Repurposing Nucleoside Analogs for Human Coronaviruses

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ABSTRACT Coronavirus disease 2019 (COVID-19) is a serious illness caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2 or CoV-2). Some reports claimed certain nucleoside analogs to be active against CoV-2 and thus needed confirmation. Here, we evaluated a panel of compounds and identified novel nucleoside analogs with antiviral activity against CoV-2 and HCoV-OC43 while ruling out others. Of significance, sofosbuvir demonstrated no antiviral effect against CoV-2, and its triphosphate did not inhibit CoV-2 RNA polymerase.

KEYWORDS SARS-CoV-2, HCoV-OC43, antiviral agents, nucleoside analogs, remdesivir, sofosbuvir, emtricitabine, lamivudine, COVID-19, Coronaviridae

Coronavirus disease 2019 (COVID-19) has emerged as a global pandemic with significant morbidity and mortality caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2 or CoV-2). CoV-2-infected individuals usually develop mild to severe flulike symptoms, whereas other individuals (particularly the elderly harboring underlying chronic health complications, such as diabetes and heart disease, or immunocompromised individuals) are particularly prone to develop severe to fatal clinical outcomes (1, 2). SARS-CoV-2 is an enveloped virus with a positive-sense single-stranded RNA genome, which belongs to the genus *Betacoronavirus* (3). Human coronavirus OC43 (HCoV-OC43) is also a member of the *Betacoronavirus* genus and thought to be the most commonly encountered human coronavirus (3). The search for anticoronavirus agents is highly desirable for a rapid response to this pandemic.

One of the main druggable targets for coronaviruses (including CoV-2) is the RNA-dependent RNA polymerase (RdRp). This viral polymerase displays similar catalytic mechanisms and some key conserved amino acids in the active site among various positive-sense RNA viruses (4). Nucleoside analogs are a well-established class of antiviral agents for treatment of many human viruses. Widely used antiviral drugs are nucleoside analogs, including emtricitabine (FTC; HIV-1/HBV [hepatitis B virus]), lamivudine (3TC; HIV-1/HBV), tenofovir disoproxil fumarate (TDF; HIV-1/HBV), tenofovir alafenamide (TAF; HIV/HBV), entecavir (HBV), telbivudine (HBV), and sofosbuvir (HCV [hepatitis C virus]) (5–8).

In this study, we evaluated a defined library of compounds consisting of approved and experimental antiviral nucleoside analogs for their potential *in vitro* antiviral activity against SARS-CoV-2 and HCoV-OC43 (Fig. 1). We selected several reported anti-HCV agents, such as 2′-*C*-methylcytidine (compound 2) (9), sofosbuvir (compound 4), a novel prodrug of sofosbuvir (compound 3) (10), and two 2′-dihalogeno nucleoside prodrugs (compounds 6 and 7) (11, 12). We also selected ALS-8112 (compound 5) (13), the active form of lumicitabine, a drug developed until recently for the treatment of respiratory syncytial virus (RSV) (14). Note that our group previously demonstrated that ALS-8112 is also an inhibitor of Nipah virus replication (15). Finally, 7-deaza-7-fluoro-2′-*C*-methyladenosine (compound 1), a nucleoside showing anti-yellow fever virus

