

Orally delivered MK-4482 inhibits SARS-CoV-2 replication in the Syrian hamster model

Kyle Rosenke

National Institutes of Health

Frederick Hansen

NIH

Benjamin Schwarz

National Institute of Allergy and Infectious Diseases

Friederike Feldmann

National Institute of Allergy and Infectious Diseases, National Institutes of Health

Elaine Haddock

NIH/NIAID Rocky Mountain Laboratories

Rebecca Rosenke

NIAID/NIH

Kimberly Meade-White

NIH

Atsushi Okumura

National Institute of Allergy and Infectious Diseases

Shanna Leventhal

NIH

David Hawman

NIH

Emily Ricotta

NIH <https://orcid.org/0000-0001-9928-8275>

Catharine Bosio

National Institute of Allergy and Infectious Diseases

Greg Saturday

NIH/NIAID/RML

Heinz Feldmann

National Institute of Allergy and Infectious Diseases <https://orcid.org/0000-0001-9448-8227>


Michael Jarvis (✉ michael.jarvis@plymouth.ac.uk)

University of Plymouth

Keywords: COVID-19, MK-4482, orally administered nucleoside analog, SARS-CoV-2 replication, Syrian hamster model

Posted Date: October 8th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-86289/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Version of Record: A version of this preprint was published at Nature Communications on April 16th, 2021. See the published version at <https://doi.org/10.1038/s41467-021-22580-8>.

1 **Orally delivered MK-4482 inhibits SARS-CoV-2 replication in the Syrian**
2 **hamster model**

3 **Kyle Rosenke¹, Frederick Hansen¹, Benjamin Schwarz², Friederike Feldmann³, Elaine**
4 **Haddock¹, Rebecca Rosenke³, Kimberly Meade-White¹, Atsushi Okumura¹ Shanna Leventhal¹,**
5 **David W. Hawman¹, Emily Ricotta⁴, Catharine M. Bosio², Greg Saturday³, Heinz Feldmann^{1*},**
6 **Michael A. Jarvis^{1,5,6*}**

7 *Corresponding authors: Michael A. Jarvis and Heinz Feldmann, Rocky Mountain Laboratories,
8 903 S 4th Street, Hamilton, MT, US-59840; Tel: (406)-375-7410; Email:
9 michael.jarvis@plymouth.ac.uk and feldmannh@niaid.nih.gov

10 **Affiliations:** ¹Laboratory of Virology, ²Laboratory of Bacteriology and ³Rocky Mountain
11 Veterinary Branch, Division of Intramural Research, National Institute of Allergy and Infectious
12 Diseases, National Institutes of Health, Hamilton, MT, USA;
13 ⁴Laboratory of Clinical Immunology and Microbiology, Division of Intramural Research, National
14 Institute of Allergy and Infectious Diseases, National Institute of Health; Bethesda, MD, USA
15 ⁵University of Plymouth; and ⁶The Vaccine Group Ltd, Plymouth, Devon, UK;

16
17 **One sentence summary:** MK-4482 inhibits SARS-CoV-2 replication in Syrian hamster model

18 **Conflict of Interest Statement:** The authors have declared that no conflict of interest exists.

19

20

21 **The COVID-19 pandemic progresses unabated in many regions of the world. An effective**
22 **antiviral against SARS-CoV-2 that could be administered orally for use following high-risk**
23 **exposure would be of substantial benefit in controlling the COVID-19 pandemic. Herein, we**
24 **show that MK-4482, an orally administered nucleoside analog, inhibits SARS-CoV-2 replication**
25 **in the Syrian hamster model. The inhibitory effect of MK-4482 on SARS-CoV-2 replication was**
26 **observed in animals when the drug was administered either beginning 12 hours before or 12**
27 **hours following infection in a high-risk exposure model. These data support the potential utility**
28 **of MK-4482 to control SARS-CoV-2 infection in humans following high-risk exposure as well as**
29 **for treatment of COVID-19 patients.**

30

31 **INTRODUCTION**

32 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of
33 coronavirus disease 2019 (COVID-19)¹. Following emergence of the virus in Wuhan in the Hubei
34 province of the People’s Republic of China in late 2019², COVID-19 was declared a pandemic by
35 the World Health Organization (WHO) on 11th March, 2020³. As of late September, 2020, there
36 are over 32 million confirmed cases and more than 1,000,000 deaths from COVID-19 worldwide³.
37 Myriad differences in governmental public health responses, politicization of the pandemic
38 response and societal acceptance of control measures have resulted in differing levels of success
39 in controlling the initial wave of infection around the world⁴⁻⁷. Even in those countries that have
40 achieved a higher degree of control of the initial pandemic wave, the unavoidable need to relax
41 highly stringent public health measures has resulted in a rebound of SARS-CoV-2 infections, with
42 the associated fear of a second wave arriving this winter in the Northern hemisphere⁸.

43 Currently, there are no drugs suitable for high-risk exposure use against SARS-CoV-2. The
44 nucleoside analog, GS-5734 (remdesivir), a non-obligate RNA chain terminator, has been granted
45 emergency use authorization (EUA) by the FDA for the treatment of COVID-19 patients⁹. This EUA
46 was based on the demonstration of a decreased time to recovery in patients hospitalized for
47 severe COVID-19, and was recently expanded to include all hospitalized adult and pediatric
48 patients, irrespective of disease severity^{9,10}. In preclinical animal studies, which are more
49 amenable than clinical trials for assessment against high-risk exposure, GS-5734 administered 12
50 hours after SARS-CoV-2 infection was shown to lower lung viral load and lung pathology, although
51 treatment had no effect on shedding from the upper respiratory tract¹¹. However, currently GS-
52 5734 can be administered only via the intravenous route, which makes its application to the
53 control of high-risk exposure challenging.

54 MK-4482 (known previously as EIDD-2801) is an orally administered bioavailable prodrug
55 (5'-isopropylester form) of the cytidine nucleoside analogue EIDD-1931 (β -D-N⁴-hydroxycytidine;
56 NHC)¹². Using a high throughput screen of nucleoside analogs, EIDD-1931, the active compound
57 resulting from hydrolysis of MK-4482, was identified as a broad activity inhibitor of influenza A
58 and respiratory syncytial viruses, with initial functional assays showing the drug to function as a
59 RNA mutagen rather than chain terminator¹³. Originally developed for treatment of hepatitis C
60 virus (HCV) in early the 2000s¹⁴, recent studies indicated potent activity of EIDD-1931 against
61 SARS-CoV-2 in multiple cell types, including biologically relevant epithelial cells *in vitro*, and
62 against MERS-CoV-1 and SARS-CoV-1 coronaviruses in mouse models when administered shortly
63 before as well as following infection¹⁵.

