Inhibitors of the Tick-Borne, Hemorrhagic Fever-Associated Flaviviruses

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No antiviral therapies are available for the tick-borne flaviviruses associated with hemorrhagic fevers: Kyasanur Forest disease virus (KFDV), both classical and the Alkhurma hemorrhagic fever virus (AHFV) subtype, and Omsk hemorrhagic fever virus (OHFV). We tested compounds reported to have antiviral activity against members of the Flaviviridae family for their ability to inhibit AHFV replication. 6-Azauridine (6-azaU), 2′-C-methylcytidine (2′-CMC), and interferon alpha 2a (IFN-α2a) inhibited the replication of AHFV and also KFDV, OHFV, and Powassan virus. The combination of IFN-α2a and 2′-CMC exerted an additive antiviral effect on AHFV, and the combination of IFN-α2a and 6-azaU was moderately synergistic. The combination of 2′-CMC and 6-azaU was complex, being strongly synergistic but with a moderate level of antagonism. The antiviral activity of 6-azaU was reduced by the addition of cytidine but not guanosine, suggesting that it acted by inhibiting pyrimidine biosynthesis. To investigate the mechanism of action of 2′-CMC, AHFV variants with reduced susceptibility to 2′-CMC were selected. We used a replicon system to assess the substitutions present in the selected AHFV population. A double NS5 mutant, S603T/C666S, and a reduced level of replication which was increased when M644V was also present, although the replication of this triple mutant was still below that of the wild type. The S603 and C666 residues were predicted to lie in the active site of the AHFV NS5 polymerase, implicating the catalytic center of the enzyme as the binding site for 2′-CMC.

Currently, no antiviral therapies are approved for any of the tick-borne hemorrhagic fever flaviviruses, and patient treatment is limited to supportive care. A formalin-inactivated vaccine to protect against KFDV infection is licensed for use in India; however, vaccine uptake is low, and current batches appear to offer suboptimal protection (9). In the United States, KFDV and OHFV are classified as select agents and KFDV is an NIAID biodefense category B priority pathogen considered to pose a risk to national security. Research on these agents in the United States is conducted under biological safety level 4 (BSL-4).

The tick-borne flaviviruses are poorly characterized compared to some other members of the Flaviviridae family. HCV, for example, has been the focus of intense research, culminating in the approval of protease inhibitors for the treatment of hepatitis C (10). The effects of direct-acting antivirals, like the protease inhibitor telaprevir (11), the NSSA inhibitor daclatasvir (12), the non-nucleoside polymerase inhibitor VX-222 (13), and 2′-modified nucleoside analogues (14, 15), on HCV all have been well characterized. Inhibitors of host cell enzymes, such as α-glucosidase (16), hsp90 (17), and cellular kinases (18), also have been reported to inhibit HCV. Lycorine, an alkaloid compound found in plants (19), ezetimibe (20), and ribavirin (21) all inhibit various members of the Flaviviridae. 6-Azauridine (6-azaU) blocks cellular pyrimidine biosynthesis and inhibits several flaviviruses by inhibit-
ing orotidine monophosphate (OMP) decarboxylase (21–23). We tested these compounds for their ability to inhibit the growth of tick-borne hemorrhagic fever viruses in cell culture. Here, we report the characterization of 3 compounds with antiviral activities. To understand the mechanism of action of one of these, 2′-C-methylcytidine (2′-CMC), we generated mutant viruses able to grow in its presence. We also describe the first reverse genetics system for AHFV, which we used to study the variants associated with reduced susceptibility to 2′-CMC.

MATERIALS AND METHODS

Biosafety. All work with infectious virus and full-length viral RNA was conducted in a BSL-4 laboratory at the Centers for Disease Control and Prevention (CDC; Atlanta, GA). All laboratory staff adhered to international practices appropriate for this biosafety level. Experiments involving AHFV cDNA were approved by the CDC Institutional Biosafety Committee.

Cells, viruses, and compounds. A549, Vero E6, HeLa, and HT-1080 cells were obtained from the CDC core facility and maintained in Dulbecco’s modified Eagle medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% (vol/vol) fetal calf serum (FCS; HyClone; Life Technologies, Waltham, MA, USA) and penicillin-streptomycin (Life Technologies). HuH-7 cells were obtained from Apath, LLC (Brooklyn, NY, USA), and propagated in DMEM, 10% FCS, and 1× nonessential amino acids (Life Technologies). Wild-type KFDV (strain P9605), KFDV (strain AHFV 200300001), OHFV (strain Bogoluvovska), and Powassan virus (PWWV; strain Byers) were from the CDC Viral Special Pathogens Branch reference collection and were passaged once in Vero E6 cells before use. 2′-Modified nucleoside analogs were from Carbonsynth (Comp-ton, United Kingdom). 6-azaU, 6-azauridine triacetate, castanospermine, N-butyldeoxyxojirimycin (NB-DNJ), 1-deoxynojirimycin hydrochloride (DONH), galdanamyctin, 17-dimethylaminoethylamino-17-demethoxygaldanamyctin (17-DMAG), 17-(allylaminio)-17-demethoxygaldanamyctin (17-AAQ), uridine, cytidine, and guanosine were from Sigma-Aldrich (St. Louis, MO, USA). Telaprevir, dasabuvir (BMS-790052), VX-222, dasatanib, erlotinib, gefitinib, and lапatinib were from Selleck Chemicals (Houston, TX, USA). Human interferon alpha 2a (IFN-α2a) was from PBL Interferon Source (Piscataway, NJ, USA).

