Small molecule antiviral β-D-N4-hydroxycytidine inhibits a proofreading-intact coronavirus with a high genetic barrier to resistance

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ABSTRACT

Coronaviruses (CoVs) have emerged from animal reservoirs to cause severe and lethal disease in humans, but there are currently no FDA approved antivirals to treat these infections. One class of antiviral compounds, nucleoside analogues, mimics naturally occurring nucleosides to inhibit viral replication. While these compounds have been successful therapeutics for several viral infections, mutagenic nucleoside analogues, such as ribavirin and 5-fluorouracil, have been ineffective at inhibiting CoVs. This has been attributed to the proofreading activity of the viral 3'-5' exoribonuclease (ExoN). β-D-N4-hydroxycytidine (NHC, EIDD-1931; Emory Institute for Drug Development) has recently been reported to inhibit multiple viruses. Here, we demonstrate that NHC inhibits both murine hepatitis virus (MHV) (EC₅₀=0.17 μM) and Middle East respiratory syndrome CoV (MERS-CoV) (EC₅₀= 0.56 μM) with minimal cytotoxicity. NHC inhibited MHV lacking ExoN-proofreading activity similarly to WT MHV, suggesting an ability to evade or overcome ExoN activity. NHC inhibited MHV only when added early during infection, decreased viral specific infectivity, and increased the number and proportion of G:A and C:U transition mutations present after a single infection. Low-level NHC resistance was difficult to achieve and was associated with multiple transition mutations across the genome in both MHV and MERS-CoV. These results point toward a virus-mutagenic mechanism of NHC inhibition in CoVs and indicate a high genetic barrier to NHC resistance. Together, these data support further development of NHC for treatment of CoVs and suggest a novel mechanism of NHC interaction with the CoV replication complex that may shed light on critical aspects of replication.
The emergence of coronaviruses (CoVs) into human populations from animal reservoirs has demonstrated their epidemic capability, pandemic potential, and ability to cause severe disease. However, no antivirals have been approved to treat these infections. Here, we demonstrate the potent antiviral activity of a broad-spectrum ribonucleoside analogue, β-D-N⁴-hydroxycytidine (NHC), against two divergent CoVs. Viral proofreading activity does not markedly impact sensitivity to NHC inhibition, suggesting a novel interaction between a nucleoside analogue inhibitor and the CoV replicase. Further, passage in the presence of NHC generates only low-level resistance, likely due to the accumulation of multiple, potentially deleterious, transition mutations. Together, these data support a mutagenic mechanism of inhibition by NHC and further support the development of NHC for treatment of CoV infections.
INTRODUCTION

The emergence of severe acute respiratory syndrome (SARS) in 2002 and Middle East respiratory syndrome (MERS) in 2012 has underscored the ability of coronaviruses (CoVs) to cause lethal disease in humans (1, 2). MERS-CoV continues to infect humans in the Middle East and four additional human CoVs (HCoVs), HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1, continue to circulate globally and cause respiratory disease (3-6). The continued circulation of SARS- and MERS-like CoVs that can replicate efficiently in primary human airway cells in bat populations further demonstrates the potential for CoVs to emerge and cause severe disease in the future (7-10). While SARS-CoV and MERS-CoV outbreaks have been controlled largely through public health measures (11-13), the potential for future outbreaks highlights the need for safe and effective therapeutics to combat CoV infections. There are currently no approved therapeutics or vaccines for any human CoV infection. Previous efforts to treat CoV infections with existing antivirals did not conclusively benefit clinical outcome; thus, the current standard of care remains mostly supportive (14-16).

Several targets for direct-acting antivirals are being investigated to treat CoV infections (17-19). Because the viral replication machinery performs an essential role in genome replication, therapeutics approved to treat multiple different viral infections are aimed at this target (20). Many approved antivirals are classified as nucleoside analogues, compounds that mimic natural nucleosides to inhibit viral replication (21). Inhibition by nucleoside analogues can be accomplished through a variety of mechanisms. Common mechanisms of action include incorporation of the analogue by the viral polymerase to induce premature termination of strand.
synthesis or loss of essential genetic information through mutagenesis (22-25). A previous study reported that the nucleoside analogues ribavirin (RBV) and 5-fluorouracil (5-FU) did not potently inhibit CoVs, and this finding was attributed to the proofreading capabilities of the viral 3’-5’ exoribonuclease (ExoN) (26). Recent reports have demonstrated the inhibition of WT CoVs by nucleoside analogues such as galidesivir (BCX4430) and remdesivir (GS-5734) (27-29). While these compounds have shown efficacy against CoVs, administration of multiple compounds simultaneously may be required to effectively treat CoV infections and control the emergence of drug resistance, as has been demonstrated for other viral infections (30).