64 In the present study, we determined the half-maximal inhibitory concentration (IC₅₀)
65 value for EIDD-1931 in tissue culture and subsequently assessed the potential of MK-4482
66 following oral administration to control SARS-CoV-2 in the highly susceptible Syrian hamster
67 model^{16,17}. We show that MK-4482, when administered either starting at 12 hours prior to SARS-
68 CoV-2 infection, or even 12 hours post-infection, significantly decreased viral lung loads and
69 pathology, but did not affect shedding from the upper respiratory tract. These findings support
70 the potential of MK-4482 as an orally administered drug for high-risk exposure and possibly
71 therapeutic use in humans.

72

73 **RESULTS**

74 First, we determined the *in vitro* inhibitory effect of EIDD-1931 on SARS-CoV-2 replication in Calu-
75 3 cells, a disease-relevant human lung epithelial cell line. Cells were pretreated with differing
76 drug concentrations and the effect on viral RNA load in tissue culture supernatant was
77 determined at 24 hours after infection by quantitative reverse transcriptase polymerase chain
78 reaction (RT-PCR) (Figure 1A). EIDD-1931 treatment resulted in a decrease in SARS-CoV-2
79 replication by approximately 3-logs (880-fold) when compared to no drug controls (Figure 1A).
80 Viability was also assessed over the differing concentrations, demonstrating only minimal cellular
81 toxicity at the highest drug concentration (Figure 1C). The half-maximal inhibitory concentration
82 (IC₅₀) value for EIDD-1931 was shown to be at sub-micromolar levels in Calu-3 cells at 414.6 nM
83 (Figure 1B).

84 Having verified *in vitro* efficacy and determined the IC₅₀ value of EIDD-1931, we next
85 assessed efficacy of the MK-4432 prodrug in the Syrian hamster model, which is regarded as a
86 preclinical model of mild disease, with animals having self-limiting pneumonia^{16,17}. Given the
87 possibility for oral dosing, we were interested in the utility of MK-4432 as a treatment following
88 high-risk exposure. Two groups of hamsters (n=6 per group) were treated with MK-4432
89 (250mg/kg) by oral gavage 12 hours and 2 hours before (pre-infection group) or 12 hours post-
90 infection (post-infection group). Animals were then dosed every 12 hours with MK-4432
91 (250mg/kg). A control group was treated using the same route and timing as the pre-infection
92 group with vehicle only (see schematic; Figure 2A). Hamsters were infected intranasally with
93 SARS-CoV-2 using a dose of 5x10² TCID₅₀ (100 times infectious dose 50; ID₅₀). The ID₅₀ value was
94 determined in a separate study concerned with further refinement of the Syrian hamster SARS-
95 CoV-2 model¹⁷. Treatment in all groups was continued for 3 consecutive days and hamsters in all
96 groups were euthanized on day 4 post-infection.

97 Disease manifestation in Syrian hamsters following SARS-CoV-2 infection is transient with
98 only mild clinical signs^{16,17}, and no discernible difference in disease manifestation based on
99 clinical symptoms was observed between any group over the course of the study. Virus shedding
100 was measured with oral swabs collected on day 2 and 4 post-infection. Levels of viral RNA in the
101 oral cavity were similar between all groups at these two time points of analysis (approx. 10⁸ and
102 10⁷, for day 2 and 4 post-infection, respectively), and decreased from day 2 to 4 (Figure 2B). Lung
103 tissue samples were collected at day 4 post-infection for analysis. In contrast to levels of
104 shedding, a 1-log decrease in viral RNA was detected in the lungs of pre-infection and post-
105 infection groups, respectively, when compared to the vehicle control group (Figure 2C). This

106 corresponded to a 2-log decrease in infectious virus in the lungs of the MK-4482 treated groups
107 when compared to the vehicle controls (Figure 2D).

108 Lung samples were taken for histopathological analyses, and results are shown in Figure
109 3A to F. Analysis revealed pulmonary lesions consisting of a moderate-marked broncho-
110 interstitial pneumonia centered on terminal bronchioles and extending into the adjacent alveoli.
111 Multifocal necrotic epithelial cells and moderate numbers of infiltrating neutrophils and
112 macrophages with abundant luminal cellular exudate in the bronchi and bronchioles were also
113 present. Alveolar septa were expanded by edema fluid and leucocytes. Moderate type II
114 pneumocyte hyperplasia was noted in more consolidated areas with abundant alveolar
115 macrophages, cellular exudate and edema. Blood vessels were surrounded by moderate
116 numbers of lymphocytes that multifocally aggregated in vascular tunics and elevated the
117 overlying epithelium. Low numbers of syncytial cells were noted in the bronchioles and alveoli.
118 These described lesions affected between 20-50% of pulmonary tissue in the vehicle control
119 groups and while the pre-infection and post-infection treatment groups had similar lesions, they
120 were significantly less abundant. One animal in each of the pre- and post-infection treatment
121 groups had no lesions at all. Pneumonia in the remaining animals affected roughly 5-15% of the
122 lung tissue, but lesions were minimal to mild.

123 Immunoreactivity against SARS-COV2 antigen was used to further compare the lung
124 samples between the three different treatment groups (Figure 3G to I). Antigen staining was
125 observed in bronchial and bronchiolar epithelium, type I and II pneumocytes as well as a small
126 number of pulmonary macrophages. A positive pixel analysis on whole lung slides demonstrated
127 a significant difference in viral antigen present among the three groups. The total number of

128 positive pixels was divided by the area of lung scanned to determine a percentage of lung
129 containing viral antigen. This analysis revealed that the vehicle controls contained significantly
130 more antigen than the treated groups, with the vehicle controls having on average 4.71 times
131 more antigen signal than pre-infection treatment animals and 3.68 times more signal than post-
132 infection treatment animals. Post-infection treatment animals exhibited a slightly higher antigen
133 signal than pre-infection treatment animals, but the difference was not significant (Figure 4A).

