“Inhibitors of the tick-borne, hemorrhagic fever-associated flaviviruses.”

Mike Flint¹, Laura K. McMullan¹, Kimberly A. Dodd¹, Brian H. Bird¹, Marina Khristova¹, Stuart T. Nichol¹ & Christina F. Spiropoulou¹#

¹ Viral Special Pathogens Branch, Centers for Disease Control and Prevention, Atlanta, Georgia, USA
² School of Veterinary Medicine, University of California Davis, Davis, California, USA

#Corresponding author. Viral Special Pathogens Branch, Division of High Consequence Pathogens and Pathology, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Road MS G-14, Atlanta, GA 30333, USA. Tel: +1-404-639 1294. E-mail address: ccs8@cdc.gov

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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-α2a (IFNα) inhibited the replication of AHFV and also KFDV, OHFV and Powassan virus. The combination of IFNα and 2'-CMC exerted an additive antiviral effect on AHFV and the combination of IFNα 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 triple mutant S603T/C666S/M644V were more resistant to 2'-CMC than the wild-type replicon. The S603T/C666S mutant had a reduced level of replication which was increased when M644V was also present, though the replication of this triple mutant was still below that of 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.
Members of the Flaviviridae family are enveloped, positive-sense, single-stranded RNA viruses and include important pathogens such as hepatitis C virus (HCV), dengue virus (DENV) and West Nile virus (WNV)(1). There are four genera in the family: Flavivirus (type species: yellow fever virus, YFV), Pestivirus (bovine viral diarrhea virus 1), Hepacivirus (HCV) and Pegivirus (pegivirus A). The Flavivirus genus includes over 50 species that are either mosquito-borne, tick-borne or have no known vector. Flaviviruses that cause human disease can also be divided into those predominantly associated with encephalitis (including WNV and Japanese encephalitis virus) and those that cause a more viscerotropic disease and hemorrhagic fever (YFV and DENV). Of the tick-borne flaviviruses, 2 cause hemorrhagic fevers in humans, Kyasanur Forest disease virus (KFDV), both classical and the Alkhurma hemorrhagic fever virus (AHFV) subtype (called Alkhumra virus in some reports), and Omsk hemorrhagic fever virus (OHFV) (2, 3).

KFDV was first isolated from a sick monkey in 1957 after an outbreak of severe febrile illness in residents of the Kyasanur Forest area of southwest India (4, 5). Large numbers of monkeys in the forest had died from a disease with hemorrhagic symptoms. In humans, KFDV hemorrhagic fever has a case fatality rate of 2-10% and is a significant public health issue in the endemic area. AHFV is a variant of KFDV, with 89% homology at the nucleotide level (6) and has been the cause of several outbreaks of severe hemorrhagic fever in Saudi Arabia (7). AHFV case fatalities have been reported to be as high as 25%, though this may be an overestimate due to undiagnosed mild and asymptomatic infections. OHFV circulates in western Siberia and has a relatively low case fatality rate, at 0.4-2.5% (8). The only tick-borne flaviviruses present in the Americas are deer-tick virus, and its close relative Powassan virus (POWV) (2). POWV generally causes a mild or subclinical disease, with rare cases of encephalitis.

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 a NIAID biodefense category B priority pathogen considered to pose a risk to national security. Research on these agents in the U.S. 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 NS5A inhibitor daclatasvir (12), the non-nucleoside polymerase inhibitor VX-222 (13) and 2'-modified nucleoside analogues (14, 15) on HCV have all been well characterized. Inhibitors of host-cell enzymes such as α-glucosidase (16), hsp90 (17) and cellular kinases (18) have also 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, inhibiting 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 laboratorians 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% (v/v) fetal calf serum (FCS; HyClone, Thermo Scientific, Waltham, MA, USA) and penicillin-streptomycin (Life Technologies). Huh7 cells were obtained from Apath, LLC (Brooklyn, NY, USA) and propagated in DMEM, 10% FCS and 1x non-essential amino acids (Life Technologies). Wild-type KFDV (strain P9605), KFDV (strain AHFV 20030001), OHFV (strain Bogoluvovska) and Powassan virus (POWV, 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 Carbosynth (Compton, UK). 6-azaU, 6-azauridine triacetate, castanospermine, N-butyldoxyojirimycin (NB-DNJ), 1-deoxyojirimycin hydrochloride (DONH), geldanamycin, 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), uridine and guanosine were from Sigma-Aldrich (St. Louis, MO, USA). Telaprevir, daclatasvir (BMS-790052,) VX-222, dasatanib, erlotinib, gefitinib and lapatinib were from Selleck Chemicals (Houston, TX, USA). Human interferon alpha 2a (IFNα2a) was from PBL interferon source (Piscataway, NJ, USA).
Assay for the inhibition of cytopathic effect (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% dimethylsulfoxide (DMSO, Sigma-Aldrich) and added to the wells to yield a final DMSO concentration of 0.5% (v/v). 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. IFNα2a was diluted in DMEM supplemented with 10% FCS and titrated in a 10-point, 10-fold dilution series. 1 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 80-100% 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 semi-log 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 EC₅₀ values for different viruses, with a P value of 0.05 being used to assign significance.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) assay

