

## **A nanoluciferase SARS-CoV-2 for rapid neutralization testing and screening of anti-infective drugs for COVID-19**

Xuping Xie<sup>1,#,\*</sup>, Antonio E. Muruato<sup>1,2,#</sup>, Xianwen Zhang<sup>1</sup>, Kumari G. Lokugamage<sup>2</sup>, Camila R. Fontes-Garfias<sup>1</sup>, Jing Zou<sup>1</sup>, Jianying Liu<sup>2</sup>, Ping Ren<sup>3</sup>, Mini Balakrishnan<sup>4</sup>, Tomas Cihlar<sup>4</sup>, Chien-Te K. Tseng<sup>2</sup>, Shinji Makino<sup>2</sup>, Vineet D. Menachery<sup>2,3,5</sup>, John P. Bilello<sup>4\*</sup>, and Pei-Yong Shi<sup>1,5,6,7\*</sup>

<sup>1</sup>Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston TX, USA

<sup>2</sup>Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston TX, USA

<sup>3</sup>Department of Pathology, University of Texas Medical Branch, Galveston TX, USA

<sup>4</sup>Gilead Sciences, Inc., Foster City, CA, USA

<sup>5</sup>Institute for Human Infections and Immunity, University of Texas Medical Branch, Galveston, TX, USA

<sup>6</sup>Sealy Institute for Vaccine Sciences, University of Texas Medical Branch, Galveston, TX, USA

<sup>7</sup>Sealy Center for Structural Biology & Molecular Biophysics, University of Texas Medical Branch, Galveston, TX, USA

#X.X. and A.E.M. contributed equally to this study

\*Correspondence: X.X. (xuxie@UTMB.edu), J.P.B. (john.bilello@gilead.com), or P.-Y.S. (peshi@UTMB.edu)

**Running title:** A rapid neutralization assay and screen of anti-infective drugs for COVID-19

**Keywords:** SARS-CoV-2, COVID-19, serological diagnosis, vaccine, antiviral, drug repurpose, coronavirus

1 **Abstract**

2 A high-throughput platform would greatly facilitate COVID-19 serological testing and  
3 antiviral screening. Here we report a nanoluciferase SARS-CoV-2 (SARS-CoV-2-Nluc) that is  
4 genetically stable and replicates similarly to the wild-type virus in cell culture. We demonstrate  
5 that the optimized reporter virus assay in Vero E6 cells can be used to measure neutralizing  
6 antibody activity in patient sera and produces results in concordance with a plaque reduction  
7 neutralization test (PRNT). Compared with the low-throughput PRNT (3 days), the SARS-CoV-  
8 2-Nluc assay has substantially shorter turnaround time (5 hours) with a high-throughput testing  
9 capacity. Thus, the assay can be readily deployed for large-scale vaccine evaluation and  
10 neutralizing antibody testing in humans. Additionally, we developed a high-throughput antiviral  
11 assay using SARS-CoV-2-Nluc infection of A549 cells expressing human ACE2 receptor (A549-  
12 hACE2). When tested against this reporter virus, remdesivir exhibited substantially more potent  
13 activity in A549-hACE2 cells compared to Vero E6 cells ( $EC_{50}$  0.115 vs 1.28  $\mu$ M), while this  
14 difference was not observed for chloroquine ( $EC_{50}$  1.32 vs 3.52  $\mu$ M), underscoring the  
15 importance of selecting appropriate cells for antiviral testing. Using the optimized SARS-CoV-2-  
16 Nluc assay, we evaluated a collection of approved and investigational antivirals and other anti-  
17 infective drugs. Nelfinavir, rupintrivir, and cobicistat were identified as the most selective  
18 inhibitors of SARS-CoV-2-Nluc ( $EC_{50}$  0.77 to 2.74  $\mu$ M). In contrast, most of the clinically  
19 approved antivirals, including tenofovir alafenamide, emtricitabine, sofosbuvir, ledipasvir, and  
20 velpatasvir were inactive at concentrations up to 10  $\mu$ M. Collectively, this high-throughput  
21 platform represents a reliable tool for rapid neutralization testing and antiviral screening for  
22 SARS-CoV-2.

23

24 **Introduction**

25           Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan,  
26   China in late 2019<sup>1,2</sup> and caused global pandemic of coronavirus disease 2019 (COVID-19).  
27   Two other human coronaviruses emerged in the past two decades and caused severe  
28   respiratory syndrome, including SARS-CoV in 2002 and Middle East respiratory syndrome  
29   (MERS-CoV) in 2012<sup>3</sup>. In addition, four endemic human coronaviruses (*i.e.*, OC43, 229E, NL63,  
30   and HKU1) cause common cold respiratory diseases. For COVID-19 diagnosis, nucleic acid-  
31   based RT-PCR assays have been used to identify individuals with acute viral infection. The RT-  
32   PCR assay is essential for detecting and contact tracing to control viral transmission. Given the  
33   unknown extent of asymptomatic infections, rapid and reliable serological assays are urgently  
34   needed to determine the real scale of local community infections. In addition, the ability to  
35   quickly measure neutralizing antibody levels is required to determine the protective immunity of  
36   previously infected individuals, to identify convalescent donors with protective antibodies for  
37   plasma therapy, and to evaluate various vaccines under development. Although various  
38   serological assay platforms have been developed [*e.g.*, lateral flow immunoassay, ELISA,  
39   microsphere immunoassay, and vesicular stomatitis virus (VSV) pseudotyped with SARS-CoV-2  
40   spike], the conventional plaque reduction neutralization test (PRNT) remains the gold standard  
41   of serological diagnosis because it directly measures the neutralizing antibody levels required to  
42   block an authentic viral infection. However, the low throughput and long assay turnaround time  
43   make PRNT impossible for large scale diagnosis, representing a critical gap for COVID-19  
44   response and countermeasure development.

45           The goals of this study were to (i) develop a rapid neutralization assay that maintains the  
46   gold standard of PRNT for serological COVID-19 diagnosis, (ii) establish a high-throughput  
47   assay for reliable antiviral screening, and (ii) screen exploratory and FDA-approved anti-  
48   infective drugs for potential COVID-19 repurposing. We established a nanoluciferase SARS-  
49   CoV-2 (SARS-CoV-2-Nluc) as a platform for rapid serodiagnosis and high-throughput drug

50 screening. When used to test COVID-19 patient sera, the rapid neutralization assay yielded  
51 results commensurate with the conventional PRNT. A version of the SARS-CoV-2-Nluc infection  
52 assay has also been developed for high throughput screening of antivirals and validated using  
53 known SARS-CoV-2 inhibitors such as remdesivir and chloroquine. The developed assay was  
54 employed to test a collection of approved and investigational anti-infective drugs, including  
55 established antivirals against HIV and HCV.

56

## 57 **Results**

58 **A stable SARS-CoV-2-Nluc.** Using an infectious cDNA clone of SARS-CoV-2 (strain  
59 2019-nCoV/USA\_WA1/2020)<sup>4</sup>, we engineered nanoluciferase (Nluc) gene at the OFR7 of the  
60 viral genome (Fig. 1a). Seven cDNA fragments spanning the SARS-CoV-2 genome were ligated  
61 *in vitro* to generate a full-genome Nluc cDNA. A T7 promoter was engineered to *in vitro*  
62 transcribe the full-length Nluc viral RNA. The RNA transcript was highly infectious after  
63 electroporation into Vero E6 cells (African green monkey kidney epithelial cells), producing 10<sup>7</sup>  
64 PFU/ml of virus. The infectious clone-derived SARS-CoV-2-Nluc developed plaques slightly  
65 larger than the wild-type recombinant SARS-CoV-2 (Fig. 1b). The SARS-CoV-2-Nluc and wild-  
66 type SARS-CoV-2 exhibited similar replication kinetics in Vero E6 cells (Fig. 1c), indicating that  
67 insertion of Nluc gene does not affect the viral replication *in vitro*.

68 To examine the stability of SARS-CoV-2-Nluc, we continuously cultured the virus for five  
69 passages on Vero E6 cells (1-2 days per passage). The passage 5 (P5) virus produced similar  
70 plaque morphology (Fig. 1d), replication kinetics (Fig. 1e), and luciferase profile as the P1 virus  
71 (Fig. 1f). Next, we performed RT-PCR to verify the retention of Nluc gene in the P1 and P5 viral  
72 genomes using two primers spanning the insertion junctions (nucleotides 25,068-28,099 of viral  
73 genome). The RT-PCR products derived from both P1 and P5 SARS-CoV-2-Nluc were 156-bp  
74 larger than that from the wild-type recombinant SARS-CoV-2 (Fig. 1g, lanes 1-3). The 156-bp

75 difference is due to the substitution of ORF7 (368 bp) with Nluc gene (513 bp). Digestion of the  
76 RT-PCR products with BsrGI (located upstream of the Nluc insertion) and PacI (located at the  
77 C-terminal region of Nluc) generated distinct DNA fragments between the Nluc and wild-type  
78 viruses, whereas the P1 and P5 viruses produced identical digestion patterns (Fig. 1g, lanes 4-  
79 6). Furthermore, we confirmed the retention of Nluc reporter by sequencing the P1 and P5 RT-  
80 PCR products (Fig. 1h). Compared with the infectious clone-derived wild-type SARS-CoV-2<sup>4</sup>,  
81 both P1 and P5 reporter viruses contained five single nucleotide mutations that led to amino  
82 acid changes in different viral proteins (Fig. 1h). These mutations may account for the slightly  
83 larger plaques of SARS-CoV-2-Nluc. No other mutations were recovered from the passaged  
84 viruses. Altogether, the results demonstrate that SARS-CoV-2-Nluc stably maintains the  
85 reporter gene after five rounds of passaging on Vero E6 cells.

#### 86 **Human angiotensin-converting enzyme (hACE2) as a receptor for SARS-CoV-2.**

87 We explored SARS-CoV-2-Nluc to study virus entry, serological diagnosis, and antiviral  
88 screening. Infection of Vero E6 cells with SARS-CoV-2-Nluc [multiplicity of infection (MOI) 1.0]  
89 produced a robust Nluc profile that peaked at 24 h post-infection (p.i.; Fig. 2a). As early as 1 h  
90 p.i., the Nluc signal was >10 fold above the background, suggesting that Nluc signals at early  
91 timepoints may be used to study virus entry. Thus, we evaluated the function of hACE2 in virus  
92 entry by pre-incubating Vero E6 cells with anti-hACE2 polyclonal antibodies for 1 h, followed by  
93 SARS-CoV-2-Nluc infection (Fig. 2b). The anti-hACE2 antibodies inhibited Nluc signal at 6 h p.i.  
94 in a dose-responsive manner (Fig. 2c). As a negative control, pre-treatment with antibodies  
95 against hDPP4 (a receptor for MERS-CoV infection) did not suppress Nluc activity (Fig. 2c),  
96 indicating the role of hACE2 in SARS-CoV-2 entry. To further evaluate these results, we  
97 compared the efficiencies of virus entry between naïve A549 (a human alveolar epithelial cell  
98 line) and A549 stably expressing hACE2 (A549-hACE2; Fig. 2d). At various MOIs, the Nluc  
99 signals (collected at 24 h p.i.) from A549-hACE2 cells were ~100-fold higher than those from the

100 naïve A549 cells (Fig. 2e). Collectively, the results support hACE2 as a receptor for SARS-  
101 COV-2 entry.