134 To evaluate the pharmacokinetics of MK-4482 in the animals, MK-4482 and the EIDD-
135 1931 metabolite were measured in clarified lung homogenate by liquid chromatography and
136 mass spectrometry (LCMS) at the point of necropsy. Since SARS-CoV-2 is a respiratory disease,
137 levels of drug in lung tissue are expected to be the best indicator of therapeutic potential. All
138 treated animals displayed detectable levels of EIDD-1931 in the lung and levels were similar
139 across treatment groups (pre-infection: 18.80 ± 5.97 nmol/g_{lung}, post-infection 17.56 ± 5.49
140 nmol/g_{lung}) (Table 1) (Figure 4B). In line with its demonstrated rapid hydrolysis to EIDD-1931
141 following absorption, MK-4482 was not detected in the tissue^{12,15}. Volume/concentration is
142 difficult to estimate in tissues due to non-homogenous drug distribution and organ hydration. On
143 average, water content of the lung is approximately 80% by weight and this number can be used
144 to calculate a conservative estimated EIDD-1931 concentration in the tissue under the
145 assumptions of homogenous distribution and hydration¹⁸. These estimates suggest a
146 concentration of 15.04 ± 4.78 μ M in the pre-infection group and 14.05 ± 4.39 μ M in the post-
147 infection group at the point of necropsy (12 hours post final MK-4482 dose) (Table 1) (Figure 4B).
148 These values compare well with previous studies where a single oral dose of MK-4482 at

149 128mg/kg in ferrets (compared to 250mg/kg in our study) resulted in EIDD-1931 lung
150 concentrations of 10.7 ± 1.2 nmol/g¹².

151 **DISCUSSION**

152 In the present study, we used the established Syrian hamster animal model^{16,17} to assess the
153 inhibitory effect of the nucleoside analog MK-4482 on SARS-CoV-2 replication *in vivo*. Our study
154 shows the capacity of MK-4482 to substantially reduce the replication of SARS-CoV-2 in the lungs
155 based on both viral RNA genome copy number and levels of infectious virus. Importantly, this
156 control of virus replication was associated with markedly reduced lung pathology. MK-4482 has
157 been shown to inhibit the replication of other related human coronaviruses, MERS-CoV and
158 SARS-CoV-1 in mouse models¹⁵. Our current study is the first demonstration of inhibition of SARS-
159 CoV-2 and amelioration of lung disease by MK-4482 in any animal model.

160 Currently, only a single drug (GS-5734) has been given EUA for treatment of SARS-CoV-2
161 induced COVID-19 disease⁹. Rather than having an impact on mortality, the EUA was based on a
162 demonstration of reduced recovery time for hospitalized patients with COVID-19¹⁰. In a study
163 performed in the rhesus macaque model, GS-5734 administered at 12 hours post-infection was
164 shown to lower both the peak infectious titers of SARS-CoV-2 in bronchoalveolar lavage (BAL)
165 and virus genome copy number in the lung at day 7 post-infection by approximately 2-
166 logs¹¹. Currently, there is no data showing the efficacy of GS-5734 against SARS-CoV-2 in the
167 Syrian hamster model, but treatment starting a day prior to infection and continued twice daily
168 thereafter resulted in significant improvement of SARS-CoV-2 infection in adenovirus 5-hACE2
169 transduced mice¹⁹. However, the hamster and macaque models appear relatively comparable,
170 with both being associated with a rapid increase in SARS-CoV-2 replication in the lung and other

171 respiratory tissues and mild clinical disease^{16,17,20}. Given these similarities, MK-4482 should likely
172 be considered as a potential additional treatment option for COVID-19 patients.

173 Similar to GS-5734, MK-4482 exhibits broad inhibition of divergent RNA viruses^{12-15,21-23}.
174 Although both drugs are nucleoside analogs, MK-4482 has been shown to function as a RNA
175 mutagen inducing genome catastrophe^{15,24}, while GS-5734 is a non-obligate RNA chain
176 terminator²⁵. The function of MK-4482 as an RNA mutagen may raise concerns regarding ‘off-
177 target’ mutagenic toxicity. However, even at an EIDD-1931 dose of 500mg/kg, treatment of mice
178 in a MERS-CoV model did not increase mutation rates of the ISG15 mRNA transcript, a gene highly
179 induced during MERS-CoV infection, whilst viral genes accumulated mutations¹⁵. Incorporation
180 of ribonucleosides has also been shown to be highly selective for RNA compared to host DNA²⁶.
181 Consistent with this level of safety, the guanosine ribonucleoside analog, ribavirin, which has
182 several mechanisms of action including one of RNA mutation/error catastrophe, has been used
183 for decades in patients, including infants with severe lower respiratory tract infections²⁷. If
184 deemed safe, MK-4482 would join GS-5734 as the second broadly direct acting antiviral to target
185 emerging RNA viruses, and in this case, specifically SARS-CoV-2.

186 Infectious disease pathology is a complex interplay between the pathogen and the host.
187 Consequently, strategically planned combination therapy may be more effective than the use of
188 single drugs. Combinations of drugs with different mechanisms of action would be preferable.
189 Such combination therapy has been shown to be highly effective for the control of other viral
190 pathogens, notably human immunodeficiency and hepatitis C virus infection^{28,29}. Therefore, the
191 combination of MK-4482, an RNA mutagen, with the non-obligate RNA chain terminator, GS-
192 5734, may yield additional efficacy in the treatment of SARS-CoV-2 infections. Additional

193 combination partners could be potent neutralizing antibodies³⁰. In addition, immune response
194 modifying drugs such as dexamethasone have been shown to be effective for the later
195 deleterious host responses associated with COVID-19 disease³¹. The combination of such a
196 therapeutic with direct antivirals, such as MK-4482 and GS-5734, may increase treatment
197 efficacy, especially in more severe cases.

198 GS-5734 is currently only administered by the intravenous route. A clear advantage of
199 MK-4482 is the capacity for oral administration, which opens up the possibility for use of the drug
200 as a post-exposure treatment prior to symptom onset. Our data suggest that initiation of
201 treatment within 12 hours of a productive exposure resulting in infection significantly reduces
202 SARS-CoV-2 replication and associated pathology in the lung target organ. Consistent with this
203 idea, direct acting antivirals, including GS-5734 have been shown to be most effective in
204 modifying disease outcome when administered early following infection³². If adequately priced
205 for widespread global use, we believe this post-exposure application MK-4482 could substantially
206 affect the course of the pandemic.

