

Flaviviruses Are Sensitive to Inhibition of Thymidine Synthesis Pathways

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Dengue virus has emerged as a global health threat to over one-third of humankind. As a positive-strand RNA virus, dengue virus relies on the host cell metabolism for its translation, replication, and egress. Therefore, a better understanding of the host cell metabolic pathways required for dengue virus infection offers the opportunity to develop new approaches for therapeutic intervention. In a recently described screen of known drugs and bioactive molecules, we observed that methotrexate and floxuridine inhibited dengue virus infections at low micromolar concentrations. Here, we demonstrate that all serotypes of dengue virus, as well as West Nile virus, are highly sensitive to both methotrexate and floxuridine, whereas other RNA viruses (Sindbis virus and vesicular stomatitis virus) are not. Interestingly, flavivirus replication was restored by folinic acid, a thymidine precursor, in the presence of methotrexate and by thymidine in the presence of floxuridine, suggesting an unexpected role for thymidine in flavivirus replication. Since thymidine is not incorporated into RNA genomes, it is likely that increased thymidine production is indirectly involved in flavivirus replication. A possible mechanism is suggested by the finding that p53 inhibition restored dengue virus replication in the presence of floxuridine, consistent with thymidine-less stress triggering p53-mediated antiviral effects in infected cells. Our data reveal thymidine synthesis pathways as new and unexpected therapeutic targets for antiviral drug development.

Dengue virus (DENV) poses a significant human health risk for approximately 40% of the world's population (<http://www.who.int/mediacentre/factsheets/fs117/en/>). DENV occurs as four serotypes, with increasing and widespread circulation of all serotypes considered a contributing factor for the rising incidence of dengue disease. A particularly severe complication is dengue hemorrhagic fever (DHF), which results in approximately 22,000 deaths annually and of which the incidence is currently on the rise (<http://www.who.int/csr/disease/dengue/impact/en/index.html>). However, even nonfatal, self-limiting DENV infection can result in a severe, painful disease that is also known as “break-bone fever.” No vaccine or antiviral therapeutics are licensed to address DENV infections, and treatment is currently limited to supportive care.

Many studies are ongoing with the hopes of identifying effective novel anti-DENV therapeutics and/or potential therapeutic targets. Several viral factors have been explored as therapeutic targets, including the viral protease, helicase, and RNA-dependent RNA polymerase (reviewed in reference 1). As an alternative to directly targeting viral proteins, it is conceivable to target host cell metabolic pathways that are required for viral replication. Flaviviruses, small RNA viruses that include DENV as well as West Nile virus (WNV), yellow fever virus, and Japanese encephalitis virus, are highly dependent on the host cell to provide support for viral entry, replication, assembly, and egress. Examples of such support include host cholesterol biosynthesis, required for flavivirus entry, and the host kinases c-Src and c-Yes, required for DENV and WNV maturation and egress, respectively (2–6). Three DENV-specific therapeutics are currently in clinical trials (clinicaltrials.gov): celgosivir, which interferes with the folding of DENV NS1 protein and triggers the host unfolded protein response (7), and chloroquine and anti-Rh₀-D antibodies, which block viral entry and increase platelet counts, respectively, during DHF (8, 9). Of note, all three therapeutics affect host cell processes and are not directed solely against viral factors.

To identify additional host cell pathways that are required for DENV replication, we previously described a high-content cell-based screen of a library of bioactive small molecules, including established drugs (10). Here, we further characterize two of the most effective small-molecule inhibitors of DENV identified in that screen, the antimetabolites methotrexate (MTX) and floxuridine (FUDR). We demonstrate that these compounds inhibit the replication of multiple flaviviruses in multiple cell types. Our results reveal an unanticipated sensitivity of flaviviruses to the inhibition of thymidine synthesis, suggesting that these cellular pathways contain viable targets for the development of antivirals that interfere with host-virus interactions.

MATERIALS AND METHODS

Cells and viruses. Low-passage-number HEK293 cells were obtained from Microbix, Vero and HeLa cells were obtained from the ATCC, Huh7 cells were obtained from F. Chisari (Scripps Research Institute), and the BHK-DENV replicon was obtained from M. Diamond (Washington University, St. Louis, MO). HEK293 cells were cultured in minimal essential medium Eagle (Cellgro) supplemented with nonessential amino acids, L-glutamine, 10% heat-inactivated fetal bovine serum (FBS), and antibiotics (100 units/ml penicillin and 100 g/ml streptomycin). All other cell types described were cultured in Dulbecco's modification of Eagle's medium (Cellgro) supplemented with 10% heat-inactivated FBS and antibiotics (100 units/ml penicillin and 100 g/ml streptomycin).

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DENV2 (strain New Guinea C), DENV3 (strain H87), DENV4 (strain H241), and Sindbis virus (strain AR-339) were obtained from the ATCC. DENV1 (strain West-Pac) was obtained from R. Putnak (Walter Reed Army Institute of Research). DENV2 (strain S221) was obtained from S. Shresta (La Jolla Institute). All DENV serotypes and strains as well as WNV (strain 385-99) (11) were grown, and titers were determined, as previously described (10). Herpes simplex virus 1 (HSV-1; strain F1) was obtained from A. Hill (Oregon Health and Science University). Vaccinia virus (VACV; strain Western Reserve) was obtained from M. Slifka (Oregon Health and Science University). Vesicular stomatitis virus (VSV; strain Indiana) was obtained from J. Hiscott (VGTI Florida) (12).