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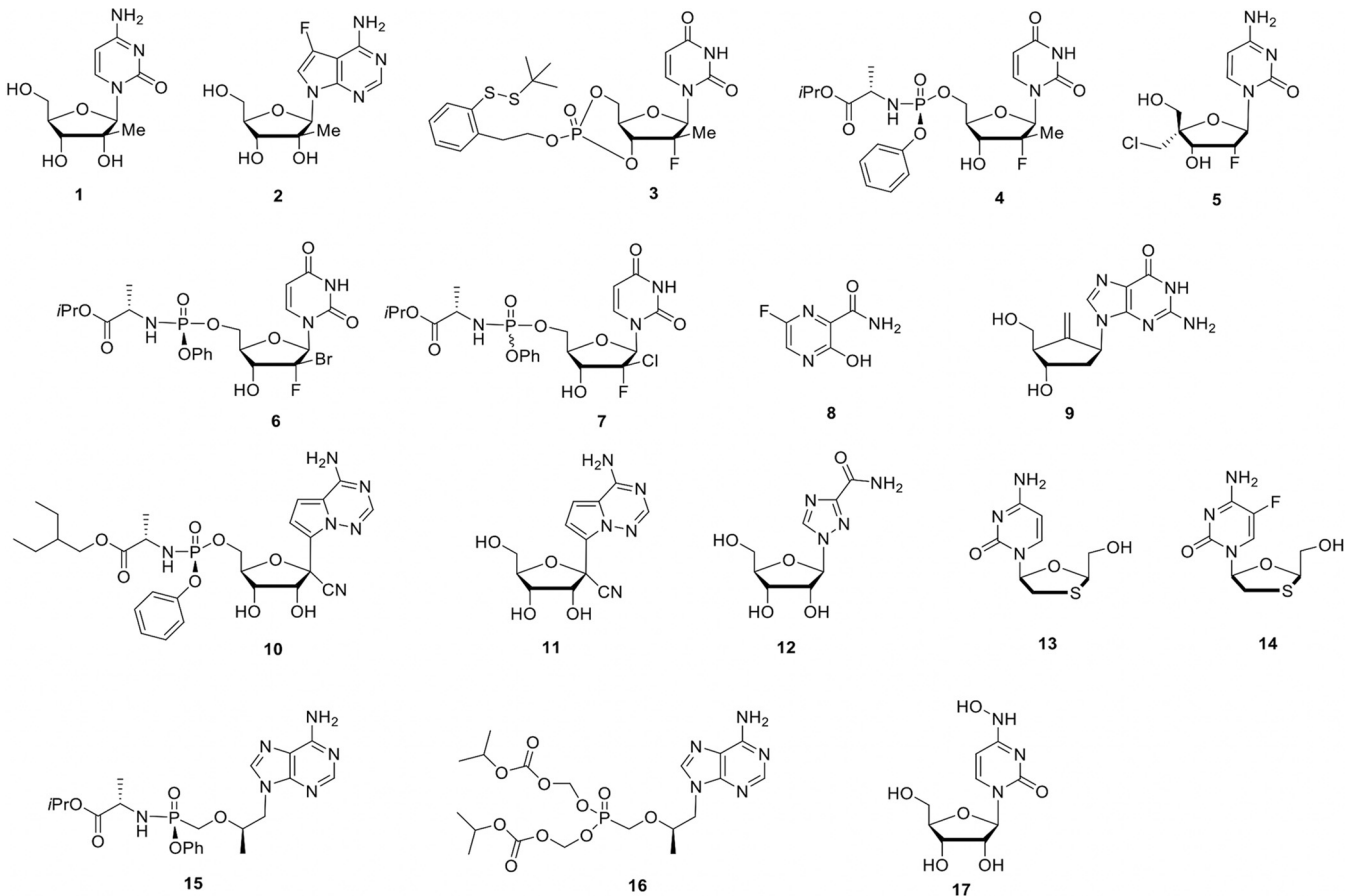
FIG 1 Chemical structures of nucleoside analogs evaluated in culture.

activity, was also evaluated (16, 17). Remdesivir (compound 10) (18) and β-D-N^4-hydroxycytidine (NHC; compound 17) were used as positive drug controls for these studies (19). Additional compounds were selected from our in-house library of nucleoside analogs based on their effectiveness against HCV *in vitro*.

All compounds were evaluated for their cell toxicity profiles using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] method (20). Briefly, we determined the half-maximal cytotoxic concentration (CC_{50}) value in a panel of different cell lines, including human peripheral blood mononuclear cells (PBM), human hepatoma cells (Huh-7), human lymphoblastic cells (CEM), and African green monkey kidney cells (Vero CCL-81 cells; ATCC) by treatment of exponentially growing culture of each cell line for 4 days (Table 1). Based on the cytotoxicity data, we chose the nontoxic concentration (range, 2.5 to 20 μM) as the highest concentration for our antiviral assays in subsequent studies to avoid any minor toxicity of the compounds.