207

208 **MATERIALS AND METHODS**

209 **Biosafety and ethics**

210 Work with infectious SARS-CoV-2 was approved by the Institutional Biosafety Committee (IBC)
211 and performed in high biocontainment at Rocky Mountain Laboratories (RML), NIAID, NIH.
212 Sample removal from high biocontainment followed IBC-approved Standard Operating Protocols.
213 Animal work was approved by the Institutional Animal Care and Use Committee and performed

214 by certified staff in an Association for Assessment and Accreditation of Laboratory Animal Care
215 International accredited facility. Work followed the institution's guidelines for animal use, the
216 guidelines and basic principles in the NIH Guide for the Care and Use of Laboratory Animals, the
217 Animal Welfare Act, United States Department of Agriculture and the United States Public Health
218 Service Policy on Humane Care and Use of Laboratory Animals. Syrian hamsters were group
219 housed in HEPA-filtered cage systems enriched with nesting material and were provided with
220 commercial chow and water *ad libitum*. Animals were monitored at least twice daily.

221 **Virus and cells**

222 SARS-CoV-2 isolate nCoV-WA1-2020 (MN985325.1) was kindly provided by the Centers for
223 Disease Control and Prevention, Atlanta, GA, USA³³ and propagated once at RML in Vero E6 cells
224 in high glucose DMEM (Sigma) supplemented with 2% fetal bovine serum (Gibco), 1 mM L-
225 glutamine (Gibco), 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco). The virus stock used
226 was free of contaminations and confirmed to be identical to the initial deposited Genbank
227 sequence (MN985325.1). Vero E6 cells were maintained in high glucose DMEM supplemented
228 with 10% fetal calf serum, 1 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin.

229 **Syrian hamster study design**

230 Hamsters were divided into groups for either pre-infection or post-infection MK-4482 treatment
231 (n=6 per group). Groups were then treated with MK-4482 (250 mg/kg) [MedChemExpress
232 dissolved in 10 % polyethylene glycol (PEG)-400; 2.5% Cremophor RH40 in water] at 12 hours and
233 2 hours prior to infection (pre-infection group) or 12 hours following infection (post-infection
234 group). Treatment was then maintained with 12 hour dosing until the completion of the study 84

235 hours post-infection (day 4). A third group consisted of vehicle control animals that received the
236 same dosing schedule and volume as the pre-infection group. All groups were infected
237 intranasally with 5×10^2 TCID₅₀ of SARS-CoV-2 (25 μ L/nare). Animals were monitored twice daily
238 for disease signs and progression. All procedures were performed on anesthetized animals. Oral
239 swabs were collected on days 2 and 4 post-infection. Animals were euthanized on day 4 post-
240 infection and lung tissues were collected at necropsy for pathology and virology.

241 **Liquid chromatography and mass spectrometry (LCMS)**

242 LCMS grade water, methanol, acetonitrile and acetic acid were purchased through Fisher
243 Scientific. All synthetic standards for molecular analysis were purchased from MedChemExpress.
244 Clarified lung homogenates were gamma-irradiated (2 megarads) for removal from
245 biocontainment according to IBC-approved protocol³⁴. Standard curves of MK-4482 and EIDD-
246 1931 were made in lung homogenate from uninfected animals and subjected to irradiation to
247 account for molecular degradation. Samples were prepared for analysis by adding 300 μ L of
248 methanol to 100 μ L of homogenate and incubating at 4°C for 30 minutes to precipitate
249 macromolecules. Samples were centrifuged at 16,000 x g at 4°C and the supernatant was
250 transferred to a sample vial for LCMS analysis. Samples were separated by HILIC chromatography
251 on a Sciex ExionLC™ AC system. Samples were injected onto a Waters XBridge® Amide column
252 (130Å, 3.5 μ m, 3 mm X 100 mm) and eluted using a binary gradient from 95 % acetonitrile, 0.8 %
253 acetic acid, 10 mM ammonium acetate to 50 % acetonitrile, 0.8 % acetic acid, 10 mM ammonium
254 acetate over 8 min. Analytes were measured using a Sciex 5500 QTRAP® mass spectrometer in
255 positive mode with electrospray ionization (CUR: 40, CAD: Med, ISV: 2500, Temp: 450, GS1: 50,
256 GS2: 50). Multiple reaction monitoring (MRM) was performed using the optimized conditions in

257 Table 2. To ensure signal fidelity triggered spectra were compared back to synthetic standards.
258 Previously published MRM signals for biological nucleosides were utilized to confirm minimal
259 interference at the retention time of interest³⁵. All analytes were quantified against an 8-point
260 calibration curve of the respective synthetic standard prepared in the target matrix and
261 processed in the same manner as experimental samples. Limits of quantification in lung
262 homogenate after irradiation was 5 ng/mL for EIDD-1931 and 50 pg/mL for MK-4482.

263 **Virus load**

264 RNA was extracted from swabs using the QIAamp Viral RNA kit (Qiagen) according to the
265 manufacturer's instructions. Tissues were homogenized in RLT buffer and RNA was extracted
266 using the RNeasy kit (Qiagen) according to the manufacturer's instructions. For detection of viral
267 RNA, 5 µL RNA was used in a one-step real-time RT-PCR against the N gene which detects genomic
268 and subgenomic RNA¹⁷ using the Rotor-Gene probe kit (Qiagen) according to instructions of the
269 manufacturer. In each run, standard dilutions of RNA standards counted by droplet digital PCR
270 were run in parallel, to calculate copy numbers in the samples.

271 **Virus titration**

272 Virus isolation was performed on lung tissues by homogenizing the tissue in 1 mL DMEM using a
273 TissueLyser (Qiagen) and inoculating Vero E6 cells in a 96-well plate with 200 µL of a 1:10 dilution
274 series of the cleared homogenate. One hour after inoculation of cells, the inoculum was removed
275 and replaced with 200 µL DMEM (Sigma-Aldrich) supplemented with 2% fetal bovine serum, 1
276 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin. Six days after inoculation,

277 cytopathogenic effect was scored and the TCID₅₀ was calculated using the Reed-Muench
278 method³⁶.