β-D-N4-hydroxycytidine (NHC) (EIDD-1931, Emory Institute for Drug Development), a cytidine analogue, has recently been shown to inhibit multiple viruses, including chikungunya virus, Venezuelan equine encephalitis virus, respiratory syncytial virus, hepatitis C virus, norovirus, influenza A and B viruses, and Ebola virus (31-36). Previous reports have demonstrated an increased introduction of transition mutations in viral genomes after treatment as well as a high genetic barrier to resistance (31, 36). Antiviral activity of NHC has also been reported against the human α-CoV HCoV-NL63, as well as the β-CoV SARS-CoV (38-40). Neither NHC mechanism of action nor NHC resistance have been described for any CoVs to date.

In this study, we investigated NHC inhibition and resistance in two divergent β-CoVs, murine hepatitis virus (MHV) and MERS-CoV. We show that NHC potently inhibits WT MHV and MERS-CoV with minimal cytotoxicity. We also demonstrate that MHV ExoN proofreading activity has a limited but measurable effect on sensitivity to NHC. We observe an NHC inhibition profile consistent with a mutagenic mechanism of action featuring an accumulation of transition mutations, indicative of a high genetic barrier to resistance.
RESULTS

NHC inhibits MHV and MERS-CoV replication with minimal cytotoxicity.

NHC (Fig. 1) has potent broad-spectrum antiviral activity against many RNA viral families (31-36). We first determined if NHC also inhibits CoV replication using a dose-response experiment with two divergent β-CoVs: the model CoV, MHV, and the epidemically circulating zoonotic CoV, MERS-CoV. NHC treatment resulted in a dose-dependent reduction in viral titer for MHV (Fig. 2A) and MERS-CoV (Fig. 2B). This inhibition resulted in a 50% effective concentration (EC50) of 0.17 μM for MHV (Fig. 2C) and 0.56 μM for MERS-CoV (Fig. 2D). We detected negligible changes in DBT-9 cell viability out to 200 μM (Fig. 2E) and CC50 values above 10 μM in Vero cells (Fig. 2F). The antiviral activity was not due to cytotoxicity, as the selectivity index was >1000 for MHV and >20 for MERS-CoV. Together, these results confirm potent inhibition of β-CoVs by NHC.

NHC inhibition profile in CoVs is consistent with mutagenesis.

To better understand the mechanism through which NHC inhibits CoV replication, we performed a time-of-drug addition assay to determine at what point in the viral replication cycle NHC acts (37). We added 16 μM (~100x EC50 concentration) NHC at the indicated times pre- or post-infection of cells with WT MHV at a MOI of 1 PFU/cell and quantified viral replication after a single infectious cycle. Compared to the vehicle (DMSO) control, NHC significantly inhibited MHV replication when added at or before six hours post-infection (Fig. 3A), suggesting that NHC acts at early stages of the viral replication cycle. We next determined the effect of NHC on MHV RNA levels and compared to effect on infectious viral titer. RNA levels were reduced by
approximately 10-fold at the highest tested concentration of NHC in both MHV-infected cell
monolayers (Fig. 3B) and supernatants (Fig. 3C). In contrast, viral titer was reduced up to 5,000-
fold at this concentration. We therefore calculated the ratio of infectious virus per viral RNA
genome copy number normalized to the untreated control (specific infectivity) after NHC
treatment and found that the specific infectivity of WT MHV was reduced in a dose-dependent
manner after treatment with increasing concentrations of NHC (Fig. 3D). Together, these data
are consistent with a mutagenic mechanism of NHC anti-CoV activity.

**NHC treatment increases transition mutations present across the MHV genome.**

To directly test the effect of NHC treatment on mutational burden, we treated WT MHV with
increasing concentrations of NHC and performed full-genome next-generation sequencing
(NGS) on viral populations released after a single round of infection. Our data demonstrate a
dose-dependent increase in mutations present at low frequencies (<5 % of viral population)
across the genome after treatment with increasing concentrations of NHC (Fig. 4A-C). Further
analysis of the types of mutations introduced by NHC revealed an increase in the total number of
transition mutations with increasing NHC concentrations (Fig. 4D-F). The relative proportion of
G:A and C:U transitions among all observed mutations was increased by 13-15% in the presence
of 2 μM NHC and 36-40% in the presence of 4 μM NHC compared to the vehicle control (Fig.
4G, H). Conversely, the relative proportion of A:G and U:C transitions was decreased with
increasing NHC concentrations compared to the vehicle control (Fig. 4G, H). Together, these
results demonstrate that NHC treatment during a single round of WT MHV infection causes
predominantly G:A and C:U transition mutations that are detectable at low frequencies across the
genome. These data further support a mutagenic mechanism of action for NHC inhibition of WT
NHC inhibition is modestly enhanced in the absence of ExoN proofreading.

Mutagenic nucleoside analogues, such as RBV and 5-fluorouracil (5-FU), have been ineffective at potently inhibiting WT CoVs due to the ExoN proofreading activity (26). A proofreading-deficient MHV mutant, ExoN(-), displays increased sensitivity to previously tested nucleoside analogues, indicating that proofreading dampens inhibition by these compounds (26, 38, 39).