TABLE 1 Primer and probe sequences

| Primer/probe	Sequence
SARS-CoV-2	
Forward primer	5'-GAC CCC AAA ATC AGC GAA AT-3'
Reverse primer	5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'
Probe	5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3'
HCoV-OC43	
Forward primer	5-ATG TTA GGC CGA TAA TTG AGG ACT AT-3'
Reverse primer	5'-FAM-CAT ACT CTG ACG GTC ACA AT-BHQ1-3'
Probe	5'-AAT GTA AAG ATG GCC GCG TAT T-3'
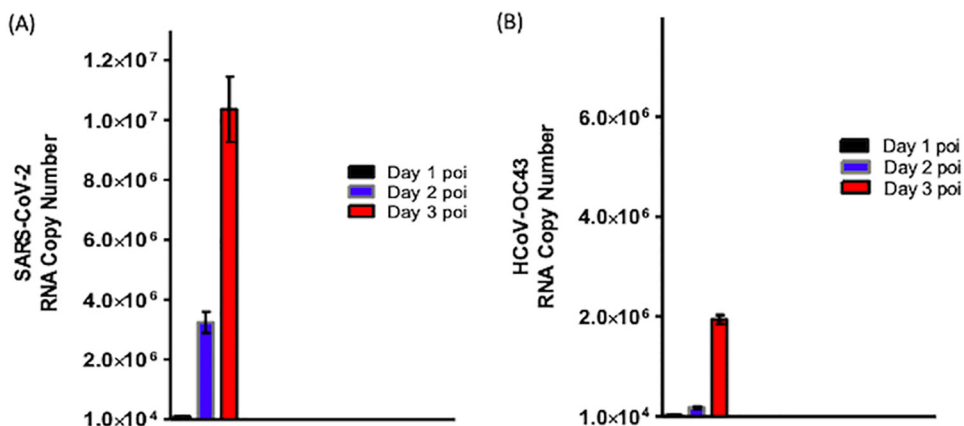
FIG 2 Virus replication kinetic in cell culture. Replication kinetics of SARS-CoV-2 in Vero cells (A) and HCoV-OC43 in Huh-7 cells (B).

HCoV-OC43 was obtained from ATCC (Manassas, VA), and SARS-CoV-2 was provided by BEI Resources (NR-52281: USA-WA/2020). HCoV-OC43 and SARS-CoV-2 were propagated in Huh-7 and Vero cells, respectively, and titrated by the 50% tissue culture infective dose (TCID$_{50}$) method, followed by storage of aliquots at $-80°C$ until further use.

To determine the kinetic replication for each virus in cell culture, a confluent monolayer of each cell line in a 96-well microplate was inoculated at a multiplicity of infection (MOI) of 0.1 for SARS-CoV-2 (21) and HCoV-OC43, and the yield of progeny virus production was assessed at various time points using a specific reverse transcription-quantitative PCR (qRT-PCR) for each virus. Two separate qRT-PCR assays were established to quantify the yield of SARS-CoV-2 and HCoV-OC43 from our antiviral assays in the supernatant of the infected cells through the different time points postinfection (Fig. 2). Briefly, a one-step qRT-PCR was conducted in a final volume of 20 μl containing extracted viral RNA, probe/primer mix (Table 1), and qScript-Tough master mix (Quantibio, USA). Quantitative PCR measurement was performed using the StepOnePlus real-time PCR system (Roche, Germany) according to the manufacturer's protocol.

To determine the best time point for the virus-yield assay, a kinetic replication of SARS-CoV-2 and HCoV-OC43 in Vero and Huh-7 cells, respectively, was performed, and the yield of progeny virus was assessed from the supernatant of viral infected cells at different-interval time points using specific qRT-PCR for each virus as mentioned earlier. We determined that 48 and 72 h postinfection were the optimum time points for SARS-CoV-2 and HCoV-OC43, respectively (Fig. 2), because there was no observed cell death and cytopathic effect on infected cells; and, more important, a significant increase in the virus RNA copy numbers that were harvested from the supernatant of the infected cells was observed at that time for SARS-CoV-2 and HCoV-OC43.