279 **Histopathology and immunohistochemistry**

280 Histopathology and immunohistochemistry were performed on hamster lung tissues. Tissues
281 were fixed in 10 % Neutral Buffered Formalin with two changes, for a minimum of 7 days
282 according to IBC-approved SOP. Tissues were placed in cassettes and processed with a Sakura
283 VIP-6 Tissue Tek, on a 12-hour automated schedule, using a graded series of ethanol, xylene, and
284 PureAffin. Embedded tissues were sectioned at 5 µm and dried overnight at 42°C prior to
285 staining. Specific anti-CoV immunoreactivity was detected using Sino Biological Inc. SARS-
286 CoV/SARS-CoV-2 nucleocapsid antibody (Sino Biological cat#40143-MM05) at a 1:1000 dilution.
287 The secondary antibody was the Vector Laboratories ImPress VR anti-mouse IgG polymer (cat#
288 MP-7422). The tissues were then processed for immunohistochemistry using the Discovery Ultra
289 automated stainer (Ventana Medical Systems) with a ChromoMap DAB kit (Roche Tissue
290 Diagnostics cat#760-159). The tissues slides were scanned with the Aperio ScanScope XT (Aperio
291 Technologies, Inc.) and the entire section analyzed with the ImageScope Positive Pixel Count
292 algorithm (version 9.1)³⁷. All tissue slides were analyzed by a board-certified veterinary
293 pathologist.

294 **Statistical analyses**

295 Statistical analysis was performed in R version 4.0.2. The difference in viral load, infectious
296 titers and antigen positivity between study arms was assessed by ANOVA followed by a Kruskal-
297 Wallis test and a pairwise Wilcoxon rank sum test to correct for multiple comparisons.

298 **Data Availability**

299 All raw data (RT-PCR, infectious titers, pathology) is available upon reasonable request.

300 **AUTHOR CONTRIBUTIONS**

301 K. Rosenke, F. Hansen, H. Feldmann and M. A. Jarvis contributed to the design, execution and
302 data analysis, and writing of the manuscript

303 B. Schwarz and C. M. Bosio contributed to the metabolite analysis and editing

304 G. Saturday and R. Rosenke contributed to histological and pathology support and analysis

305 F. Feldmann, E. Haddock, K. Meade-White, A. Okumura, S. Leventhal, D. W. Hawman
306 contributed experiment support and data analysis

307 E. Ricotta contributed to data analysis

308 All authors reviewed and contributed to preparation of the final manuscript

309

310 **ACKNOWLEDGEMENTS**

311 We are thankful to the animal caretakers and histopathology group of the Rocky Mountain
312 Veterinary Branch (NIAID, NIH) for their support with animal related work, and Anita Mora (Visual
313 and Medical Arts Unit, NIAID, NIH) for help with the display items. This work was funded by the
314 Intramural Research Program of the National Institutes of Allergy and Infectious Diseases (NIAID)
315 and National Institutes of Health (NIH). MAJ is funded through The Vaccine Group Ltd, and the
316 University of Plymouth.

317

318

319

320 **DISCLAIMER**

321 The opinions, conclusions and recommendations in this report are those of the authors and do
322 not necessarily represent the official positions of the National Institute of Allergy and Infectious
323 Diseases (NIAID) at the National Institutes of Health (NIH), The Vaccine Group Ltd or the
324 University of Plymouth.

325 **REFERENCES**

- 326 1. Lu, R., *et al.* Genomic characterisation and epidemiology of 2019 novel coronavirus:
327 implications for virus origins and receptor binding. *Lancet* **395**, 565-574 (2020).
- 328 2. Chen, N., *et al.* Epidemiological and clinical characteristics of 99 cases of 2019 novel
329 coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet* **395**, 507-513
330 (2020).
- 331 3. WHO. Coronavirus disease (COVID-2019) situation reports. Vol. 2020 (2020).
- 332 4. Gibney, E. Whose coronavirus strategy worked best? Scientists hunt most effective
333 policies. *Nature* **581**, 15-16 (2020).
- 334 5. Cowling, B.J., *et al.* Impact assessment of non-pharmaceutical interventions against
335 coronavirus disease 2019 and influenza in Hong Kong: an observational study. *Lancet*
336 *Public Health* **5**, e279-e288 (2020).
- 337 6. Lee, D. & Choi, B. Policies and innovations to battle Covid-19 - A Case study of South
338 Korea. *Health Policy Technol* (2020).

- 339 7. Hou, Z., *et al.* Cross-Country Comparison of Public Awareness, Rumors, and Behavioral
340 Responses to the COVID-19 Epidemic: Infodemiology Study. *J Med Internet Res* **22**,
341 e211143 (2020).
- 342 8. Middleton, J., Lopes, H., Michelson, K. & Reid, J. Planning for a second wave pandemic
343 of COVID-19 and planning for winter : A statement from the Association of Schools of
344 Public Health in the European Region. *Int J Public Health* (2020).
- 345 9. FDA, U. Reissue Letter for Emergency Use Authorization (EUA) for emergency use of
346 Veklury (remdesivir). (2020).
- 347 10. Beigel, J.H., *et al.* Remdesivir for the Treatment of Covid-19 - Preliminary Report. *N Engl*
348 *J Med* (2020).
- 349 11. Williamson, B.N., *et al.* Clinical benefit of remdesivir in rhesus macaques infected with
350 SARS-CoV-2. *Nature* **585**, 273-276 (2020).
- 351 12. Toots, M., *et al.* Characterization of orally efficacious influenza drug with high resistance
352 barrier in ferrets and human airway epithelia. *Sci Transl Med* **11**(2019).
- 353 13. Yoon, J.J., *et al.* Orally Efficacious Broad-Spectrum Ribonucleoside Analog Inhibitor of
354 Influenza and Respiratory Syncytial Viruses. *Antimicrob Agents Chemother* **62**(2018).
- 355 14. Stuyver, L.J., *et al.* Ribonucleoside analogue that blocks replication of bovine viral
356 diarrhea and hepatitis C viruses in culture. *Antimicrob Agents Chemother* **47**, 244-254
357 (2003).
- 358 15. Sheahan, T.P., *et al.* An orally bioavailable broad-spectrum antiviral inhibits SARS-CoV-2
359 in human airway epithelial cell cultures and multiple coronaviruses in mice. *Sci Transl*
360 *Med* **12**(2020).