Thus, we tested the sensitivity of ExoN(-) MHV to NHC inhibition. Our results indicate that NHC decreases viral titer of both WT and ExoN(-) MHV in a dose-dependent manner, but that ExoN(-) MHV demonstrates a statistically significant increase in sensitivity to NHC inhibition compared to WT MHV (Fig. 5A). However, this difference is reflected in only a modest decrease in EC₉₀ concentration by approximately 2-fold for ExoN(-) (0.72 μM) compared to WT MHV (1.59 μM) (Fig. 5B). The minimal change in sensitivity to NHC observed for ExoN(-) MHV indicates that NHC potency is only marginally affected by ExoN proofreading activity.

Passage in the presence of NHC yields low-level resistance associated with multiple transition mutations.

To better understand the development and impact of NHC resistance in CoVs, we passaged two lineages of WT MHV in thirty times in the presence of increasing concentrations of NHC and tested the sensitivity of passage 30 (p30) MHV populations to NHC inhibition. We found that the lineage 1 (MHV p30.1) viral population showed no change in sensitivity to NHC compared to WT MHV (Fig. 6A). However, lineage 2 (MHV p30.2) showed a decrease in sensitivity to NHC inhibition in a titer-reduction assay, especially at higher concentrations of compound. We
observed a modest, approximately 2-fold, increase in EC\textsubscript{90} values for MHV NHC passage viruses (MHV p30.1 EC\textsubscript{90} = 2.61 \mu M; MHV p30.2 EC\textsubscript{90} = 2.41 \mu M; WT MHV EC\textsubscript{90} = 1.53 \mu M) (Fig. 6B). This suggests that MHV passage resulted in minimal resistance to NHC. We next sought to determine if passaging WT MHV in the presence of NHC altered the replication capacity of these viruses. We found that both lineages showed a delay in replication but ultimately reached similar peak titers as WT MHV (Fig. 6C). This delay in replication suggests that MHV p30 viruses are less fit than WT MHV.

To identify mutations associated with these phenotypes after passage, we sequenced complete genomes of MHV p30.1 and MHV p30.2. Both lineages passaged in the presence NHC had accumulated over 100 consensus mutations distributed across the genome (Fig. 6D, E; Table S1). By comparison, a previous study reported that WT MHV accumulated only 23 total mutations after 250 passages in the absence of drug (39). Further analysis of the p30 MHV mutational profile demonstrated that slightly more of the total mutations in both lineages were synonymous changes that did not result in an amino acid change as opposed to nonsynonymous changes that did alter amino acid sequence (Fig. 6F; Table S1). Additionally, the vast majority of mutations in both lineages were transition mutations resulting in a purine-to-purine or pyrimidine-to-pyrimidine change (Fig. 6G). Both lineages contained only two transversion mutations resulting in a purine-to-pyrimidine or pyrimidine-to-purine change. Though all possible transition mutation types were detected in both viral lineage populations, the majority in both passage lineages were G:A transitions (Fig. 6H), which is consistent with the MHV NGS data (Fig. 4). To determine if the mutational profile at p30 was consistent with an earlier passage, we analyzed the whole genome of both lineage 1 and 2 at passage 19 (p19). Both lineages demonstrated fewer mutations at p19 than at p30, but the profiles of synonymous vs.
nonsynonymous changes and the transition mutations were similar (Fig. S1; Table S2). To determine whether the lack of robust resistance to NHC was broadly applicable across \( \beta \)-CoVs, we assessed the capacity of MERS-CoV to evolve resistance to NHC. Like MHV, we passaged two lineages of MERS-CoV 30 times in the presence of increasing concentrations of NHC and tested the sensitivity of these lineages to inhibition by NHC. Compared to WT MERS-CoV passaged in the absence of drug, both MERS-CoV NHC p30.1 and p30.2 exhibited decreased sensitivity to NHC inhibition (Fig. 7A). This correlated with modestly increased EC\( _{90} \) values for the passage lineages (WT MERS-CoV EC\( _{90} \) = 1.31 \( \mu \)M; MERS-CoV p30.1 EC\( _{90} \) = 3.04 \( \mu \)M; MERS-CoV p30.2 EC\( _{90} \) = 2.12 \( \mu \)M) (Fig. 7B), corresponding to approximately 2-fold resistance. Similar to MHV, we observed no substantial shift in dose response curve for MERS-CoV, indicating minimal acquired resistance. NHC p30 viruses replicated similarly to WT p30 MERS-CoV (Fig. 7C). We sequenced both lineages of MERS-CoV p30 population virus and detected 27 consensus mutations in MERS-CoV NHC p30.1 (Fig. 7D; Table S3) and 41 consensus mutations in MERS-CoV NHC p30.2 (Fig. 7E; Table S3) that were randomly distributed across the genome. Both MERS-CoV NHC p30.1 and MERS-CoV NHC p30.2 accumulated nonsynonymous and synonymous mutations in roughly equal proportions (Fig. 7F). Like in MHV, the mutations detected in MERS-CoV p30 lineages were predominantly transition mutations (Fig. 7G). Further analysis of these mutations revealed that the predominant type of transition was lineage-dependent. The majority of transition mutations in MERS-CoV NHC p30.1 were G:A transitions, as was observed in both p30 MHV lineages, whereas MERS-CoV NHC p30.2 contained a similar number of each type (Fig. 7H). These results indicate that MERS-CoV can achieve low-level resistance to NHC and that development of resistance is associated with the accumulation of multiple transition mutations. Together, our data suggest...
NHC acts as a mutagen and that it poses a high genetic barrier to resistance for β-CoVs.