102 **A rapid neutralization assay for COVID-19 diagnosis.** The robust early Nluc signals  
103 after SARS-COV-2-Nluc infection (Fig. 2a) prompted us to develop a rapid neutralization assay.  
104 Fig. 3a depicts the flowchart of SARS-COV-2-Nluc neutralization assay in a 96-well format. After  
105 incubating serum samples with SARS-COV-2-Nluc at 37°C for 1 h, the virus/serum mixtures  
106 were added to Vero E6 cells (pre-seeded in a 96-well plate) at an MOI of 0.5. At 4 h p.i., Nluc  
107 signals were measured to determine the serum dilution that neutralized 50% of Nluc activity  
108 (NT<sub>50</sub>). We chose 4 h p.i. as the assay end time because the Nluc signal at this timepoint was  
109 >100 fold above the background (Fig. 2a). The total assay time to completion was 5 h (1 h  
110 virus/serum incubation plus 4 h viral infection). Following this protocol, we tested twenty-one  
111 COVID-19-positive sera from RT-PCR-confirmed patients and nine COVID-19-negative human  
112 sera (collected before COVID-19 emergence; Fig. 3b). All COVID-19-positive sera (samples 1-  
113 21) showed positive NT<sub>50</sub> of 66 to 7237, while all COVID-19-negative sera (samples 22-30)  
114 showed negative NT<sub>50</sub> <20, the lowest tested serum dilution. Fig. 3c shows three representative  
115 neutralization curves: Nluc signals were suppressed by the positive sera in an inverse dilution-  
116 dependent manner. The results suggest that SARS-COV-2-Nluc could be used for rapid  
117 neutralization testing.

118 To validate the Nluc neutralization results, we performed conventional PRNT on the  
119 same set of patient sera. The twenty-one COVID-19-positive samples exhibited PRNT<sub>50</sub> of 80 to  
120 3200, and the nine COVID-19-negative samples showed PRNT<sub>50</sub> <20 (Fig. 3b). The  
121 neutralization results between the Nluc virus and PRNT assays had a correlation coefficient ( $R^2$ )  
122 of 0.8395 (Fig. 3d). Notably, the NT<sub>50</sub> values from the Nluc assay are on average 3-fold higher  
123 than the PRNT<sub>50</sub> values from the plaque assay. Overall, the results indicate that the SARS-CoV-

124 2-Nluc neutralization assay detects neutralizing antibodies in COVID-19 patient sera with a  
125 higher sensitivity than the conventional PRNT assay.

126 **A high-throughput antiviral assay for SARS-CoV-2.** Reporter viruses have been  
127 commonly used for antiviral screening<sup>5-11</sup>. Therefore, we developed a 96-well format antiviral  
128 assay using the SARS-CoV-2-Nluc reporter virus. Vero E6 cells were initially used in our assay  
129 development because this cell line is highly susceptible to SARS-CoV-2 infection<sup>1</sup>. Since  
130 COVID-19 is a respiratory disease, we also tested A549 (a human alveolar epithelial cell line)  
131 for the assay development. However, due to the low permissiveness of A549 for SARS-CoV-2-  
132 Nluc infection, we included A549-hACE2 cells to enhance viral infection in our assay (Fig. 2e).  
133 Two SARS-CoV-2 inhibitors that received the emergency use authorization in US for COVID-19  
134 at the time of assay development, chloroquine phosphate (a malaria drug) and remdesivir (an  
135 antiviral adenosine analog prodrug)<sup>12</sup>, were used to evaluate the assay in both Vero E6 and  
136 A549-hACE2 cells (Fig. 4). In a 3-day cytotoxicity assay, chloroquine showed CC<sub>50</sub> of >50 µM  
137 on both cells, whereas remdesivir had CC<sub>50</sub> of >50 µM and 32.5 µM in Vero E6 and A549-  
138 hACE2 cells, respectively (Fig. 4a,b). For testing antiviral activity, we optimized the assay  
139 conditions (12,000 Vero or A549-hACE2 cells per well and MOI 0.025) to allow for multiple  
140 rounds of viral replication in 48 h p.i. without developing significant cytopathic effect (CPE). Both  
141 chloroquine and remdesivir inhibited Nluc activity in a dose-dependent manner (Fig. 4c,d).  
142 Importantly, the EC<sub>50</sub> value for remdesivir in A549-hACE2 cells (0.115 µM) was >10-fold lower  
143 than that in Vero E6 cells (1.28 µM), while the potency of chloroquine was only marginally  
144 different between the two cell lines (EC<sub>50</sub> 1.32 vs 3.52 µM; Fig. 4e). This result underscores the  
145 importance of using biologically relevant cells for antiviral testing. Thus, we chose A549-hACE2  
146 for the following high-throughput antiviral screening of additional compounds.

147 **Testing of clinically relevant anti-infective drugs for antiviral activity against**  
148 **SARS-CoV-2.** A broad selection of forty clinically approved and investigational antivirals and

149 other anti-infective drugs were tested for anti-SARS-CoV-2-Nluc activities in A549-hACE2 cells.  
150 Based on their indication and/or mode of action, the tested drugs belong to four categories,  
151 including (i) antiviral nucleoside/nucleotide analogs, (ii) HIV antivirals, (iii) HCV antivirals, and  
152 (iv) other primarily anti-infective drugs.

153 **(i) Nucleoside/nucleotide analog drugs.** Ten nucleoside analogs with antiviral  
154 activities against other viruses were evaluated for activity against SARS-CoV-2-Nluc (Table 1).  
155 Only remdesivir showed SARS-CoV-2-Nuc activity with an  $EC_{50}$  and  $CC_{50}$  of 0.115 and 32.7  $\mu\text{M}$ ,  
156 respectively, and selectivity index ( $SI = CC_{50}/EC_{50}$ ) of 284. No other nucleoside analogs,  
157 including sofosbuvir or any other 2'C-methyl substituted anti-HCV nucleosides or their prodrugs,  
158 exhibited anti-SARS-CoV-2 activity at concentrations up to 10  $\mu\text{M}$ . The results agree with  
159 previous reports demonstrating potent inhibition of SARS-CoV-2 by remdesivir in physiologically  
160 relevant airway epithelial cells<sup>13</sup>, and lack of SARS-CoV-2 inhibition by favipiravir and/or  
161 ribavirin<sup>14-16</sup>.

162 **(ii) HIV antivirals.** Fifteen clinically approved antiretrovirals, including protease inhibitors  
163 (PIs), nucleoside/nucleotide reverse-transcriptase inhibitors (NRTIs), non-nucleoside reverse  
164 transcriptase inhibitors (NNRTIs), and an integrase strand-transfer inhibitor (INSTI), were  
165 assessed for their activities against SARS-CoV-2-Nluc (Table 2). Among the nine FDA-  
166 approved HIV PIs tested, nelfinavir was the only compound that inhibited SARS-CoV-2-Nluc  
167 with a sub-micromolar potency ( $EC_{50}$  0.77  $\mu\text{M}$ ), albeit with a relatively narrow SI of 16. Factoring  
168 in human plasma protein binding of nelfinavir<sup>17</sup>, the projected protein adjusted potency ( $paEC_{50}$   
169 ~30  $\mu\text{M}$ ) is significantly above the clinically achievable plasma concentration of the drug (Table  
170 2). Of the remaining PIs, five were inactive (amprenavir, ritonavir, indinavir, darunavir, and  
171 atazanavir with  $EC_{50}$  >10  $\mu\text{M}$ ) and three exhibited rather weak antiviral activity (lopinavir,  
172 saquinavir, and tipranavir with  $EC_{50}$  of 8-9  $\mu\text{M}$  and SI of 3-4).

173 Among the HIV RT inhibitors, all three NRTIs (emtricitabine, tenofovir alafenamide, and  
174 rovafovir) were inactive against SARS-CoV-2-Nluc with  $EC_{50}$  >10  $\mu\text{M}$  (Table 2). The two



175 NNRTIs (rilpivirine and efavirenz) exhibited poor SI <3.9. Bictegravir, a drug targeting HIV  
176 integrase, was inactive against SARS-CoV-2-Nluc with  $EC_{50} >10 \mu\text{M}$  (Table 2).

177 **(iii) HCV antivirals.** Nine FDA-approved HCV drugs with diverse modes of action  
178 targeting viral protease, polymerase (both nucleotide and non-nucleoside inhibitors), or NS5A  
179 protein were tested. None of them showed any anti-SARS-CoV-2-Nluc activities with  $EC_{50} >10$   
180  $\mu\text{M}$  (Table 3).

181 **(iv) Other classes of drugs.** Ten additional clinically validated drugs, six of which are  
182 anti-infective medicines, were tested against SARS-CoV-2-Nluc (Table 4). Rupintrivir, a human  
183 rhinovirus (HRV) 3CLpro cysteine protease inhibitor, inhibited SARS-CoV-2-Nluc with  $EC_{50}$  1.87  
184  $\mu\text{M}$ , representing a 156-fold lower potency than that against HRV<sup>18</sup>. Niclosamide (an  
185 antihelminthic drug) showed anti-SARS-CoV-2-Nluc activity ( $EC_{50}$  0.715  $\mu\text{M}$ ) with low selectivity  
186 (SI 1.8). As described in Fig. 4, chloroquine exhibited selective inhibition of anti-SARS-CoV-2-  
187 Nluc ( $EC_{50}$  1.32  $\mu\text{M}$  and SI >37.9). Presatovir, a respiratory syncytial virus (RSV) fusion  
188 inhibitor, showed an  $EC_{50}$  of 2.53  $\mu\text{M}$  and SI of >37.9. The  $EC_{50}$  of presatovir against SARS-  
189 CoV-2 is 7,000-fold less potent than against RSV<sup>19</sup>, establishing that clinical exposures are  
190 below the  $EC_{50}$  determined for SARS-CoV-2<sup>20</sup>, precluding the potential for COVID-19 therapy.  
191 Cobicistat, a selective mechanism-based inhibitor of CYP3A enzymes, weakly inhibited SARS-  
192 CoV-2-Nluc ( $EC_{50}$  2.7  $\mu\text{M}$ ) with a modest SI of 17.3. Oseltamavir carboxylate and baloxavir, two  
193 approved drugs targeting influenza A virus neuraminidase and endonuclease, respectively,  
194 were inactive against SARS-CoV-2-Nluc with  $EC_{50} >10 \mu\text{M}$ . Nivocasan, an inhibitor of cellular  
195 caspases 1, 8, and 9 (treatment for hepatic fibrosis and non-alcoholic steatohepatitis related to  
196 HCV infection), as well as two inhibitors of Bruton's tyrosine kinase (BTK; treatment for  
197 lymphoma and leukemia) were also inactive against SARS-CoV-2 with  $EC_{50} >10 \mu\text{M}$  (Table 4).  
198 Taken together, only remdesivir, chloroquine, and rupintrivir have antiviral activity against  
199 recombinant SARS-CoV-2-Nluc.