Immunofluorescence assay (IFA). HEK293 cells were infected with DENV2 at a multiplicity of infection (MOI) of 0.5 PFU/cell in cell medium containing 10 μ M MTX, 10 μ M FUDR, or 1% dimethyl sulfoxide (DMSO) (vol/vol). Cells were fixed 48 h postinfection (p.i.) and stained with a monoclonal antibody specific for DENV/WNV envelope protein (clone 4G2; ATCC), an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Santa Cruz), and 4',6-diamidino-2-phenylindole (DAPI).

Dose-response curves. HEK293 cells were infected with DENV2 at an MOI of 3 PFU/cell in cell medium containing increasing concentrations of MTX or FUDR (in a constant 1% DMSO). Supernatants were collected at 48 h p.i., and titers were determined on Vero cells. Fifty percent inhibitory concentration (IC₅₀) values were calculated using Prism software (GraphPad).

Cell viability. HEK293 cells were incubated in cell medium containing increasing concentrations of MTX or FUDR in a constant 1% (vol/vol) DMSO. After 48 h, Cell Titer Glo reagent (Promega) was added and luminescence was measured per the manufacturer's instructions. Fifty percent cytotoxic concentration (CC₅₀) values were calculated using Prism software. In some experiments (where indicated), Cell Titer Fluor reagent (Promega) was substituted for Cell Titer Glo to measure cell viability.

Huh7, Vero, and HeLa infections. Cells were infected with DENV2 at an MOI of 3 PFU/cell in cell medium containing 10 μ M MTX, 10 μ M FUDR, or 1% (vol/vol) DMSO as a control. Supernatants were collected at 48 h postinfection, and titers were determined on Vero cells.

Flavivirus multistep growth curves. HEK293 cells were infected with DENV or WNV at an MOI of 0.1 PFU/cell in cell medium containing 1 μ M MTX, 1 μ M FUDR, or 1% (vol/vol) DMSO as a control. Supernatants were collected at indicated times postinfection, and titers were determined on Vero cells.

HSV-1, vaccinia virus, Sindbis virus, and VSV infections. HEK293 cells were infected with each virus at an MOI of 3 PFU/cell in cell medium containing increasing concentrations of MTX or FUDR in a constant 1% (vol/vol) DMSO. Supernatants were collected at 24 h (Sindbis virus and VSV) or 48 h (HSV-1 and VACV) postinfection and assayed for infectious virus by a standard plaque assay on Vero cells.

DENV replicon experiments. BHK cells harboring a DENV replicon expressing a *Renilla* luciferase reporter were treated with increasing concentrations of ribavirin, MTX, or FUDR (or DMSO as a negative control). At 48 h posttreatment, cells were lysed and *Renilla* luciferase activity was measured using the dual-luciferase reporter assay (Promega).

Folinic acid treatment. HEK293 cells were infected with DENV2 at an MOI of 3 PFU/cell in cell medium containing 1 μ M MTX and increasing concentrations of folinic acid (leucovorin). Supernatants were collected, and infectious virus was quantified as described above.

Thymidine/uridine treatment. HEK293 cells were infected with DENV2 at an MOI of 3 PFU/cell in the presence of 1 μ M FUDR and increasing concentrations of uridine or thymidine. Supernatants were collected at 48 h p.i., and titers were determined on Vero cells.

Pifithrin- α treatment. HEK293 cells were infected with DENV2 at an MOI of 3 PFU/cell in the presence of 1 μ M FUDR and/or 10 μ M pifithrin- α . Supernatants were collected at 48 h p.i., and titers were determined on Vero cells.

Mouse studies. All mouse studies were approved by the OHSU West Campus Institutional Animal Use and Care Committee. The AG129 mouse model of DENV infection has been previously characterized (13). AG129 mice were obtained from M. Slifka (Oregon Health and Science University) and bred at the Vaccine and Gene Therapy Institute. Subcutaneous osmotic pumps (Alzet) were implanted into the mice to deliver MTX, FUDR, or 50% (vol/vol) DMSO vehicle alone at a constant rate. Mice were infected intraperitoneally (i.p.) with the S221 strain of DENV2. C57BL/6 mice, purchased from Jackson Laboratories, were utilized as a mouse model of WNV infection. Mice were injected i.p. with MTX, FUDR, or 10% (vol/vol) DMSO vehicle alone and then infected subcutaneously (s.c.) with WNV at a 90% lethal dose (LD₉₀) 24 h later. Mice received additional injections of drugs or the vehicle control 24 h p.i. and then every 48 h thereafter.

RESULTS

MTX and FUDR inhibit DENV replication. In the high-throughput screen previously described (10), as well as in confirmatory experiments in a 96-well plate format, immunofluorescent detection of DENV envelope protein was used to assess virus replication. At a concentration of 10 μ M, both MTX and FUDR strongly inhibited the accumulation of DENV envelope protein in infected cells at 48 h p.i. (Fig. 1A). Both MTX and FUDR inhibited DENV replication in a dose-dependent manner with IC₅₀ values of 0.09 and 0.06 μ M, respectively (Table 1). We further confirmed the inhibitory effects of MTX and FUDR by performing a dose-response experiment on HEK293 cells and quantifying virus released into the supernatant 48 h p.i. (Fig. 1B). Using this assay, viral release into the supernatant was reduced by approximately 3 logs at concentrations above 2 μ M with an IC₅₀ of 0.17 μ M for MTX and 0.22 μ M for FUDR (Table 1).

At 10 μ M, the concentration used in the primary screen, little reduction of cell nuclei was observed (Fig. 1A), suggesting that the anti-DENV effect was not due to cytotoxicity. In addition, effects of the compounds on cell viability were determined using Cell Titer Glo (Promega) (Fig. 1C). While the CC₅₀ value of FUDR was more than 100 μ M, the CC₅₀ value for MTX was 12.42 μ M. For both compounds, the IC₅₀ values were thus at least 10-fold lower than the CC₅₀, indicating a therapeutic index within a range appropriate for further consideration.