DISCUSSION
In this study, we demonstrate that NHC potently inhibits the divergent β-CoVs MHV and MERS-CoV. Our data are consistent with a virus mutagenic mechanism of action as evidenced by a decrease in specific infectivity and an increase in G:A and C:U transition mutations present at low frequencies across the genome after treatment with NHC. We also demonstrate that robust resistance to NHC is difficult to achieve in both MHV and MERS-CoV. Both WT MHV and ExoN(-) MHV are sensitive to NHC inhibition, suggesting that NHC is able to overcome ExoN-mediated proofreading to inhibit WT CoVs and that it interacts with CoVs differently than other previously tested nucleoside analogues.

Utility of the broad spectrum antiviral NHC as a pan-CoV therapeutic.
Early work with NHC focused on the mutagenic effects of this compound in multiple bacterial systems (40-42). More recently, the antiviral properties of this compound have been reported for multiple RNA viruses, including chikungunya virus, Venezuelan equine encephalitis virus, respiratory syncytial virus, hepatitis C virus, norovirus, influenza A and B viruses, and Ebola virus (31-36). NHC has also been shown potently inhibited SARS-CoV and HCoV-NL63 (43, 44), suggesting potential utility in treating CoV infections (17). Based on previous studies, NHC appears to primarily inhibit viral replication by mutagenesis (31, 34). Serial passaging in the presence of NHC led to low-level resistance for VEEV, but no detectable resistance for RSV, IAV, or bovine viral diarrhea virus, indicating a high barrier to resistance (31, 34, 36). Consistent with these previous studies, we demonstrate that NHC is mutagenic in CoVs and that serial
passaging yields low-level, approximately 2-fold resistance. Low-level resistance has also been observed for remdesivir, another nucleoside analogue that potently inhibits CoVs.

Approximately 6-fold resistance to remdesivir is conferred by two mutations in the CoV RdRp (38). This study further expands the known antiviral spectrum of NHC to include MHV and MERS-CoV, two genetically divergent β-CoVs and supports NHC development as a broad-spectrum CoV antiviral.

NHC inhibition may circumvent ExoN-mediated proofreading.

NHC is the first mutagenic nucleoside analogue demonstrated to potently inhibit proofreading-intact CoVs. Previous studies have demonstrated that viruses lacking ExoN proofreading activity, or ExoN(-) viruses, are more sensitive to inhibition by nucleoside analogues, especially RBV and 5-FU (26, 38, 39, 45). This increased sensitivity has been attributed to an inability of ExoN(-) to efficiently remove incorrect nucleosides (46). However, we observed a minimal change in NHC sensitivity between WT MHV and ExoN(-) MHV, especially by EC₉₀. This suggests that NHC interacts with the CoV replicase differently than other previously tested nucleoside analogues. One explanation is that NHC may evade removal by the proofreading ExoN. Studies investigating nucleosides that inhibit DNA viruses have suggested an inability of the viral exonuclease to efficiently excise some nucleoside analogues (47, 48). Further, a previous study suggested that the T4 DNA exonuclease activity was incapable of removing NHC (49). While the SARS-CoV ExoN efficiently removes 3' terminal mismatches regardless of type (46, 50), the effect of NHC on this activity has not been investigated. Interestingly, mismatches readily observed during single nucleotide elongation by the SARS-CoV polymerase in the absence of drugs correspond to mismatches that would lead to the G:A and C:U transitions...
observed after NHC treatment (46). This suggests that the CoV polymerase could be naturally more prone to make these types of errors, which are then magnified by NHC. This could lead to a scenario where ExoN cannot prevent dipping below the error threshold, ultimately resulting in lethal mutagenesis and similar inhibition of both WT MHV and ExoN(-) MHV (51).