Assay for the inhibition of CPE. One day prior to infection, A549 cells were seeded in opaque white 96-well plates (Costar 3917; Corning Inc., Corning, NY, USA) at 20,000 cells per well. For initial testing, small molecules were solubilized in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich) and added to the wells to yield a final DMSO concentration of 0.5% (vol/vol). Compounds were tested in quadruplicate at a concentration of 50 μM unless indicated otherwise. For small-molecule concentration-response curves, a 10-point, 2-fold dilution series was used, with each compound concentration tested in quadruplicate. IFN-α2a was diluted in DMEM supplemented with 10% FCS and titrated in a 10-point, 10-fold dilution series. One h after the addition of compound, cells were mock infected or infected with virus at a multiplicity of infection (MOI) of 0.5. Cells were then incubated at 37°C in 5% CO2 for 3 days to yield 80 to 100% cytopathic effect (CPE) in virus control wells. Cell viability was determined using CellTiter-Glo (Promega, Madison, WI, USA) according to the manufacturer’s instructions. GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used to fit a 4-parameter equation to semilog plots of the concentration-response data and to derive the concentration of compound that inhibited 50% of the virus-induced cell death (EC50). The 50% cytotoxic concentration (CC50) was derived similarly for the mock-infected cells, and the selectivity index (SI) was calculated by dividing CC50 by EC50. Ordinary one-way analysis of variance (ANOVA) was used to compare mean EC50s for different viruses, with a P value of 0.05 being used to assign significance.

qRT-PCR assay. A549 cells were seeded, compounds were added, and cells were infected as described for the CPE inhibition assay. The medium was removed 24 h postinfection, and lysis buffer (MagMax total RNA isolation kit; Life Technologies) was added. Cell lysates were γ irradiated with 2 × 106 rads, and RNA was extracted using a MagMax-96 deep-well magnetic particle processor (Life Technologies). Quantitative reverse-transcription PCR (qRT-PCR) was performed with the Express one-step superscript qRT-PCR kit (Life Technologies) and analyzed on an Applied Biosciences 7500 real-time PCR machine (Life Technologies). AHFV RNA was quantitated using forward (5′-CTGATGTTTGGCTACTAGGA GGAG-3′) and reverse (5′-CAGCTCCATGTTGTGCTACTGC-3′) primers and a probe oligonucleotide (5′-GAAATGGACGAGCTAGGAG CCGAACGTGAC-3′) from Integrated DNA Technologies (Coralville, IA). Glycerolaldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control gene; pilot experiments demonstrated that its expression was not significantly altered after 24 h of viral infection (data not shown). Levels of GAPDH RNA were determined using a human control primer-probe set (Life Technologies). Viral RNA levels were normalized to GAPDH RNA and expressed relative to infected, vehicle-only controls. EC50s were determined as in the CPE inhibition assay.

Virus titer reduction assay. A549 cells were seeded, compounds were added, and cells were infected as described for the CPE inhibition assay. Twenty-four h later, the cells were frozen at −80°C. To determine virus titers, cells were frozen and thawed 3 times, and the resulting supernatants were used to determine 50% tissue culture infective doses (TCID50) using A549 cells (8 wells per dilution). Three days postinfection, the cell monolayers were scored for CPE and endpoint virus titers were calculated using the method of Reed and Muench (24).

In vitro combination experiments. For combination studies, checkerboard matrices of compound dilutions were generated and added to A549 cells seeded as described for the CPE inhibition assay. For the compound titrated in the horizontal direction, eight 2-fold serial dilutions were generated. In the vertical direction, six 2-fold serial dilutions were made. Concentration-response curves for the individual compounds were determined simultaneously. 2′-CMC and 6-azaU were diluted in DMEM, starting from twice the EC50 as determined in the CPE inhibition assay. IFN-α2a was diluted from a starting concentration of 10,000 IU/ml in DMEM supplemented with 0.1% bovine serum albumin (BSA; Sigma-Aldrich). One h after the addition of compounds, cells were infected with AHFV at an MOI of 0.5 and incubated for 3 days at 37°C and 5% CO2. The antiviral effects of compounds in combination were determined as described in the CPE inhibition assay and analyzed using MacSynergy II software (25, 26). Results were expressed as the mean synergy and antagonism volumes (μM3) calculated at the 95% confidence level from 3 independent experiments, each with at least 4 replicates. The extent of synergy or antagonism was defined as volumes of μM3: <25, insignificant; 25 to 50, minor but significant; 50 to 100, moderate; and >100, strong synergy or antagonism.