200

## 201 Discussion

202 We developed a stable reporter SARS-CoV-2-Nluc variant for rapid neutralization  
203 testing. Since neutralizing titer is a key parameter to predict immunity, the rapid SARS-CoV-2-  
204 Nluc neutralization assay will enable many aspects of COVID-19 research, including  
205 epidemiological surveillance, vaccine development, and antiviral discovery. Although the current  
206 assay was performed in a 96-well format, given the magnitude and dynamic range of Nluc  
207 signal, it can be readily adapted to a 384- or 1536-well format for large-scale testing. Notably,  
208 when diagnosing patient sera, the SARS-CoV-2-Nluc assay generated NT<sub>50</sub> value on average  
209 3-fold higher than the conventional PRNT<sub>50</sub>. The higher sensitivity of the SARS-CoV-2-Nluc  
210 assay might be due to different endpoint readouts (plaque counts versus luminescence signal of  
211 Nluc that could accumulate in cells). Importantly, compared with the conventional PRNT assay,  
212 our reporter neutralization test has shortened the turnaround time from 3 days to 5 h and  
213 increased the testing capacity. Despite the strengths of high throughput and speed, the current  
214 rapid neutralization assay must be performed in a biosafety level 3 (BSL-3) facility, representing  
215 a major limitation. Experiments are ongoing to attenuate SARS-CoV-2-Nluc so that the assay  
216 could be performed in a BSL-2 laboratory. Aligned with the same premise, BSL-2 lab compatible  
217 neutralization assays have been reported using VSV pseudotyped with SARS-CoV-2 spike  
218 protein<sup>21,21</sup>.

219 We additionally optimized and validated the recombinant SARS-CoV-2-Nluc for high-  
220 throughput antiviral screening. Our results demonstrate that cell type could significantly affect a  
221 compound's EC<sub>50</sub> value, underscoring the importance of using biologically relevant cells for drug  
222 discovery. The extent of EC<sub>50</sub> discrepancy from different cells was dependent on the  
223 compound's mode of action. Remdesivir EC<sub>50</sub> values differed by >10-fold when the assay used  
224 Vero E6 and A549-hACE2 cells. In another study, remdesivir was shown to be even more  
225 potent (EC<sub>50</sub> 0.01 μM) when tested on primary human airway epithelial (HAE) cells<sup>13</sup>. The  
226 potency differences seen between cell types are due to the differential metabolism of remdesivir

227 in various cells. Host metabolic enzymes are required to convert the remdesivir prodrug to a  
228 monophosphate substrate, which is further metabolized by host kinases to its active  
229 triphosphate form that incorporates into viral RNA for chain termination. Vero E6 cells are less  
230 efficient in forming the active triphosphate than A549-hACE2 and primary HAE cells<sup>13,22</sup>, leading  
231 to higher EC<sub>50</sub> values. The antiviral activity of chloroquine was more consistent between the two  
232 cell lines tested, indicating that its mode of action is independent of host metabolism. This  
233 highlights the need for careful and appropriate interpretation of *in vitro* antiviral data for  
234 compounds with different mechanisms of action such as remdesivir and chloroquine, which may  
235 appear similar in some cell types but are substantially different in cells that are more clinically  
236 relevant for SARS-CoV-2 infection.

237 Remdesivir has received the FDA EUA for COVID-19 treatment and is being tested in  
238 additional clinical trials, including combination therapies. In a double-blind, randomized,  
239 placebo-controlled trial involving 1,063 patients hospitalized with COVID-19, patients receiving  
240 remdesivir experienced a shortened recovery time of 11 days as compared with 15 days for  
241 patients in the placebo group<sup>23</sup>. Besides SARS-CoV-2, remdesivir was also shown to potently  
242 inhibit SARS-CoV and MERS-CoV in cell culture and animal models<sup>13,24-27</sup>. For chloroquine,  
243 inconsistent results were obtained from several clinical studies with small patient numbers<sup>28-30</sup>. A  
244 recent retrospective multicenter study involving >1,400 patients showed that treatment with  
245 hydroxychloroquine, azithromycin, or both, compared with no treatment, was not associated  
246 with significant differences in fatality rate among hospitalized patients<sup>31</sup>. These and other  
247 controversial results prompted recent decision by FDA to revoke the EUA for chloroquine and  
248 hydroxychloroquine (<https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-revokes-emergency-use-authorization-chloroquine-and>).  
249

250 Using the validated SARS-CoV-2-Nluc/A549-hACE2 infection assay, we screened a  
251 collection of 40 clinically relevant antivirals and anti-infective drugs. In addition to remdesivir and

252 chloroquine used for the assay validation, nelfinavir (HIV protease inhibitor), rupintrivir (HRV  
253 protease inhibitor), and cobicistat (a pharmacoenhancer and inhibitor of CYP450) were  
254 identified as the most potent and selective inhibitors among the tested compounds with  $EC_{50}$   
255 values ranging from 0.77 to 2.74  $\mu$ M and SI >15-fold. In studies with HIV *in vitro*, a 40-fold shift  
256 in the antiviral  $EC_{50}$  was reported when assays were conducted in the presence of 50% human  
257 serum<sup>17</sup>, an effect also likely relevant for COVID-19. Based on their antiviral potencies  
258 established *in vitro*, it is unlikely that nelfinavir or cobicistat would exert major clinical effects in  
259 COVID-19 patients at the current clinically approved doses, since their systemic free drug levels  
260 based on total plasma concentration and established plasma protein binding are below their  
261 measured *in vitro*  $EC_{50}$  for SARS-CoV-2-Nluc<sup>39,45</sup>. Rupintrivir is a selective covalent inhibitor of  
262 HRV 3CLpro cysteine protease<sup>18</sup>, and thus may inhibit SARS-CoV-2 through blocking the main  
263 3CLpro cysteine protease activity. Rupintrivir has potent activity *in vitro* against HRV that is  
264 approximately 100-fold better compared to SARS-CoV-2<sup>32</sup>. It has been tested clinically as an  
265 intranasal spray for the treatment of HRV-associated common cold<sup>33</sup>, but there is no clinical  
266 experience with either systemic or inhaled administration of rupintrivir. Hence, further studies  
267 would be required to better understand rupintrivir's mode of action, efficacy in animal models,  
268 and potential clinical benefits in COVID-19 patients depending on the route of administration.

269 Several antiviral drugs approved for the treatment of HIV or HCV have been suggested  
270 to be potentially useful for the treatment of COVID-19<sup>34,35</sup>. These include in particular, sofosbuvir  
271 either alone<sup>35,36</sup> or in combination with velpatasvir<sup>37</sup>, in addition to HIV NNRTIs tenofovir<sup>38</sup> and  
272 emtricitabine<sup>34,35</sup>. Their activities against SARS-CoV-2 were postulated primarily based on  
273 computational modeling of their interactions with the viral RdRp. Our results clearly demonstrate  
274 the lack of antiviral activity of this group of drugs against SARS-CoV-2; therefore, these drugs  
275 do not justify clinical studies in COVID-19 patients.

276           In summary, we have developed a stable recombinant SARS-CoV-2-Nluc for use in  
277 rapid neutralization testing and high-throughput antiviral drug discovery. Using the optimized  
278 and validated high-throughput infection assay, we screened a collection of approved and  
279 investigational antivirals and other anti-infective drugs. Among the tested agents, rupintrivir was  
280 identified as a selective *in vitro* inhibitor of SARS-CoV-2 that might be considered for further  
281 studies to fully establish its potential for the treatment of COVID-19.

282 **References**

- 283
- 284 1 Zhou, P. *et al.* A pneumonia outbreak associated with a new coronavirus of probable bat  
285 origin. *Nature* **579**, 270-273, doi:10.1038/s41586-020-2012-7 (2020).
- 286 2 Zhu, N. *et al.* A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N Engl*  
287 *J Med* **382**, 727-733, doi:10.1056/NEJMoa2001017 (2020).
- 288 3 de Wit, E., van Doremalen, N., Falzarano, D. & Munster, V. J. SARS and MERS: recent  
289 insights into emerging coronaviruses. *Nat Rev Microbiol* **14**, 523-534,  
290 doi:10.1038/nrmicro.2016.81 (2016).
- 291 4 Xie, X. *et al.* An Infectious cDNA Clone of SARS-CoV-2. *Cell Host Microbe* **27**, 841-848  
292 e843, doi:10.1016/j.chom.2020.04.004 (2020).
- 293 5 Puig-Basagoiti, F. *et al.* High-throughput assays using luciferase-expressing replicon,  
294 virus-like particle, and full-length virus for West Nile virus drug discovery. *Antimicrob.*  
295 *Agent. Chemother.* **49**, 4980-4988 (2005).
- 296 6 Zou, G., Xu, H. Y., Qing, M., Wang, Q. Y. & Shi, P. Y. Development and characterization  
297 of a stable luciferase dengue virus for high-throughput screening. *Antiviral Res* **91**, 11-  
298 19, doi:10.1016/j.antiviral.2011.05.001 (2011).
- 299 7 Shan, C. *et al.* An Infectious cDNA Clone of Zika Virus to Study Viral Virulence,  
300 Mosquito Transmission, and Antiviral Inhibitors. *Cell Host Microbe* **19**, 891-900,  
301 doi:10.1016/j.chom.2016.05.004 (2016).
- 302 8 Scobey, T. *et al.* Reverse genetics with a full-length infectious cDNA of the Middle East  
303 respiratory syndrome coronavirus. *Proc Natl Acad Sci U S A* **110**, 16157-16162,  
304 doi:10.1073/pnas.1311542110 (2013).
- 305 9 Almazan, F. *et al.* Coronavirus reverse genetic systems: infectious clones and replicons.  
306 *Virus Res* **189**, 262-270, doi:10.1016/j.virusres.2014.05.026 (2014).
- 307 10 Hou, Y. J. *et al.* SARS-CoV-2 Reverse Genetics Reveals a Variable Infection Gradient in  
308 the Respiratory Tract. *Cell* **182**, 1–18 (2020).
- 309 11 Roberts, R. S., Yount, B. L., Sims, A. C., Baker, S. & Baric, R. S. Renilla luciferase as a  
310 reporter to assess SARS-CoV mRNA transcription regulation and efficacy of anti-SARS-  
311 CoV agents. *Adv Exp Med Biol* **581**, 597-600, doi:10.1007/978-0-387-33012-9\_108  
312 (2006).
- 313 12 Wang, M. *et al.* Remdesivir and chloroquine effectively inhibit the recently emerged  
314 novel coronavirus (2019-nCoV) in vitro. *Cell Res* **30**, 269-271, doi:10.1038/s41422-020-  
315 0282-0 (2020).
- 316 13 Pruijssers, A. J. *et al.* Remdesivir potently inhibits SARS-CoV-2 in human lung cells and  
317 chimeric SARS-CoV expressing the SARS-CoV-2 RNA polymerase in mice. *bioRxiv* doi:  
318 <https://doi.org/10.1101/2020.04.27.064279> (2020).
- 319 14 Choy, K. T. *et al.* Remdesivir, lopinavir, emetine, and homoharringtonine inhibit SARS-  
320 CoV-2 replication in vitro. *Antiviral Res* **178**, 104786, doi:10.1016/j.antiviral.2020.104786  
321 (2020).
- 322 15 Jeon, S. *et al.* Identification of antiviral drug candidates against SARS-CoV-2 from FDA-  
323 approved drugs. *Antimicrob Agents Chemother*, doi:10.1128/AAC.00819-20 (2020).
- 324 16 Liu, J. *et al.* A 2'-deoxy-2'-fluoro-2'-C-methyl uridine cyclopentyl carbocyclic analog and  
325 its phosphoramidate prodrug as inhibitors of HCV NS5B polymerase. *Nucleosides*  
326 *Nucleotides Nucleic Acids* **31**, 277-285, doi:10.1080/15257770.2012.658131 (2012).
- 327 17 Molla, A. *et al.* Human serum attenuates the activity of protease inhibitors toward wild-  
328 type and mutant human immunodeficiency virus. *Virology* **250**, 255-262,  
329 doi:10.1006/viro.1998.9383 (1998).
- 330 18 Kawatkar, S. P. *et al.* Design and structure-activity relationships of novel inhibitors of  
331 human rhinovirus 3C protease. *Bioorg Med Chem Lett* **26**, 3248-3252,  
332 doi:10.1016/j.bmcl.2016.05.066 (2016).