Several nucleosides including the mutagenic RBV have multiple demonstrated mechanisms other than direct incorporation into the genome (52, 53). Thus, another explanation for the unique potency of NHC in the presence of an active proofreading ExoN is that it may inhibit viral replication by additional mechanisms beyond mutagenesis. Indeed, previous reports have suggested that NHC may also interfere with the RNA secondary structure or virion release to cause inhibition (31, 36). Further, exogenous C or U in the presence of NHC could rescue viral replication in HCV, Chikungunya virus, RSV, and Influenza A virus (32, 34, 36), indicating that NHC competes with exogenous nucleosides at some stage prior to viral inhibition. These results raise the possibility that NHC could inhibit a process that results in similar inhibition of these viruses by a mechanism unrelated to ExoN. Thus, future studies will be important to investigate the role of proofreading in NHC inhibition of CoVs to shed light on intricacies of NHC inhibition of the CoV replication complex.

NHC mutagenesis may hinder emergence of robust resistance to NHC.

The decrease in specific infectivity along with the accumulation of transitions across the CoV genome support a mutagenic mechanism of action for NHC in CoVs. NHC resistance in CoVs was modest and difficult to achieve, as we obtained approximately 2-fold resistance after 30 passages. Resistance was associated with multiple mutations. Interestingly, MERS-CoV accumulated less mutations over 30 passages than MHV. While differences in viral mutation
rates could be the driver of this difference, previous studies have suggested that MHV does not have a higher mutation rate than MERS-CoV (54-56). The differences in mutation accumulation between MHV and MERS-CoV may be a product of different passage conditions. While MHV was passaged with a consistent transfer volume, MERS-CoV passage volumes were adjusted over time to sustain viral replication under escalating selection for drug resistance. The constant volume passaging conditions may have more severely bottlenecked MHV populations and fixed more mutations in the genome than the variable volume passaging conditions applied to MERS-CoV (57). Alternatively, this difference could also reflect a difference in mutational robustness of the MHV and MERS-CoV genomes, though this proposition would need to be investigated further (58, 59). While a portion of the mutations that accumulated over passage likely contribute to NHC resistance, other mutations, such as those in ns2 or nsp2, which encode proteins dispensable for viral replication in cell culture, may be merely tolerated because of their limited effect on viral fitness in the context of our passage conditions (60-62). Few common mutations arose in both MHV and MERS-CoV passage series, (Supp. Table 1-3), suggesting that multiple pathways to low-level NHC resistance exist in CoVs. Interestingly, for both MHV and MERS-CoV, the p30 lineage that demonstrated a greater change in sensitivity to NHC was the lineage that had fewer overall mutations (Fig. 6, 7). Both MHV passage lineages replicated less well than WT MHV, suggesting that the accumulation of mutations during passage may negatively impact viral fitness and the ability of MHV to evolve robust resistance to NHC. Further, the MHV lineage that did not result in changed sensitivity to NHC by p30 (MHV p30.1) had fewer mutations present at consensus by p19 than the other lineage (Fig. S1). Thus, it is possible that the accumulation of deleterious mutations counteracts potential benefits of resistance mutations (63). If this is the case, mutations promoting NHC resistance would need to arise early during
passage to help mitigate the accumulation of excess deleterious mutations. Alternatively, the
inability to evade inhibition by NHC may lead to the accumulation of a greater number of NHC-
associated transitions and ultimately a higher mutational burden that may impact viral fitness
(64, 65). Together, our results support the hypothesis that establishment of resistance to NHC in
CoVs requires a delicate balance of resistance-promoting mutations, viral fitness, and
accumulation of deleterious mutations. Thus, defining the roles of individual NHC resistance-
associated mutations will be an important goal for future studies. Overall, our results support
further development of NHC as a broad-spectrum antiviral for treatment of CoV infections and
contribute new insights into important aspects of CoV replication.
MATERIALS AND METHODS

Cell culture. Murine astrocytoma delayed brain tumor (DBT) (66) and Vero cells (ATCC CCL-81) were maintained at 37°C in Dulbecco’s modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1% penicillin and streptomycin (Gibco), and 0.1% amphotericin B (Corning).

Viruses. All work with MHV was performed using the recombinant WT strain MHV-A59 (GenBank accession number AY910861 (67)). MERS-CoV stocks were generated from cDNA clones (GenBank accession number JX869059(68)).

Compounds and cell viability studies. NHC was synthesized at the Emory Institute for Drug Development and prepared as a 20 mM stock solution in dimethyl sulfoxide (DMSO). Cell viability was assessed using CellTiter-Glo (Promega) in 96-well plates according to the manufacturer’s instructions. DBT and Vero cells were incubated with indicated concentration of compound at 37°C for 24 hours (DBT) or 48 hours (Vero). Cell viability was determined using a Veritas Microplate Luminometer (Promega) or GloMax (Promega) with values normalized to those of vehicle-treated cells.