DENV inhibition is cell type independent. Every immortalized cell line possesses its own physiological properties, with factors such as species origin, cell lineage, and immortalization method contributing to potential differences. To determine whether the anti-DENV2 properties of MTX and FUDR were confined to the HEK293 cells used in our screen, we tested the molecules in several different cell lines. As with HEK293 cells, both MTX and FUDR reduced the viral titer in supernatants of infected Huh7 (human hepatocarcinoma) and Vero (African green monkey kidney epithelial) cells at 48 h p.i. (Fig. 2A).

The CC₅₀ values of MTX and FUDR were calculated for Huh7 and Vero cells using the Cell Titer Fluor reagent (Fig. 2B and C; Table 2). As with HEK293 cells, the IC₅₀ values were approximately 10-fold or more lower than the CC₅₀ (Table 2).

MTX and FUDR are effective against multiple flaviviruses and DNA viruses but not against all RNA viruses. Numerous flaviviruses, including strains from all four serotypes of DENV, are capable of causing severe human disease. To determine if MTX and/or FUDR would inhibit multiple members of the flavivirus family, we conducted a multistep growth curve analysis of the replication of DENV1, DENV2, DENV3, DENV4, and WNV in

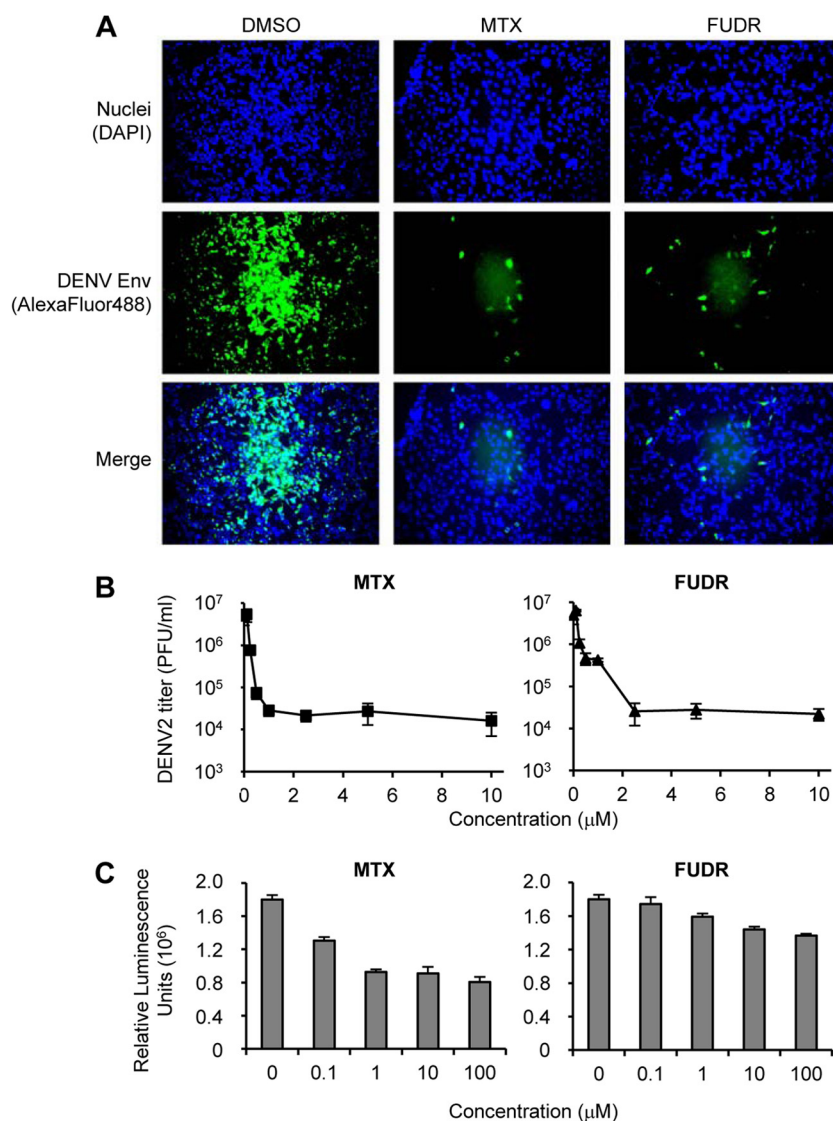


FIG 1 Characterization of the anti-DENV efficacy of MTX and FUDR. (A) HEK293 cells were treated with DMSO only, 10 µM MTX, or 10 µM FUDR and infected with DENV2 at an MOI of 0.5 PFU/cell. Cells were analyzed 48 h p.i. by immunofluorescence for the presence of DENV-Env protein. (B) HEK293 cells were treated with increasing doses of MTX or FUDR as indicated and infected with DENV at an MOI of 3 PFU/cell. Supernatant was collected 48 h p.i., and the titers of infectious virions were determined. (C) HEK293 cells were treated with increasing doses of MTX or FUDR as indicated, and cell viability was assessed after 48 h using Cell Titer Glo reagent.

the presence of 1 µM (each) antimetabolite. With each virus tested, both MTX and FUDR reduced viral titers by 1 to 2 logs (Fig. 3). Consistent with this observation, it was previously reported that MTX inhibits the replication of another flavivirus, yellow fever virus, vaccine strain 17D (14).