A549 cells were seeded, compounds were added, and cells were infected as for the CPE inhibition assay. The medium was removed 24 h post-infection, and lysis buffer (MagMax Total RNA isolation kit, Life Technologies) was added. Cell lysates were γ-irradiated with 2 x 10⁶ rads, and RNA was extracted using a MagMax-96 Deep Well Magnetic Particle Processor (Life Technologies). 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’-CTGAATGGTTGCTCTAGGGAGGAG-3’) and reverse (5’-CAGTCCCATGTTTGTCTCACCTCT-3’) primers and a probe oligonucleotide (5’- CGAAGTGGAGTCATGGAGACGGAACGTGACA-3’) from Integrated DNA Technologies (Coralville, IA). Glyceraldehyde 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. EC50 values were determined as in the CPE inhibition assay.

**Virus titer reduction assay**

A549 cells were seeded, compounds were added, and cells were infected as for the CPE inhibition assay. 24 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 infectious doses (TCID50) using A549 cells (8 wells per dilution). Three days post-infection, 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 for the CPE inhibition assay. For the compound titrated in the horizontal direction, 8 2-fold serial dilutions were generated. In the vertical direction, 6 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. IFNα2a 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 in the CPE inhibition assay and analyzed using MacSynergy II software (25, 26). Results were expressed as the mean synergy and antagonism volumes (µM²) calculated at 95% confidence from 3 independent experiments, each with at least 4 replicates. The extent of synergy or antagonism was defined as volumes of µM²: <25 were insignificant; 25-50, minor but significant; 50-100, moderate and values >100 indicated 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 Mini Kit, Life Technologies). A 4 kb fragment spanning the C-terminus of NS4A, NS4B, NS5 and the 3'-UTR was amplified by one-step RT-PCR using the primers Alk7F and alkG1R and the method described previously (27). The resulting amplicons 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 (accession # JF416954) and variants were quantified using the probabilistic variant detection function of the CLC Genomics Workbench 6 (CLCbio, Aarhus, Denmark) with a 1% minimum. The mean coverage at each base was 7497 reads.
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 (QuikChange 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 Rsrl 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 a minimal T7 promoter lacking two Gs 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 x 10⁶ cells/ml in Resuspension buffer (Life Technologies), and 625 ng of replicon RNA added to 100 μL cells. Electroporation conditions were 1600 V, 20 ms, 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 post-transfection. 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 H1MD 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 post-transfection.
**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% (w/v) paraformaldehyde for 10 minutes at room temperature and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min at room temperature. AHFV proteins were detected with anti-AHFV hyperimmune mouse ascites (1:1000 dilution in PBS supplemented with 2% w/v BSA) and goat anti-mouse Alexa 488 (1:1000; Life Technologies). Cell nuclei were stained with DAPI.

**Modeling resistant variants**

A homology model was obtained using one-to-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 ID 2HFZ) (29). Percentage identity between the 2 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 1000-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 NSSA 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). We therefore tested a panel of 2'-C- and 2'-O-methylated nucleoside analogues for their ability to inhibit AHFV. Dasatinib (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 IFNα2a. In subsequent testing, the EC50 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 with reduced cytotoxicity has been reported (19), and we did not study lycorine itself further. The characterization of the antiviral activities of 2'-CMC, 6-azaU and IFNα2a is 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 EC50 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 EC50 of 2.5 µM (Table 1) (EC50 values 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 EC50 of 160 µM (Table 1).