Nucleoside analogue sensitivity studies and generation of EC_{50} curves. Subconfluent monolayers of DBT cells were infected with MHV at a multiplicity of infection (MOI) of 0.01 PFU per cell for 1 hour at 37°C. The inoculum was removed and replaced with media containing the indicated compound concentration. Cell supernatants were harvested 24 hours post-infection. Titers were determined by plaque assay as described previously (69). Subconfluent monolayers of Vero cells were infected with an MOI of 0.01 PFU/cell of MERS-CoV. After virus adsorption for 30 minutes at 37°C, the inoculum was removed. Cells were washed with PBS and incubated with medium containing the indicated concentrations of NHC or DMSO (vehicle control). After
48 h, supernatant was collected and titers were determined by plaque assay as described previously (70). EC50 and EC90 values and curves were generated using the nonlinear regression curve fit in GraphPad Prism software (La Jolla, CA).

**Time-of-drug addition assay.** Subconfluent monolayers of DBT cells were treated with media containing DMSO or 16 μM NHC (~100x EC50) at the indicated time pre- or post-infection. Cells were infected with WT MHV at an MOI of 1 PFU/cell for 1 hour at 37°C. Virus inoculum was removed and fresh medium was replaced. Culture supernatant was harvested 12 hours post-infection, and viral titer was determined by plaque assay.

**Quantification of viral genomic RNA.** Subconfluent DBT cells were infected with WT MHV at an MOI of 0.01 PFU/cell. Inoculum was removed after 1 h incubation at 37°C and medium containing indicated concentrations of NHC was added. Total RNA from cells and supernatant RNA was harvested using TRIzol reagent (Invitrogen) after 20 hours. Both total RNA and supernatant RNA were extracted by phase separation. Total RNA was purified by ethanol precipitation and supernatant RNA was purified using the PureLink RNA mini kit (Invitrogen) according to manufacturer’s protocol. Total RNA was reverse transcribed using SuperScript III (Invitrogen) to generate cDNA that was quantified by quantitative polymerase chain reaction (qPCR) as previously described (26). Data are presented as 2^ΔΔCT, where ΔΔCT denotes the change in the threshold cycle for the viral target (nsp10) normalized to the control (GAPDH) before and after drug treatment. Supernatant RNA was quantified using one-step reverse transcriptase quantitative PCR (qRT-PCR) as previously described (45). Data are presented as the fold change in genome RNA copies normalized to vehicle control.

**Determination of specific infectivity.** Subconfluent DBT cells were infected with WT MHV at a MOI of 0.01 PFU/cell. Inoculum was removed after 1 h incubation at 37°C and medium
containing indicated concentrations of NHC was added. Supernatant RNA was harvested using the TRIzol reagent (Invitrogen) after 20 hours, followed by extraction and quantification as described above. Viral titer was determined by plaque assay. The specific infectivity was calculated as PFU divided by supernatant genome RNA copy number. This ratio was then normalized to the vehicle control.

**NGS studies.** Subconfluent DBT cells were infected with WT MHV at an MOI of 0.01 PFU/cell and treated with the indicated concentrations of NHC. Supernatant was collected 24 hours post-infection. Purified viral RNA was submitted to GENEWIZ for library preparation and sequencing. Briefly, after quality controls, viral RNAs were randomly fragmented using heat. Libraries were prepared and sequenced on the Illumina HiSeq platform. GENEWIZ performed base-calling and read demultiplexing. Trimmomatic was used to trim adapter contaminants and reads shorter than 36 basepairs and filter low quality bases (Q-score <30) (71). The paired-end fastq reads were then aligned to the MHV genome using Bowtie2 to generate a SAM file (72). SAMtools was used to process the resultant alignment file and calculate coverage depth at each nucleotide, generating a sorted and indexed BAM file. LoFreq was used to call substitution variants, including low-frequency variants, and generate a variant file (73). The Bash shell and Excel were used to further process and analyze the resultant vcf file. A frequency of 0.001 was used as a cutoff for variants, consistent with previous reports (74).

Absolute numbers of mutations are reported for each NHC treatment. The percentage of the total mutations for each specific mutation type was calculated using these numbers. The difference in percentage for each class of mutation after treatment as compared with vehicle control is referred to as the relative proportion of these mutations.

**MHV population passage in the presence of NHC.** WT MHV was passaged in triplicate in
increasing concentrations of NHC, from 1 μM to a maximum of 5 μM. Infection was initiated for passage 1 at MOI = 0.1 PFU/cell. Viral supernatants were harvested from each viral lineage and frozen when the cell monolayer demonstrated 80% cytopathic effect (CPE) or after 24 hrs. A constant volume of 16 μL was used to initiate subsequent passages. All three lineages were maintained until passage 16 when lineage 3 demonstrated no visible CPE upon multiple attempts at varying concentrations. Lineage 1 and 2 were maintained until passage 30. After each passage, total RNA was harvested from infected cell monolayers using the TRIzol reagent. Viral RNA was extracted from passage 19 and passage 30 samples and reverse transcribed using SuperScript III, followed by generation of 12 PCR amplicons to cover the whole genome. Dideoxy amplicon sequencing was performed by GENEWIZ and analyzed to identify mutations present at greater than 50% of total using MacVector. Viral mutation maps depicting the identified mutations were generated using MacVector.