- 333 19 Perron, M. *et al.* GS-5806 Inhibits a Broad Range of Respiratory Syncytial Virus Clinical  
334 Isolates by Blocking the Virus-Cell Fusion Process. *Antimicrob Agents Chemother* **60**,  
335 1264-1273, doi:10.1128/AAC.01497-15 (2015).
- 336 20 Marty, F. M. *et al.* A Phase 2b, Randomized, Double-blind, Placebo-Controlled  
337 Multicenter Study Evaluating Antiviral Effects, Pharmacokinetics, Safety, and Tolerability  
338 of Presatovir in Hematopoietic Cell Transplant Recipients with Respiratory Syncytial  
339 Virus (RSV) Infection of the Lower Respiratory Tract. *Clin Infect Dis*,  
340 doi:10.1093/cid/ciz1167 (2019).
- 341 21 Nie, J. *et al.* Establishment and validation of a pseudovirus neutralization assay for  
342 SARS-CoV-2. *Emerg Microbes Infect* **9**, 680-686, doi:10.1080/22221751.2020.1743767  
343 (2020).
- 344 22 Gordon, C. J. *et al.* Remdesivir is a direct-acting antiviral that inhibits RNA-dependent  
345 RNA polymerase from severe acute respiratory syndrome coronavirus 2 with high  
346 potency. *J Biol Chem* **295**, 6785-6797, doi:10.1074/jbc.RA120.013679 (2020).
- 347 23 Beigel, J. H. *et al.* Remdesivir for the Treatment of Covid-19 - Preliminary Report. *N Engl*  
348 *J Med*, doi:10.1056/NEJMoa2007764 (2020).
- 349 24 de Wit, E. *et al.* Prophylactic and therapeutic remdesivir (GS-5734) treatment in the  
350 rhesus macaque model of MERS-CoV infection. *Proc Natl Acad Sci U S A* **117**, 6771-  
351 6776, doi:10.1073/pnas.1922083117 (2020).
- 352 25 Williamson, B. N. *et al.* Clinical benefit of remdesivir in rhesus macaques infected with  
353 SARS-CoV-2. *bioRxiv* doi: <https://doi.org/10.1101/2020.04.15.043166> (2020).
- 354 26 Sheahan, T. P. *et al.* Comparative therapeutic efficacy of remdesivir and combination  
355 lopinavir, ritonavir, and interferon beta against MERS-CoV. *Nat Commun* **11**, 222,  
356 doi:10.1038/s41467-019-13940-6 (2020).
- 357 27 Sheahan, T. P. *et al.* Broad-spectrum antiviral GS-5734 inhibits both epidemic and  
358 zoonotic coronaviruses. *Sci Transl Med* **9**, doi:10.1126/scitranslmed.aal3653 (2017).
- 359 28 Gao, J., Tian, Z. & Yang, X. Breakthrough: Chloroquine phosphate has shown apparent  
360 efficacy in treatment of COVID-19 associated pneumonia in clinical studies. *Biosci*  
361 *Trends* **14**, 72-73, doi:10.5582/bst.2020.01047 (2020).
- 362 29 Gautret, P. *et al.* Clinical and microbiological effect of a combination of  
363 hydroxychloroquine and azithromycin in 80 COVID-19 patients with at least a six-day  
364 follow up: A pilot observational study. *Travel Med Infect Dis* **34**, 101663,  
365 doi:10.1016/j.tmaid.2020.101663 (2020).
- 366 30 Molina, J. M. *et al.* No evidence of rapid antiviral clearance or clinical benefit with the  
367 combination of hydroxychloroquine and azithromycin in patients with severe COVID-19  
368 infection. *Med Mal Infect* <https://doi.org/10.1016/j.medmal.2020.03.006> (2020).
- 369 31 Rosenberg, E. S. *et al.* Association of Treatment With Hydroxychloroquine or  
370 Azithromycin With In-Hospital Mortality in Patients With COVID-19 in New York State.  
371 *JAMA*, doi:10.1001/jama.2020.8630 (2020).
- 372 32 Patick, A. K. *et al.* In vitro antiviral activity of AG7088, a potent inhibitor of human  
373 rhinovirus 3C protease. *Antimicrob Agents Chemother* **43**, 2444-2450 (1999).
- 374 33 Hayden, F. G. *et al.* Phase II, randomized, double-blind, placebo-controlled studies of  
375 rupintrivir nasal spray 2-percent suspension for prevention and treatment of  
376 experimentally induced rhinovirus colds in healthy volunteers. *Antimicrob Agents*  
377 *Chemother* **47**, 3907-3916, doi:10.1128/aac.47.12.3907-3916.2003 (2003).
- 378 34 Copertino Jr., D. C. *et al.* Antiretroviral Drug Activity and Potential for Pre-Exposure  
379 Prophylaxis Against COVID-19 and HIV Infection. *ChemRxiv*  
380 <https://doi.org/10.26434/chemrxiv.12250199.v1> (2020).
- 381 35 Elfiky, A. A. Ribavirin, Remdesivir, Sofosbuvir, Galidesivir, and Tenofovir against SARS-  
382 CoV-2 RNA dependent RNA polymerase (RdRp): A molecular docking study. *Life Sci*  
383 **253**, 117592, doi:10.1016/j.lfs.2020.117592 (2020).

- 384 36 Jácome, R., Campillo-Balderas, J. A., León, S. P. d., Becerra, A. & Lazcano, A.  
385 Sofosbuvir as a Potential Alternative to Treat the SARS-CoV-2 Epidemic. *Sci Rep* **10**  
386 (2020).
- 387 37 Izzi, A., Messina, V., Rinaldi, L. & Maggi, P. Editorial - Sofosbuvir/Velpatasvir as a  
388 Combination With Strong Potential Activity Against SARS-CoV2 (COVID-19) Infection:  
389 How to Use Direct-Acting Antivirals as Broad-Spectrum Antiviral Agents. *Eur Rev Med*  
390 *Pharmacol Sci* **24**, 5193-5194 (2020).
- 391 38 Chien, M. *et al.* Nucleotide Analogues as Inhibitors of SARS-CoV-2 Polymerase. *bioRxiv*  
392 doi: 10.1101/2020.03.18.997585 (2020).
- 393 39 Siegel, D. *et al.* Discovery and Synthesis of a Phosphoramidate Prodrug of a  
394 Pyrrolo[2,1-f][triazin-4-amino] Adenine C-Nucleoside (GS-5734) for the Treatment of  
395 Ebola and Emerging Viruses. *J Med Chem* **60**, 1648-1661,  
396 doi:10.1021/acs.jmedchem.6b01594 (2017).
- 397 40 Cho, A. *et al.* Discovery of the first C-nucleoside HCV polymerase inhibitor (GS-6620)  
398 with demonstrated antiviral response in HCV infected patients. *J Med Chem* **57**, 1812-  
399 1825, doi:10.1021/jm400201a (2014).
- 400 41 Carroll, S. S. *et al.* Robust antiviral efficacy upon administration of a nucleoside analog  
401 to hepatitis C virus-infected chimpanzees. *Antimicrob Agents Chemother* **53**, 926-934,  
402 doi:10.1128/AAC.01032-08 (2009).
- 403 42 Sofia, M. J. Nucleotide prodrugs for HCV therapy. *Antivir Chem Chemother* **22**, 23-49,  
404 doi:10.3851/IMP1797 (2011).
- 405 43 Deval, J. *et al.* Molecular Basis for the Selective Inhibition of Respiratory Syncytial Virus  
406 RNA Polymerase by 2'-Fluoro-4'-Chloromethyl-Cytidine Triphosphate. *PLoS Pathog* **11**,  
407 e1004995, doi:10.1371/journal.ppat.1004995 (2015).
- 408 44 Langley, D. R. *et al.* Inhibition of hepatitis B virus polymerase by entecavir. *J Virol* **81**,  
409 3992-4001, doi:10.1128/JVI.02395-06 (2007).
- 410 45 De Clercq, E. Clinical potential of the acyclic nucleoside phosphonates cidofovir,  
411 adefovir, and tenofovir in treatment of DNA virus and retrovirus infections. *Clin Microbiol*  
412 *Rev* **16**, 569-596, doi:10.1128/cmr.16.4.569-596.2003 (2003).
- 413 46 Vanderlinden, E. *et al.* Distinct Effects of T-705 (Favipiravir) and Ribavirin on Influenza  
414 Virus Replication and Viral RNA Synthesis. *Antimicrob Agents Chemother* **60**, 6679-  
415 6691, doi:10.1128/AAC.01156-16 (2016).
- 416 47 Eron, J. J. *et al.* Once-daily versus twice-daily lopinavir/ritonavir in antiretroviral-naïve  
417 HIV-positive patients: a 48-week randomized clinical trial. *J Infect Dis* **189**, 265-272,  
418 doi:10.1086/380799 (2004).
- 419 48 Zhu, L. *et al.* Pharmacokinetics and inhibitory quotient of atazanavir/ritonavir versus  
420 lopinavir/ritonavir in HIV-infected, treatment-naïve patients who participated in the  
421 CASTLE Study. *J Antimicrob Chemother* **67**, 465-468, doi:10.1093/jac/dkr490 (2012).
- 422 49 Sadler, B. M. & Stein, D. S. Clinical pharmacology and pharmacokinetics of amprenavir.  
423 *Ann Pharmacother* **36**, 102-118, doi:10.1345/aph.10423 (2002).
- 424 50 Bardsley-Elliot, A. & Plosker, G. L. Nelfinavir: an update on its use in HIV infection.  
425 *Drugs* **59**, 581-620, doi:10.2165/00003495-200059030-00014 (2000).
- 426 51 Lea, A. P. & Faulds, D. Ritonavir. *Drugs* **52**, 541-546; discussion 547-548,  
427 doi:10.2165/00003495-199652040-00007 (1996).
- 428 52 Zeldin, R. K. & Petruschke, R. A. Pharmacological and therapeutic properties of  
429 ritonavir-boosted protease inhibitor therapy in HIV-infected patients. *J Antimicrob*  
430 *Chemother* **53**, 4-9, doi:10.1093/jac/dkh029 (2004).
- 431 53 Stein, D. S. *et al.* A 24-week open-label phase I/II evaluation of the HIV protease  
432 inhibitor MK-639 (indinavir). *AIDS* **10**, 485-492, doi:10.1097/00002030-199605000-  
433 00006 (1996).



