

Remdesivir potently inhibits SARS-CoV-2 in human lung cells and chimeric SARS-CoV expressing the SARS-CoV-2 RNA polymerase in mice.

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1 SUMMARY

2 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in 2019 as the causative agent
3 of the novel pandemic viral disease COVID-19. With no approved therapies, this pandemic illustrates the
4 urgent need for safe, broad-spectrum antiviral countermeasures against SARS-CoV-2 and future emerging
5 CoVs. We report that remdesivir (RDV), a monophosphoramidate prodrug of an adenosine analog,
6 potently inhibits SARS-CoV-2 replication in human lung cells and primary human airway epithelial
7 cultures ($EC_{50} = 0.01 \mu\text{M}$). Weaker activity was observed in Vero E6 cells ($EC_{50} = 1.65 \mu\text{M}$) due to their
8 low capacity to metabolize RDV. To rapidly evaluate *in vivo* efficacy, we engineered a chimeric SARS-
9 CoV encoding the viral target of RDV, the RNA-dependent RNA polymerase, of SARS-CoV-2. In mice
10 infected with chimeric virus, therapeutic RDV administration diminished lung viral load and improved
11 pulmonary function as compared to vehicle treated animals. These data provide evidence that RDV is

12 potently active against SARS-CoV-2 *in vitro* and *in vivo*, supporting its further clinical testing for
13 treatment of COVID-19.

14

15 Keywords: COVID-19, SARS-CoV-2, coronavirus, antivirals, remdesivir, RdRp, RNA-dependent RNA
16 polymerase, therapeutic, mouse.

17

18 **INTRODUCTION**

19 Coronaviruses (CoV) are genetically diverse positive sense RNA viruses that circulate in animals and
20 humans. In the past 20 years, three new human CoV have emerged: severe acute respiratory syndrome
21 CoV (SARS-CoV-1) in 2002, Middle East respiratory syndrome (MERS)-CoV in 2012 and current
22 pandemic SARS-CoV-2, the causative agent of COVID-19 (de Wit et al., 2016; Zhou et al., 2020b).
23 While four endemic human CoV (HCoV-OC43, -229E, -NL63, and -HKU1) typically cause mild
24 respiratory diseases with common cold-like symptoms, SARS-CoV-1, MERS-CoV, and SARS-CoV-2
25 cause severe respiratory disease with respective mortality rates of 11% (Chan-Yeung and Xu, 2003), 35%
26 (Arabi et al., 2017), and an estimated 3% (Chen, 2020). The development of effective broad-spectrum
27 antivirals has been hampered by viral diversity, the capacity of CoVs to adaptively overcome negative
28 selective pressures, and the ability to actively counteract drugs through the action of a proofreading
29 exoribonuclease. We previously reported that remdesivir (RDV), a monophosphoramidate prodrug of the
30 C-adenosine analog GS-441524, potently inhibits replication of a broad spectrum of pre-pandemic bat
31 CoVs and human epidemic CoVs in primary human lung cell cultures (Agostini et al., 2018; Brown et al.,
32 2019; Sheahan et al., 2017). Biochemical analysis of the mechanism of inhibition of the SARS-CoV-2,
33 SARS-CoV-1, and MERS-CoV RNA-dependent RNA polymerase (RdRp) revealed that incorporation of
34 the active metabolite RDV triphosphate (RDV-TP) was more efficient than the natural substrate ATP and
35 led to delayed chain termination three nucleotides downstream of incorporation (Gordon et al., 2020a,
36 2020b). Prolonged passaging of murine hepatitis virus (MHV), a group 2a CoV, in the presence of GS-
37 441524 resulted in low level resistance through mutations in the RdRp, further implicating this protein as
38 the drug target (Agostini et al., 2018). RDV showed both prophylactic and therapeutic efficacy in mouse
39 models of SARS and MERS and against MERS-CoV challenge in a rhesus macaque model (Sheahan et
40 al., 2017, 2020a; Wit et al., 2020). Here we report that RDV potently inhibits SARS-CoV-2 replication
41 Calu3 human lung cells with sub-micromolar EC₅₀ and in primary human airway epithelial cultures
42 (HAEs) with nanomolar EC₅₀. Notably, we have detected comparably lower potency of RDV in
43 established human and monkey cell lines due to their lower metabolic capacity to activate the compound.

44 Mice infected with chimeric SARS-CoV-1 encoding the SARS-CoV-2 RdRp and treated therapeutically
45 with RDV show decreased viral loads in the lungs and increased pulmonary function. These data
46 emphasize the potential of RDV as a promising countermeasure against the ongoing COVID-19
47 pandemic.

48

49 RESULTS

50 **Structural model of remdesivir incorporation by the SARS-CoV-2 polymerase and conservation of**
51 **the active site across human CoV.** Drug function and performance is heavily influenced by
52 microvariation in target genes across virus families, biodistribution in the organism, and, importantly,
53 host cell and tissue expression patterns that influence drug stability and metabolism. We previously
54 modeled RDV on a homology model of SARS-CoV-2 based on the cryo-EM structure of SARS-CoV-1
55 polymerase complex (Gordon et al., 2020b; Kirchdoerfer and Ward, 2019). Composed of nsp12, nsp7 and
56 nsp8, the model was consistent with biochemical findings predicting efficient incorporation of RDV-TP
57 into the growing RNA strand and provided an explanation for the observed delayed chain termination
58 after the incorporation of three additional nucleotides. We have since refined this model using the recently
59 released cryo-EM structure of the SARS-CoV-2 polymerase complex (Gao et al., 2020). The major
60 qualitative change is a more complete picture of the N-terminal NiRAN domain of nsp12, which was not
61 resolved in the SARS-CoV-1 structure. The current model of the pre-incorporation state, with RDV-TP,
62 RNA primer and template strands and catalytic metals was well-optimized with a series of constrained
63 energy minimizations and conformational searches, as described previously (**Fig. 1A**). Bound to the two
64 catalytic Mg²⁺ ions, RDV-TP is coordinated by two basic residues (R553 and R555). The ribose 2'OH
65 forms hydrogen bonds to T680 and N691, and the 1'CN resides in a shallow pocket formed by T687 and
66 A688 (**Fig. 1B**). The interaction with T680 distinguishes CoVs from other structurally related families,
67 including noroviruses, picornaviruses, and the flaviviruses. While key residues including D623, S682, and
68 N691, are conserved across all these virus families and have been shown to govern positioning of the
69 NTP into the active site, the role of T680 appears to be novel. While writing this manuscript, another
70 model of RDV-TP in the SARS-CoV-2 active site (Shannon et al., 2020) was published which predicts a
71 role for S682 as well. The position of T680 relative to N691 strongly implies it will contribute to the
72 recognition of the ribose 2'OH, likely diminishing the role of S682 as a result, consistent with earlier
73 predictions (Kirchdoerfer and Ward, 2019).

74 Modeling of the RDV resistance mutations identified in MHV (Agostini et al., 2018) onto homologous
75 residues V557 and F480 in the SARS-CoV-2 RdRp structure reveals that V557L shifts the position of the

76 template base, which in turn shifts the positioning of the incoming NTP (**Fig. S1 A, B**). This will impact
77 RDV activity in that it alters the position of the 1'CN in the pocket. Because the model predicts no direct
78 interaction of F480 with the NTP, primer, or template, the effect of the F480L mutation is more difficult
79 to discern. The F480L mutation could potentially induce a subtle change to the 1'CN binding pocket (**Fig.**
80 **S1 C, D**). Alignment of nsp12 sequences from SARS-CoV-2 used in other studies of RDV shows
81 complete conservation of nsp12 nucleotide sequences, predicting the comparable antiviral activity of
82 RDV against these isolates (**Fig. S2**). We next modeled the active sites of the six other human CoVs
83 SARS-CoV-1 (**Fig. S3A**), MERS-CoV (**Fig. S3B**), HCoV-OC43 (**Fig. S3C**), -229E (**Fig. S3D**), -NL63
84 (**Fig. S3E**), and -HKU1 (**Fig. S3F**). The models show that SARS-CoV-2 is identical to SARS-CoV-1 out
85 to a radius of 18 Å from the active site. Differences detected on the periphery of the active site of the
86 MERS-CoV and HCoV-OC43, -229E, -NL63, and HKU1 correspond to residues that do not directly
87 interact RDV-TP. Together, these data demonstrate high structural conservation of the RdRp active site
88 interacting with RDV-TP across all seven known human CoV strains.