- 361 16. Chan, J.F., *et al.* Simulation of the clinical and pathological manifestations of Coronavirus
362 Disease 2019 (COVID-19) in golden Syrian hamster model: implications for disease
363 pathogenesis and transmissibility. *Clin Infect Dis* (2020).
- 364 17. Rosenke, K., *et al.* Defining the Syrian hamster as a highly susceptible preclinical model
365 for SARS-CoV-2 infection. *BioRxiv* (2020). doi:
366 <https://doi.org/10.1101/2020.09.25.314070>
- 367 18. Lange, N.R. & Schuster, D.P. The measurement of lung water. *Crit Care* **3**, R19-R24
368 (1999).
- 369 19. Sun, J., *et al.* Generation of a Broadly Useful Model for COVID-19 Pathogenesis,
370 Vaccination, and Treatment. *Cell* **182**, 734-743 e735 (2020).
- 371 20. Munster, V.J., *et al.* Respiratory disease in rhesus macaques inoculated with SARS-CoV-
372 2. *Nature* **585**, 268-272 (2020).
- 373 21. Urakova, N., *et al.* beta-d-N (4)-Hydroxycytidine Is a Potent Anti-alphavirus Compound
374 That Induces a High Level of Mutations in the Viral Genome. *J Virol* **92**(2018).
- 375 22. Ehteshami, M., *et al.* Characterization of beta-d-N(4)-Hydroxycytidine as a Novel
376 Inhibitor of Chikungunya Virus. *Antimicrob Agents Chemother* **61**(2017).
- 377 23. Barnard, D.L., *et al.* Inhibition of severe acute respiratory syndrome-associated
378 coronavirus (SARSCoV) by calpain inhibitors and beta-D-N4-hydroxycytidine. *Antivir*
379 *Chem Chemother* **15**, 15-22 (2004).
- 380 24. Agostini, M.L., *et al.* Small-Molecule Antiviral beta-d-N (4)-Hydroxycytidine Inhibits a
381 Proofreading-Intact Coronavirus with a High Genetic Barrier to Resistance. *J Virol*
382 **93**(2019).

- 383 25. Warren, T.K., *et al.* Therapeutic efficacy of the small molecule GS-5734 against Ebola
384 virus in rhesus monkeys. *Nature* **531**, 381-385 (2016).
- 385 26. Suzuki, T., Moriyama, K., Otsuka, C., Loakes, D. & Negishi, K. Template properties of
386 mutagenic cytosine analogues in reverse transcription. *Nucleic Acids Res* **34**, 6438-6449
387 (2006).
- 388 27. Graci, J.D. & Cameron, C.E. Mechanisms of action of ribavirin against distinct viruses.
389 *Rev Med Virol* **16**, 37-48 (2006).
- 390 28. Maeda, K., Das, D., Kobayakawa, T., Tamamura, H. & Takeuchi, H. Discovery and
391 Development of Anti-HIV Therapeutic Agents: Progress Towards Improved HIV
392 Medication. *Curr Top Med Chem* **19**, 1621-1649 (2019).
- 393 29. Naggie, S. & Muir, A.J. Oral Combination Therapies for Hepatitis C Virus Infection:
394 Successes, Challenges, and Unmet Needs. *Annu Rev Med* **68**, 345-358 (2017).
- 395 30. Wang, C., *et al.* A human monoclonal antibody blocking SARS-CoV-2 infection. *Nat*
396 *Commun* **11**, 2251 (2020).
- 397 31. Group, R.C., *et al.* Dexamethasone in Hospitalized Patients with Covid-19 - Preliminary
398 Report. *N Engl J Med* (2020).
- 399 32. Sheahan, T.P., *et al.* Broad-spectrum antiviral GS-5734 inhibits both epidemic and
400 zoonotic coronaviruses. *Sci Transl Med* **9**(2017).
- 401 33. Harcourt, J., *et al.* Severe Acute Respiratory Syndrome Coronavirus 2 from Patient with
402 Coronavirus Disease, United States. *Emerg Infect Dis* **26**, 1266-1273 (2020).

- 403 34. Feldmann, F., Shupert, W.L., Haddock, E., Twardoski, B. & Feldmann, H. Gamma
404 Irradiation as an Effective Method for Inactivation of Emerging Viral Pathogens. *Am J*
405 *Trop Med Hyg* **100**, 1275-1277 (2019).
- 406 35. Du, Y., *et al.* Development and evaluation of a hydrophilic interaction liquid
407 chromatography-MS/MS method to quantify 19 nucleobases and nucleosides in rat
408 plasma. *Biomed Chromatogr* **31**(2017).
- 409 36. Reed, L.J. & Muench, H. A simple method of estimating fifty percent endpoints.
410 *American Journal of Epidemiology* **27**, 493-497 (1938).
- 411 37. Baseler, L.J., *et al.* An Acute Immune Response to Middle East Respiratory Syndrome
412 Coronavirus Replication Contributes to Viral Pathogenicity. *Am J Pathol* **186**, 630-638
413 (2016).
- 414