Next, we evaluated our compounds for their potential *in vitro* inhibitory effects against each virus using a virus-yield inhibition assay with qRT-PCR, as mentioned above. The resultant inhibitory effect of each test compound was calculated as a percentage of virus-yield inhibition. Briefly, a monolayer of Vero cells was prepared in a 96-well plate. The cells were then treated with a single nontoxic dose of each compound in triplicate and infected with CoV-2 at an MOI of 0.1 (21) and for HCOV-OC43 with 1 h of incubation followed by removal of inoculum and overlaying with cell culture medium containing the compounds. As a vehicle control, cells in three wells were treated with 0.1% dimethyl sulfoxide diluted in cell culture medium. The plate was kept at 37°C for 48 h in the presence of 5% CO_2, which was followed by a virus-yield inhibition assay using qRT-PCR on harvested supernatants from the wells. We identified four nucleoside analogs (compounds 1 to 3 and 11), along with our positive controls (compounds 10 and 17), that yielded a strong inhibitory effect (>90% virus-yield decrease) from a nontoxic single dose against *in vitro* replication of CoV-2. In addition,
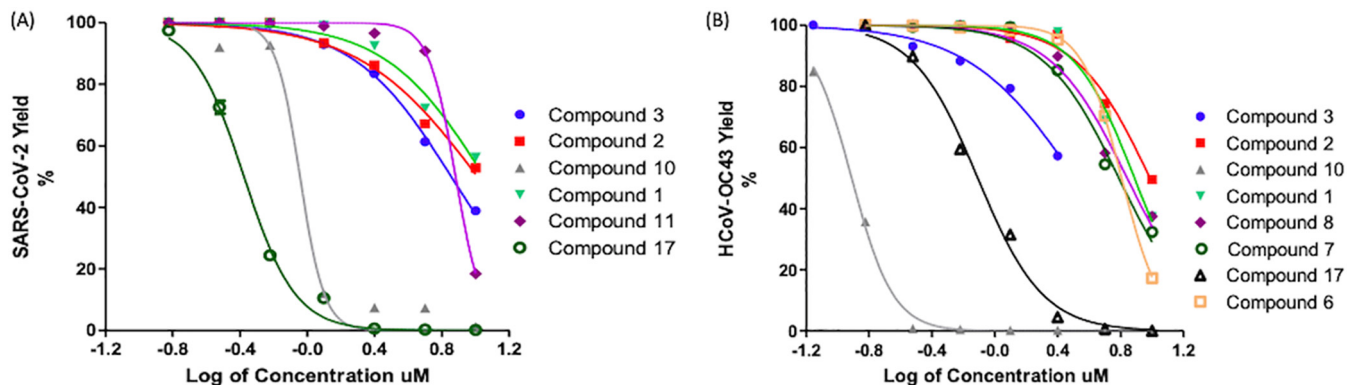
FIG 3 Dose-response antiviral activity. Dose response study of selected nucleoside analogs against SARS-CoV-2 in Vero cells (A) and HCoV-OC43 in Huh-7 cells (B). All experiments were done in triplicate.

these four compounds showed marked inhibitory effects against HCoV-OC43. Interestingly, compounds 6 and 7 exhibited antiviral activity against HCoV-OC43 but not against CoV-2. All active compounds were chosen for a subsequent dose-response study to determine their potency in culture (Fig. 3). Selected compounds were further assessed in a dose-dependent manner for their antiviral activity against SARS-CoV-2 and HCoV-OC43 using a virus-yield inhibition assay by determining the viral RNA copy number in collected supernatants 2 days posttreatment compared to the results from infected but untreated cells and noninfected and untreated cells as necessary controls (Table 2). All experiments were performed in triplicate, and each experiment was repeated three times independently to achieve reliable and statistically meaningful results.

The median effective concentration (EC_{50}) and the concentration with 90% of inhibitory effect (EC_{90}) were calculated using GraphPad Prism for Mac, version 7 (GraphPad Software Inc., San Diego, CA) and reported as the mean $\pm$ standard deviation. The selectivity index (SI) for each effective compound was calculated as CC_{50}/EC_{50}.