89 **Remdesivir and GS-441524 potently inhibit SARS-CoV-2 replication.** Remdesivir (RDV) and its
90 parent nucleoside analog GS-441524 inhibit CoVs and multiple other viruses (Agostini et al., 2018; Cho
91 et al., 2012; Lo et al., 2017; Sheahan et al., 2017; Warren et al., 2016). Previous reports (Choy et al.,
92 2020; Wang et al., 2020; Runfeng et al., 2020) suggest RDV inhibits SARS-CoV-2, but comparative
93 studies of anti-SARS-CoV-2 activity using authentic compound in physiologically relevant cell lines are
94 lacking. We first compared SARS-CoV-2 replication in established cell lines to determine which cell
95 types could potentially be suitable for studying RDV efficacy against SARS-CoV-2. Viral yields were
96 determined at 24, 48, and 72 hours post-infection (hpi) in Vero E6, Vero CCL-81 (Vero), Huh7, and
97 Calu3 2B4 (Yoshikawa et al., 2010) cells (**Fig. 2A**). Vero E6 and Vero cells supported highest levels of
98 SARS-CoV-2 replication, consistent with a previous study (Harcourt et al.). Maximum yields were
99 detected at 48 hpi in Vero E6 cells (>6 logs at MOI = 0.1 and 0.01 PFU/cell), 24 hpi in Vero cells infected
100 at MOI = 0.1 PFU (>5 logs), 48 hpi in Vero cells infected at MOI = 0.01 PFU/cell (>5 logs), 72 hpi in
101 Calu3 2B4 (>4 logs at MOI = 0.1 PFU/cell), and 48 hpi in Huh7 cells (>4 logs at MOI = 0.1 PFU/cell, <2
102 logs at MOI = 0.01 PFU/cell). These results indicate that Vero E6, Vero, and Calu3 2B4 cells support
103 varying levels of SARS-CoV-2 replication and cell type should be chosen for a given study depending on
104 study goals.

105 To determine if RDV and GS-441524 inhibit SARS-CoV-2 replication in established cell lines, Calu3
106 2B4 human lung adenocarcinoma cells and Vero E6 African green monkey kidney cells were infected
107 with the SARS-CoV-2 clinical isolate 2019-nCoV/USA-WA1/2020 and treated with a range of RDV or
108 GS-441524 concentrations. Supernatants were harvested at time points corresponding to peak viral

109 replication for each cell type, and infectious viral titer and viral genome copy number in the supernatant
110 were quantified by plaque assay and RT-qPCR, respectively. RDV and GS-441524 potently inhibited
111 SARS-CoV-2 replication in a dose-dependent manner in both cell types (**Fig. 2; Table 1**). In Calu3 cells,
112 both compounds displayed dose-dependent inhibition of viral replication as determined by plaque assay
113 (**Fig. 2B**) and RT-qPCR (**Fig. 2C**). RDV inhibited SARS-CoV-2 with an $EC_{50} = 0.28 \mu\text{M}$ and $EC_{90} = 2.48$
114 μM . The parent compound GS-441524 was less potent: $EC_{50} = 0.62 \mu\text{M}$, $EC_{90} = 1.34 \mu\text{M}$ (**Fig. 2D; Table**
115 **1**). EC_{50} values determined by quantification of viral genome copies were roughly two-fold higher than
116 those obtained by quantification of infectious virus (**Fig. 2E; Table 1**). Both compounds also displayed
117 dose-dependent inhibition of viral replication in Vero E6 cells as determined by infectious viral titer and
118 genome copy number (**Fig 2F**). RDV inhibited SARS-CoV-2 with $EC_{50} = 1.65 \mu\text{M}$ and $EC_{90} = 2.40 \mu\text{M}$,
119 while GS-441524 was more potent ($EC_{50} = 0.47 \mu\text{M}$, $EC_{90} = 0.71 \mu\text{M}$) (**Fig. 2G; Table 1**). Relative
120 potency based on genome copies was similar to that assessed by quantification of infectious viral titer in
121 Vero E6 cells (**Fig. 2H; Table 1**). Thus, RDV inhibits SARS-CoV-2 more potently in Calu3 2B4 than in
122 Vero E6 cells.

123 **RDV is a highly potent antiviral inhibitor of SARS-CoV-2 in primary human airway epithelial**
124 **(HAE) cultures.** Primary HAE cultures grown on air-liquid interface recapitulate the cellular complexity
125 and physiology of the human conducting airway (Sims et al., 2005). Therefore, we evaluated antiviral
126 activity of RDV in this biologically relevant model. In RDV treated HAE, we observed a dose-dependent
127 reduction in infectious virus production, with >100-fold inhibition at the highest tested concentration
128 (**Fig. 3A**). Importantly, RDV demonstrate potent antiviral activity with EC_{50} values of 0.0010 and 0.009
129 μM in two independent experiments (**Fig. 3B**). We previously reported that RDV is not cytotoxic at doses
130 at or below 10 μM in this culture system, supporting the conclusion that the observed antiviral effect was
131 virus-specific (Sheahan et al., 2017). Together, these data demonstrate that RDV is potently antiviral
132 against SARS-CoV-2 in primary human lung cultures with a selectivity index of >1000.

133 **Antiviral activities of RDV and GS-441524 correlate with RDV-TP metabolite levels.** Cell type
134 specific expression of genes that metabolize ribonucleoside analogs can have a profound impact on
135 activity (Eriksson, 2013; Koczor et al., 2012). Table 1 and prior studies (Bojkova et al., 2020; Choy et al.,
136 2020; Jeon et al., 2020) demonstrate the antiviral activity of RDV against SARS-CoV-2 is highly variable
137 in different cell culture models. Both RDV and GS-441524 undergo intracellular conversion to the active
138 metabolite RDV-TP involving several metabolic steps (**Fig. S4**) and the efficiency of each step might
139 differ between cell types. Therefore, to reconcile the differences in antiviral activity of RDV and GS-
140 441524 observed in our and other studies, we compared intracellular RDV-TP concentrations in Vero E6,
141 Calu3 2B4, and HAEs following incubation with the two compounds. RDV-TP levels per million cells

142 produced after 8- to 48-hour treatment with RDV were substantially higher in primary HAE cultures than
143 either Calu3 2B4 or Vero E6. (**Fig 4; Table 1; Tables S1, S2**). Given the primary nature of HAE cultures,
144 we used cells from two independent donors with similar demographic profiles. RDV-TP was efficiently
145 formed in both donor cultures following incubation with RDV with a difference of < 3-fold from each
146 other. The lowest levels of RDV-TP were observed following RDV treatment of Vero E6 cells and were
147 approximately 4- and 20-fold lower than those observed in Calu3 2B4 and HAE cultures, respectively.
148 The levels of GS-441524 as well as the intermediate mono- and di-phosphorylated metabolites (RDV-MP
149 and RDV-DP) were readily detected in Calu3 2B4 cultures following treatment with RDV, but were
150 below the limit of quantification in Vero E6 cells at all time points tested (**Table S1**). In addition,
151 incubation of Vero E6 cells with GS-441524 yielded 4-fold higher RDV-TP levels compared to
152 incubation with RDV corresponding to higher antiviral potency of GS-441524 relative to RDV, which is
153 not observed with either Calu3 or HAE cultures. (**Table S1, S2**). In conclusion, the RDV-TP levels in the
154 different cell types directly correlated with the antiviral potencies of RDV and GS-441524 against SARS-
155 CoV-2 with the HAE cultures producing substantially higher levels of RDV-TP that translated into
156 markedly more potent antiviral activity of RDV (**Table 1**). Importantly, the metabolism of RDV in Vero
157 E6 cells appeared altered and was less efficient particularly in comparison with the HAE cultures,
158 indicating that Vero E6 cells might not be an adequate cell type to characterize the antiviral activity of
159 RDV and potentially also other nucleotide prodrug-based antivirals.