Selection of 2′-CMC-resistant variants. To select for AHFV variants with reduced susceptibility to 2′-CMC, a 75-cm2 flask of A549 cells was infected with AHFV at an MOI of 1 in the presence of 25 μM 2′-CMC. Virus was passed according to the schedule shown in Table 4, using 75-cm2 flasks and transferring 1 ml of medium each time. At the indicated passages, 1-ml samples of the cell culture media were taken, 10 μg of carrier RNA (Life Technologies) was added, and total RNA was extracted (PureLink RNA minikit; Life Technologies). A 4-kb fragment spanning the C terminus of NS4A, NS4B, NS5, and the 3′ untranslated region (UTR) was amplified by one-step RT-PCR using the primers Alk7F and AlkG1R and the method described previously (27). The resulting amplions were treated with RNase cocktail enzyme mix (Life Technologies) for 20 min at room temperature to remove any viral RNA before being transferred from BSL-4 to BSL-2 in TRizol (Life Technologies), purified, and subjected to next-generation sequencing using the PacBio platform (Pacific Biosciences, Menlo Park, CA, USA). Sequencing reads were assembled using a reference sequence (GenBank accession number JF416954), and variants were quantified using the probabilistic variant detection function of the CLC Genomics Workbench 6 (CLCbio, Aarhus, Den-
AHFV reporter replicon. cDNA encoding the AHFV reporter replicon was synthesized and cloned in two pieces by GenScript (Piscataway, NJ, USA). Mutations were introduced into the NS5 sequence by site-directed mutagenesis (QuickChange II; Agilent Technologies, Santa Clara, CA, USA). A mutant with an inactivated polymerase (pol− mutant) was generated by mutating the GDD motif of NS5 to RTA, an RsrII restriction site, to facilitate identification by restriction digestion. All mutants were verified by Sanger sequencing. To assemble the reporter replicon, each fragment was amplified by PCR and used in overlapping extension PCR to generate the replicon cDNA with minimal T7 promoter lacking two G residues at the 5′ end of the sense strand. These amplicons were used as templates to transcribe capped replicon RNA using the T7 mMESSAGE mMACHINE system (Life Technologies). RNA was assessed by agarose gel electrophoresis and quantitated by spectrophotometry. Replicon RNA was introduced into Huh7 cells by electroporation using the Neon transfection system (Life Technologies). Huh7 cells were resuspended at 5 × 10⁶ cells/ml in resuspension buffer (Life Technologies), and 625 ng of replicon RNA was added to 100 μl cells. Electroporation conditions were 1,600 V, 20 ms, and 1 pulse, and 5 μl of the transfected cell mix was added to 100 μl of medium per well of a 96-well plate. Compounds were added 4 h posttransfection. For harvesting, the medium was removed, the cells were washed once with phosphate-buffered saline (PBS), and reporter lysis buffer (Promega) was added. Samples were frozen at −80°C and thawed once before luciferase activity was assayed (BrightGlo luciferase assay system; Promega). Luminescence was measured on a Synergy H1M plate reader (BioTek, Winooski, VT, USA). To account for potential differences in transfection efficiency, luciferase signals were normalized to those from cells harvested 4 h posttransfection.

Immunofluorescence. To detect AHFV proteins expressed by the reporter replicon, Huh7 cells were electroporated with replicon RNA and incubated for 48 h. Cells were washed with PBS, fixed with 2% (wt/vol) paraformaldehyde for 10 min at room temperature, and permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 10 min at room temperature. AHFV proteins were detected with anti-AHFV hyperimmune mouse ascites (1:1,000 dilution in PBS supplemented with 2% [wt/vol] BSA) and goat anti-mouse Alexa 488 (1:1,000; Life Technologies). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

Modeling resistant variants. A homology model was obtained using one-2-one threading through the Phyre2 Protein Fold Recognition Server (28), using the AHFV RNA-dependent RNA polymerase (RdRp) sequence and the structure of the WNV RdRp domain (Protein Data Bank entry 2HFZ) (29). Percent identity between the two sequences was 63%, and the confidence value for the threading returned by Phyre2 was 100%. The UCSF Chimera package was used for molecular graphics (30).

RESULTS
Identification of inhibitors of AHFV replication. We found that the human lung epithelial cell line A549 supported robust growth of the tick-borne flaviviruses AHFV, KFDV, OHFV, and POWV, yielding 100- to 1,000-fold higher titers than Vero E6 cells. This growth was accompanied by a CPE that, for AHFV, resulted in 95% cell death 3 days after infection at an MOI of 0.5. We took advantage of this CPE to test if compounds reported to have an antiviral effect against other members of the Flaviviridae could inhibit AHFV-induced cell death. Compounds were initially tested at 50 μM and 10 μM unless otherwise noted. The viability of compound-treated but mock-infected cultures was determined simultaneously.

Compounds that did not specifically inhibit AHFV-induced CPE included the HCV protease inhibitor telaprevir, the NS5A inhibitor daclatasvir, and the non-nucleoside polymerase inhibitor VX-222 (data not shown). Similarly, inhibitors of α-glucosidase (castanospermine, NB-DNJ, and DONH), hsp90 (geldanamycin, 17-DMAG, and 17-AAG), and cellular kinases (dasatinib, erlotinib, gefitinib, and lapatinib at 1 or 10 μM) exhibited no specific antiviral activity. Ezetimibe and ribavirin also exhibited no specific anti-AHFV activity (data not shown).