In this study, the reference compounds remdesivir and NHC displayed EC_{50} values

TABLE 2 Antiviral activity and cytotoxicity of compounds against SARS-CoV-2 and HCoV-OC43 in different cell lines

	Antiviral activity against (μM):							
	SARS-CoV-2		HCoV-OC43					
	Vero cells		Huh7 cells		Cytotoxicity (CC_{50} [μM])			
Compound	EC_{50}	EC_{90}	EC_{50}	EC_{90}	PBM	CEM	Vero	Huh7
1 (2'-MeC)	9.2 $\pm$ 0.1	29.6 $\pm$ 0.4	10 $\pm$ 0.7	15.1 $\pm$ 0.1	65.4	84.3	>100	>100
2	7.6 $\pm$ 0.4	28.8 $\pm$ 0.8	6.7 $\pm$ 1.1	13.8 $\pm$ 0.2	72.4	63.9	>100	38
3 (sofosbuvir cyclic phosphate prodrug)	6.3 $\pm$ 0.1	18.9 $\pm$ 0.2	>2[a]	ND[b]	58.4	31.6	16.4	2.9
4 (sofosbuvir)	>20	>20	>20	>20	>100	>100	>100	>100
5 (ALS-8112, lumicitabine)	>20	>20	>20	>20	4.2	2.8	>100	4.2
6	>20	>20	6.8 $\pm$ 0.2	12.8 $\pm$ 0.4	>100	>100	>100	72.4
7	>20	>20	5.9 $\pm$ 0.6	18.6 $\pm$ 0.3	>100	>100	>100	>100
8 (favipiravir)	>20	>20	6.8	>10	>100	>100	>100	>100
9 (entecavir)	>20	>20	>20	>20	21.5	>100	>100	>100
10 (remdesivir)	1.0 $\pm$ 0.1	3.5 $\pm$ 0.3	0.04 $\pm$ 0.1	0.09 $\pm$ 0.09	4.5	11.6	>100	2.1
11 (parent nucleoside of remdesivir, GS-441524)	8.2 $\pm$ 0.4	13.2 $\pm$ 0.8	>10	>20	>100	>100	>100	>100
12 (ribavirin)	>20	>20	20.6	>20	>100	7.5	>100	22.1
13 (3TC)	>20	>20	>20	>20	>100	>100	>100	>100
14 (FTC)	>20	>20	>20	>20	>100	>100	>100	>100
15 (TAF)	>20	>20	>20	>20	50.8	13.67	36.4	>100
16 (TDF)	>20	>20	>20	>20	44.4	33.9	>100	>100
17 (NHC)	0.3 $\pm$ 0.2	0.8 $\pm$ 0.1	0.8 $\pm$ 0.03	1.8 $\pm$ 0.1	44.3	3.5	12.6	80.3

[a]To avoid the cytotoxicity, 2 μM was chosen as the highest concentration for antiviral assays for compound 3 for HCoV-OC43 in Huh-7 cells.
[b]ND, not determined.
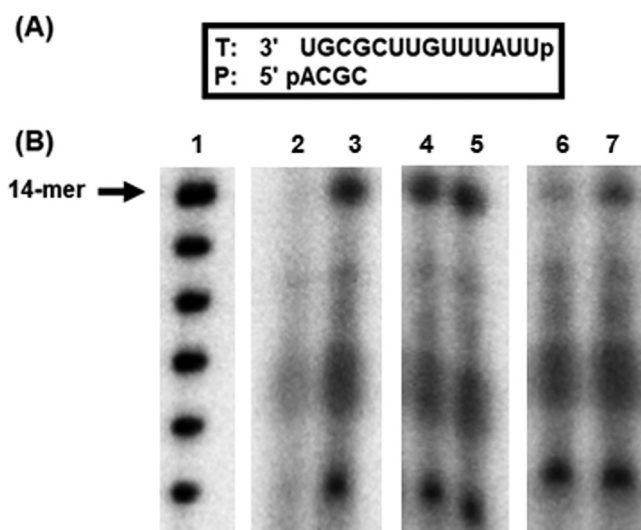
FIG 4 SARS-CoV-2 RdRp inhibition by triphosphate forms of sofosbuvir and remdesivir. (A) RNA 4-mer primer/14-mer template used in the RdRp reactions. (B) Full-length 14-mer RNA product syntheses by SARS-CoV-2 RdRp in the presence/absence of each compound. Respective lanes were labeled as indicated: 1, size marker; 2, no enzyme control; 3, SARS-CoV-2 RdRp control reaction without inhibitors; 4 and 5, SARS-CoV-2 RdRp reactions with 10 μM and 1 μM sofosbuvir triphosphate; 6 and 7, SARS-CoV-2 RdRp reactions with 10 μM and 1 μM remdesivir triphosphate.