MERS-CoV population passage in the presence of NHC. Three parallel, independent passage series of WT MERS-CoV were performed on Vero cells in the presence of gradually increasing concentrations of NHC up to a maximum concentration of 6.5 μM to select for drug-resistant mutant viruses. Virus adaptation to NHC-supplemented complete culture medium was assessed by monitoring progression of characteristic MERS-CoV CPE. Volumes of transferred culture supernatants were adjusted empirically to balance continuous selective pressure against culture extinction. Each of triplicate lineages in the MERS-CoV passage experiment was sustained through passage 30. However, the third lineage was severely impaired in replication and was excluded from further analysis. Total infected-cell MERS-CoV RNA purified from monolayers infected with terminal-passage (p30) culture supernatant was used to generate RT-PCR products for consensus Sanger sequencing of the complete viral genome (Genewiz). Changes in passaged
virus nucleotide and deduced amino acid sequences were identified via alignment with the WT parental virus genomic sequence using MacVector.

Virus replication assays. Subconfluent monolayers of DBT (MHV) or Vero (MERS-CoV) cells were infected with WT or NHC-passaged viral populations at an MOI of 0.01 PFU/cell for 1 hour (MHV) or 30 minutes (MERS-CoV). Inocula were removed and cells were washed with PBS before addition of pre-warmed media. Supernatants were harvested at indicated times post-infection, and titers were determined by plaque assay.

Statistics. Statistical tests were performed using GraphPad Prism 7 software (La Jolla, CA) as described in the respective figure legends.
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Fig. 1. Chemical structure of EIDD-1931, β-D-N4-hydroxycytidine (NHC).

Fig. 2. NHC inhibits MHV and MERS-CoV with minimal cytotoxicity. (A) Change in MHV and (B) MERS-CoV titer relative to vehicle control after treatment with increasing concentrations of NHC. The data represent the results of 6 independent experiments, each with 3 replicates. Error bars represent standard error of the mean (SEM). (C) Change in titer data from (A) represented as percent of vehicle control. WT MHV EC_{50} = 0.17 μM. (D) Change in titer data from (B) represented as percent of vehicle control. WT MERS-CoV EC_{50} = 0.56 μM. (E) DBT-9 cell viability as a percent of DMSO control across NHC concentrations. No cytotoxicity was detected up to 200 μM. The data represent the results of 2 independent experiments, each with 2 replicates (MHV). Error bars represent standard error of the mean (SEM). (F) Vero cell viability as a percent of DMSO control across NHC concentrations. Less than 50% cytotoxicity was detected up to 10 μM. The data represent the results of 2 independent experiments, each with 3 replicates. Error bars represent standard error of the mean (SEM).

Fig. 3. NHC inhibition profile of MHV is consistent with mutagenesis. (A) Treatment with 16 μM NHC (~100X EC_{50}) significantly inhibits MHV replication during a single infection when added before 6 h p.i. (B) Both MHV titer and monolayer RNA copies decrease after treatment with increasing concentrations of NHC. (C) NHC treatment results in a decrease in supernatant MHV RNA. (D) Data from (C) represented as the ratio of infectious WT MHV to genomic MHV RNA present in supernatant, or specific infectivity, normalized to vehicle control. NHC treatment results in a decrease in specific infectivity of MHV. All data in this figure represent the results of 2 independent experiments, each with 3 replicates. Error bars represent standard error of the mean (SEM). Statistical significance compared to DMSO control was determined by one-
Fig. 4. NHC treatment drives increase in low-frequency G:A and C:U transition mutations in WT MHV during a single infection. (A) Distribution and frequency of variants across the genome detected by NGS after vehicle treatment, (B) 2 μM NHC treatment, or (C) 4 μM NHC treatment. Log10 depth of coverage at each genomic position is depicted by the line; frequency of individual mutations spread across the genome are represented by dots. (D) Number of mutations in WT MHV after infection in the presence of (D) vehicle, (E) 2 μM NHC, or (F) 4 μM NHC presented by type. Transition mutations are shown in grey and transversion mutations are shown in white. (G) Change in relative proportion of each mutation type after treatment with (G) 2 μM NHC, or (H) 4 μM NHC compared to vehicle control. The relative proportions of G:A and C:U transitions increase with increasing concentrations of NHC treatment and are denoted by green shading.

Fig. 5. Sensitivity of ExoN(-) MHV to inhibition by NHC. (A) Change in viral titer for WT MHV and ExoN(-) MHV relative to vehicle control after treatment with NHC. ExoN(-) is more sensitive to NHC than WT. The data represent the results of 3 independent experiments, each with 3 replicates. Error bars represent SEM. Statistical significance compared to WT MHV was determined by Wilcoxon test and is denoted **, P < 0.01. (B) Change in viral titer data from (A) represented as percent of vehicle control. WT EC90 = 1.59 μM, ExoN(-) EC90 = 0.72 μM. ExoN(-) MHV is approximately 2-fold more sensitive to NHC than WT MHV.
Fig. 6. Resistance and mutational profile of MHV after 30 passages in the presence of NHC.