Different 2′-modified nucleosides differ in their potency against HCV (14, 15). Therefore, we tested a panel of 2′-O-C- and 2′-O-methylated nucleoside analogues for their ability to inhibit AHFV. Dasatanib (10 μM) was used as a positive control for cell death. At 50 μM, none of the tested nucleosides were cytotoxic (Fig. 1B), and 2′-CMC and 2′-CMA demonstrated an antiviral effect, with 2′-CMC being the more potent (Fig. 1A). Other compounds with a specific anti-AHFV effect were lycorine (at 1 μM), 6-azaU, and IFN-α2a. In subsequent testing, the

![FIG 1 2′-Modified nucleoside analogues inhibit Alkhurma hemorrhagic fever virus (AHFV). (A) Inhibition of AHFV-induced cell death. 2′-Modified nucleoside analogues are dissolved in DMSO and added to A549 cells to a final concentration of 50 μM and 0.5% DMSO. Cells were infected with AHFV at a multiplicity of infection (MOI) of 0.5 and incubated for 3 days, and cell viability was determined. (B) Effect of 2′-modified nucleosides on cell viability. A549 cells were treated as described for panel A but were mock infected. Cell viability was measured 3 days after the addition of compound. Cells were treated with 10 μM dasatanib as a positive control to inhibit cell viability. Mean values from four replicate wells are shown, and error bars indicate the standard errors of the means. A representative of three independent experiments is shown. Abbreviations: DMSO, dimethyl sulfoxide; 2′-CMA, 2′-C-methylcytidine; 2′-CMC, 2′-C-methylcytidine; 2′-CMG, 2′-C-methylguanine; 2′-CMU, 2′-C-methyluridine; 2′-OMA, 2′-O-methyladenine; 2′-OMC, 2′-O-methylcytidine; 2′-OMG, 2′-O-methylguanine; 2′-OMU, 2′-O-methyluridine.](image-url)
EC_{50} for lycorine against AHFV was determined to be 1.7 μM, but it had a detectable cytotoxic effect and an SI of 9 (Table 1). An analog of lycorine with anti-WNV activity but reduced cytotoxicity has been reported (19), and we did not study lycorine itself further. The antiviral activities of 2′-CMC, 6-azaU, and IFN-α2a are described below.

Characterization of AHFV inhibitors. The activity of 2′-CMC against AHFV was investigated further. In the assay for the inhibition of AHFV CPE, the EC_{50} of 2′-CMC was determined to be 15.3 μM (Table 1). Its antiviral activity was confirmed using qRT-PCR for viral RNA, with an EC_{50} of 2.5 μM (Table 1) (EC_{50} in our qRT-PCR assay were consistently ~7-fold lower than those determined in the CPE assay, probably due to the shorter time period used in the qRT-PCR assay). The cytotoxicity of 2′-CMC was determined in mock-infected cells using a cell viability assay and by qRT-PCR for GAPDH; SI values were >3.5 and >20, respectively. 2′-CMC inhibited AHFV production in a concentration-dependent manner, with 25 μM reducing virus titer by ~4 logs (Fig. 2A). We also tested the anti-AHFV activity of PSI-6130, a derivative of 2′-CMC that was over 4-fold more potent than 2′-CMC against the HCV replicon but was a poor inhibitor of WNV, DENV, and YFV (31). PSI-6130 only weakly inhibited AHFV CPE, with an EC_{50} of 160 μM (Table 1).

The EC_{50} of 6-azaU was 1.9 μM in the CPE inhibition assay and 0.33 μM as measured by qRT-PCR (Table 1). 6-azaU was relatively well tolerated by the cells, with no apparent cytotoxic effect and an SI of >154 as determined by qRT-PCR. 6-azaU also mediated a marked concentration-dependent inhibition of AHFV titters (Fig. 2B). An orally absorbable produg of 6-azaU, 6-azaU triacetate, has been used to treat severe psoriasis. 6-azaU triacetate also inhibited AHFV-induced CPE but was ~4-fold less potent (Table 1).

For many years, pegylated IFN-α2a or IFN-α2b was a component of the standard of care for hepatitis C. We tested the nonpegylated form of IFN-α2a for its ability to inhibit AHFV replication. IFN-α2a exhibited antiviral activity with a somewhat flat dose-response curve; EC_{50} were 684 and 12.4 IU/ml as measured by the CPE inhibition and qRT-PCR assays, respectively (Table 1). This activity is similar to that against the HCV replicon (32). IFN-α2a produced no detectable cytotoxic effect at up to 160,000 IU/ml. In the AHFV titer assay, however, IFN-α2a was less effective than 2′-CMC or 6-azaU, with a maximal inhibition of ~1.5 logs at 208,000 IU/ml (Fig. 2C).

To confirm that the inhibitory activities of 2′-CMC, 6-azaU, and IFN-α2a were not specific to A549 cells, each compound was tested for its ability to inhibit AHFV replication in HeLa, HT-1080, Huh7, and VeroE6 cells. Each compound inhibited AHFV replication in a dose-dependent manner, as measured by qRT-PCR, in all cell lines tested (data not shown).

