 0.5°C for 5sec each.

Negative controls were included with each virus qRT-PCR experiment to ensure no cross-contamination or background noise: Qiagen RNeasy kit's RLT buffer extract, no-virus cells-only lysate extract, PCR kit-included water. Melt curves were analyzed to ensure the correct viral target amplification.

Statistics, Analysis and Data Presentation

Microsoft Excel was used to graph preliminary data and run simple statistics (average, standard deviation) and normalize qRT-PCR copy numbers to percent inhibition. Prism (GraphPad Software, Boston, MA) was used to calculate IC50 values and standard deviations using Nonlinear Regression – Variable Slope (four parameter) analysis. Prism was used to generate final graph figures and picture statistics.

Results

Lack of 4'-FlU toxicity below 500μM in Vero cells has been previously reported [6]. Additionally, lack of toxicity below 100μM was shown for A549, Huh-7, and BHK-21 cells [6] and below 200μM for HEp-2, MDCK, BHK-T7, and BEAS-2B cells [1]. We observed no signs of cell toxicity during treatment of Vero cells with 4'-FlU up to 100μM, the maximum concentration used. In agreement with previous studies on multiple other RNA viruses, we observed inhibition against all flaviviruses tested (Fig. 1). The most sensitive virus was DENV-2 NGC with an IC50 of 0.42 ± 0.16 μM. The majority of other tested viruses showed IC50 values slightly over 1 μM: YFV-17D IC50 1.23 ± 0.32 μM, DENV-3 H87 IC50 2.15 ± 0.14 μM, ZIKV MR766 (old world) IC50 2.34 ± 0.26 μM, DENV-4 H42 IC50 3.56 ± 0.67 μM. Two viruses showed inhibition at somewhat higher concentrations: ZIKV PRVABC59 (new world) IC50 8.00 ± 0.15 μM and DENV-1 HI1 IC50 8.76 ± 1.47 μM. Viral inhibition of 90% or greater was seen at 10μM concentration for DENV2-NGC, DENV3-H87, YFV-17D, and ZIKV MR766. At 10μM ZIKV PRVABC59 showed 84% inhibition but required 100μM to reach 99%. At 10μM DENV4-H42 showed 71% inhibition but required 100μM to achieve 99%. The least sensitive virus, DENV1-HI1 was inhibited by 82% at 100μM.

Comparing our inhibition results for DENV-2 NGC (IC50 of 0.42 ± 0.16 μM) with the previous report [8] from Wong et al. DENV-2 (IC50 of 2.7 μM), shows a substantial difference in sensitivity. However, Wong et al. used a DENV luciferase reporter construct in an infectivity assay while we used a qRT-PCR assay to measure viral transcripts in infected cells, which we feel is more appropriate for measuring the activity of a replication inhibitor. Further, Wong et al. did not provide any details of the DENV-2 strain used in their assay or provide any measure of error in their IC50 determination [8].

Comparison of the inhibition sensitivities between viruses in this study showed a roughly 20-fold difference between the most and least sensitive viruses that is difficult to explain by experimental variability. We considered if time from infection to RNA harvest could be an important variable. However, all of the DENV strains were harvested at 72 hours and both ZIKV strains were harvested at 48 hours, while some of the largest differences in sensitivity were between the different DENV strains as well as between the two ZIKV strains. Flavivirus RNA polymerase proteins are highly conserved and show no obvious differences between highly sensitive and less sensitive viruses. This suggests the possibility that differences in sensitivity may potentially be linked to other viral replication proteins or secondary structure of the viral RNA genome.

Discussion

The results shown here extend the remarkably broad-spectrum inhibition of RNA viruses by 4'-FlU to include members of the *Aedes* mosquito-transmitted flaviviruses, including the four serotypes of DENV, YFV and ZIKV. We did not test other clinically important flaviviruses, such as West Nile virus, but we expect, based on the breadth of 4'-FlU activity and the conservation of polymerase proteins and replication strategy that West Nile, Saint Louis encephalitis and other flaviviruses may be sensitive as well. We tested only the 17D vaccine strain of YFV rather than a pathogenic isolate, so we cannot make any claims beyond the vaccine strain. We used a qRT-PCR viral replication assay system, directly measuring viral RNA levels in infected cells, rather than a plaque/infectivity assay to avoid potential artifacts related to entry and exit stages of the viral lifecycle. In these assays, 4'-FlU was added at the same time as infection occurred, so we have no information on inhibition when treatment is delayed, although in other viral systems delayed treatment with 4'-FlU can still be effective [4, 5]. As the assays reported here were conducted in cell culture, we have no evidence if 4'-FlU might be a useful inhibitor in DENV, YFV or ZIKV animal models or in human patients. However, the sensitivities for most of the viruses tested here are in the range of concentrations achieved *in vivo* in other non-human 4'-FlU viral inhibition model systems [1–7].