To determine if the inhibition of viral replication by these compounds is flavivirus specific, we further examined whether MTX and

FUDR inhibit the replication of HSV-1, VACV, Sindbis virus, or VSV using plaque assays. At 10 µM, both MTX and FUDR inhibited the DNA viruses tested, with the titer of HSV-1 reduced by several orders of magnitude and VACV by about 1 log (Fig. 4A and B). These findings agree with previous reports demonstrating that each drug inhibits DNA viruses (15–18). Of interest, neither drug affected the replication of either Sindbis virus or VSV, both RNA viruses (Fig. 4C and D).

Mechanisms of DENV inhibition. Both MTX and FUDR are widely used therapeutics for the treatment of certain cancers. As such, the antimetabolite mechanisms causing interference with the life cycle of cancer cells have been characterized for both compounds (Fig. 5A and B) (19, 20). We hypothesized that as antimetabolites, both molecules likely inhibited flavivirus replication by interfering with nucleic acid synthesis and thus genomic replication. To test this hypothesis, we utilized BHK cells that stably

TABLE 1 IC₅₀ and CC₅₀ values for MTX and FUDR in HEK293 cells

Treatment	Concn (µM)		
	IC ₅₀ (IFA)	IC ₅₀ (plaque assay)	CC ₅₀ (Cell Titer Glo)
MTX	0.09	0.17	12.42
FUDR	0.06	0.22	>100

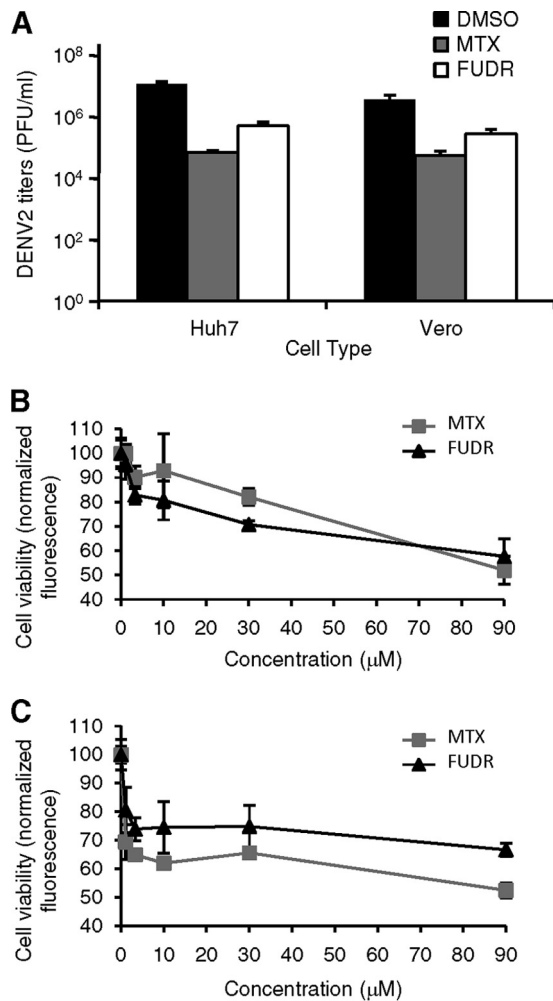


FIG 2 Anti-DENV efficacy is not HEK293 specific. (A) Huh7 or Vero cells were treated with DMSO only, 10 μ M MTX, or 10 μ M FUDR and infected with DENV2 at an MOI of 3 PFU/cell. Supernatants were collected 48 h p.i., and titers of infectious virions were determined. (B and C) Huh7 (B) or Vero (C) cells were treated with increasing doses of MTX or FUDR as indicated, and cell viability was assessed after 48 h using the Cell Titer Fluor reagent.

express a replicon with the nonstructural proteins of DENV along with luciferase as a read-out (21). When these cells were treated with MTX, FUDR, or the positive-control ribavirin, luciferase levels were decreased (Fig. 5C), suggesting that both compounds affect genome replication and/or gene expression of DENV.

MTX is an inhibitor of the enzyme dihydrofolate reductase (DHFR) (Fig. 5A) (20). To determine if the reduction of DENV2 replication is dependent on DHFR inhibition, we treated HEK293 cells with MTX in the presence or absence of folic acid (leucovorin). Folic acid can readily be converted to tetrahydrofolate independently of DHFR, bypassing the effects of blocking the enzyme (18). Indeed, DENV2 levels in the presence of both MTX and folic acid were comparable to those levels in the absence of MTX (Fig. 5D), demonstrating the involvement of DHFR in the anti-DENV2 activity of MTX.

FUDR functions against cancer using two mechanisms, by acting directly as a uracil analog and by inhibiting thymidylate synthase (Fig. 5B) (19, 22). If the antiviral activity of FUDR was due to it functioning

as a nucleoside analog, we reasoned that the addition of exogenous uridine would be able to compete with FUDR, thereby reducing its efficacy. However, the addition of various doses of uridine was unable to reduce the effects of FUDR on DENV2 titers (Fig. 5E). We then tested the ability of thymidine added exogenously, thus bypassing the requirement for thymidylate synthase (23), to counteract the inhibitory effects of FUDR on DENV2 replication. Indeed, DENV2 replication in the presence of both FUDR and thymidine was comparable to that observed in the absence of FUDR (Fig. 5F). These data suggest that FUDR interferes with DENV2 replication through the inhibition of thymidylate synthase.