Activity against other pathogenic tick-borne flaviviruses. We next tested 2′-CMC, 6-azaU, and IFN-α2a for their ability to inhibit the replication of other highly pathogenic tick-borne flaviviruses, the hemorrhagic-fever viruses KFDV and OHFV and the encephalitis-associated POWV. The EC_{50}s for each compound for each virus were determined using the CPE inhibition assay (Table 2). 2′-CMC inhibited CPE induced by all of the tested viruses. 6-azaU and 6-azaU triacetate similarly inhibited virus-induced CPE. IFN-α2a inhibited AHFV and KFDV less well than POWV and OHFV (the order of susceptibility, ranked from most to least susceptible, was POWV = OHFV > AHFV = KFDV).

In vitro combination studies. Combinations of drugs are used to treat HCV and HIV to help curtail the emergence of resistant mutants and to achieve greater antiviral effects at lower doses. We determined the effects of combinations of compounds on AHFV using the Bliss independence model and MacSynergy II software (33–35). Checkboard titrations of pairs of compounds and each compound alone were tested for their ability to inhibit AHFV. The calculated theoretical additive antiviral effect was subtracted from the observed effect to reveal a statistically significant greater-than-expected (synergistic) or less-than-expected (antagonistic) antiviral effect. No significant cytotoxicity was produced by the tested combinations in mock-infected cells (<15% cytotoxicity at any dilution). The combination of IFN-α2a with 2′-CMC demonstrated no significant synergy or antagonism, indicating that this combination was additive (Table 3). When IFN-α2a was combined with 6-azaU, a moderate level of synergy was apparent (60.16 μM^2). In contrast, the combination of 2′-CMC and 6-azaU yielded a strongly synergistic interaction (228.81 μM^2) along with a moderate antagonism (~59.07 μM^2). Volumes of >100 μM^2 are predicted to be significant in vivo, suggesting that of those tested here, only the combination of 2′-CMC and 6-azaU exerts a synergistic effect in patients.

Mechanisms of action. The antiviral activity of 6-azaU has been attributed to its inhibition of OMP decarboxylase and consequent inhibition of cellular pyrimidine synthesis. To test if AHFV inhibition by 6-azaU occurred through this mechanism, we determined whether the addition of uridine, cytidine, or...
guanosine to the culture medium blocked 6-azaU activity. A dose-dependent inhibition of the antiviral effect was observed with increasing concentrations of uridine or cytidine but not guanosine (Fig. 3). This is consistent with the hypothesis that 6-azaU inhibits AHFV through the inhibition of cellular pyrimidine synthesis.

To investigate the mechanism by which 2’-CMC inhibits AHFV, variants with reduced susceptibility were selected by passing the virus 21 times in the presence of increasing concentrations of 2’-CMC for decreasing periods of time (Table 4). Virus was also passaged without compound as a mock-selected control. The titer of this mock-selected virus remained constant at approximately 4 × 10^8 TCID50/ml over the course of the experiment. In contrast, the titer of AHFV grown in the presence of 2’-CMC was 3 × 10^8 TCID50/ml after 8 passages and fell 10-fold, to 3 × 10^7, at passage 17 before recovering at passage 21. This suggested that selection occurred between passages 8 and 17 and that a mutant able to replicate in the presence of 2’-CMC arose. Therefore, we determined the susceptibility of each virus population to 2’-CMC.

Mock-selected virus was consistently susceptible to 2’-CMC over the course of the experiment, with an EC50 of around 8 × 10^9 M. In contrast, virus cultured in the presence of 2’-CMC was sensitive to inhibition at passage 8 (EC50, 10.4 × 10^9 M), but by passage 17 it was at least 6-fold less susceptible (EC50, 4.4 × 10^10 M).

Since 2’-CMC inhibits the NS5B polymerase of HCV, we amplified and deep-sequenced PCR products including the NS4B and NS5 regions of the selected AHFV populations from passages 8, 17, and 21. Two substitutions from the reference sequence arose only in the mock-selected viruses and were not investigated further (Table 5). Three substitutions were associated with growth in the presence of 2’-CMC. The virus quasispecies at passage 8 was identical to the reference sequence, but at passage 17, two substitutions, S603T and C666S (NS5 amino acid residue numbering), were present in the majority of the sequence reads (96 and 98%, respectively). A third variant, M644V, was identified in 36% of the reads at passage 17 and in 82% of reads at passage 21.

To determine if the mutations observed by deep sequencing were present in the same viral genomes, the PCR product amplification and sequencing were determined for each virus population. The results showed that the mutations were present in the same viral genomes, confirming the successful selection of variants resistant to 2’-CMC.