While all of the flaviviruses tested were sensitive at some level to treatment with 4'-FlU, the different inhibition curves and IC50 values warrant further discussion. The most sensitive virus, DENV-2 NGC with an IC50 of 0.42 ± 0.16 μM was 20 times more sensitive than the least sensitive virus, DENV-1 HI1 with an IC50 of 8.76 ± 1.47 μM. This level of difference is difficult to explain by experimental variability and likely has an underlying biological mechanism. However, examination of the viral NS5 polymerase protein sequence shows a high level of conservation that does not correlate with sensitivity. Among the viruses tested, the two most divergent NS5 polymerases, DENV-2 NGC and YFV-17D, share 60% amino acid identity and show the highest sensitivity to 4'-FlU. On the other hand, the most sensitive, DENV-2 NGC, and least sensitive, DENV-1 HI1, viruses have NS5 polymerases that share 80% amino acid identity. There are no single amino acid changes in NS5 that appear to explain the differences in sensitivity, suggesting that there may be critical determinants of flavivirus 4'-FlU sensitivity that lie outside of the NS5 protein, possibly in the associated NS3 helicase or in the structure of the RNA genome. Cryo-electron microscopy shows the close association of DENV NS5, NS3 and the RNA stem-loop A region at the 5' beginning of the viral genome in the replication complex [18]. When 4'-FlU is incorporated, the 4' F substitution causes a sugar pucker, constraining the structure of the ribose sugar, leading to changes in base pairing stability with the replicating genomic RNA [19], potentially causing steric hindrance in the nascent RNA and altering the formation and function of the NS5/NS3/RNA complex.

Resistance studies in other viral systems are directly relevant to the discussion of the mechanism of 4'-FlU inhibition. Wong et al. found 4'-FlU resistance-associated mutations in the finger region of the Venezuelan equine encephalitis virus nsP4 polymerase [8] and Yin et al. found resistance-associated mutations in both the finger and palm regions of the chikungunya virus nsP4 polymerase [7]. However, in a study of 4'-FlU and influenza virus, resistance-associated mutations were found not only in the PB1

(polymerase) but also in the PB2 (methyl transferase) and the PA (endonuclease) proteins [2], supporting the idea that the mechanism of inhibition involves the quaternary structure of the entire multi-protein and RNA replication complex. Interestingly, none of these studies investigating 4'-FlU resistance in different virus systems found high level resistance, and all resistant phenotypes showed attenuated pathogenicity. Given that the mechanism of 4'-FlU inhibition may involve oligomeric replication complexes consisting of multiple protein and RNA units, this may be an example of a dominant-negative interaction where the presence of even a small percentage of sensitive (wild type) components renders resistant variant components sensitive as well, suppressing the development of high-level resistance [20]. Additionally, resistance mutations in vector-borne viruses may be further constrained by selection during replication in the invertebrate vector [21–23].

Abbreviations

4'-fluoro uridine (4'-FlU), dengue virus (DENV 1-4), yellow fever virus (YFV), Zika virus (ZIKV), Eagle's Minimum Essential Medium (EMEM), Dulbecco's Modified Eagle Medium (DMEM), multiplicity of infection (MOI), reverse transcription (RT), polymerase chain reaction (PCR)

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing financial interests; however, SFM serves as an editorial board member of Virology Journal.

Funding

LMP and SFM were supported by National Institute of General Medical Sciences (R15GM153057).

Authors' contributions

LMP and SFM conceived the study and designed the experimental approach. LMP conducted the

experiments, performed data analysis and statistical evaluations. Both authors wrote, edited, and approved the final manuscript.

Acknowledgements

We would like to thank Yancey Hrobowski for useful discussions.

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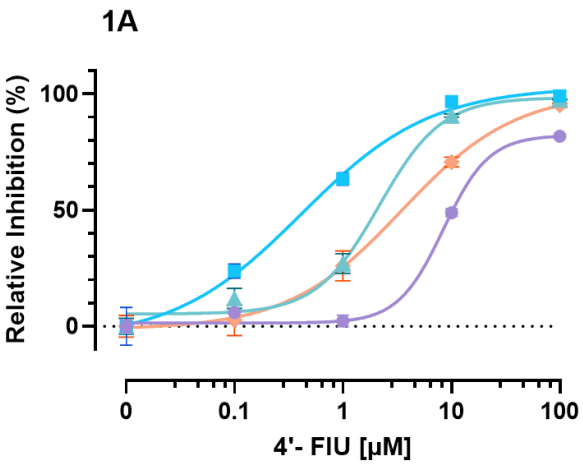
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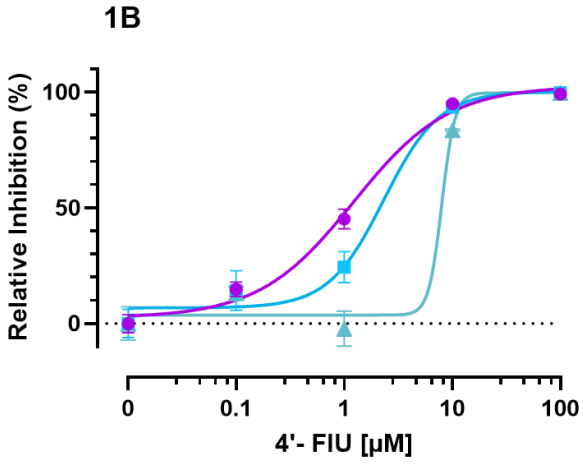
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Figures



Flavivirus	IC_{50} ± SD [μM]
DENV1-HI1	8.76 ± 1.47
DENV2-NGC	0.42 ± 0.16
DENV3-H87	2.15 ± 0.14
DENV4-H42	3.56 ± 0.67



Flavivirus	IC_{50} ± SD [μM]
YFV-17D	1.23 ± 0.32
ZIKV MR766	2.34 ± 0.26
ZIKV PRVABC59	8.00 ± 0.15

Figure 1

Dose response inhibition curves. Percent inhibition of **(A)**DENV serotypes and **(B)** YFV and ZIKV flaviviruses with IC50 value ± standard deviation. IC50 values and standard deviations were calculated using Nonlinear Regression – Variable Slope (four parameter) analysis.