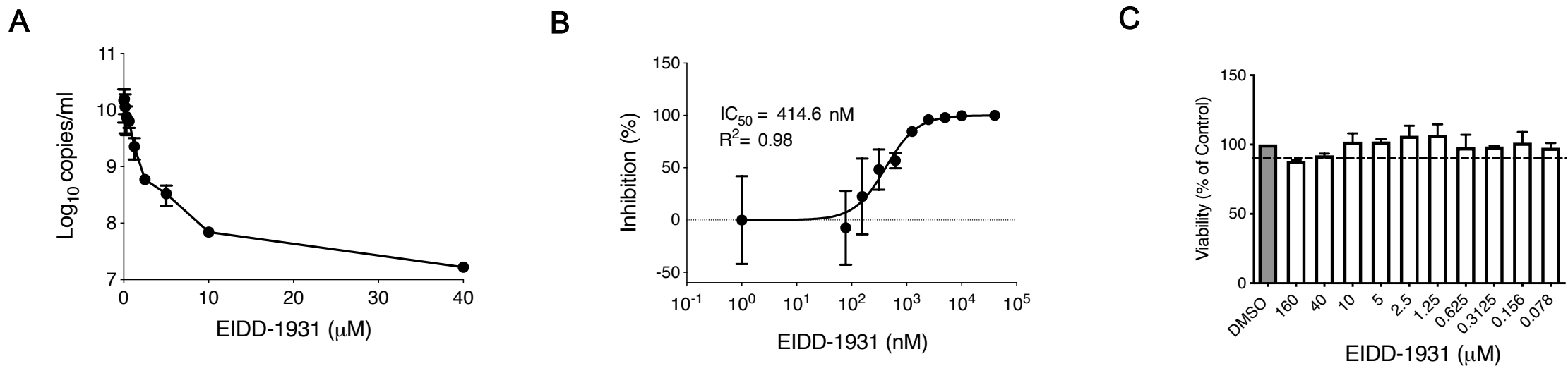


Figure 1: EIDD-1931 inhibits SARS-CoV-2 replication in human lung epithelial Calu-3 cells. Cells were pretreated for 1 hour with differing EIDD-1931 concentrations, followed by infection with SARS-CoV-2 at a MOI of 0.01 for 1 hour. After 1 hour, media was replaced, and cells were cultured in the presence of drug for 24 hours at 37°C in a 5% CO₂ incubator. **(A)** Virus yield in the cell supernatant was measured by quantitative RT-PCR of clarified culture supernatant by using primer and probe sets to quantify total viral RNA (N gene; genomic and subgenomic RNA). **(B)** IC₅₀ values were determined using results from the RT-PCR following log-based transformation of drug concentrations and normalization to percentage inhibition based on diluent alone controls by fitting to drug-dose response curves using Prism software. **(C)** Absence of toxicity (>90% viability; shown by dotted line) at highest EIDD-1931 concentration used for analysis of SARS-CoV-2 replication (40μM) was confirmed using CellTiter-Glo® 2.0 Assay (Promega, Corp., Madison, WI, USA) as per manufacturer’s protocol. For A to C, means are shown ± standard deviation.

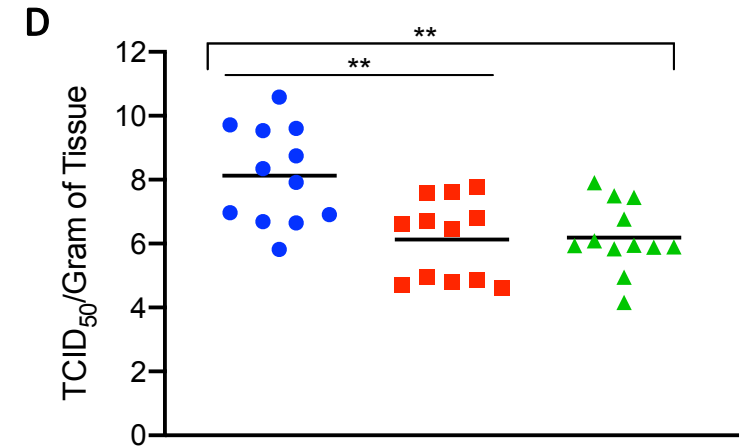
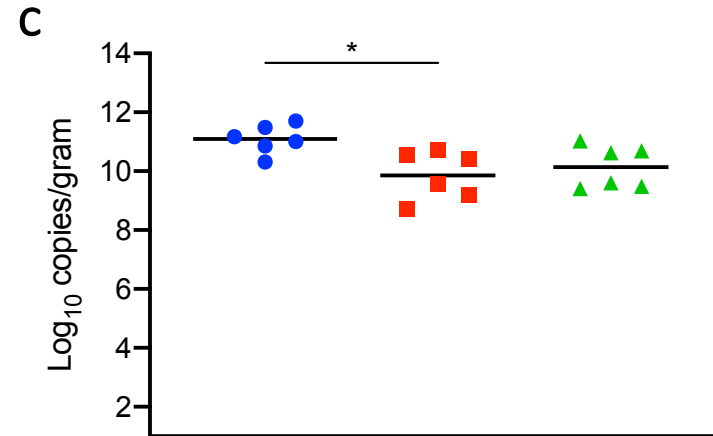
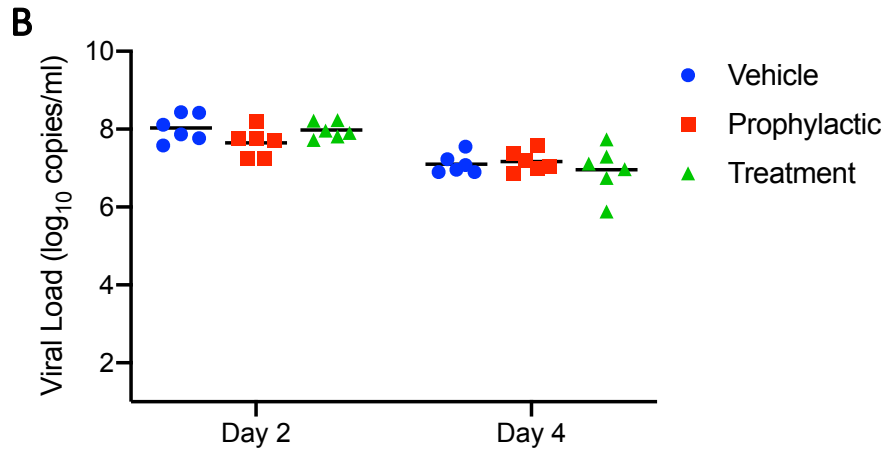
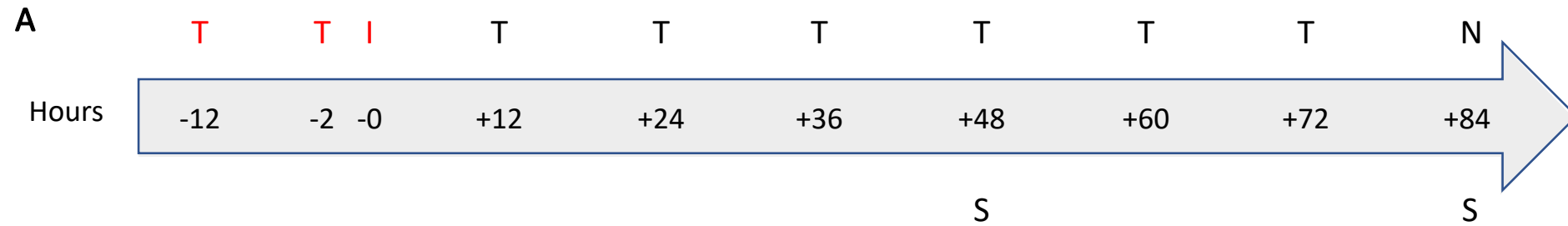