160 **RDV is active against the SARS-CoV-2 RdRp *in vivo*.** To determine whether RDV exerts antiviral
161 effect on SARS-CoV-2 *in vivo*, we constructed a chimeric mouse-adapted SARS-CoV-1 variant encoding
162 the target of RDV antiviral activity, the RdRp, of SARS-CoV-2 (SARS1/SARS2-RdRp) (**Fig. 5A**).
163 Although other chimeric replicase ORF recombinant CoVs have shown to be viable (Stobart et al., 2013),
164 this is the first demonstration that the RdRp from a related but different CoV can support efficient
165 replication of another. After recovery and sequence-confirmation (**Fig. 5B**) of recombinant chimeric
166 viruses with and without nanoluciferase reporter, we compared SARS-CoV-1 and SARS1/SARS2-RdRp
167 replication and sensitivity to RDV in Huh7 cells. Replication of both viruses was inhibited similarly in a
168 dose-dependent manner by RDV (SARS-CoV-1 mean $EC_{50} = 0.007 \mu\text{M}$; SARS1/SARS2-RdRp mean
169 $EC_{50} = 0.003 \mu\text{M}$) (**Fig. 5C and D**). We then sought to determine the therapeutic efficacy of RDV against
170 the SARS1/SARS2-RdRp in mouse models employed for previous studies of RDV (Sheahan et al., 2017).
171 Mice produce a serum esterase absent in humans, carboxyl esterase 1c (*Ces1c*), that dramatically reduces
172 half-life of RDV. Thus, to mirror pharmacokinetics observed in humans, mouse studies with RDV must
173 be performed in transgenic C57Bl/6 *Ces1c*^{-/-} mice (Sheahan et al., 2017). We infected female C57Bl/6
174 *Ces1c*^{-/-} mice with 10^3 PFU SARS1/SARS2-RdRp and initiated subcutaneous treatment with 25 mg/kg

175 RDV BID at one day post-infection (dpi). This regimen was continued until study termination. While
176 weight loss did not differ between vehicle- and RDV-treated animals (**Fig. 5E**), lung hemorrhage at five
177 dpi was significantly reduced with RDV treatment (**Fig. 5F**). To gain insight into physiologic metrics of
178 disease severity, we measured pulmonary function daily by whole body plethysmography (WBP). The
179 WBP metric, PenH, is a surrogate marker of pulmonary obstruction (Menachery et al., 2015a).
180 Therapeutic RDV significantly ameliorated loss of pulmonary function observed in the vehicle-treated
181 group (**Fig. 5G**). Importantly, RDV treatment dramatically reduced lung viral load (**Fig. 5H**). Taken
182 together, these data demonstrate that therapeutically administered RDV can reduce virus replication and
183 improve pulmonary function in an ongoing infection with a chimeric SARS-CoV-1/SARS-CoV-2 virus
184 encoding the target of RDV, the RdRp.

185

186 **DISCUSSION**

187 The COVID-19 pandemic has gravely illustrated the need for countermeasures against emerging epidemic
188 and pandemic CoVs. Broad-spectrum antiviral drugs, antibodies, and vaccines are needed to combat the
189 current pandemic and those that will emerge in the future. RDV shows potent activity against an array of
190 genetically diverse CoVs as well as against unrelated emerging viruses like Ebola (Agostini et al., 2018;
191 Brown et al., 2019; Sheahan et al., 2017, 2020a; Warren et al., 2016). In this study, we demonstrate that
192 RDV and its parent nucleoside GS-441524 are active against SARS-CoV-2 in a physiologically relevant
193 cell line and that RDV exerts substantially higher antiviral activity in primary human airway cultures.
194 Potency was directly related to the intracellular concentration of pharmacologically active triphosphate
195 metabolite, which was markedly higher in primary HAE cultures compared to human lung cells (Calu3
196 2B4) and monkey kidney cells (Vero E6). Our data are consistent with recent studies demonstrating
197 important contributions of natural variation in host and tissue specific gene expression patterns and
198 microbiome specific contributions to drug metabolism, stability, and bioavailability in different tissues
199 (Eriksson, 2013; Koczor et al., 2012). Modeling of RDV onto the SARS-CoV-2 RdRp revealed that the
200 positioning of the RDV into the active site closely resembled that of cognate natural substrate ATP,
201 consistent with efficient incorporation into RNA during replication of the viral genome. RDV decreased
202 viral loads and improved lung function in mice infected with SARS1/SARS2-RdRp chimeric virus when
203 treated at 1 dpi. This is the first rigorous demonstration of potent inhibition of SARS-CoV-2 in
204 continuous and primary human lung cultures and first study suggesting efficacy of RDV against SARS-
205 CoV-2 in mice.

206 Previous studies of RDV anti-SARS-CoV-2 activity reported EC₅₀ values of 0.77 μM as determined by
207 quantification of genome copy number (Wang et al., 2020), 23.15 μM as determined by TCID₅₀, 26.90 as
208 determined by RNA copy number (Choy et al., 2020), and 0.651 μM as determined by cytopathic effect
209 (CPE) (Runfeng et al., 2020), all in Vero E6 cells. The potency of RDV in Vero E6 cells (EC₅₀ 1.65 μM)
210 observed in our study is comparable to values reported by Wang *et al.* and Runfeng *et al.*, but greater than
211 reported by Choy *et al.* Sequence comparison of the nsp12 from the Seattle, WA isolate used in this study
212 versus SARS-CoV-2 isolates used in the previously mentioned studies assessing RDV potency did not
213 reveal consensus changes in nsp12 sequence, suggesting that any isolate-specific variation in RDV
214 sensitivity is not likely due to differences in the RDV-TP interaction with the RdRp. Therefore, the
215 differences in EC₅₀ may be partially explained by intrinsic differences of SARS-CoV-2 virus isolates,
216 quantification methods, and assay conditions such as incubation period and virus input.

217 Although Vero E6 cells support robust replication of SARS-CoV-2 as illustrated here and elsewhere, our
218 study emphasized the extreme caution that should be exercised when interpreting drug efficacy and
219 potency experiments performed using Vero cell lineages. Nucleoside analog efficacy is greatly dependent
220 on metabolism into the active form. In contrast to GS-441524, RDV contains a protective group which
221 facilitates cellular uptake of the nucleoside analog and addition of the first phosphate group, which
222 accelerates conversion to the active triphosphate (Mehellou et al., 2018). Consistent with previous reports
223 (Agostini et al., 2018), RDV showed enhanced inhibition of SARS-CoV-2 over GS-441524 in Calu3 2B4
224 cells. In contrast, RDV was two-fold less potent than GS-441524 in Vero E6 cells. Relative potency of
225 the two compounds was directly linked to intracellular concentration of the active triphosphate
226 metabolite, suggesting an altered uptake and/or intracellular metabolism of RDV, consistent with a
227 previous report describing inefficient metabolism of the nucleotide prodrug sofosbuvir in Vero cells
228 (Mumtaz et al., 2017). Drug potency in Vero E6 was similar whether quantified by infectious virus or
229 genome copy number. In Calu3 cells, the potency determined by RT-qPCR was about two-fold lower
230 than when quantified by plaque assay. It is possible that RT-qPCR, which was developed to detect
231 nucleocapsid (N) RNA, also detects packaged subgenomic RNAs and defective genomes in addition to
232 full-length genomes. This would result in underestimation of the reduction in infectious titer. Notably, the
233 potency of RDV against SARS-CoV-1 encoding the SARS-CoV-2 RdRp in Huh7 cells was more than
234 100-fold higher than that of RDV against bona-fide SARS-CoV-2 in Huh7 and Calu3 cells. This
235 difference could be due to infectivity, which is driven by the SARS-CoV-1 instead of the SARS-CoV-2
236 spike protein. In addition, SARS-CoV-1 infects Huh7 cells at low frequency at the MOI used in this study
237 and does not appear to spread throughout the culture over the course of the experiment. The number of
238 Huh7 cells in which virus replicates is relatively lower compared to Calu3 cells, possibly enhancing
239 potency of RDV in Huh7 compared to Calu3. Interestingly, the antiviral potency of RDV against SARS-

240 CoV-2 in HAE cultures was comparable to SARS-CoV-1 and MERS-CoV (Sheahan et al., 2017), which
241 is consistent with the high conservation of the RdRp active site across these different CoVs. Together,
242 these results emphasize the need for careful selection and use of multiple cell types and methods to study
243 potency and efficacy of nucleoside analogs and other antiviral compounds.