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**TABLE 2** Activity of inhibitors against selected pathogenic tick-borne flaviviruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>2’-CMC EC50 (µM)</th>
<th>6-azaU EC50 (µM)</th>
<th>IFN-α2a EC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KFDV</td>
<td>7.2 ± 2.7</td>
<td>2.0 ± 1.3</td>
<td>8.0 ± 6.6</td>
</tr>
<tr>
<td>OHFV</td>
<td>3.2 ± 1.1</td>
<td>1.9 ± 0.5</td>
<td>6.5 ± 2.5</td>
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<tr>
<td>POWV</td>
<td>5.5 ± 1.3</td>
<td>1.7 ± 0.3</td>
<td>5.4 ± 1.5</td>
</tr>
</tbody>
</table>

* Mean EC50 ± the standard deviations from at least 3 independent determinations are shown in µM (in IU/ml for IFN-α2a).

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**TABLE 3** Antiviral effects of compounds in combination

<table>
<thead>
<tr>
<th>Compound A</th>
<th>Compound B</th>
<th>Mean volb (µM)</th>
<th>Synergy</th>
<th>Antagonism</th>
<th>Interaction</th>
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</thead>
<tbody>
<tr>
<td>IFN-α2a</td>
<td>2’-CMC</td>
<td>21.85</td>
<td>−0.03</td>
<td>Additive</td>
<td></td>
</tr>
<tr>
<td>IFN-α2a</td>
<td>6-azaU</td>
<td>60.16</td>
<td>−22.18</td>
<td>Moderate</td>
<td></td>
</tr>
<tr>
<td>2’-CMC</td>
<td>6-azaU</td>
<td>228.81</td>
<td>−59.07</td>
<td>Strong</td>
<td>synergistic</td>
</tr>
</tbody>
</table>

* Values represent the mean synergy or antagonism volume at the 95% confidence level from 3 independent experiments, each performed with at least 4 replicates.
The G700S variant was found in a single clone by Sanger se-

Amino acid substitutions in AHFV that reduced susceptibility to 2′-CMC. To test if the mutations in AHFV grown under 2′-CMC selection were responsible for resistance, we developed a reporter replicon system for AHFV (Fig. 4A). This replicon included the first 27 amino acids of the C protein fused to the N terminus of firefly luciferase, the encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES), the first 21 amino acids of the E1 protein fused to the N terminus of NS1, and the genes encoding the remaining nonstructural proteins. A T7 promoter sequence was introduced before the 5′UTR so that the replicon was replication competent (pol+), indicating that the replicon was replication competent (Fig. 4B). Consistent with this, AHFV proteins were detected by immunofluorescence 48 h posttransfection only with the wild-type replicon (Fig. 4C).

Mutations found in the 2′-CMC-selected AHFV were introduced into the replicon, and their effect on replication in the presence of this compound was assessed. 2′-CMC inhibited the luciferase signal from the wild-type replicon in a dose-dependent manner, as expected (Fig. 5A). Similarly, it inhibited expression from the single mutants S603T, C666S, and M644V and the double mutants S603T/M644V and C666S/M644V. 2′-CMC did not, however, inhibit luciferase expression by the double mutant S603T/C666S or the triple mutant S603T/M644V/C666S. Thus, the presence of both S603T and C666S was required for resistance to 2′-CMC.

To assess the replication capacity of each mutant, we compared the luciferase signals 48 h posttransfection to those after treatment with 2′-CMC (Fig. 5B). The C666S and M644V mutants yielded signals that were similar to those of the wild type. In contrast, the signal from the S603T mutant was approximately 200-fold less than that of the wild-type replicon, suggesting that this substitution caused a dramatic defect in replication. Similarly, lower levels of luciferase expression were observed with the double mutants S603T/C666S, S603T/M644V, and C666S/M644V. However, the luciferase signal from the triple mutant, S603T/M644V/C666S, was greater than that of the double mutants, though it was still approximately 20-fold less than that of the wild-type sequence. Thus, the M644V change is able to somewhat compensate for the reduced replication caused by the S603T/C666S substitutions.

The G700S variant was found in a single clone by Sanger se-

### TABLE 5 Substitutions associated with AHFV growth in the presence of 2′-CMC

<table>
<thead>
<tr>
<th>Selection type and nucleotide change</th>
<th>Polyprotein numbering</th>
<th>NS5 numbering</th>
<th>Passage no.</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>C8028T</td>
<td>Noncoding</td>
<td>8</td>
<td>22</td>
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<tr>
<td></td>
<td></td>
<td>Noncoding</td>
<td>17</td>
<td>69</td>
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<td></td>
<td></td>
<td></td>
<td>21</td>
<td>58</td>
</tr>
<tr>
<td>T9595C</td>
<td>Y3164H</td>
<td>Y651H</td>
<td>8</td>
<td>0</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>17</td>
<td>67</td>
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<tr>
<td></td>
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<td></td>
<td>21</td>
<td>65</td>
</tr>
<tr>
<td>2′-CMC</td>
<td>T9451A</td>
<td>S3116T</td>
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<tr>
<td></td>
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<td>S603T</td>
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<td>98</td>
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### TABLE 4 Selection AHFV variants with reduced susceptibility to 2′-CMC

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<th>Passage no.</th>
<th>2′-CMC (µM)</th>
<th>Duration of selection (days)</th>
<th>Activity by selection typea</th>
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<td></td>
<td></td>
<td>Titer (TCID50/ml)</td>
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<td>Titer (TCID50/ml)</td>
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<td>50</td>
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| Mean EC50s ± standard deviations are from 3 independent determinations. ND, not done.
sequencing. The mutant with this change was susceptible to 2'-CMC inhibition and, in the absence of compound, gave luciferase signals similar to those of the wild type (data not shown). Since this substitution was found in only a small proportion of the clones, it was not investigated further.