The EC50 of 6-azaU was 1.9 µM in the CPE inhibition assay and 0.33 µM 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 titers (Fig. 2B). An orally absorbable prodrug of 6-azaU, 6-azaU triacetate, has been used as a treat severe psoriasis. 6-azaU triacetate also inhibited AHFV-induced CPE, but was ~4-fold less potent (Table 1).

For many years, pegylated IFNα2a or IFNα2b were components of the standard of care for hepatitis C. We tested the non-pegylated form of IFNα2a for its ability to inhibit AHFV replication. IFNα2a exhibited antiviral activity, with a somewhat flat dose-response; EC50 values 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). IFNα2a produced no detectable cytotoxic effect at up to 160,000 IU/mL. In the AHFV titer assay, however,
IFNα2a 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 IFNα2a 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

Next, we tested 2′-CMC, 6-azaU, 6-azaU-triacetate and IFNα2a 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 EC50 values 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. IFNα2a inhibited AHFV and KFDV less well than POWV and OHFV (the order of susceptibility, ranked from most susceptible to least, being 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). Checkerboard 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 IFNα2a with 2'-CMC demonstrated no significant synergy or antagonism, indicating that this combination was additive (Table 3). When IFNα2a was combined with 6-azaU, a moderate level of synergy was apparent (60.16 µM²). In contrast, the combination of 2'-CMC and 6-azaU yielded a strongly synergistic interaction (228.81 µM²), along with a moderate antagonism (-59.07 µM²). Volumes of > 100 µM² are predicted to be significant in vivo, suggesting that of those tested here, only the combination of 2'-CMC and 6-azaU might exert 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 a reduced susceptibility were selected by passaging 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 x 10⁸ TCID₅₀/mL over the course of the experiment. In contrast, the titer of AHFV grown in the presence of 2'-CMC was 3 x 10⁸ TCID₅₀/mL after 8 passages, and fell 10-fold to 3 x 10⁷ 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. We therefore 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 µM. In contrast,
virus cultured in the presence of 2'-CMC was sensitive to inhibition at passage 8 (EC50 10.4 µM), but by passage 17 was at least 6-fold less susceptible (EC50 > 44 µM).

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 differences 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 amplified from the 2'-CMC-selected virus at passage 21 was cloned and six clones were subjected to Sanger sequencing. Of these six, all encoded both the S603T and C666S substitutions. Five of them also contained M644V, and one of these also had NS5 G700S (data not shown).

**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 EMCV IRES, the first 21 amino acids of the E1 protein fused to the N-terminus of NS1 and the genes encoding the remaining non-structural proteins. A T7 promoter sequence was introduced before the 5' untranslated region so that the amplicon could be used as a template for in vitro transcription. When replicon RNA was electroporated into Huh7 cells, luciferase was expressed from the wild-type replicon sequence, but not from a mutant in which the polymerase was inactivated by mutagenesis (pol-),
indicating that the replicon was replication competent (Fig. 4B). Consistent with this, AHFV proteins were detected by immunofluorescence 48 h post-transfection with only 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 nor 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 hours post-transfection, without 2′-CMC (Fig. 5B). The C666S and M644V mutants yielded signals that were similar to 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. The luciferase signal from the triple mutant, S603T/M644V/C666S was however, greater than that of the double mutants, though 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 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 wild-type (data not shown). Since this substitution was only found in 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 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.
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 (IFNα2a) 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 may be 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), leading some to conclude that the pathway is not druggable. Potentially, however, combining an inhibitor of pyrimidine biosynthesis with inhibitors acting through different mechanisms might 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 also showing antagonism at some concentrations. The effect of this combination in patients is difficult to predict, but 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 IFNα2a inhibited the replication of all the tick-borne, hemorrhagic fever-associated flaviviruses tested, though it did not reduce viral titers as effectively as 6-azaU or 2'-CMC (Fig. 2). A Russian language manuscript previously reported that larifan, an inducer of IFN, was active against OHFV (41). Due to its short half-life, IFN would likely 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 inhibits 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, valopcitabine or 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 log_{10} 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 IIb trial. This combination reduced viral load by >4 log_{10} 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 non-toxic 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 IIb 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, sofosbuvir or Sovaldi) has recently been approved. These experiences in the HCV field suggest that it may be possible to generate analogues of 2'-CMC effective against AHFV and other flaviviruses, though guanosine derivatives should probably be avoided.