5-Fluorouracil (5FU), an intermediate of FUDR along the pathway toward incorporation into RNA (Fig. 5B), is also commercially available and used as an antimetabolic drug in the treatment of certain cancers. We tested the ability of 5FU to inhibit DENV2 replication in HEK293 cells at concentrations known to be effective for FUDR. At both 1 and 10 μ M, 5FU did not inhibit DENV2 replication (Fig. 5G). Further, a dose-response curve demonstrated no significant effect on DENV2 replication levels at concentrations up to 100 μ M 5FU (Fig. 5H). This result further supports our conclusion that FUDR does not inhibit flavivirus replication through interference with RNA metabolism.

The anti-DENV activity of FUDR is p53 dependent. As an RNA virus, DENV2 does not directly incorporate deoxythymidine into its genome, thus suggesting that any antiviral effects of thymidylate synthase inhibition would be indirect. One possible mechanism for this antiviral effect is the p53 pathway, as thymidine deprivation results in the accumulation of p53 and the expression of p53-regulated genes (24–26). Activation of p53 has been shown as possessing antiviral properties against a broad range of viruses *in vitro* and *in vivo*. The antiviral activity of p53 has been attributed both to its inhibition of the cell cycle and contribution to virus-induced apoptosis and to its ability to induce antiviral, interferon (IFN)-stimulated genes (27–29). Interestingly, the accumulation of p53 following FUDR treatment could be prevented through the addition of exogenous thymidine (26), potentially explaining the FUDR-overriding effects that we observed using exogenous thymidine (Fig. 5F).

HEK293 cells, utilized in the initial high-throughput screen as well as the majority of subsequent experiments, contain the adenovirus E1B protein, capable of reducing but not totally eliminating p53 activity (30). In contrast, p53 is efficiently degraded in HeLa cells due to the human papillomavirus E6 protein (31) and cannot be detected (32), making this cell line essentially a “functional knockout” of p53. We therefore treated HeLa cells with FUDR and infected them with DENV2. Unlike the other diverse cell lines tested (Fig. 2), comparable levels of DENV were mea-

TABLE 2 IC₅₀ and CC₅₀ values for MTX and FUDR in Huh7 and Vero cells

Cell line	Treatment	Concn (μ M)	
		IC ₅₀ (plaque assay)	CC ₅₀ (Cell Titer Fluor)
Huh7	MTX	1	>90
	FUDR	15	>90
Vero	MTX	0.2	>90
	FUDR	0.1	>90

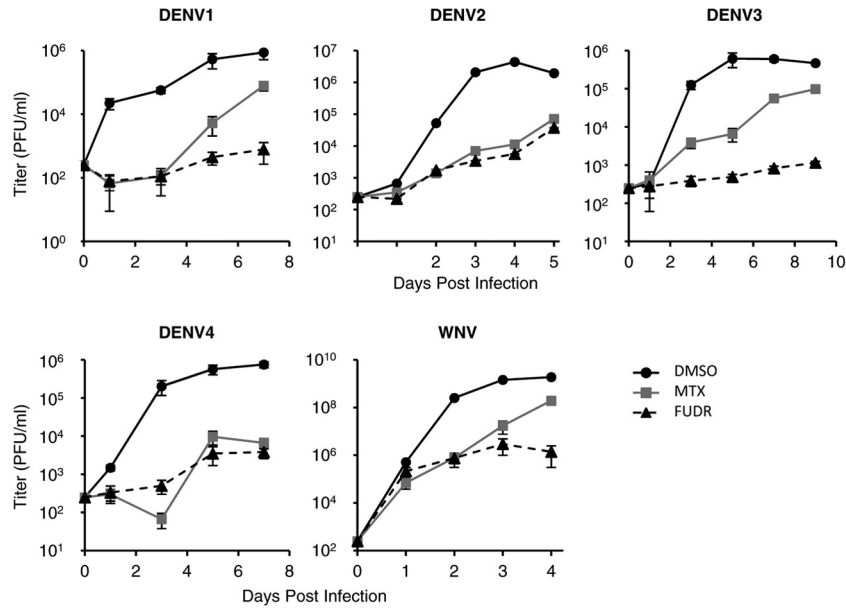


FIG 3 MTX and FUDR have activity against multiple flaviviruses. HEK293 cells were treated with DMSO vehicle, 1 μ M MTX, or 1 μ M FUDR and infected with representative strains of each DENV serotype as well as WNV at an MOI of 0.1 PFU/cell. Samples of the supernatant were taken at the indicated times p.i., and titers of infectious virions were determined.

sured in the supernatant of cells infected in the presence and in the absence of FUDR (Fig. 6A).

The lack of FUDR efficacy in HeLa cells cannot definitively be attributed to p53, as there are a number of differences between this cell line and the others used in this study. To directly address the role of p53 in FUDR activity, we utilized pifithrin- α , a known chemical inhibitor of p53 (33). HEK293 cells were infected with DENV2 in the presence of FUDR and/or pifithrin- α . After 48 h, cells treated with FUDR alone demonstrated a reduced amount of DENV2 in the supernatant (as previously demonstrated) whereas cells treated with pifithrin- α and the same dose of FUDR did not (Fig. 6B). Therefore, functional p53 must be present for FUDR to

reduce DENV2 replication. In contrast to the findings with FUDR, pifithrin- α did not restore DENV replication levels in the presence of MTX (Fig. 6B), suggesting that in addition to depletion of thymidine, MTX affects DENV replication by its known effects on purine or methionine synthesis.