- 434 54 Anderson, P. L. *et al.* Indinavir plasma protein binding in HIV-1-infected adults. *AIDS* **14**,  
435 2293-2297, doi:10.1097/00002030-200010200-00010 (2000).
- 436 55 Singh, K. *et al.* Pharmacokinetics and safety of saquinavir/ritonavir and omeprazole in  
437 HIV-infected subjects. *Clin Pharmacol Ther* **83**, 867-872, doi:10.1038/sj.clpt.6100375  
438 (2008).
- 439 56 Surleraux, D. L. *et al.* Discovery and selection of TMC114, a next generation HIV-1  
440 protease inhibitor. *J Med Chem* **48**, 1813-1822, doi:10.1021/jm049560p (2005).
- 441 57 MacGregor, T. R. *et al.* Pharmacokinetic characterization of different dose combinations  
442 of coadministered tipranavir and ritonavir in healthy volunteers. *HIV Clin Trials* **5**, 371-  
443 382, doi:10.1310/RRX7-49ME-27V7-MWWV (2004).
- 444 58 Saag, M. S. *et al.* Efficacy and safety of emtricitabine vs stavudine in combination  
445 therapy in antiretroviral-naïve patients: a randomized trial. *JAMA* **292**, 180-189,  
446 doi:10.1001/jama.292.2.180 (2004).
- 447 59 Custodio, J. M. *et al.* Pharmacokinetics and Safety of Tenofovir Alafenamide in HIV-  
448 Uninfected Subjects with Severe Renal Impairment. *Antimicrob Agents Chemother* **60**,  
449 5135-5140, doi:10.1128/AAC.00005-16 (2016).
- 450 60 Ruane, P. J. *et al.* Antiviral activity, safety, and pharmacokinetics/pharmacodynamics of  
451 tenofovir alafenamide as 10-day monotherapy in HIV-1-positive adults. *J Acquir Immune*  
452 *Defic Syndr* **63**, 449-455, doi:10.1097/QAI.0b013e3182965d45 (2013).
- 453 61 Ray, A. S. *et al.* Intracellular metabolism of the nucleotide prodrug GS-9131, a potent  
454 anti-human immunodeficiency virus agent. *Antimicrob Agents Chemother* **52**, 648-654,  
455 doi:10.1128/AAC.01209-07 (2008).
- 456 62 Goebel, F. *et al.* Short-term antiviral activity of TMC278--a novel NNRTI--in treatment-  
457 naïve HIV-1-infected subjects. *AIDS* **20**, 1721-1726,  
458 doi:10.1097/01.aids.0000242818.65215.bd (2006).
- 459 63 Adkins, J. C. & Noble, S. Efavirenz. *Drugs* **56**, 1055-1064; discussion 1065-1056,  
460 doi:10.2165/00003495-199856060-00014 (1998).
- 461 64 Gallant, J. E. *et al.* Antiviral Activity, Safety, and Pharmacokinetics of Bictegravir as 10-  
462 Day Monotherapy in HIV-1-Infected Adults. *J Acquir Immune Defic Syndr* **75**, 61-66,  
463 doi:10.1097/QAI.0000000000001306 (2017).
- 464 65 Sheng, X. C. *et al.* Discovery of GS-9256: a novel phosphinic acid derived inhibitor of the  
465 hepatitis C virus NS3/4A protease with potent clinical activity. *Bioorg Med Chem Lett* **22**,  
466 1394-1396, doi:10.1016/j.bmcl.2011.12.038 (2012).
- 467 66 Yang, H. *et al.* Preclinical characterization of the novel hepatitis C virus NS3 protease  
468 inhibitor GS-9451. *Antimicrob Agents Chemother* **58**, 647-653, doi:10.1128/AAC.00487-  
469 13 (2014).
- 470 67 Taylor, J. G. *et al.* Discovery of the pan-genotypic hepatitis C virus NS3/4A protease  
471 inhibitor voxilaprevir (GS-9857): A component of Vosevi((R)). *Bioorg Med Chem Lett* **29**,  
472 2428-2436, doi:10.1016/j.bmcl.2019.03.037 (2019).
- 473 68 Shih, I. H. *et al.* Mechanistic characterization of GS-9190 (Tegobuvir), a novel  
474 nonnucleoside inhibitor of hepatitis C virus NS5B polymerase. *Antimicrob Agents*  
475 *Chemother* **55**, 4196-4203, doi:10.1128/AAC.00307-11 (2011).
- 476 69 Lazerwith, S. E. *et al.* Discovery of GS-9669, a thumb site II non-nucleoside inhibitor of  
477 NS5B for the treatment of genotype 1 chronic hepatitis C infection. *J Med Chem* **57**,  
478 1893-1901, doi:10.1021/jm401420j (2014).
- 479 70 Link, J. O. *et al.* Discovery of ledipasvir (GS-5885): a potent, once-daily oral NS5A  
480 inhibitor for the treatment of hepatitis C virus infection. *J Med Chem* **57**, 2033-2046,  
481 doi:10.1021/jm401499g (2014).
- 482 71 Link, J. O. *et al.* Discovery of velpatasvir (GS-5816): A potent pan-genotypic HCV NS5A  
483 inhibitor in the single-tablet regimens Vosevi((R)) and Epclusa((R)). *Bioorg Med Chem*  
484 *Lett* **29**, 2415-2427, doi:10.1016/j.bmcl.2019.04.027 (2019).

- 485 72 Xu, J., Shi, P. Y., Li, H. & Zhou, J. Broad Spectrum Antiviral Agent Niclosamide and Its  
486 Therapeutic Potential. *ACS Infect Dis* **6**, 909-915, doi:10.1021/acsinfecdis.0c00052  
487 (2020).
- 488 73 Takano, T., Katoh, Y., Doki, T. & Hohdatsu, T. Effect of chloroquine on feline infectious  
489 peritonitis virus infection in vitro and in vivo. *Antiviral Res* **99**, 100-107,  
490 doi:10.1016/j.antiviral.2013.04.016 (2013).
- 491 74 Xu, L. *et al.* Cobicistat (GS-9350): A Potent and Selective Inhibitor of Human CYP3A as  
492 a Novel Pharmacoenhancer. *ACS Med Chem Lett* **1**, 209-213, doi:10.1021/ml1000257  
493 (2010).
- 494 75 Takahashi, K. *et al.* In vitro and in vivo activities of T-705 and oseltamivir against  
495 influenza virus. *Antivir Chem Chemother* **14**, 235-241,  
496 doi:10.1177/09563202020301400502 (2003).
- 497 76 Noshi, T. *et al.* In vitro characterization of baloxavir acid, a first-in-class cap-dependent  
498 endonuclease inhibitor of the influenza virus polymerase PA subunit. *Antiviral Res* **160**,  
499 109-117, doi:10.1016/j.antiviral.2018.10.008 (2018).
- 500 77 Ratziu, V. *et al.* A phase 2, randomized, double-blind, placebo-controlled study of GS-  
501 9450 in subjects with nonalcoholic steatohepatitis. *Hepatology* **55**, 419-428,  
502 doi:10.1002/hep.24747 (2012).
- 503 78 Bond, D. A. & Woyach, J. A. Targeting BTK in CLL: Beyond Ibrutinib. *Curr Hematol*  
504 *Malign Rep* **14**, 197-205, doi:10.1007/s11899-019-00512-0 (2019).
- 505 79 Mossel, E. C. *et al.* Exogenous ACE2 expression allows refractory cell lines to support  
506 severe acute respiratory syndrome coronavirus replication. *J Virol* **79**, 3846-3850,  
507 doi:10.1128/JVI.79.6.3846-3850.2005 (2005).
- 508 80 Xie, X. *et al.* An infectious cDNA clone of SARS-CoV-2. *Cell Host Microbe*,  
509 doi:10.1016/j.chom.2020.04.004 (2020).
- 510

511 **Table 1. Nucleoside and nucleotide analogs against SARS-CoV-2-Nluc**  
 512

Compound name	EC <sub>50</sub> (μM) <sup>a</sup>	CC <sub>50</sub> (μM) <sup>a</sup>	SI <sup>b</sup>	Nucleoside/tide analog	Reference
Remdesivir (GS-5734)	0.115 ± 0.007	32.7 ± 5.2	284	1'-CN-C-adenosine	39
GS-6620	>10	>50	-	1'CN, 2'Me-C-adenosine	40
MK-0608	>10	>50	-	2'Me-7-deaza-adenosine	41
PSI-352938	>10	>50	-	2'Me-2'F-guanosine	42
Sofosbuvir	>10	>50	-	2'Me, 2'F-uridine	16
ALS-8112	>10	>50	-	2'F, 4'Cl-Me-cytidine	43
Entecavir	>10	>50	-	Carbocyclic deoxyguanosine	44
Cidofovir	>10	>50	-	Acyclic cytidine phosphonate	45
Favipiravir (T-705)	>10	>50	-	Modified nucleobase	46
Ribavirin	>10	>50	-	Ribofuranosyl	-