(A) Change in viral titer for WT MHV, MHV NHC passage 30 (p30) lineage 1 (MHV p30.1), and MHV NHC p30 lineage 2 (MHV p30.2) relative to vehicle controls after treatment with NHC. MHV NHC p30.2 is less sensitive to NHC than WT MHV while MHV p30.1 shows no change in sensitivity. The data represent the results of 2 independent experiments, each with 3 replicates. Error bars represent SEM. Statistical significance compared to WT MHV was determined by ratio paired t test and is denoted *, P < 0.05.

(B) Change in viral titer data from (A) represented as percent of vehicle control. WT MHV EC$_{90}$ = 1.53 μM; MHV p30.1 EC$_{90}$ = 2.61 μM, MHV p30.2 EC$_{90}$ = 2.41 μM. (C) Replication kinetics of NHC passage viruses. MHV p30.1 and p30.2 are delayed in replication compared to WT MHV but ultimately reach similar peak titers. The data represent the results of 2 independent experiments, each with 3 replicates. Error bars represent standard deviation (SD).

(D) MHV p30.1 accumulated a total of 162 consensus mutations across the genome detectable by Sanger sequencing. Of these mutations, 81 were synonymous, 64 were nonsynonymous, and 17 were noncoding. (E) MHV p30.2 accumulated 102 total mutations across the genome. Of these mutations, 54 were synonymous, 42 were nonsynonymous, and 7 were noncoding. (F) Each lineage accumulated more synonymous changes than nonsynonymous or noncoding changes over passage. (G) Breakdown of transition and transversion mutations present in each lineage after passage. MHV p30.1 and p30.2 mutations were predominantly transitions. (H) Breakdown of the types of transition mutations present in each lineage across passage. G:A transitions were the most abundant for both MHV p30.1 and p30.2.
Fig. 7. Resistance and mutational profile of MERS-CoV after 30 passages in the presence of NHC. (A) Change in viral titer relative to vehicle controls after treatment with NHC for WT MERS-CoV passaged 30 times in the absence of drug, MERS-CoV NHC passage 30 lineage 1 (MERS-CoV p30.1), and MERS-CoV NHC passage 30 lineage 2 (MERS-CoV p30.2) relative to vehicle controls after treatment with NHC. Both MERS-CoV p30.1 and p30.2 are less sensitive to NHC than WT MERS-CoV. The data represent the results of 2 independent experiments, each with 3 replicates. Error bars represent SEM. (B) Change in viral titer data from (A) represented as percent of vehicle control. WT MERS-CoV EC₉₀ = 1.31 μM, MERS-CoV p30.1 EC₉₀ = 3.04 μM, MERS-CoV p30.2 EC₉₀ = 2.12 μM. (C) Replication kinetics of NHC passage viruses. WT MERS-CoV, MERS-CoV p30.1, and MERS-CoV p30.2 replicate with similar kinetics and reach similar peak titers. The data represent the results of 2 independent experiments, each with 3 replicates. Error bars represent SEM. (D) MERS-CoV p30.1 accumulated 27 total mutations across the genome. Of these mutations, 14 were synonymous and 13 were nonsynonymous. (E) MERS-CoV p30.2 accumulated 41 total mutations. Of these mutations, 17 were synonymous, and 24 were nonsynonymous. (F) Both MERS-CoV p30.1 and p30.2 accumulated a similar numbers of nonsynonymous and synonymous changes during passage. (G) MERS-CoV p30.1 and p30.2 acquired predominantly transitions. (H) The types of transition mutations present in each lineage across passage. MERS-CoV p30.1 acquired relatively more G:A transitions, whereas MERS-CoV p30.2 acquired similar numbers of each transition type.
Change in Viral Titer (log_{10} PFU/ml)

WT
ExoN(-)

% Inhibition

ExoN(-)
WT

A
B
Change in Viral Titer (log_{10} PFU/ml)

% Inhibition

Time post-infection (h)

Viral Titer (log_{10} PFU/mL)

WT MHV
MHV p30.1
MHV p30.2

162 total mutations:
64 Nonsynonymous
81 Synonymous
17 Noncoding

102 total mutations:
42 Nonsynonymous
54 Synonymous
7 Noncoding

Transition type

A:G G:A C:U U:C

Number of mutations

MHV lineage

Nonsynonymous
Synonymous
Noncoding

MHV p30.1
MHV p30.2
Change in Viral Titer (Log_{10} PFU/mL)

% Inhibition

Viral Yield (Log_{10} PFU/mL)

MERS-CoV p30.1

27 total mutations:
13 Nonsynonymous
14 Synonymous

MERS-CoV p30.2

41 total mutations:
24 Nonsynonymous
17 Synonymous

E: MERS-CoV p30.2

F: MERS-CoV lineage

G: MERS-CoV lineage

H: Transition type