MTX and FUDR do not prolong survival in murine models of flaviviral infection. To test the efficacy of MTX and FUDR *in vivo*, we utilized the AG129 mouse model of DENV infection. AG129 mice were implanted with subcutaneous osmotic pumps that released a constant level of MTX, FUDR, or DMSO alone as a control. They were then infected with the S221 mouse-adapted strain of DENV2. In this experiment, all of the mice succumbed to infection rapidly with no enhancement of survival observed in the mice receiving MTX or FUDR (data not shown). In addition, uninfected mice that received MTX demonstrated dramatically enhanced weight loss (data not shown), a result of the toxicity of the high concentrations (20 mg/kg of body weight or greater) of MTX used in these experiments.

We next tested the efficacy of MTX and FUDR in the murine model of WNV infection. C57BL/6 mice were infected subcutaneously with WNV. They were additionally given i.p. injections of MTX, FUDR, or DMSO alone on the day prior to infection as well as days 1, 3, 5, and 7 postinfection. As with DENV, neither MTX nor FUDR significantly prolonged survival in these mice at the doses tested (Fig. 7).

DISCUSSION

We report here the surprising finding that flaviviruses are highly susceptible to inhibitors of thymidine synthesis. FUDR is metabolized in the cell to a product that inhibits thymidine synthase, and the addition of exogenous thymidine was able to overcome the inhibition of DENV replication. MTX inhibits DHFR, upstream of thymidine synthesis. While exogenous thymidine alone did not rescue DENV replication in the presence of MTX, other critical

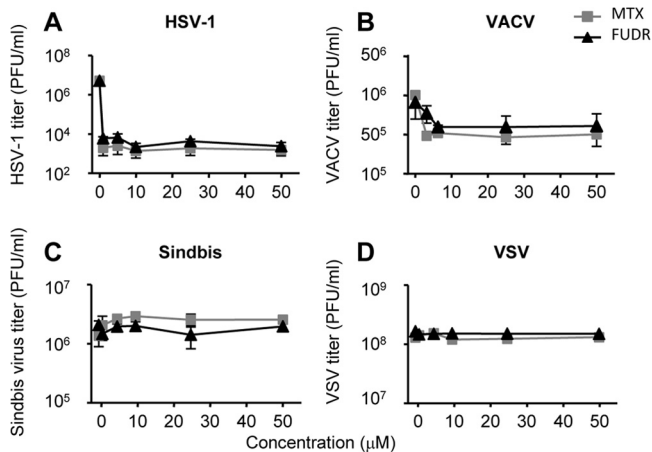


FIG 4 MTX and FUDR are not effective against all viruses. HEK293 cells were treated with increasing doses of MTX or FUDR as indicated and infected with HSV-1, VACV, Sindbis virus, or VSV at an MOI of 3 PFU/cell. Supernatants were collected at 24 h (Sindbis virus and VSV) or 48 h (HSV-1 and VACV), and titers of infectious virions were determined.