513  
 514 <sup>a</sup>Values are mean ± standard deviation of two independent replicate experiments in A549-  
 515 hACE2 cells

516 <sup>b</sup>Selectivity index (SI) = CC<sub>50</sub> / EC<sub>50</sub>  
 517

518 **Table 2. HIV drugs against SARS-CoV-2-Nluc**

519

Inhibitor class	Compound name	EC <sub>50</sub> (μM) <sup>a</sup>	CC <sub>50</sub> (μM) <sup>a</sup>	SI <sup>b</sup>	Exposure (μM) <sup>c</sup>	Plasma protein binding (%) <sup>d</sup>	Reference
HIV protease (aspartyl)	Lopinavir	9.00 ± 0.42	31.5 ± 2.5	3.5	15.6 / 8.8	98-99	47, 48
	Amprenavir	>10	>50	-	-	90	49
	Nelfinavir	0.77 ± 0.32	12.0 ± 1.3	15.7	8.3 / 2.6	>98	50, e
	Ritonavir	>10	36.9 ± 1.7	-	-	98-99	51, 52
	Indinavir	>10	>50	-	-	61	53, 54
	Saquinavir	8.95 ± 0.31	35.1 ± 11.7	3.9	3.7 / 0.65	98	55, e
	Darunavir	>10	>50	-	-	95	56, e
	Atazanavir	>10	>50	-	-	86	48
	Tipranavir	8.65 ± 0.16	28.4 ± 0.5	3.3	130 / 30.8	99.9	57
HIV NRTI	Emtricitabine (FTC)	>10	>50	-	C <sub>max</sub> 7.9	4	58, e
	Tenofovir alafenamide (TAF)	>10	>50	-	C <sub>max</sub> 0.4	80	59, 60
	Rovafovir (GS-9131)	>10	>50	-	-	-	61
HIV NNRTI	Rilpivirine	7.80 ± 1.04	14.6 ± 1.6	1.9	0.83 / 0.30	99.7	62, e
	Efavirenz	>9.6	37.6 ± 10.7	<3.9	12.9 / 5.6	99.5-99.8	63
HIV integrase	Bictegravir <sup>†</sup>	>10	>50	-	-	>99	64

520

521 <sup>a</sup>Values are mean ± standard deviation of two independent replicates in A549-hACE2 cells

522 <sup>b</sup>SI = CC<sub>50</sub> / EC<sub>50</sub>

523 <sup>c</sup>Values represent C<sub>max</sub> / C<sub>min</sub> for human exposures in the clinic based on approved dosing

524 schedules

525 <sup>d</sup>Data from literature as cited

526 <sup>e</sup>Information from product description

527 **Table 3. HCV drugs against SARS-CoV-2-Nluc**

528

Inhibitor class	Compound name	EC <sub>50</sub> (μM) <sup>a</sup>	CC <sub>50</sub> (μM) <sup>a</sup>	Reference
HCV protease (serine)	GS-9256	>10	31.8 ± 10.9	<sup>65</sup>
	GS-9451	>10	>50	<sup>66</sup>
	Voxilaprevir	>10	16.0 ± 1.2	<sup>67</sup>
HCV nucleoside RdRp	Sofosbuvir	>10	>50	<sup>16</sup>
HCV non-nucleoside RdRp	GS-9130	>10	>50	-
	Tegobuvir	>10	17.9 ± 3.1	<sup>68</sup>
	Radalbuvir	>10	>50	<sup>69</sup>
HCV NS5A	Ledapisvir	>10	>50	<sup>70</sup>
	Velpatasvir	>10	>50	<sup>71</sup>

529 <sup>a</sup>Values are mean ± standard deviation of two independent replicates in A549-hACE2 cells

530

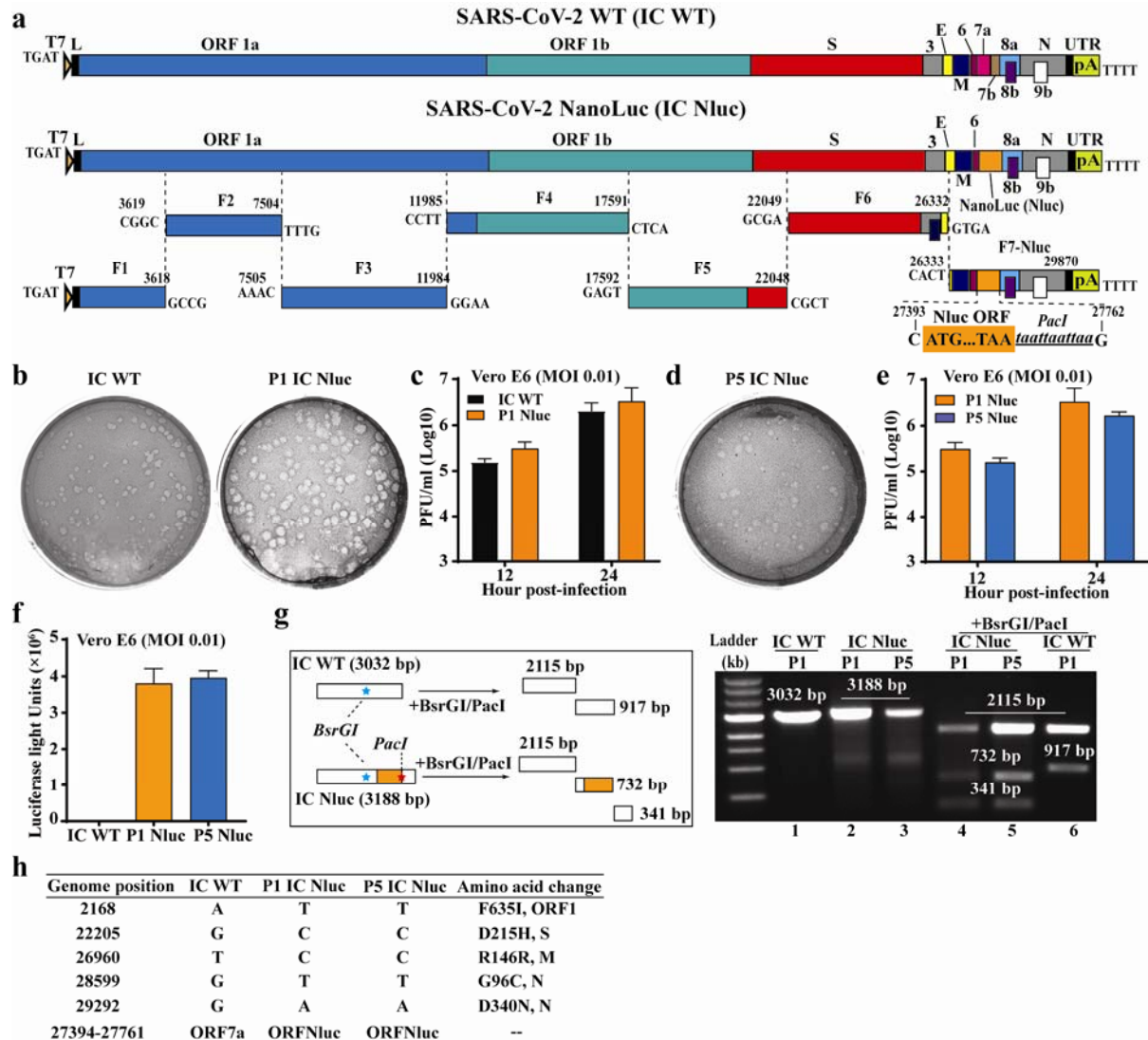
531 **Table 4. Other drug classes against SARS-CoV-2-Nluc**

532

Inhibitor class	Compound name	EC <sub>50</sub> (μM) <sup>a</sup>	CC <sub>50</sub> (μM) <sup>a</sup>	SI <sup>b</sup>	Reference
HRV protease (serine)	Rupintrivir	1.87 ± 0.47	>50	>26.7	<sup>78</sup>
Anthelmintic	Niclosamide	0.715 ± 0.332	1.28 ± 0.23	1.8	<sup>72</sup>
Antimalarial / amebicide	Chloroquine	1.32 ± 0.36	>50	>37.9	<sup>73</sup>
RSV fusion	Presatovir	2.53 ± 0.69	34.0 ± 6.5	13.5	<sup>19</sup>
CYP3A inhibitor	Cobicistat	2.74 ± 0.20	47.3 ± 2.5	17.3	<sup>74</sup>
Influenza neuraminidase	Oseltamivir carboxylate	>10	>50	-	<sup>75</sup>
Influenza endonuclease	Baloxavir	>10	47.0 ± 1.3	-	<sup>76</sup>
Caspases 1, 8, & 9	Nivocasan (GS-9450)	>10	>50	-	<sup>77</sup>
BTK	Tirabrutinib	>10	>50	-	<sup>78</sup>
	Ibrutinib <sup>l</sup>	>10	>50	-	<sup>78</sup>

533 <sup>a</sup>Values are mean ± standard deviation of two independent replicates in A549-hACE2 cells

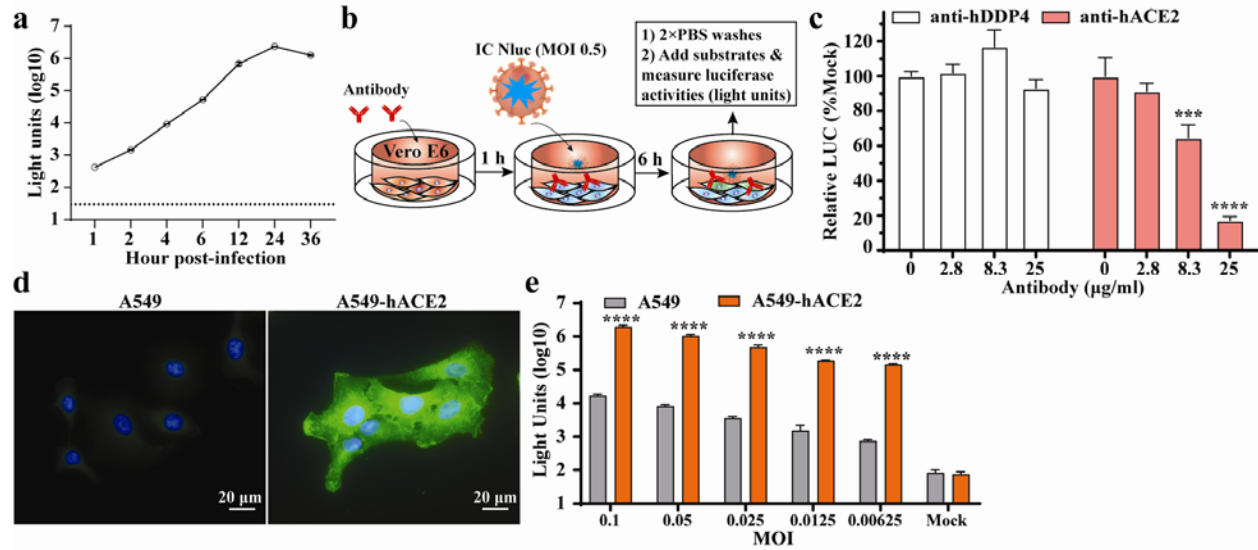
534 <sup>b</sup>SI = CC<sub>50</sub> / EC<sub>50</sub>