244 The target of RDV antiviral activity is the viral RdRp. To mirror the pharmacokinetic exposures observed
245 in humans, RDV studies in mice must be performed in *Ces1c*^{-/-} animals (Sheahan et al., 2017). In
246 addition, SARS-CoV-2 does not readily infect WT mice due to incompatibilities between virus spike and
247 the murine ortholog of ACE2, which serves as the SARS-CoV-2 entry receptor (Wan et al., 2020). The
248 breeding a doubly transgenic (hACE2, *Ces1c*^{-/-}) mice for use in RDV efficacy studies is ongoing. To
249 rapidly assess the therapeutic efficacy of RDV against SARS-CoV-2, we constructed recombinant SARS-
250 CoV-1 chimeric virus encoding the SARS-CoV-2 RdRp (SARS1/SARS2-RdRp). Virus entry, tropism,
251 and pathogenesis of this chimeric virus are driven by parental mouse-adapted SARS-CoV-1 virus. Similar
252 to our previous studies with SARS-CoV-1 (Sheahan et al., 2017), we now show that therapeutic
253 administration of RDV one dpi can both reduce viral load and improve pulmonary function in mice. The
254 kinetics of SARS-CoV-1 replication and disease are notably compressed in mice as compared to humans
255 where virus titer peaks 10-15 days after onset of symptoms (Hung et al., 2004; Peiris et al., 2004). By
256 comparison, initial reports suggest that SARS-CoV-2 replication peaks around 5-6 days after symptom
257 onset, just prior to onset of dyspnea (Pan et al., 2020; Zhou et al., 2020a). A recent preprint described the
258 therapeutic efficacy of RDV against SARS-CoV-2 in rhesus macaques, where RDV treatment reduced
259 respiratory pathology and viral loads in bronchoalveolar lavage fluid (Williamson et al., 2020). Prior to
260 the emergence of pandemic SARS-CoV-2, RDV was evaluated in phase 1 clinical trials as well as phase 2
261 randomized controlled trial trials to treat acute Ebola virus disease in the Democratic Republic of Congo
262 (DRC), and human safety data are available (Mulangu et al., 2019). Thus, our preclinical development of
263 RDV positioned RDV for immediate compassionate use of RDV for severely ill COVID-19 patients.
264 While early results are promising (Grein et al., 2020), Phase III randomized controlled trials for the
265 treatment of patients with COVID-19 are ongoing globally and will ultimately determine efficacy, safety,
266 and optimal dosing of RDV in patients with different stages of COVID-19.

267 Despite worldwide drug discovery efforts and over 300 active clinical evaluations of potential treatments,
268 no effective countermeasure currently exists to combat COVID-19 (Sanders et al., 2020) or likely future
269 CoV pandemics. Large-scale deployment of antiviral monotherapies creates high risk for emergence of
270 drug resistance. Our previous work demonstrates that CoV resistance to RDV is generated slowly and is
271 conferred by two mutations in the RdRp. In addition, RDV-resistant CoVs exhibit reduced replication
272 capacity and are also more sensitive to another potentially active nucleoside analog inhibitor β -D-N4-

273 hydroxycytidine (NHC; EIDD-1931/2801) (Agostini et al., 2019; Sheahan et al., 2020b). Therapies
274 combining direct-acting antivirals (DAAs) such as RDV and NHC, along with other DAAs such as
275 antibodies and protease inhibitors that target different stages of the viral replication cycle, could be
276 considered for counteracting resistance if it emerges in patients treated with antiviral monotherapy. In
277 addition, attention should be given to combining DAAs with anti-inflammatory drugs to potentially
278 extend the treatment window during which DAAs can improve outcomes. With SARS-CoV-1-, SARS-
279 CoV-2-, and MERS-like CoVs continuing to circulate in bat species, more outbreaks of novel CoVs are
280 expected (Menachery et al., 2015b, 2016). Identification and evaluation of broadly efficacious, robust
281 anti-CoV therapies are thus urgently needed in the present and future.

282

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298

299 **DECLARATION OF INTERESTS**

300 The authors affiliated with Gilead Sciences, Inc. are employees of the company and own company stock.
301 The other authors have no conflict of interest to report.

302

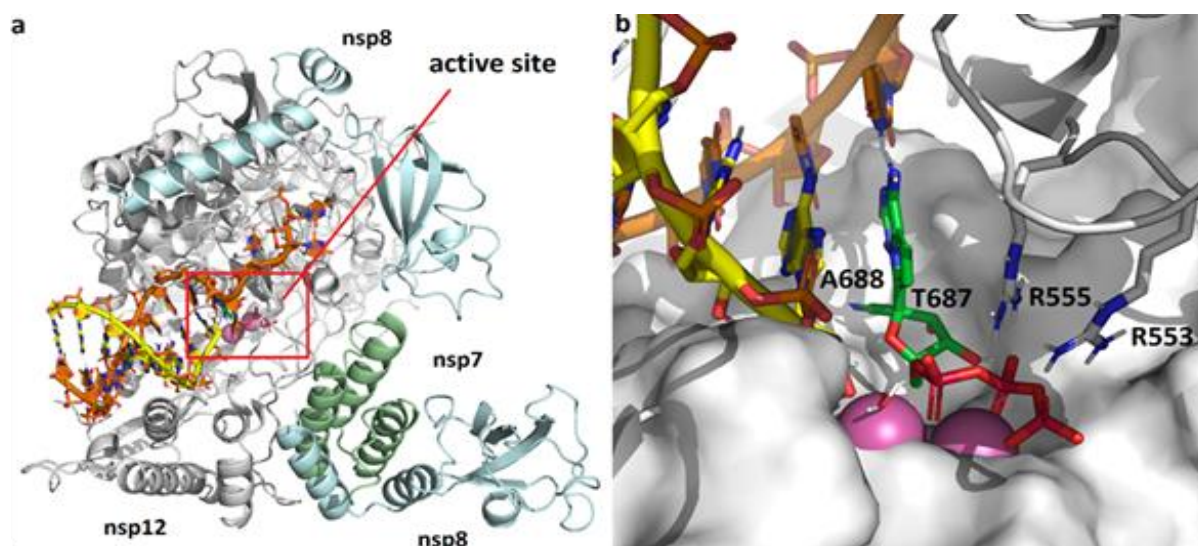
303 **AUTHOR CONTRIBUTIONS**

304 A.J.P., T.P.S., conceived, designed, and performed experiments and management and coordinated
305 responsibility for the research activity planning and execution. A.J.P., J.K.P., J.P.B, and T.P.S. wrote the
306 manuscript. A.S.G., A.S., S.R.L, K.H.D., B.L.Y., M.L.A., L.J.S., J.D.C., X.L., T.M.H., K.G., D.R.M.,
307 A.J.B., R.L.G., J.K.P., V.D.P., J.P., B.M., D.B., and E.M. performed experiments. T.P.S, D.R.M, R.S.B.,
308 A.J.P., J.D.C., and M.R.D. secured funding. D.P.P, N.J.T, T.C. provided reagents. J.D.C., J.Y.F., J.P.B.,
309 D.P.P, T.C., R.S.B., M.R.D edited the manuscript and provided expertise and feedback.