For HCV, the substitution S282T, in the active site of NS5B, is associated with 2'-CMC resistance (15, 36). We were interested to see where the substitutions associated with AHFV resistance to 2'-CMC might be located. Since the structure of AHFV NS5 has not been solved, the AHFV NS5 sequence was modeled on the

**FIG 4** Reporter replicon system for AHFV. (A) Schematic of the subgenomic, luciferase reporter, AHFV replicon. The T7 promoter is followed by the 5' untranslated region of AHFV and then the N-terminal 27 amino acids of the AHFV C protein (C*) fused to firefly luciferase (FF-luc), the EMCV IRES, and the signal sequence from the AHFV E1 protein (E1ss) and the nonstructural genes NS1, 2A, 2B, 3, 4A, 4B, and 5. The location of the NS5 polymerase active site is indicated (GDD). (B) Replicon luciferase signals over time. Wild-type or polymerase-inactivated (pol−) replicons were electroporated into Huh7 cells, and luciferase activity was determined at various times posttransfection. To account for differences in transfection efficiency, luciferase signals were normalized to those obtained at 4 h posttransfection. Mean values from four replicate wells are shown, and error bars indicate the standard deviations. (C) Immunofluorescence of Huh7 cells electroporated with pol− or wild-type replicons, fixed and stained at 48 h posttransfection. Cell nuclei are blue; green staining indicates AHFV proteins.

**FIG 5** Amino acid substitutions associated with AHFV growth in the presence of 2'-CMC assessed using the reporter replicon. (A) Susceptibility of mutant AHFV replicons to 2'-CMC. Wild-type or mutant replicon RNA was electroporated into Huh7 cells, 2'-CMC was added 4 h posttransfection, and the cultures were incubated for 48 h before harvesting for luciferase assay. Luciferase signals were normalized to that at 4 h posttransfection and are shown as percentages of the normalized signal obtained for that replicon incubated without 2'-CMC. Mean values from three experiments, performed with RNA transcribed from two independently generated templates, is shown with error bars representing the standard errors of the means. (B) Replication capacity of mutant replicons. Mutant replicons were electroporated into Huh7 cells, and cells were incubated for 48 h before harvesting for luciferase assay. Mean values from three experiments, performed with RNA transcribed from two independently generated templates, is shown with error bars representing the standard errors of the means.
published structure of the RdRp domain of WNV NS5 (29). This analysis predicted that AHFV S603 is located in the NS5 RdRp active site, positioned analogously to HCV S282 (Fig. 6B). The C666S change is immediately C terminal to the GDD motif, so it too lies in the active site. The M644V change was predicted to lie away from the active site (Fig. 6C).

Altogether, these data suggest that both the S603T and C666S amino acid changes are required for resistance to 2′-CMC and are located in the active site of the AHFV RdRp domain. This combination, however, is associated with a severe replication defect, which can be partially compensated for by the M644V substitution.

**DISCUSSION**

Currently, no antiviral therapies exist for KFDV, either classical or the AHFV subtype, and OHFV, the highly pathogenic tick-borne flaviviruses that cause human hemorrhagic fevers. We tested compounds previously reported to inhibit members of the *Flaviviridae* family for their ability to inhibit these viruses; two small molecules (6-azaU and 2′-CMC) and one biologic (IFN-α2a) inhibited all three.

6-azaU inhibits cellular pyrimidine biosynthesis by inhibition of OMP decarboxylase. It has long been known to inhibit diverse viruses in culture, though it has generally performed poorly in animal models (37, 38), probably because the antiviral effect is overcome by nucleosides in the diet. Inhibitors of other enzymes in the pyrimidine biosynthetic pathway are similarly active in culture but not in vivo (39, 40), suggesting that the pathway may not contain suitable antiviral targets. Potentially, however, combining an inhibitor of pyrimidine biosynthesis with inhibitors acting through different mechanisms may improve antiviral efficacy in vivo. In particular, an inhibitor of pyrimidine biosynthesis might increase the efficacy of a pyrimidine nucleoside inhibitor. We found that the combination of 6-azaU and 2′-CMC exhibited a complex interaction with a high level of synergy, but it also showed antagonism at some concentrations. The effect of this combination in patients is difficult to predict, but it could be synergistic at certain doses.

Pegylated IFN-α was a component of the standard of care for treating hepatitis C for many years. We found that IFN-α2a inhibited the replication of all of the tick-borne, hemorrhagic fever-associated flaviviruses tested, although it did not reduce viral titers as effectively as 6-azaU or 2′-CMC (Fig. 2). A Russian-language study previously reported that larifan, an inducer of IFN, was active against OHFV (41). Due to its short half-life, IFN likely would have to be administered immediately prior to, or shortly after, infection.