Understanding the mechanism by which 2'-CMC inhibits AHFV replication is an important step towards 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 5-10-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 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 probably inhibits AHFV replication in the same way it does HCV, by binding 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, though 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 a 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, 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 (Dodd et al, submitted for publication) and plan to test the compounds for antiviral activity in these models. These compounds, even if not clinically effective, may be 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.
We are indebted to Tim Flietstra for advice on statistical analyses, to Mike Frace and Lori Rowe for help with PacBio sequencing and to Tanya Klimova for assistance with editing this manuscript.
REFERENCES


Table 1: Activities of AHFV inhibitors. Compounds were tested for their ability to inhibit AHFV by measuring inhibition of AHFV-induced CPE or measuring viral RNA by qRT-PCR. ND = not done.

<table>
<thead>
<tr>
<th></th>
<th>Inhibition of CPE</th>
<th>qRT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC_{50} (µM)</td>
<td>CC_{50} (µM)</td>
</tr>
<tr>
<td>2'-CMC</td>
<td>15.3 ± 3.7</td>
<td>&gt;50</td>
</tr>
<tr>
<td>PSI-6130</td>
<td>160 ± 64</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Lycorine</td>
<td>1.7 ± 0.5</td>
<td>16.2 ± 7.0</td>
</tr>
<tr>
<td>6-azaU</td>
<td>1.9 ± 0.2</td>
<td>&gt;50</td>
</tr>
<tr>
<td>6-azaU triacetate</td>
<td>7.3 ± 1.1</td>
<td>&gt;66</td>
</tr>
<tr>
<td>IFNα2^a</td>
<td>684 ± 499</td>
<td>&gt;160,000</td>
</tr>
</tbody>
</table>

^a Mean EC_{50} and CC_{50} values ± the standard deviation from at least three independent determinations are shown. SI = CC_{50} / EC_{50}.

^b Values for IFN are in IU/mL.
Table 2: Activity of inhibitors against selected pathogenic tick-borne flaviviruses. EC$_{50}$ values for the indicated compounds were determined in an assay for CPE inhibition.

<table>
<thead>
<tr>
<th></th>
<th>2’-CMC</th>
<th>6-azaU</th>
<th>6-azaU-triacetate</th>
<th>IFN$\alpha$2a</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>
<td>863 ± 450</td>
</tr>
<tr>
<td>OHFV</td>
<td>3.2 ± 1.1</td>
<td>1.9 ± 0.5</td>
<td>6.5 ± 2.5</td>
<td>135 ± 130</td>
</tr>
<tr>
<td>POWV</td>
<td>5.5 ± 1.3</td>
<td>1.7 ± 0.3</td>
<td>5.4 ± 1.5</td>
<td>7.8 ± 3.0</td>
</tr>
</tbody>
</table>

* Mean EC$_{50}$ values ± the standard deviation from at least 3 independent determinations are shown in µM, or in IU/mL for IFN$\alpha$2a.
Table 3: Antiviral effects of compounds in combination.

<table>
<thead>
<tr>
<th>Compound A</th>
<th>Compound B</th>
<th>Mean synergy volume (µM²)²</th>
<th>Mean antagonism volume (µM²)²</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNα2a</td>
<td>2'-CMC</td>
<td>21.85</td>
<td>-0.03</td>
<td>Additive</td>
</tr>
<tr>
<td>IFNα2a</td>
<td>6-azaU</td>
<td>60.16</td>
<td>-22.18</td>
<td>Moderate synergy</td>
</tr>
<tr>
<td>2'-CMC</td>
<td>6-azaU</td>
<td>228.81</td>
<td>-59.07</td>
<td>Strong synergy, moderate antagonism</td>
</tr>
</tbody>
</table>

² Values represent the mean synergy or antagonism volume at the 95% confidence from 3 independent experiments each performed with at least 4 replicates.
Table 4: Selection AHFV variants with reduced susceptibility to 2’-CMC.