310

311 **FIGURES AND LEGENDS**

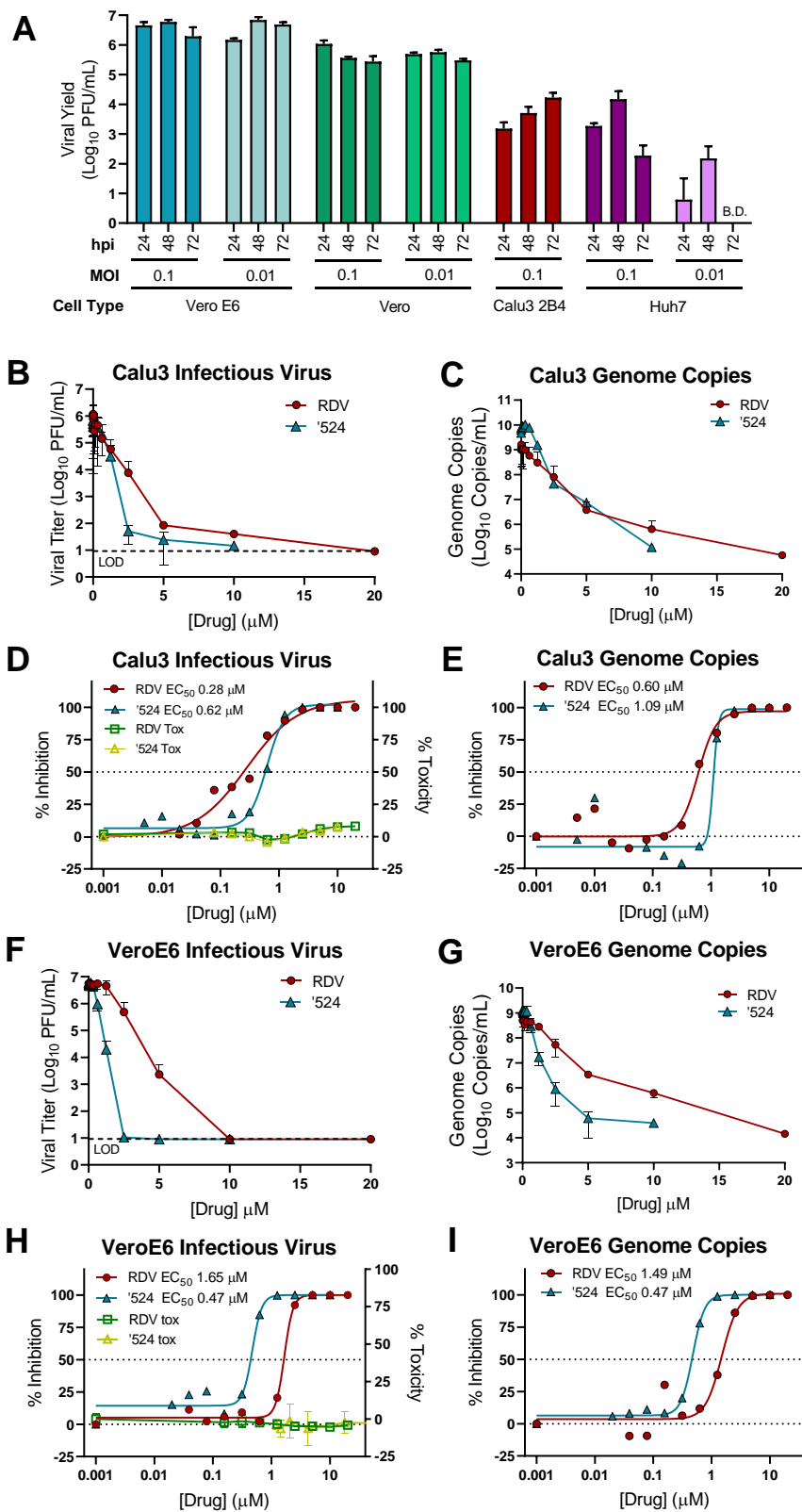
312 **Figure 1**



313

314 **Figure 1. Modeling of remdesivir onto the SARS-CoV-2 RdRp structure.** **A.** Model of SARS-CoV-2
315 polymerization complex in its elongating state. The model was based on the cryo-EM apo structures of
316 SARS-CoV-1 (PDB 6NUR) and SARS-CoV-2 (PDB 6M71). The active site is boxed in red. **B.** Enlarged
317 view of active site depicting RDV pre-incorporation. The 1'CN substituent sits in a shallow pocket formed
318 by residues T687 and A688. Bound to the two catalytic Mg²⁺ ions (pink), the triphosphate is coordinated
319 by two basic residues (R553 and R555)

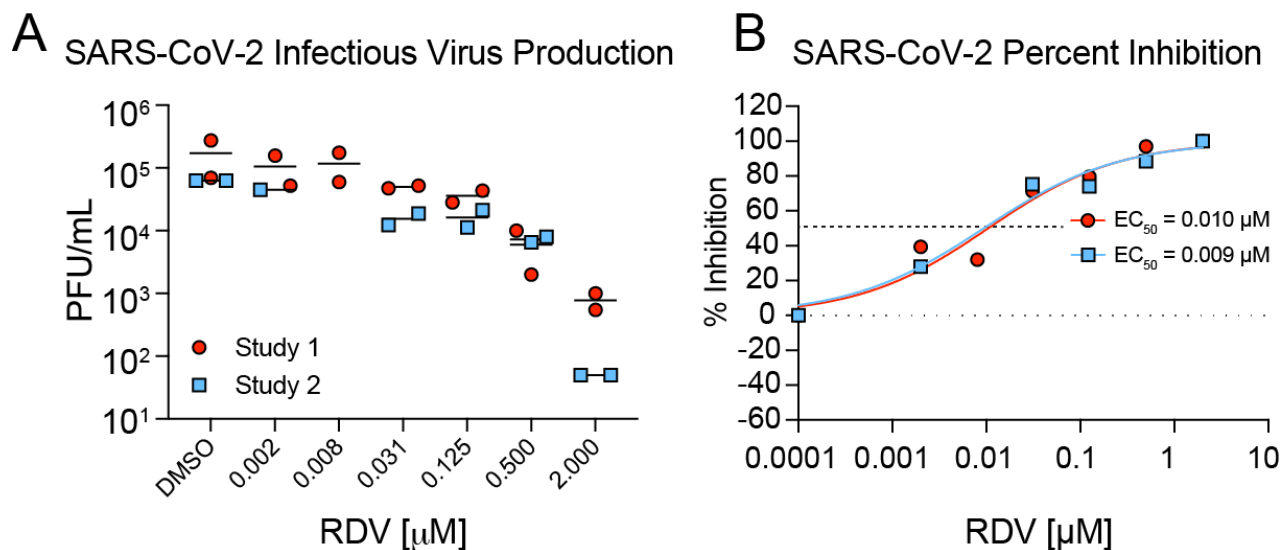
320 **Figure 2**



321

322 **Figure 2. Prodrug remdesivir (RDV) and parent nucleoside GS-441524 ('524) potently inhibit SARS-**
323 **CoV-2 replication. A.** Vero E6, Vero CCL-81 (Vero), Huh7, and Calu3 2B4 cells were infected with MOI
324 = 0.01 and/or 0.1 PFU/cell SARS-CoV-2 (2019-nCoV/USA-WA1/2020), and infectious viral titers were
325 determined by plaque assay at 0.5, 24, 48, and 72 hours post-infection (hpi). Viral yields were calculated
326 by subtracting the average 0.5 h (post-adsorption, pre-incubation) titer from each subsequent time point.
327 Data represent the average of three replicates from one experiment. Error bars indicate SD. B.D.: below
328 detection. Calu3 cells were infected with 0.1 PFU/cell SARS-CoV-2 and Vero cells were infected with 0.01
329 PFU/cell SARS-CoV-2 and treated with RDV, GS-441524 ('524), or DMSO only (control) in cell culture
330 medium. Supernatants were collected at 48 h (Vero E6) or 72 h (Calu3) post-infection. **B, C.** Reduction of
331 SARS-CoV-2 replication by RDV in Calu3 cells as determined by infectious viral titer and RT-qPCR. **D.**
332 Percent inhibition of SARS-CoV-2 replication by RDV and GS-441524 in Calu3 as determined by
333 infectious viral titer [RDV: EC₅₀ = 0.28 μM, EC₉₀ = 2.48 μM; GS-441524 EC₅₀ = 0.62 μM, EC₉₀ = 1.34
334 μM]. No significant cytotoxicity of either compound was detected in Calu3 cells. **E.** Percent inhibition of
335 SARS-CoV-2 replication by RDV and GS-441524 in Calu3 as determined RT-qPCR [RDV: EC₅₀ = 0.60
336 μM, EC₉₀ = 1.28 μM; GS-441524: EC₅₀ = 1.09 μM, EC₉₀ = 1.37 μM]. **F, G.** Reduction of SARS-CoV-2
337 replication by RDV in Vero E6 cells as determined by infectious viral titer and RT-qPCR. **H.** Percent
338 inhibition of SARS-CoV-2 replication by RDV and GS-441524 in Vero E6 cells as determined by infectious
339 viral titer [RDV: EC₅₀ = 1.65 μM, EC₉₀ = 2.40 μM; GS-441524: EC₅₀ = 0.47 μM, EC₉₀ = 0.71 μM]. No
340 significant cytotoxicity of either compound was detected in Vero E6 cells. **I.** Percent inhibition of SARS-
341 CoV-2 replication by RDV and GS-441524 in Vero E6 cells as determined RT-qPCR [RDV: EC₅₀ = 1.49
342 μM, EC₉₀ = 3.03 μM; GS-441524: EC₅₀ = 0.47 μM, EC₉₀ = 0.80 μM]. Data represent means of 2-4
343 independent experiments with 2-3 replicated each. Error bars represent SEM.

344 **Figure 3**



345
346 **Figure 3. RDV is potently antiviral against SARS-CoV-2 in primary human airway epithelial (HAE)**
347 **cultures.** HAE cultures were infected with a SARS-CoV-2 clinical isolate (2019-nCoV/USA-WA1/2020)
348 at MOI = 0.5 PFU/cell for 2 h, after which virus was removed and cultures were washed 3 times, followed
349 by incubation 37°C for 48 h. **A.** SARS-CoV-2 infectious virus production in two independent studies. Virus
350 was titered via plaque assay in apical washes at 48 h post-infection. Each symbol represents the titer from
351 a single culture, and line is drawn at the mean. **B.** Percent inhibition generated from titer data in A.