of 1.0 and 0.3 μM, respectively, against SARS-CoV-2 in Vero cells, similar to data reported in the literature (19, 22). Note, however, that appreciable toxicity was observed in human PBM and CEM cells (CC_{50}, 4.5 and 11.6 μM, respectively) for remdesivir, narrowing the therapeutic index of this compound. Cytotoxicity in the low micromolar range was also observed for NHC in our panel of cell lines (Table 2). This toxicity may be related to the high mutagenic activity of NHC and/or other factors (23, 24). Anti-RSV compound ALS-8112 (compound 5) and compounds 6 and 7 did not have a marked effect on CoV-2 replication up to 20 μM. Sofosbuvir showed weak (40% inhibition) antiviral activity at 20 μM against CoV-2 in Vero cells. Several studies using molecular modeling suggested that sofosbuvir could be used to treat COVID-19 patients (25, 26). Surprisingly, no enzymatic or cell culture antiviral data were presented in either paper to support their modeling data. To confirm that sofosbuvir is inactive against CoV-2, we determined that the active 5′-triphosphate precursor of sofosbuvir (namely, β-D-2′-fluoro-2′-methyluridine-5′-triphosphate) did not inhibit purified CoV-2 polymerase up to 10 μM (see the supplemental material and Fig. 4, lane 6) while the precursor of remdesivir inhibited the polymerase (Fig. 4, lane 7). We also confirmed that the lack of activity was not related to a cell transport or a prodrug cleavage issue by evaluating sofosbuvir against CoV-2 and HCoV-OC43 in various cells in addition to those mentioned above. We did not observe any significant activity in some cells, including human rhabdomyosarcoma (RD), human intestinal (Caco-2), and human lung (Calu3) cells, with an EC_{50} of >20 μM for all three cell lines. Note that a novel thio-prodrug of sofosbuvir (compound 3; cyclic phosphate prodrug of sofosbuvir) (Fig. 1), previously reported to be more potent than sofosbuvir itself against HCV (10), displayed an EC_{50} of 6.3 μM and against SARS-CoV-2 was ~6 times less potent than remdesivir (6.3 versus 1.0 μM). However, the activity observed for that compound is likely secondary to the toxicity noted in Vero cells (CC_{50}, 16.4 μM). This compound was tested against HCoV-OC43 in Huh-7 cells in a dose-dependent manner, but because of the toxicity of the compound observed in Huh-7 cells (CC_{50}, 2.9 μM), the highest concentration chosen for the antiviral assays was 2 μM (Table 2). 2′-C-methylcytidine (compound 1) and 7-deaza-7-fluoropurine derivative (compound 2) exhibited activities in the single-digit micromolar range (EC_{50}, 9.2 μM [SI >10] and 7.6 μM [SI >10], respectively) with no toxicity in Vero cells up to 100 μM.