<table>
<thead>
<tr>
<th>Passage #</th>
<th>2’-CMC (µM)</th>
<th>Duration of selection (days)</th>
<th>Mock-selected</th>
<th>2’-CMC-selected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Titer (TCID₅₀/mL)</td>
<td>EC₅₀ (µM)ᵃ</td>
</tr>
<tr>
<td>8</td>
<td>25</td>
<td>3 or 4</td>
<td>6 x 10⁸</td>
<td>8.8 ± 1.2</td>
</tr>
<tr>
<td>15</td>
<td>50</td>
<td>3 or 4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>17</td>
<td>50</td>
<td>2</td>
<td>2 x 10⁸</td>
<td>7.7 ± 2.4</td>
</tr>
<tr>
<td>21</td>
<td>50</td>
<td>1</td>
<td>3 x 10⁷</td>
<td>6.8 ± 2.7</td>
</tr>
</tbody>
</table>

ᵃ Mean EC₅₀ values ± the standard deviation from 3 independent determinations.
Table 5: Substitutions associated with AHFV growth in the presence of 2'-CMC.

<table>
<thead>
<tr>
<th>Selection</th>
<th>Nucleotide change</th>
<th>Polyprotein numbering</th>
<th>NSS numbering</th>
<th>Passage</th>
<th>Frequency (%)</th>
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<td>Non-coding</td>
<td>Non-coding</td>
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<td></td>
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<td>69</td>
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<td></td>
<td></td>
<td>21</td>
<td>69</td>
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<tr>
<td>Mock</td>
<td>T9595C</td>
<td>Y3164H</td>
<td>Y651H</td>
<td>8</td>
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<td></td>
<td></td>
<td>21</td>
<td>65</td>
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<tr>
<td>2'-CMC</td>
<td>T9451A</td>
<td>S3116T</td>
<td>S603T</td>
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<td>0</td>
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<td>97</td>
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<tr>
<td>2'-CMC</td>
<td>A9574G</td>
<td>M3157V</td>
<td>M644V</td>
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<td>2'-CMC</td>
<td>T9640A</td>
<td>C3179S</td>
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<td>21</td>
<td>98</td>
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</table>
Figure legends

Figure 1: 2’-modified nucleoside analogues inhibit Alkhurma hemorrhagic fever virus (AHFV). (A) Inhibition of AHFV-induced cell death. 2’-modified nucleoside analogues were 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, incubated for 3 days, and cell viability was determined. (B) Effect of 2’-modified nucleosides on cell viability. A549 cells were treated as in (A), but were mock-infected. Cell viability was measured 3 days after the addition of compound. Cells were treated with 10 µM dasatanib at as a positive control to inhibit cell viability. Mean values from four replicate wells are shown, error bars indicate the standard error of the mean. A representative of three independent experiments is shown. Abbreviations: DMSO, dimethylsulfoxide; 2’-CMA, 2’-C-methyladenine; 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.

Figure 2: Effect of compounds on AHFV titers. A549 cells were treated with (A) 2’-CMC, (B) 6-azaU, or (C) IFNα2a, and infected with AHFV at an MOI of 0.5. 24 h post-infection, the cultures were harvested, frozen and thawed three times and the viral titers in the resulting supernatants were determined. Mean titers from three biological replicates are depicted, error bars indicate standard error of the mean.

Figure 3: The antiviral effect of 6-azaU is inhibited by uridine or cytidine, but not guanosine. A549 cells were treated with 4 µM 6-azaU and diluent (black bar) or with varying concentrations of uridine (white columns), cytidine (shaded columns) or guanosine (cross-hatched columns). The cells were then infected with AHFV at an MOI of 0.5, then incubated for three days before cell viability was determined. Mean values from eight replicate wells are shown, error bars indicate the standard deviation.
Figure 4: A 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, 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 post-transfection. To account for differences in transfection efficiency, luciferase signals were normalized to those obtained at 4 h post-transfection. Mean values from four replicate wells are shown, error bars indicate the standard deviation. (C) Immunofluorescence of Huh7 cells electroporated with pol- or wild-type replicons, fixed and stained at 48 h post-transfection. Cell nuclei are blue, green staining indicates AHFV proteins.

Figure 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 post-transfection and the cultures were incubated for 48 h before harvesting for luciferase assay. Luciferase signals were normalized to that at 4 h post-transfection 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 error of the mean. (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 error of the mean.
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