352 **Table 1: Cell-specific SARS-CoV-2 potency and metabolism**

353

Analysis	RDV			GS-441524 ^a	
	Vero E6	Calu3 2B4	HAE ^b	Vero E6	Calu3 2B4
Plaque assay EC ₅₀ (μM)	1.65	0.28	0.010	0.47	0.62
Genome copy EC ₅₀ (μM)	1.49	0.60	n.d.	0.47	1.09
RDV-TP at 24 h ^c (pmol/million cells)	0.54 ± 0.15 ^d	2.17 ± 0.14 ^d	10.6 ± 5.3 ^b	2.37 ± 0.22 ^d	0.85 ± 0.16 ^d

354

355 ^aGS-441524 was not tested for antiviral potency nor RDV-TP levels in HAE cultures

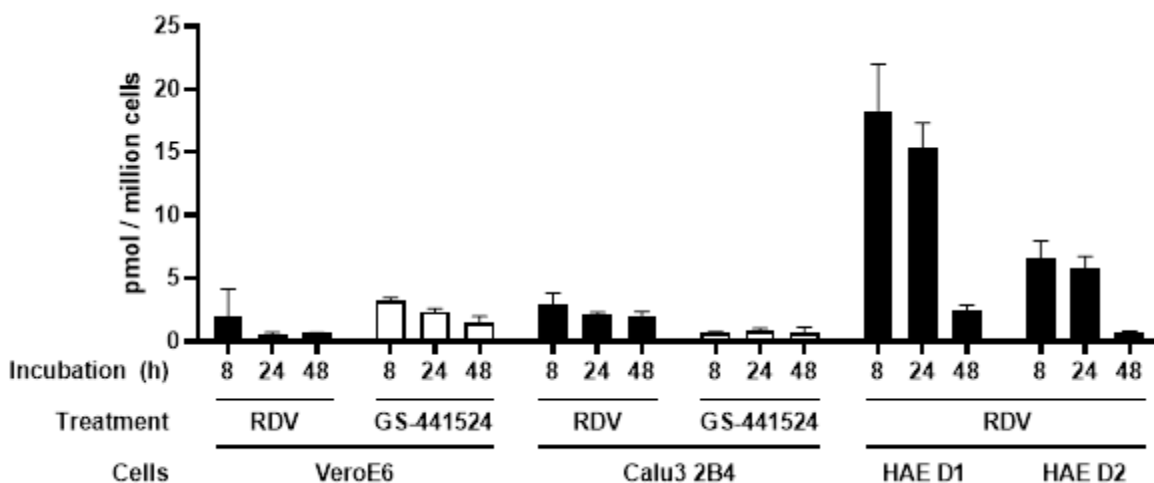
356 ^bHAE antiviral potencies and RDV-TP levels were determined independently in differentiated cultures from two donors. RDV-
357 TP levels in HAEs are presented as the mean ± SD of quadruplicate technical replicates from two donors

358 ^c Individual analyte data are presented in the supplementary information

359 ^d Values represent mean ± SD from two independent experiments with each performed with duplicate samples

360

361 **Figure 4**

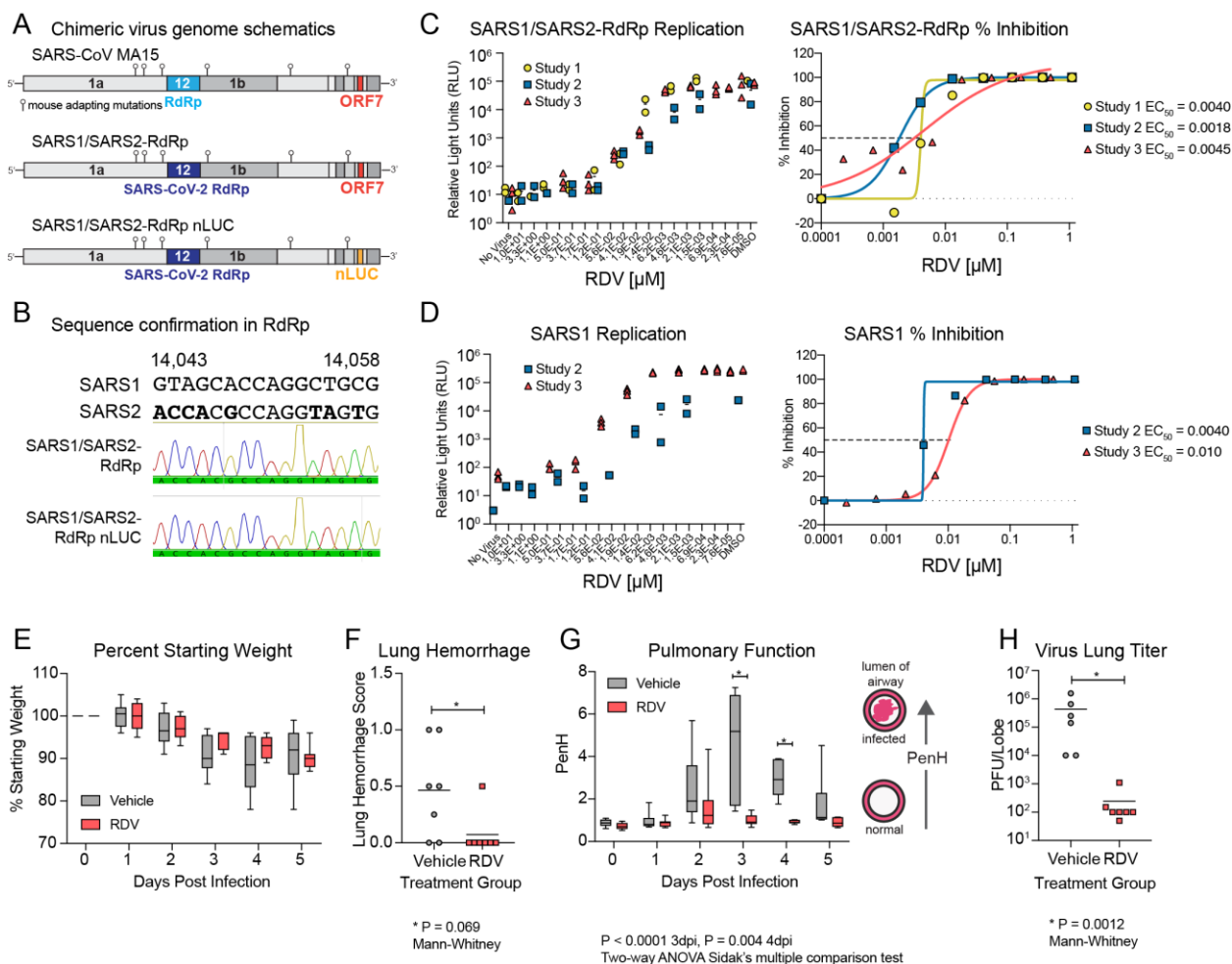


362

363 **Figure 4: RDV-TP levels in Vero E6, Calu3, and HAE cultures.** Vero E6 cells, Calu3 2B4 cells, and
364 HAE cultures were incubated with RDV or GS-441524. At 8, 24, and 48 h of treatment, whole cell extracts
365 were prepared, and RDV-TP levels were quantified by LC-MS/MS as described in Materials and Methods.
366 RDV-TP levels in Vero E6 and Calu3 2B4 cells represent mean \pm SD from two independent experiments,
367 each performed with duplicate samples. RDV-TP levels in HAEs represent the mean \pm SD of four replicates
368 for each individual donor (D1 and D2).

369 Figure 5

370



371

372 **Figure 5. Remdesivir (RDV) is active against the SARS-CoV-2 RdRp *in vivo*.** Activity of RDV against
 373 the SARS-CoV-2 RdRp was evaluated using a chimeric SARS-CoV-1 encoding the SARS-CoV-2 RdRp
 374 (SARS1/SARS2-RdRp). **A.** Schematic of the recombinant SARS-CoV-1 mouse adapted MA15 strain
 375 chimeric virus genomes generated for these studies. SARS1/SARS2-RdRp and SARS1/SARS2-RdRp-
 376 nLUC were constructed by exchanging the SARS-CoV-1 MA15 RdRp with the SARS-CoV-2 RdRp. ORF7
 377 is replaced by nanoluciferase (nLUC) in SARS2-RdRp-nLUC. **B.** Presence of the SARS-CoV-2 RdRp was
 378 confirmed by Sanger sequencing in stocks of both recombinant chimeric viruses. **C.** SARS1/SARS2-RdRp-
 379 nLUC replication in Huh7 cells in the presence of RDV (left) and associated percent inhibition (right). **D.**
 380 SARS-CoV-1 replication in Huh7 cells in the presence of RDV (left) and associated percent inhibition
 381 (right). **E.** Percent starting weight of 17-week old female *Ces1c*^{-/-} mice intranasally infected with 1×10^3
 382 PFU of SARS1/SARS2-RdRp and treated with 25mg/kg RDV subcutaneously or vehicle one day post-

383 infection (dpi) and twice daily thereafter. **F.** Lung hemorrhage at five dpi. $P = 0.069$ by Mann-Whitney test.
384 **G.** Pulmonary function by whole-body plethysmography. The PenH metric shown is a surrogate marker of
385 pulmonary obstruction. $P < 0.0001$ as determined by two-way ANOVA with Sidak's multiple comparison
386 test. **H.** Lung titer at five dpi as measured by plaque assay. $P = 0.0012$ by Mann-Whitney test. For E, G,
387 boxes encompass 25th to 75th percentile, line is drawn at the median, and whiskers represent the range.