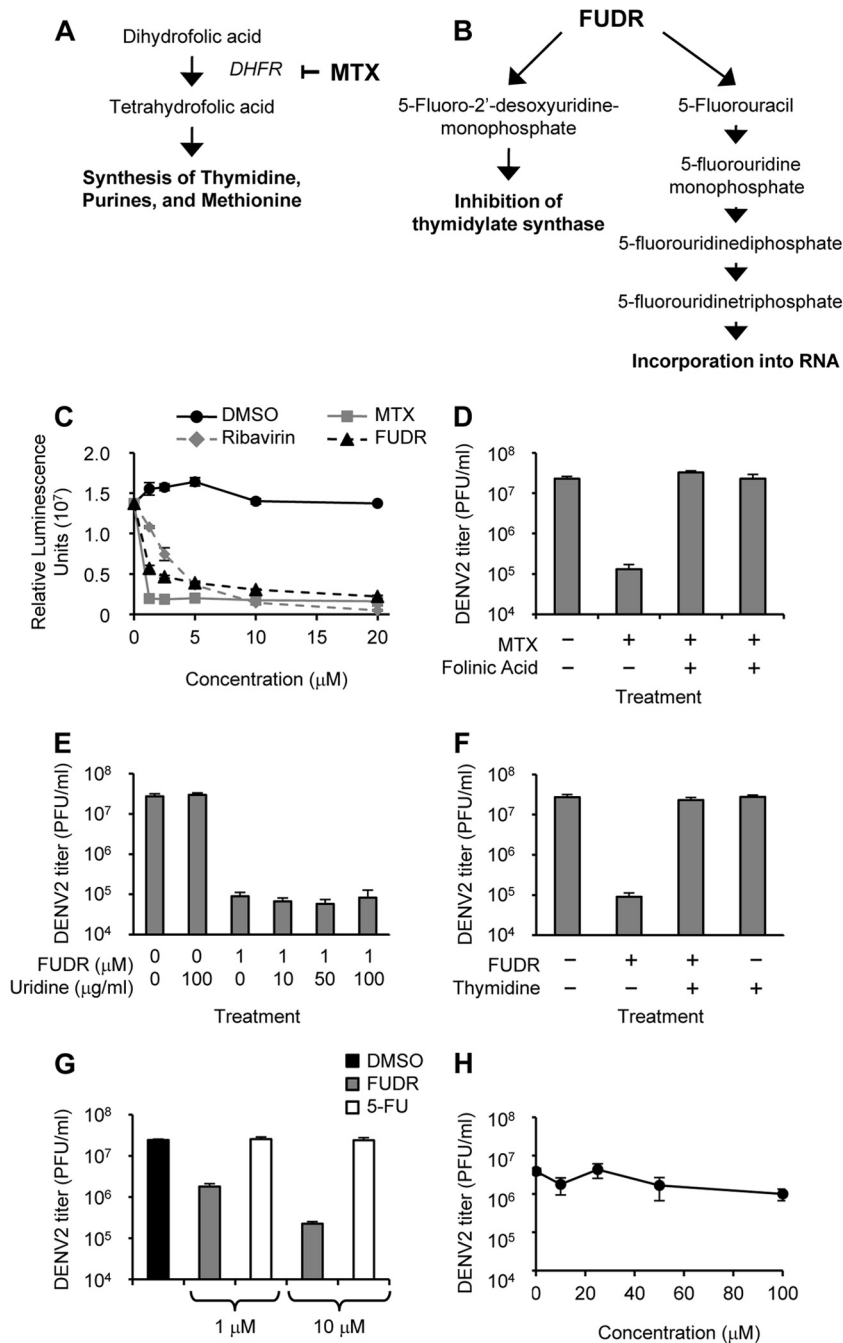


FIG 5 Mechanisms of anti-DENV activity of MTX and FUDR. (A) MTX blocks the enzyme DHFR upstream of the synthesis of thymidine, purines, and methionine. (B) FUDR is metabolized into products that inhibit thymidylate synthase or are incorporated into RNA as a uracil analog. (C) BHK cells stably maintaining a DENV replicon expressing a luciferase reporter were treated with DMSO only or increasing doses of ribavirin, MTX, or FUDR. After 48 h, luciferase was quantified via luminescence. (D) HEK293 cells were treated with combinations of MTX (1 μM) and folic acid (10 $\mu\text{g/ml}$) as indicated and infected with DENV2 at an MOI of 3 PFU/cell. Supernatants were collected 48 h p.i., and titers of infectious virions were determined. (E) HEK293 cells were treated with FUDR and increasing concentrations of uridine as indicated and infected with DENV2 at an MOI of 3 PFU/cell. Supernatants were collected 48 h p.i., and titers of infectious virions were determined. (F) HEK293 cells were treated with combinations of FUDR (1 μM) and thymidine (10 $\mu\text{g/ml}$) as indicated and infected with DENV2 at an MOI of 3 PFU/cell. Supernatants were collected 48 h p.i., and titers of infectious virions were determined. (G) HEK293 cells were treated with DMSO only, FUDR, or 5FU and infected with DENV2 at an MOI of 3 PFU/cell. Supernatants were collected 48 h p.i., and titers of infectious virions were determined. (H) HEK293 cells were treated with increasing doses of 5FU as indicated and infected with DENV2 at an MOI of 3 PFU/cell. Supernatants were collected 48 h p.i., and titers of infectious virions were determined.

molecules such as purines are also downstream of DHFR, suggesting that multiple critical metabolites are affected by DHFR inhibition. This conclusion is also supported by the inability of pifithrin- α to restore DENV replication in the presence of MTX.

As RNA viruses, flaviviruses do not directly incorporate deoxythymidine into their genome. Nonetheless, the profound changes in the host cell initiated by thymidine-less stress have a profound effect on the ability of the flaviviruses, but not the other RNA

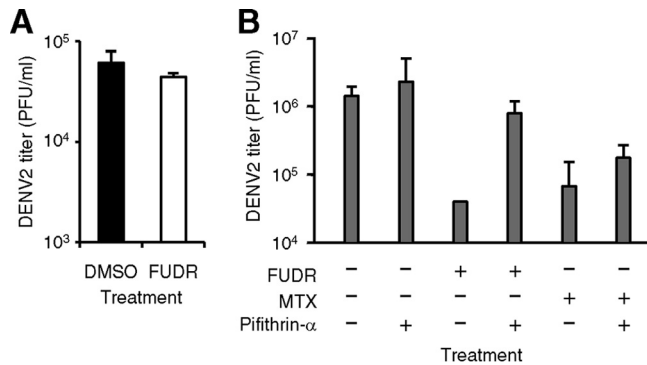


FIG 6 FUDR-mediated DENV inhibition is p53 dependent. (A) HeLa cells were treated with DMSO only or 10 μ M FUDR and infected with DENV2 at an MOI of 3 PFU/cell. Supernatants were collected 48 h p.i., and titers of infectious virions were determined. (B) HEK293 cells were treated with combinations of FUDR (1 μ M), MTX (1 μ M), and pifithrin- α (10 μ M) as indicated and infected with DENV2. Supernatants were collected 48 h p.i., and titers of infectious virions were determined.

viruses tested, to replicate their genome. This sensitivity suggests that components of the thymidine synthesis pathway could represent therapeutic targets.

The profound impact of thymidine depletion on DENV replication has not been observed previously and was unexpected given the viral RNA genome. Our results thus demonstrate the power of systematically evaluating known drugs against different viral agents by high-content screening of appropriate libraries (10). In contrast to screening small molecules having unexplored mechanisms of action, the molecular targets of approved drugs are generally known, thus implicating specific pathways in viral propagation. In this case, thymidine synthesis emerged as a significant process supporting DENV replication.

MTX and FUDR were unable to inhibit DENV or WNV replication *in vivo*. Other recent studies have documented *in vitro* inhibition of flavivirus replication by antimetabolites which were also unable to prolong survival in *in vivo* models of infection (34, 35). A few antimetabolites have successfully prolonged survival but also exhibited severe toxicity (36, 37). Similarly, we observed that MTX was toxic at high concentrations. A third antimetabolite, mercaptopurine, was identified in our initial high-throughput screen as possessing anti-DENV2 efficacy. While unrelated to thymidine synthesis, this molecule is related to another thiopurine that was recently identified as inhibitory of WNV replication *in vitro* (34). However, in follow-up evaluations, mercaptopurine demonstrated high toxicity to HEK293 cells (data not shown), and we therefore did not include it in further studies.