535  
536 **Figure 1. Development and characterization of SARS-CoV-2-Nluc.** (a) Assembly of the full-  
537 length SARS-CoV-2-Nluc cDNA. The Nanoluciferase (Nluc) gene together with a *PacI* site was  
538 placed downstream of the regulatory sequence of ORF7 to replace the ORF7 sequence. The  
539 nucleotide identities of the Nluc substitution sites are indicated. (b) Plaque morphologies of  
540 infectious clone derived P1 SARS-CoV-2-Nluc (P1 IC Nluc) and wild-type SARS-CoV-2 (IC  
541 WT). (c) Replication kinetics. Vero E6 cells were infected with infectious clone derived IC WT or  
542 P1 IC Nluc at MOI 0.01. Viruses in culture supernatants were quantified by plaque assay. (d)  
543 Plaque morphology of P5 IC Nluc. (e) Replication kinetics of P5 IC Nluc on Vero E6 cells. (f)  
544 Luciferase signals produced from SARS-CoV-2-Nluc-infected Vero E6 cells at 12 h post-

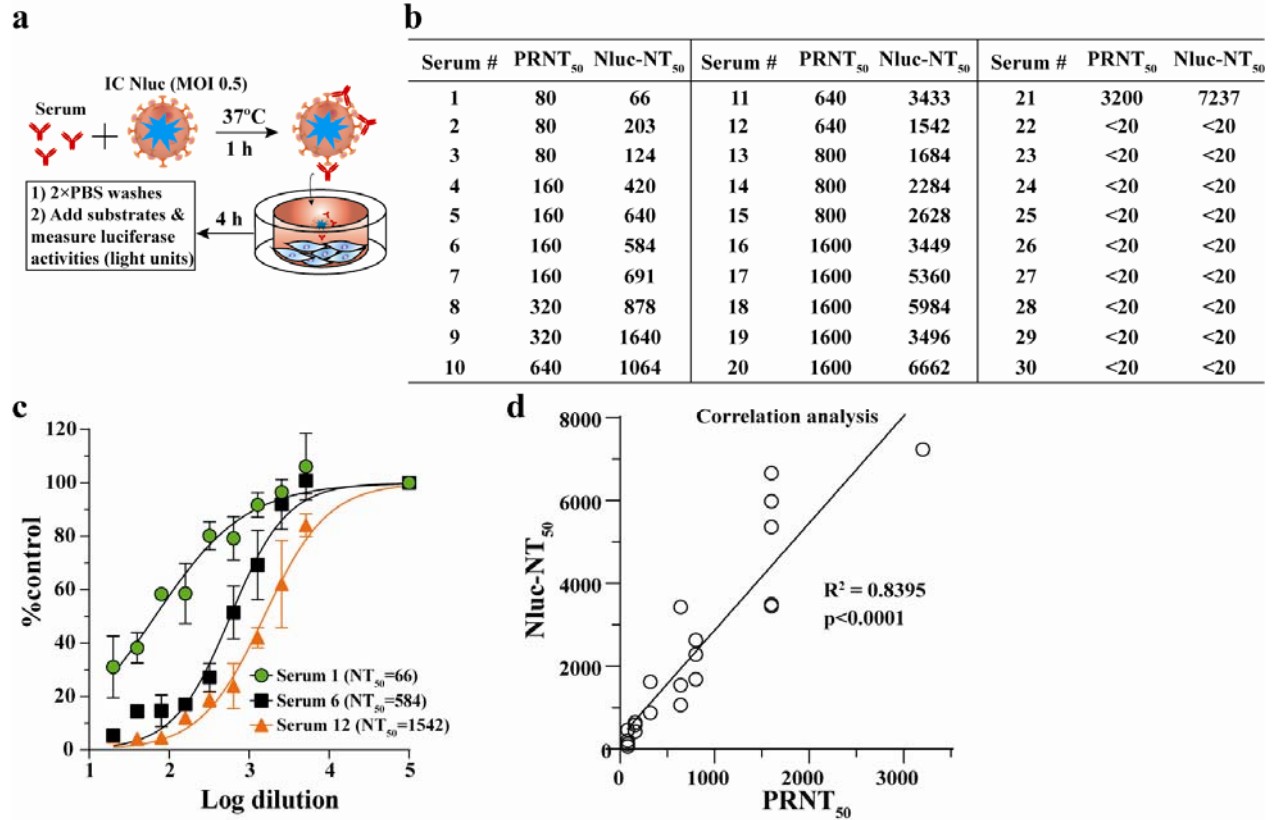
545 infection. Cells were infected with viruses at MOI 0.1. (g) Gel analysis of IC Nluc virus stability.  
546 The left panel depicts the theoretical results of RT-PCR followed by restriction enzyme  
547 digestion. The right panel shows the gel analysis of the RT-PCR products before (lanes 1–3)  
548 and after BsrGI/PacI digestion (lanes 4–6). (h) Summary of full-genome sequences of P1 and  
549 P5 IC Nluc viruses. Nucleotide and amino acid differences from the IC WT are indicated.





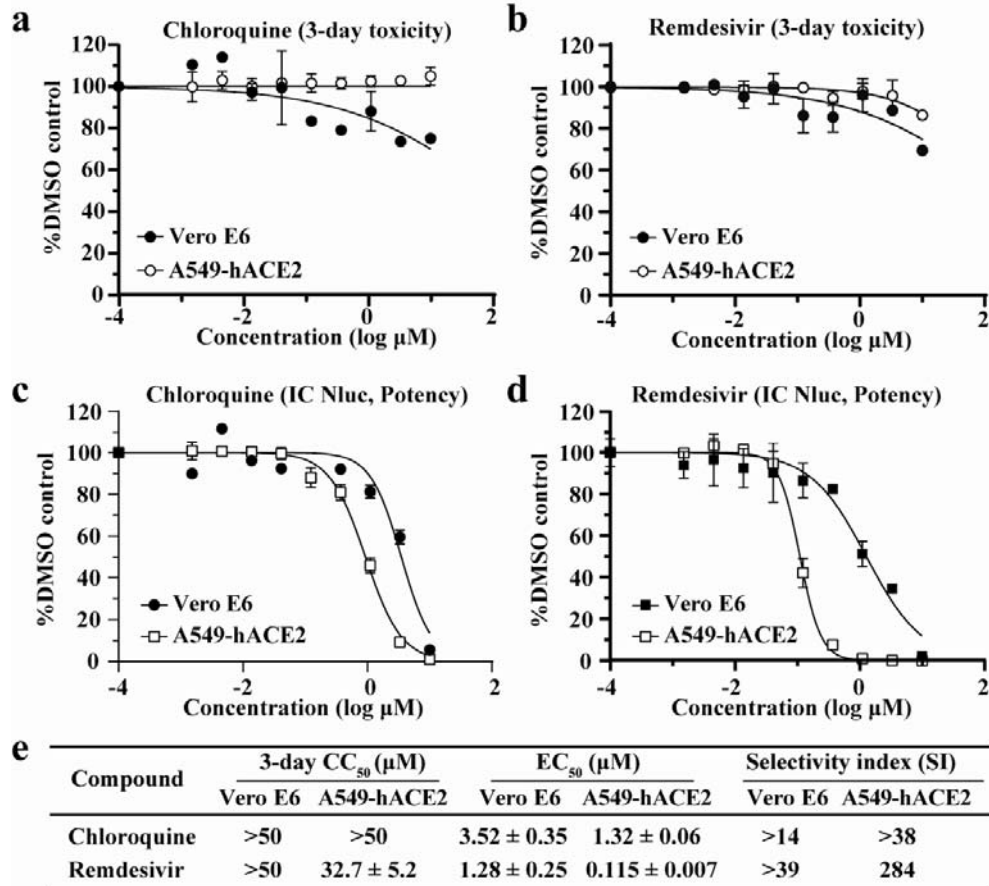
550

551 **Figure 2. Application of SARS-CoV-2-Nluc in analyzing hACE2 as an entry receptor.** (a)  
552 Replication kinetics of SARS-CoV-2-Nluc (IC Nluc) on Vero E6 cells. Cells were infected with IC  
553 Nluc at MOI 1.0. At given time points, cells were harvested for luciferase signal measurement.  
554 The means and standard deviations from three independent experiments are presented. (b)  
555 Diagram to analyze hACE2 for IC Nluc entry. (c) Relative luciferase signals following infection of  
556 cells that were preincubated with anti-hDPP4 or anti-hACE2 antibodies. The luciferase signals  
557 from antibody-treated groups were normalized to those from untreated groups. The average and  
558 standard deviation of three independent experiments are presented. (d) Immunofluorescence  
559 analysis of hACE2 expression in A549-hACE2 cells. At 24 h post-seeding, the cells were fixed  
560 and stained with anti-hACE2 polyclonal antibody. (e) Luciferase signals from IC Nluc infected-  
561 A549 and A549-hACE2 cells. Cells were infected with indicated MOIs and luciferase signals  
562 were measured at 24 h post-infection.



563

564 **Figure 3. A rapid SARS-CoV-2-Nluc-based neutralization assay.** (a) Schematic of the rapid  
 565 neutralization assay. (b) Summary of neutralizing titers as measured by PRNT and SARS-CoV-  
 566 2-Nluc neutralization (Nluc-NT) assay. Serum specimens 1-21 were from COVID-19 patients  
 567 with confirmed prior RT-PCR diagnosis. Serum specimens 22-30 were from non-COVID-19  
 568 individuals. (c) Representative neutralizing curves of the Nluc-NT assay. The means and  
 569 standard deviations from two independent experiments are shown. (d) Correlation analysis  
 570 between the Nluc-NT<sub>50</sub> and PRNT<sub>50</sub> values. The correlation efficiency  $R^2$  and  $p$  value calculated  
 571 from a linear regression analysis are shown.



572

573 **Figure 4. SARS-CoV-2-Nluc-based antiviral screening.** A three-day cytotoxicity assay was  
 574 performed for chloroquine (a) and remdesivir (b) on Vero E6 and A549-hACE2 cells. A two-day  
 575 SARS-CoV-2-Nluc infection assay (MOI 0.025) was performed to estimate the  $EC_{50}$  values of  
 576 chloroquine (c) and remdesivir (d) on Vero E6 and A549-hACE2 cells. (e) Summary of  $CC_{50}$ ,  
 577  $EC_{50}$ , and selectivity index (SI).