Several 2′-modified nucleoside analogues have been reported to inhibit the HCV replicon (14, 15). 2′-CMC also inhibited YFV and norovirus both in cell culture and in animal models (42–45). 2′-CMC inhibits the HCV polymerase, NS5B, preventing viral RNA synthesis. Due to the poor oral availability of 2′-CMC, a valine ester prodrug, valopicitabine (also called NM283), was advanced to clinical trials for HCV treatment (46). A twice-daily dose of 800 mg NM283 reduced the mean HCV load by 1.2 log10 IU/ml after 14 days, providing proof of concept for inhibitors with this mechanism. Subsequently, NM283 was evaluated in combination with pegylated IFN in a phase Ib trial. This combination reduced viral load by >4 log10 IU/ml after 28 days, but the trial was discontinued due to gastrointestinal side effects.

For the flaviviruses, 2′-CMC may be a candidate for lead optimization to generate a nontoxic analogue with greater antiviral potency. Several groups have followed this strategy to develop a nucleoside inhibitor of HCV, and multiple 2′-modified nucleosides have advanced to clinical trials for treatment of hepatitis C. Adverse effects led to the discontinuation of a phase Ib trial with a 2′-methyl guanosine nucleotide, BMS-986094 (INX-189), and to the halting of clinical development of two other guanosine derivatives, IDX184 and IDX19368. At the time of writing, RG7128,
a prodrug of PSI-6130, is being evaluated in clinical trials, and a 2'-modified uridine derivative (PSI-7977; also called sofosbuvir or Sovaldi) has recently been approved. These experiences in the HCV field suggest that it is possible to generate analogues of 2'-CMC that are effective against AHFV and other flaviviruses, although guanosine derivatives should probably be avoided.

Understanding the mechanism by which 2'-CMC inhibits AHFV replication is an important step toward the discovery of a more potent analogue. To this end, we identified several amino acid substitutions that were associated with growth of AHFV in the presence of 2'-CMC. The S282T change in the HCV NS5B polymerase conferred a 5- to 10-fold reduced susceptibility of HCV to 2'-CMC (36). S282 is located in the active site of the polymerase and helps form the surface against which nucleoside triphosphates fit when being incorporated into the RNA chain. The S282T change decreased the affinity of NS5B for 2'-CMC but was also associated with a loss of replicative fitness, to about 10% of that of the wild type. For AHFV, we found that two substitutions in the NS5 RdRp, S603T and C666S, were required to confer resistance to 2'-CMC. The S603T change appeared to be analogous to the S282T substitution in HCV, as they were predicted to be located similarly in the active site of their respective RdRp. This suggests that 2'-CMC inhibits AHFV replication in the same way it does HCV, by blocking in the active site of the polymerase and blocking viral RNA synthesis. Like HCV NS5B S282T, AHFV NS5 S603T (and the double substitution S603T/C666S) was associated with a reduced replication capacity. In the context of S603T/C666S, this was partially compensated for by the addition of M644V. Amino acid changes in HCV that might be equivalent to the C666S and M644V substitutions in AHFV NS5 have not, to our knowledge, been reported. The M644 residue was predicted to lie in a helix (α15 in the WNV structure) some 25 Å away from the GDD motif. Perhaps the M644V substitution could result in a modification of the active-site structure, although the mechanism by which this might occur is not obvious. Alternatively, M644V might enhance another function of NS5, such as an interaction with another viral or cellular protein, that could compensate for a polymerase with reduced activity. Given the likely mechanism of 2'-CMC action against AHFV and its probable binding in the NS5 RdRp active site, a rational structure-guided drug design approach, where 2'-CMC is modeled in the NS5 active site, may suggest changes to increase the affinity of the molecule and lead to the discovery of a more potent analogue.

The requirement for two amino acid changes within NS5 to confer reduced susceptibility to 2'-CMC is consistent with a high barrier to the development of resistance, as has been described for nucleoside inhibitors of HCV (47, 48). The residues in question are highly conserved, with S603 and C666 being completely conserved in all tick-borne, hemorrhagic fever flaviviruses (C666S is present in one tick-borne encephalitis virus sequence and one POWV sequence (GenBank accession numbers HM535610 and AF310950), respectively). In contrast, M644V is a frequently occurring polymorphism and is found in most of the tick-borne flaviviruses, including several AHFV sequences.

In conclusion, we have identified several compounds with activity against the tick-borne hemorrhagic fever-associated flaviviruses. We have recently characterized mouse models for AHFV and KFDV infection (K. A. Dodd, B. H. Bird, M. E. B. Jones, S. T. Nichol, and C. F. Spiropoulou, submitted for publication) and plan to test the compounds for antiviral activity in these models. These compounds, even if not clinically effective, may be useful research tools or starting points for drug development efforts. Structure-activity relationships have been established for 2'-modified nucleosides against the HCV NS5B polymerase but, to our knowledge, have not yet been investigated for other flaviviruses. The potential for a 2'-modified nucleoside to be a broad-spectrum inhibitor of flaviviruses is intriguing. Finally, the AHFV reporter replicon described here may be a useful tool for studying AHFV replication under BSL-2 containment.

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REFERENCES