388 **METHODS**

389 **Cells, viruses, and compounds.** Vero (ATCC CCL-81) and Vero E6 (ATCC CRL-1586) cells were
390 purchased from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco,
391 ThermoFisher Scientific) or 10% FCS fetal clonal serum (FCS)(HyClone, GE Life Sciences), 100 U/ml
392 penicillin and streptomycin (Gibco, ThermoFisher Scientific), and 0.25 μ M amphotericin B (Corning).
393 Human hepatoma (Huh7) cells were provided by Dr. Mark Heise at UNC Chapel Hill and grown in
394 DMEM supplemented with 10% FBS (Hyclone) and 1 \times antibiotic-antimycotic (Gibco, ThermoFisher
395 Scientific). Calu3 2B4 cells (Yoshikawa et al., 2010) were cultured in DMEM supplemented with 20%
396 FBS, 100 U/mL penicillin and streptomycin, and 0.25 μ M amphotericin B.

397 Primary HAE cell cultures used in antiviral activity assays were obtained from the Tissue Procurement
398 and Cell Culture Core Laboratory in the Marsico Lung Institute/Cystic Fibrosis Research Center at UNC.
399 All assays in this report were performed using a single HAE cell donor. Human tracheobronchial
400 epithelial cells provided by Dr. Scott Randell were obtained from airway specimens resected from
401 patients undergoing surgery under University of North Carolina Institutional Review Board-approved
402 protocols (#03-1396) by the Cystic Fibrosis Center Tissue Culture Core. Primary cells were expanded to
403 generate passage 1 cells and passage 2 cells were plated at a density of 250,000 cells per well on
404 Transwell-COL (12mm diameter) supports (Corning). Human airway epithelium cultures (HAE) were
405 generated by provision of an air-liquid interface for 6 to 8 weeks to form well-differentiated, polarized
406 cultures that resembled in vivo pseudostratified mucociliary epithelium (Fulcher et al., 2005).

407 Clinical specimens of SARS-CoV-2 from a case-patient who acquired COVID-19 during travel to China
408 and diagnosed in Washington State, USA upon return were collected as described (Holshue et al., 2020).
409 Virus isolation from this patient's specimens was performed as described in (Harcourt et al.). The
410 sequence is available through GenBank (accession number MN985325). A passage 3 stock of the SARS-
411 CoV-2 Seattle isolate was obtained from the CDC and passed twice in Vero E6 cells to generate high-titer
412 passage 5 stock for experiments described in this manuscript.

413 SARS-CoV-1 expressing GFP (GFP replaces ORF7) was created from molecular cDNA clones as
414 described (Scobey et al., 2013; Sims et al., 2005). To create SARS-CoV-1 expressing nanoluciferase
415 (nLUC), the gene for GFP was replaced with nLUC and isolated using our existing mouse adapted SARS-
416 CoV-1 (MA15) SARS-CoV-1 Urbani molecular clone (Yount et al., 2003). A synthetic cDNA encoding
417 the SARS-CoV-2 RdRp (Integrated DNA Technologies) was cloned into SARS MA15 D fragment using
418 StuI (5') and BsaI (3') via Gibson assembly. The resultant plasmids were sequence confirmed and then

419 utilized to generate recombinant virus with or without nanoluciferase as described. Recombinant virus
420 stocks were confirmed to harbor SARS-CoV-2 RdRp by Sanger sequencing.

421 Remdesivir (RDV; GS-5734) and GS-441524 were synthesized by the Department of Medicinal
422 Chemistry, Gilead Sciences (Foster City, CA).

423 **Modeling.** A model of the elongating SARS-CoV-2 polymerase complex was generated based on a
424 homology model which used the cryo-EM structure of apo SARS-CoV-1 as a template (PDB: 6NUR,
425 (Kirchdoerfer and Ward, 2019) as described previously (Gordon et al., 2020b). RNA primer and template,
426 a substrate ATP and two catalytic Mg⁺⁺ ions were oriented in the structure based on alignment to a ternary
427 x-ray structure of HCV NS5B (PDB: 4WTD, (Appleby et al., 2015). The structure was then optimized
428 with a series of constrained minimizations. To this structure we aligned the new cryo-EM structure of the
429 SARS-CoV-2 replication complex. As the SARS-CoV-2 structure does not significantly differ from the
430 SARS-CoV-1 structure, rather than a complete replacement of the model, we incorporated only those
431 residues that had not been resolved in the previous structure (residues 31-116). Additional optimization,
432 particularly of the RNA in the exit channel, was done following the previously outlined procedures. After
433 optimization of the ATP structure, RDV-TP was modeled into the active site and minimized. Models of
434 the V557L and F480L mutants and the other coronavirus models reported here were generated based on
435 this final model. All work was carried out using Prime and Macromodel (Schrödinger, LLC, New York,
436 NY, 2020). 3D coordinates of the SARS-CoV-2/RDV-TP model are provided in the Supplementary
437 Material.

438 **Sequence alignments.** Coronavirus nsp12 sequence alignment was generated using CLC Workbench
439 (Qiagen) from sequences downloaded from the NCBI website (Accession numbers MN985325.1 and
440 MT123290.1) and from GISAID's EpiFlu™ Database (Elbe and Buckland-Merrett, 2017; Shu and
441 McCauley, 2017): Accession ID EPI_ISL_402124; virus name hCoV-19/Wuhan/WIV04/2019; Location:
442 Asia / China / Hubei / Wuhan; Collection date 2019-12-30 Originating lab Wuhan Jinyintan Hospital;
443 Submitting lab Wuhan Institute of Virology, Chinese Academy of Sciences; Authors Peng Zhou, Xing-
444 Lou Yang, Ding-Yu Zhang, Lei Zhang, Yan Zhu, Hao-Rui Si, Zhengli Shi. Accession ID
445 EPI_ISL_412028; virus name hCoV-19/Hong Kong/VM20001061/2020; Location Asia / Hong Kong;
446 Collection date 2020-01-22; Originating lab Hong Kong Department of Health; Submitting lab: School of
447 Public Health, The University of Hong Kong; Authors Dominic N.C. Tsang, Daniel K.W. Chu, Leo L.M.
448 Poon, Malik Peiris.

449 **Replication in different cell types.** Vero E6, Vero, Calu3 2B4, and Huh7 cells were seeded in 24 well
450 plates and allowed to adhere for 24 h. Cells were adsorbed with 100 µl SARS-CoV-2 in gel saline for 30

451 minutes (min) at 37°C with manual rocking every 10 min. Virus inoculum was removed, cells were
452 washed in PBS, and 0.5 ml medium was added to each well. Supernatant was collected at 0, 24, 48, and
453 72 h post-infection, and infectious viral titer in supernatants was determined by plaque assay.

454 **Antiviral activity assays.** Vero E6 cells were seeded at 1×10^5 cells per well, and Calu3 2B4 cells were
455 seeded at 2×10^5 cells per well in 24-well plates (Corning). Cells were allowed to adhere for 16-24 h.
456 Drugs were dissolved in DMSO and serially diluted in DMSO to achieve 1000x final concentration.
457 Equal volumes of each 1000x concentration were further diluted 1000-fold in medium up to 2 h before
458 start of the infection. Cells were adsorbed at MOI = 0.01 PFU/cell with SARS-CoV-2 in gel saline for 30
459 min at 37°C. Plates were rocked manually to redistribute the inoculum every 10 minutes. Viral inoculum
460 was removed, and cells were washed with pre-warmed PBS+/+ (Corning) for 5 minutes. PBS+/+ was
461 removed, and medium containing dilutions of RDV, GS-441524, or vehicle (DMSO) was added. Cells
462 were incubated at 37°C. At 48 (Vero E6) or 72 (Calu3 2B4) hpi, supernatants were harvested and
463 processed for plaque assay and RT-qPCR.

464 Huh7 cells were plated at a density of 8×10^4 cells per well. Twenty-four hours later, fresh medium was
465 added. Triplicate wells of cells were infected for 1 h at 37°C with SARS1/SARS2-RdRp-nLUC or SARS-
466 CoV-1-nLUC diluted 1:100 in culture medium. Virus was removed, cultures were rinsed once with
467 medium, and fresh medium containing dilutions of RDV or vehicle (DMSO) was added. DMSO (0.05%)
468 was constant in all conditions. At 48 hpi, virus replication was measured by nLUC assay (Promega) using
469 a SpectraMax plate reader (Molecular Devices).