578 **Methods**

579 **Cell lines**

580 African green monkey kidney epithelial cells Vero E6 (ATCC®CRL-1586) were purchased from  
581 the American Type Culture Collection (ATCC, Bethesda, MD) and maintained in a high-glucose  
582 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS;  
583 HyClone Laboratories, South Logan, UT) and 1% penicillin/streptomycin (P/S). Human alveolar  
584 epithelial cell line (A549) and human embryonic kidney cells (HEK293) were maintained in a  
585 high-glucose DMEM supplemented with 10% fetal bovine serum, 1% P/S and 1% HEPES  
586 (ThermoFisher Scientific). The A549-hACE2 and HEK293-hACE2 cells that stably express  
587 human angiotensin-converting enzyme 2 (hACE2)<sup>79</sup> were grown in the culture medium  
588 supplemented with 10 µg/mL Blastidin S. Cells were grown at 37°C with 5% CO<sub>2</sub>. All culture  
589 medium and antibiotics were purchased from ThermoFisher Scientific (Waltham, MA). All cell  
590 lines were tested negative for mycoplasma.

591 **Generation of SARS-CoV-2-Nluc**

592 A subclone (F7-Nluc) was constructed by substituting the ORF7 of the viral genome with the  
593 reporter Nano<sup>R</sup>luciferase gene followed by a PacI restriction site (taattaattaa). All subclones  
594 were validated by Sanger sequencing prior to assembling the full-length clone. The full-length  
595 infectious cDNA clone of SARS-CoV-2-Nluc was generated by *in vitro* ligation of seven  
596 contiguous panel of cDNA according to a protocol as reported previously<sup>80</sup>. RNA transcript was  
597 *in vitro* synthesized by the mMESSAGING mMACHINE™ T7 Transcription Kit (ThermoFisher  
598 Scientific) and electroporated into Vero E6 cells to recover the recombinant SARS-CoV-2-Nluc  
599 by using the same protocol as described previously<sup>80</sup>. The viral stock was prepared by amplifying  
600 the SARS-CoV-2-Nluc on Vero E6 cells for one round (P1). The titer of the virus stock was  
601 determined by a standard plaque assay. All SARS-CoV-2-Nluc propagation and other virus-  
602 related work were performed at the BSL-3 facility at UTMB.

603 **RNA extraction, RT-PCR and Sanger sequencing**

604 250  $\mu$ L of culture fluids were mixed with three volume of TRIzol™ LS Reagent (Thermo Fisher  
605 Scientific). Viral RNAs were extracted per manufacturer's instructions. The extracted RNAs  
606 were dissolved in 30  $\mu$ L nuclease-free water. 11  $\mu$ L RNA samples were used for reverse  
607 transcription by using the SuperScript™ IV First-Strand Synthesis System (ThermoFisher  
608 Scientific) with random hexamer primers. Nine DNA fragments flanking the entire viral genome  
609 were amplified by PCR with specific primers. The resulting DNAs were cleaned up by the  
610 QIAquick PCR Purification Kit, and the genome sequences were determined by Sanger  
611 sequencing at GENEWIZ (South Plainfield, NJ).

612 **hACE2 antibody blocking assay**

613 15,000 Vero E6 cells per well were seeded in a white opaque 96-well plate (Corning). On the  
614 next day, cells were wash three times with PBS to remove any residual FBS and followed by 1-  
615 hour treatment with goat anti-human ACE2 antibody (R&D Systems) or anti-hDDP4 antibody  
616 (R&D Systems) (both antibodies were prepared in OptiMEM medium to the given  
617 concentrations). Afterwards, cells were infected with SARS-CoV-2-Nluc (MOI 0.5). At 6h post-  
618 infection, cells were washes twice and followed by the addition of 50  $\mu$ L Nano luciferase  
619 substrate (Promega). After 5 minutes of incubation at room temperature, luciferase signals were  
620 measured using a Synergy™ Neo2 microplate reader (BioTek) per the manufacturer's  
621 instructions.

622 **Immunofluorescence Assay**

623 Cells were seeded on a 4-well chamber slide. At 24 h post-seeding, cells were fixed and  
624 permeabilized with 0.1% Triton X-100. After 1 h-blocking with PBS+1% FBS, cellular hACE2  
625 was probed firstly by goat anti-human ACE2 antibody (R&D Systems). After three times of PBS  
626 washes, the cells were incubated with donkey anti-goat IgG conjugated with Alexa Fluor® 488

627 (ThermoFisher Scientific). Finally, the fluorescence images were acquired using the Nikon Ti2-E  
628 inverted microscope armed with a 60× objective.

### 629 **SARS-CoV-2-Nluc neutralization assay**

630 Vero E6 cells (15,000 per well in medium containing 2% FBS) were plated into a white opaque  
631 96-well plate (Corning). At 16 h post-seeding, 30  $\mu$ L of 2-fold serial diluted human sera were  
632 mixed with 30  $\mu$ L of SARS-CoV-2-Nluc (MOI 0.5) and incubated at 37°C for 1 hour. Afterwards,  
633 50  $\mu$ L of virus-sera complexes were transferred to each well of the 96-well plate. After 4 h of  
634 incubation at 37°C 5% CO<sub>2</sub>, cells were washed twice followed by the addition of Nano luciferase  
635 substrate (Promega). Luciferase signals were measured using a Synergy™ Neo2 microplate  
636 reader (BioTek) per the manufacturer's instructions. The relative luciferase signal was  
637 calculated by normalizing the luciferase signals of serum-treated groups to those of the no-  
638 serum controls. The concentration that reduces the 50% luciferase signal (NT<sub>50</sub>) were estimated  
639 by using a four-parameter logistic regression model from the Prism 8 software (GraphPad  
640 Software Inc., San Diego CA).

### 641 **Plaque reduction neutralization test (PRNT)**

642 Approximately  $1.2 \times 10^6$  Vero E6 cells were seeded to each well of 6-well plates. On the following  
643 day, 100 PFU of infectious clone-derived wild-type SARS-CoV-2 was incubated with serially  
644 diluted serum (total volume of 200  $\mu$ L) at 37°C for 1 h. The virus-serum mixture was transferred  
645 to the pre-seeded Vero E6 cells in 6-well plate. After incubation at 37°C for 1 h, 2 mL of 2% high  
646 gel temperature agar (SeaKem) in DMEM with 5% FBS and 1% P/S was added to the infected  
647 cells per well. After 2-day incubation, 2 ml of neutral red (1 g/L in PBS; Sigma) was added to the  
648 agar-covered cells. After another 5-h incubation, neutral red was removed, and individual  
649 plaques were counted for NT<sub>50</sub> calculation. Each specimen was tested in duplicates.

### 650 **SARS-CoV-2-Nluc antiviral assay**

651 Vero or A549-hACE2 cells (12,000 cells per well in phenol-red free medium containing 2% FBS)  
652 were plated into a white opaque 96-well plate (Corning). On the next day, 2-fold serial dilutions  
653 of compounds were prepared in DMSO. The compounds were further diluted 100-fold in the  
654 phenol-red free culture medium containing 2% FBS. Cell culture fluids were removed and  
655 incubated with 50  $\mu$ L of diluted compound solutions and 50  $\mu$ L of SARS-CoV2-Nluc viruses  
656 (MOI 0.025). At 48 h post-infection, 50  $\mu$ L Nano luciferase substrates (Promega) were added to  
657 each well. Luciferase signals were measured using a Synergy™ Neo2 microplate reader. The  
658 relative luciferase signals were calculated by normalizing the luciferase signals of the  
659 compound-treated groups to that of the DMSO-treated groups (set as 100%). The relative  
660 luciferase signal (Y axis) versus the  $\log_{10}$  values of compound concentration (X axis) was  
661 plotted in software Prism 8. The  $EC_{50}$  (compound concentration for reducing 50% of luciferase  
662 signal) were calculated using a nonlinear regression model (four parameters). Two experiments  
663 were performed with technical duplicates.

#### 664 **Cytotoxicity assay**

665 Vero or A549-hACE2 cells (5,000 cells per well in phenol-red free medium containing 2% FBS)  
666 were plated into a clear flat bottom 96-well plate (Nunc). On the next day, 2-fold serial dilutions  
667 of compounds were prepared in DMSO. The compounds were further diluted 100-fold. 50  $\mu$ L  
668 diluted compound solutions were added to each well of the cell plates. At 72 h post-treatment, 4  
669  $\mu$ L of Cell Counting Kit-8 (CCK-8; Sigma-Aldrich) was added to each well. After incubation at  
670 37°C for 90 min, absorbance at 450 nm was measured using the Cytation5 multi-mode  
671 microplate reader (BioTek). The relative cell viability was calculated by normalizing the  
672 absorbance of the compound-treated groups to that of the DMSO-treated groups (set as 100%).  
673 The relative cell viability (Y axis) versus the  $\log_{10}$  values of compound concentration (X axis)  
674 were plotted in software Prism 8. The  $CC_{50}$  (compound concentration for reducing 50% of cell

675 viability) were calculated using a nonlinear regression model (four parameters). Two  
676 experiments were performed with technical duplicates.

677

## 678 **Acknowledgements**

679 We also thank colleagues at UTMB for support and discussions. A.E.M. is supported by  
680 a Clinical and Translational Science Award NRSA (TL1) Training Core (TL1TR001440) from  
681 NIH. C.R.F.-G. is supported by the predoctoral fellowship from the McLaughlin Fellowship  
682 Endowment at UTMB. S.M. was supported by NIH grants AI114657 and AI146081. V.D.M. was  
683 supported by NIH grants U19AI100625, R00AG049092, R24AI120942, and STARs Award from  
684 the University of Texas System. P.-Y.S. was supported by NIH grants AI142759, AI134907,  
685 AI145617, and UL1TR001439, and awards from the Sealy & Smith Foundation, Kleberg  
686 Foundation, John S. Dunn Foundation, Amon G. Carter Foundation, Gilson Longenbaugh  
687 Foundation, and Summerfield Robert Foundation.

688

## 689 **Author contributions**

690 X.X., T.C., V.D.M., J.P.B., and P.-Y.S conceived the study. X.X., A.E.M., X.Z., K.G.L.,  
691 C.R.F.-G., J.Z., J.L., M.B., and J.P.B. performed the experiments. X.X., A.E.M., T.C., V.D.M.,  
692 J.P.B., and P.-Y.S. analyzed the results. P.R. prepared the serum specimens. C.-T.K.T. and  
693 S.M. provided critical reagents. X.X., M.B., T.C., J.P.B, and P.-Y.S wrote the manuscript.

694

## 695 **Competing interests**

696 UTMB has filed a patent on the reverse genetic system and reporter SARS-CoV-2. The  
697 authors affiliated with Gilead Sciences, Inc. are employees of the company and own company  
698 stock.