The studies here support the potential of antimetabolites as anti-DENV therapeutics but also illustrate the major challenges associated with drugs targeting host cell mechanisms. For instance, it is possible that *in vivo*, thymidine is less limiting than in tissue culture. It has been hypothesized for other antimetabolites that have failed to demonstrate *in vivo* efficacy that the animals' diet is sufficient to overcome any drug-induced deficiency (35). It is also conceivable that some nondividing cell types infected with DENV are less susceptible to the antimetabolites used than are the rapidly dividing cells used in tissue culture. Both MTX and FUDR have shown efficacy *in vivo* as anticancer drugs. As such, these drugs preferentially affect cells undergoing rapid replication. In

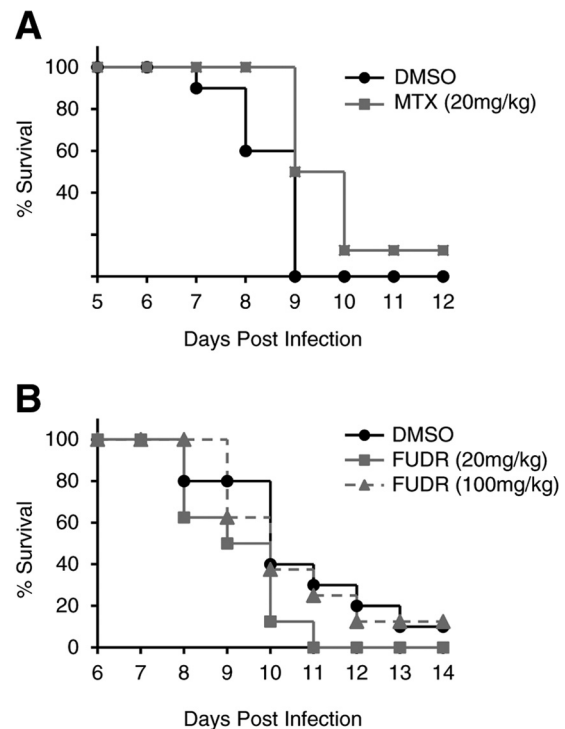


FIG 7 MTX and FUDR do not prolong survival of WNV-infected mice. C57BL/6 mice were infected s.c. with an LD₉₀ of WNV and injected i.p. daily beginning on day -1 with 10% DMSO or 20 mg/kg MTX (A) or injected i.p. every other day beginning on day -1 with 10% DMSO, 20 mg/kg FUDR, or 100 mg/kg FUDR (B).

addition to cancer cells, these drugs also affect rapidly dividing immune cells so that a beneficial effect of MTX and FUDR on infected cells may have been counteracted by a reduced immune response to DENV. *In vivo*, FUDR could conceivably be preferentially metabolized to the RNA incorporation pathway rather than the thymidylate synthase inhibition pathway (Fig. 5B), which our results have suggested is ineffective at inhibiting flaviviral replication (Fig. 5E, G, and H). The previous finding that FUDR was preferentially converted to 5FU in a murine tumor *in vivo* supports this idea, although in this study, FUDR inhibition of thymidylate synthase was also observed (22). It is possible that these limitations can be overcome by inhibiting several metabolic pathways simultaneously or by developing more-active and less-toxic inhibitors that more specifically eliminate or induce host cell pathways that modulate viral replication. Although MTX is widely used in humans, we observed significant toxicity at high concentrations in the form of dramatic weight loss in mice implanted with MTX-containing subcutaneous osmotic pumps. Thus, along with potential bioavailability issues, the adverse side effects of these drugs may outweigh desired antiviral effects.

Nonetheless, our results clearly uncover a previously unappreciated susceptibility of flaviviruses for the inhibition of thymidine synthesis. Interestingly, it has been recently demonstrated that excess thymidine, which leads to stalling of the cell cycle in S phase, strongly increased DENV production in insect cells due to acceleration of replication and assembly (38). In contrast, the same treatment did not affect DENV production in mammalian cells. Thus, it seems that excess thymidine does not affect DENV in

mammalian cells whereas depletion of thymidine drastically inhibits DENV replication at a postentry step. Our data suggest that thymidine depletion likely does not affect viral replication by depletion of nucleotides but via an indirect pathway that might involve activation of p53. While p53 is mostly known for its role as a tumor suppressor and in the induction of apoptosis, an emerging body of literature supports a role of p53 in inducing an antiviral transcriptional program. Several studies have demonstrated p53-mediated induction of interferon (IFN)-stimulated genes that inhibit viral replication *in vitro* and *in vivo* (27–29). However, we did not observe direct innate immune gene stimulation by FUDR using reporter cell lines encoding luciferase driven by either the interferon-sensitive response element or the NF- κ B transcriptional response element (data not shown), suggesting that IFN or NF- κ B activation is not the primary mechanism by which FUDR acts. It is thus likely that p53 activation prevents DENV replication by a different mechanism that could involve modulation of cell cycle or proapoptotic genes. It is in this capacity that p53 likely influences the replication of flaviviruses, which are highly dependent on host cell proteins and processes for their own replication. Further studies are needed to dissect the role of p53 in the absence of thymidine in more detail.

DENV currently poses a risk for over 40% of the world's population. The increasing range of mosquito vectors has raised concerns that this estimate could drastically increase in the future. However, there is currently no therapeutic treatment available for DENV-related diseases. Here, we demonstrate that two antimetabolites well established in the treatment of cancers, MTX and FUDR, are able to inhibit DENV replication *in vitro*. While treatment of flavivirus-infected mice with either drug did not prolong survival, this study suggests that components of the thymidine synthesis pathway represent potential therapeutic targets.

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