470 Before infection, HAE cultures (approximately 1×10^6 cells per well) were washed with phosphate-
471 buffered saline (PBS) and moved into air-liquid interface medium containing various doses of RDV
472 ranging from 0.00260 to 10 mM (final DMSO, <0.05%). Cultures were infected with SARS-CoV-2
473 clinical isolate (2019-nCoV/USA-WA1/2020) at MOI = 0.5 PFU/cell for 2 h at 37°C, after which virus
474 was removed and cultures were washed three times with PBS, followed by incubation at 37°C for 48 h.
475 The apical surface of each culture was washed with PBS and collected for virus titration, measured as
476 plaque-forming units (PFU) as previously described for SARS-CoV-1 (Scobey et al., 2013; Sims et al.,
477 2005).

478 **Cytotoxicity Assays.** Cells were seeded at a density of 15,000 cells/well (Vero E6) or 30,000 cells/well
479 (Calu3 2B4) in a white-wall clear-bottom 96-well plate (Corning) and incubated at 37°C overnight.
480 Medium was removed, and serial dilutions of drug in medium were added to each well (see “**antiviral**
481 **activity assays**”). Cytotoxicity was determined using CellTiterGlo Cell Viability Assay (Promega)
482 according to manufacturer’s instructions at 48 h (Vero E6) or 72 h (Calu3). HAE cultures were treated

483 with the same concentration range of drug in Transwell plates (Corning). Cytotoxicity in HAE was
484 previously determined by RT-qPCR of TRIzol-extracted RNA (Sheahan et al., 2017)

485 **Quantification of infectious viral titer by plaque assay.** Approximately 1×10^6 Vero E6 cells/well were
486 seeded in 6-well tissue culture plates (Corning) and allowed to grow to confluence for 48 h. Medium was
487 removed, and 200 μ L of 10-fold serial dilutions of virus-containing supernatants in gel saline were
488 adsorbed in duplicate for 30 min at 37°C. Plates were rocked manually to redistribute inoculum every 10
489 minutes. Cells were overlaid with a 1:1 mixture of 2x DMEM and 2% agar in ddH₂O and incubated at
490 37°C. Plaques were enumerated in unstained monolayers at 48-72 hpi using a light box.

491 **Quantification of viral RNA genome copy number by RT-qPCR.** Cell supernatants were harvested in
492 TRIzol LS reagent (Invitrogen), and RNA was purified following phase separation by chloroform as
493 recommended by the manufacturer. RNA in the aqueous phase was collected and further purified using
494 PureLink RNA Mini Kits (Invitrogen) according to manufacturer's protocol. Viral RNA was quantified
495 by reverse-transcription quantitative PCR (RT-qPCR) on a StepOnePlus Real-Time PCR System
496 (Applied Biosystems) using TaqMan Fast Virus 1-Step Master Mix chemistry (Applied Biosystems).
497 SARS-CoV-2 N gene RNA was amplified using forward (5'-GACCCCAAATCAGCGAAAT) and
498 reverse (5'-TCTGGTTACTGCCAGTTGAATCTG) primers and probe (5'- FAM-
499 ACCCCGCATTACGTTTGGTGGACC-BHQ1) designed by the United States Centers for Disease
500 Control and Prevention (oligonucleotides produced by IDT, cat# 10006606). RNA copy numbers were
501 interpolated from a standard curve produced with serial 10-fold dilutions of N gene RNA. Briefly, SARS-
502 CoV-2 N gene positive control plasmid (IDT, cat# 10006625) served as template to PCR-amplify a 1280
503 bp product using forward (5'-TAATACGACTCACTATAGGGATGTCTGATAATGGACCCCA) and
504 reverse (5'- TTAGGCCTGAGTTGAGTCAG) primers that appended a T7 RNA polymerase promoter to
505 the 5' end of the complete N ORF. PCR product was column purified (Promega) for subsequent *in vitro*
506 transcription of N RNA using mMACHINE T7 Transcription Kit (Invitrogen) according to
507 manufacturer's protocol. N RNA was purified using RNeasy mini kit (Qiagen) according to
508 manufacturer's protocol, and copy number was calculated using SciencePrimer.com cop number
509 calculator.

510 ***In vitro* metabolism of RDV and GS-441524.** Calu3 2B4 or Vero E6 cells were seeded in a 6-well plate
511 at 8.0×10^5 or 3.5×10^5 cells/well, respectively. Twenty-four hours later, cell culture media was replaced
512 with media containing 1 μ M RDV (GS-5734) or GS-441524 and incubated at 37°C. Differentiated HAE
513 cultures from two healthy donors (MatTek Corporation; Ashland, MA) were maintained with media
514 replacement every other day for 1 week. The HAE donors were 56- and 62-year-old females of the same
515 race. At the time of treatment, media was replaced on the basal side of the transwell HAE culture, while

516 the apical surface media was replaced with 200 μ L media containing 1 μ M RDV. At 8, 24 and 48h post
517 drug addition to all cultures, cells were washed 3 times with ice-cold tris-buffered saline, scraped into 0.5
518 mL ice-cold 70% methanol and stored at -80°C . Extracts were centrifuged at 15,000 x g for 15 minutes
519 and supernatants were transferred to clean tubes for evaporation in a MiVac Duo concentrator (Genevac).
520 Dried samples were reconstituted in mobile phase A containing 3 mM ammonium formate (pH 5) with 10
521 mM dimethylhexylamine (DMH) in water for analysis by LC-MS/MS, using a multi-stage linear gradient
522 from 10% to 50% acetonitrile in mobile phase A at a flow rate of 300 μ L/min. Analytes were separated
523 using a 50 x 2 mm, 2.5 μ m Luna C18(2) HST column (Phenomenex) connected to an LC-20ADXR
524 (Shimadzu) ternary pump system and HTS PAL autosampler (LEAP Technologies). Detection was
525 performed on a Qtrap 6500+ (AB Sciex) mass spectrometer operating in positive ion and multiple
526 reaction monitoring modes. Analytes were quantified using a 7-point standard curve ranging in
527 concentration from 0.156 to 40 pmol prepared in extracts from untreated cells. For normalization by cell
528 number, multiple untreated Calu3 or Vero E6 culture wells were counted at each timepoint. HAE cells
529 were counted at the 24-h timepoint and the counts for other timepoints were determined by normalized to
530 endogenous ATP levels for accuracy.

531 **Formulations for *in vivo* studies.** RDV was solubilized at 2.5 mg/mL in vehicle containing 12%
532 sulfobutylether- β -cyclodextrin sodium salt in water (with HCl/NaOH) at pH 5.0.

533 ***In vivo* efficacy studies.** All animal experiments were performed in accordance with the University of
534 North Carolina at Chapel Hill Institutional Animal Care and Use Committee policies and guidelines. To
535 achieve a pharmacokinetic profile similar to that observed in humans, we performed therapeutic efficacy
536 studies in *Ces1c*^{-/-} mice (stock 014096, The Jackson Laboratory), which lack a serum esterase not present
537 in humans that dramatically reduces RDV half-life (Sheahan et al., 2017). 17 week-old female *Ces1c*^{-/-}
538 mice were anaesthetized with a mixture of ketamine/xylazine and intranasally infected with 10^3 PFU
539 SARS1/SARS2-RdRp in 50 μ L. One dpi, vehicle (n = 7) and RDV (n = 7) dosing was initiated (25 mg/kg
540 subcutaneously) and continued every 12 h until the end of the study at five dpi. To monitor morbidity,
541 mice were weighed daily. Pulmonary function testing was performed daily by whole body
542 plethysmography (WBP) (Data Sciences International) (Sheahan et al., 2017). At five dpi, animals were
543 sacrificed by isoflurane overdose, lungs were scored for lung hemorrhage, and the inferior right lobe was
544 frozen at -80°C for viral titration via plaque assay on Vero E6 cells. Lung hemorrhage is a gross
545 pathological phenotype readily observed by the naked eye and driven by the degree of virus replication,
546 where lung coloration changes from pink to dark red (Sheahan et al., 2017, 2020a). For the plaque assay,
547 5×10^5 Vero E6 cells/well were seeded in 6-well plates. The following day, medium was removed, and
548 monolayers were adsorbed at 37°C for one h with serial dilutions of sample ranging from 10^{-1} to 10^{-6} .

549 Cells were overlaid with 1X DMEM, 5% Fetal Clone 2 serum, 1× antibiotic-antimycotic, 0.8% agarose.
550 Viral plaques were enumerated three days later.

551 **Mathematical and statistical analyses.** The EC₅₀ value was defined in GraphPad Prism 8 as the
552 concentration at which there was a 50% decrease in viral replication relative to vehicle alone (0%
553 inhibition). Curves were fitted based on four parameter non-linear regression analysis. All statistical tests
554 were executed using GraphPad Prism 8.

555

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