Since the global onset of COVID-19, several studies have been conducted to evaluate the effects of nucleoside analogs or their bases against CoV-2. A recent study suggested that favipiravir, an approved anti-influenza drug acting as a guanine nucleoside analog, may be a potential candidate for the treatment of SARS-CoV-2 infection, showing effective antiviral activity *in vitro* in Vero cells, with a reported EC_{50} of 61.9 μM (27). Favipiravir is being studied in two separate clinical trials in combination therapy with other antiviral agents, such as interferon-α or baloxavir marboxil (28). However, our data showed that favipiravir (compound 8) did not exhibit significant *in vitro* activity against SARS-CoV-2 and HCoV-OC43 up to 20 μM (Table 2). Therefore, this drug is unlikely to be promising for COVID-19 treatment unless large amounts are administered safely to achieve blood levels in the double-digit micromolar range. Ribavirin (compound 12), another approved nucleoside analog for HCV infections, has been considered for COVID-19 treatment. It was shown that ribavirin inhibited SARS-CoV-2 replication in Vero cells, with an EC_{50} of 109.5 μM (22); and according to our findings, this compound up to 20 μM just showed a 12% inhibitory effect against CoV-2 *in vitro* with concomitant cytotoxicity in human cells (Table 2). Notably, ribavirin has been used to treat people infected with SARS or Middle East respiratory syndrome (MERS) without successful clinical outcome (29, 30). Furthermore, there are well-known significant side effects reported for this drug, including anemia at high doses, and efficacy and safety of the drug are uncertain (30). Nevertheless, ribavirin is being used for COVID-19 treatment in combination with pegylated interferon (31). However, our cell-based data showed that ribavirin did not exhibit a marked inhibitory effect against both coronaviruses tested (Table 2). Some scientists speculated that 3TC/FTC plus TDF/TAF may contribute to lower COVID-19 incidence and severity among people living with HIV (32). However, in our hands, 3TC, FTC, TAF, and TDF (compounds 13 through 16) did not show any activity against CoV-2 or HCoV-OC43 when tested up to 20 μM. These data indicate that it is unlikely these drugs would have any clinical benefit in COVID-19-infected individuals (Table 2).

As expected, the two positive controls used, i.e., remdesivir and NHC, demonstrated activity in culture against CoV-2. Others have shown that compound 11 (GS-441524), the parent nucleoside of remdesivir, is more potent than remdesivir in Vero E6 cells, with EC_{50}s of 0.47 and 1.85 μM, respectively (33). However, in our study, compound 11 showed weak activity against SARS-CoV-2 and no activity against HCoV-OC43 (EC_{50}, 8.2 μM [SI = 12] and >10 μM) compared to remdesivir (EC_{50}, 1.0 μM [SI >100] and 0.04 μM [SI = 52] against SARS-CoV-2 and HCoV-OC43, respectively) (Table 2). Remdesivir, a nucleoside analog originally developed for HCV, was recently studied in humans for the treatment of Ebola virus (34) and showed antiviral activity against SARS-CoV-2 in culture (33). There are clinical trials showing the benefit of remdesivir in COVID-19-infected patients (35, 36). In one of the largest NIH-sponsored studies, involving 1,063 hospitalized patients, those that received the drug recovered in 11 days compared with 15 days for those who received placebo ($P < 0.0001$). The data also suggested a reduction in mortality on treatment, but it was not significant. Despite a controversial unpublished non-peer-reviewed open-label WHO SOLIDARITY trial reporting that remdesivir had no effect on mortality and apparently no clinical benefit (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/global-research-on-novel-coronavirus-2019-ncov/solidarity-clinical-trial-for-covid-19-treatments), the FDA approved Veklury (remdesivir) on 23 October 2020 as the first treatment for COVID-19 (https://www.fda.gov/news-events/press-announcements/fda-approves-first-treatment-covid-19) for use in adult and pediatric patients aged ≥12 years and weighing at least 40 kg for patients requiring hospitalization. Finally, EIDD-2801, the ester prodrug of NHC (EIDD-1931) has antiviral activity against different coronaviruses, including SARS-CoV-2 (19). The parent molecule (NHC) was active in our study (Table 2), with an EC_{50} of 0.3 μM and SI of 42 for SARS-CoV-2 and an EC_{50} of 0.8 μM and SI of 100 for HCoV-OC43. However, this molecule is known to be mutagenic (23, 24), suggesting that reproductive toxicology will be needed going forward with EIDD-2801 into the clinic.

In conclusion, the work presented here should help eliminate some of the muddied

information in the literature and provide guidance to decision makers and clinicians on which repurposed drug to advance efficiently toward FDA approval for a COVID-19 indication. Too many drugs are being tested in humans as antiviral agents without prior evaluation in cell culture. Additional modifications of repurposed nucleoside antiviral agents, with similar backbones found to be effective herein, could potentially offer improved potency and therapeutic options to reduce the global burden of COVID-19.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.04 MB.

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