Figure 2: Syrian hamster model – Study design, viral shedding, viral load, infectious titers and viral antigen. (A) Study design. Hamsters were infected with SARS-CoV-2 by the intranasal route. MK-4482 was administered either pre-infection at 12 and 2 hours prior to infection, or post-infection with treatment started 12 hours post-infection. Treatment was then continued in both treatment groups every 12 hours for 3 consecutive days until end of the experiment. Animals were euthanized on day 4 and lungs were harvested for pathology and virology. 'T' denotes treatment (red: pre-infection and black: post-infection treatments); 'I' denotes infection; 'S' denotes swab samples and 'N' indicates necropsy. (B) Viral shedding. Oral swabs were collected on days 2 and 4 post-infection to measure viral shedding, determined by RT-PCR (N gene: genomic and subgenomic) (C) Viral load in lung tissue. Lung viral loads based on RT-PCR (N gene: genomic and subgenomic) were determined as a correlate for lower respiratory tract infection. (D) Infectious virus in lung tissue. Lung samples were homogenized and titered for infectious on Vero E6 cells. Infectious titers were determined as TCID₅₀ equivalents using the Reed-Muench method³⁶. Two independent lung samples were measured from each animal. (B-D) Blue circle, vehicle control; red square, pre-infection treatment; green triangle, post-infection treatment. **Summary of Results:** (B) No statistical significance in virus shedding was found between either of the two MK-4482 treatment groups and vehicle controls. (C) There was a significant difference in lung viral loads between the pre-infection group compared to the vehicle control. Although the post-infection group trended towards lower levels, there was no significant difference between this group and vehicle control. (D) Infectious titers in the lungs were significantly different between both pre-infection and post-infection groups, compared to vehicle control group, but no significance was found between treatment groups from each other. For B to D geometric means are shown. ANOVA followed by Kruskal-Wallis analysis and a pairwise Wilcoxon test was used to analyze differences among groups. * $p < 0.05$, ** $p < 0.008$

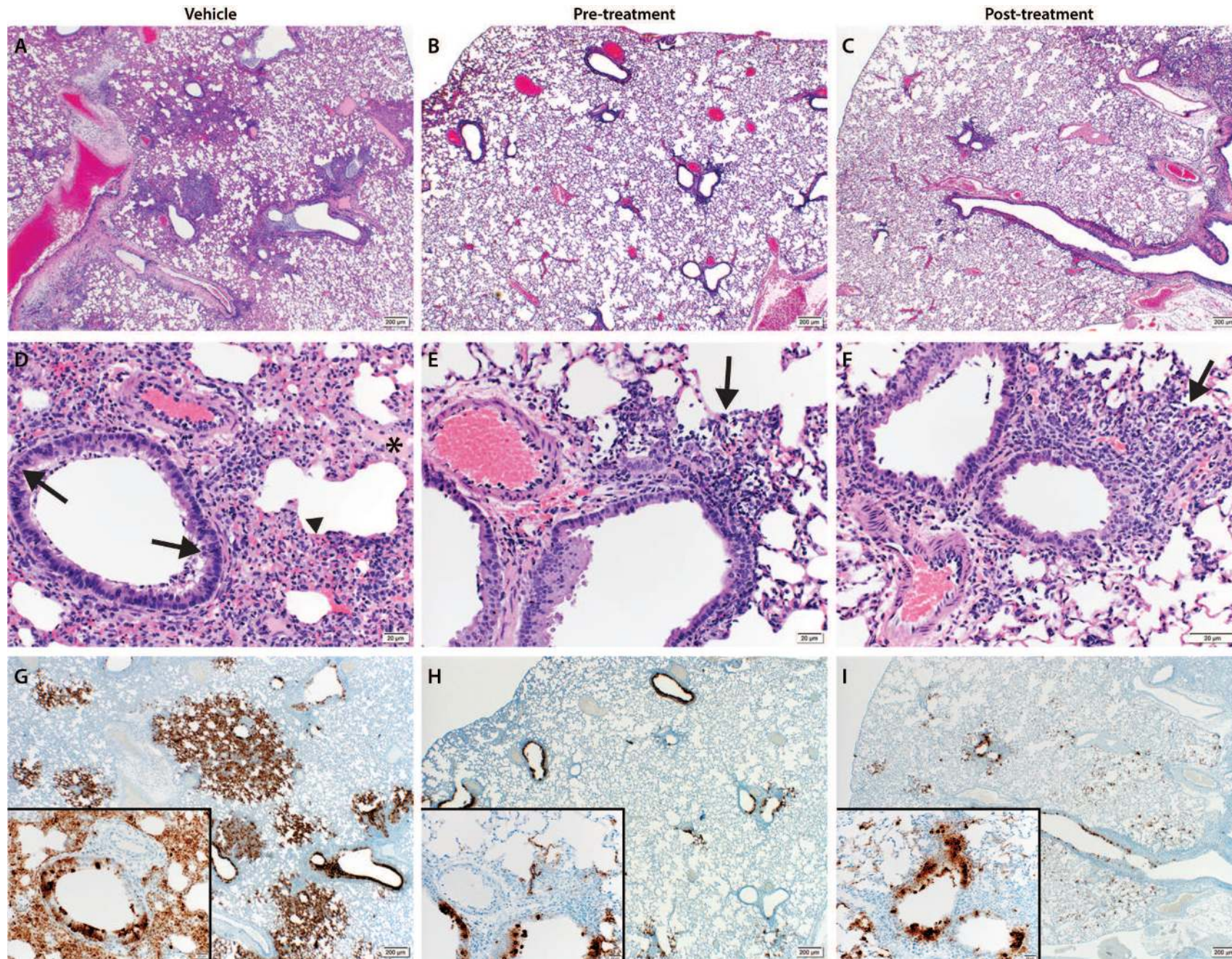


Figure 3: Pathological analysis of the lung tissue. Hematoxylin and eosin (H&E) staining was used on lung sections to examine lung pathology post-inoculation. Immunohistochemistry (IHC) was used to detect viral antigen in the same lung sections. (A, D and G) untreated vehicle control, (B, E and H) pre-infection treatment with antiviral drug MK-4482 and (C, F and I) post-infection treatment with MK-4482. (A-F) H&E stain (G, H and I) IHC for SARS-CoV-2 nucleocapsid antibody. (A) Lung 20X: multifocal, moderate broncho-interstitial pneumonia. (B and C) Lung 20X: minimal peribronchial interstitial pneumonia. (D) Lung 200X epithelial cell necrosis (arrow), edema (asterisk), interstitial pneumonia (arrowhead). (E and F) peribronchial and interstitial infiltrates (arrow). (G) Lung 20X; insert 200X: numerous immunoreactive bronchiolar epithelial cells, type I and II pneumocytes and fewer macrophages. (H and I) Lung 20X; insert 200X: scattered to moderate numbers of immunoreactive bronchiolar epithelial cells, type I and II pneumocytes and macrophages.

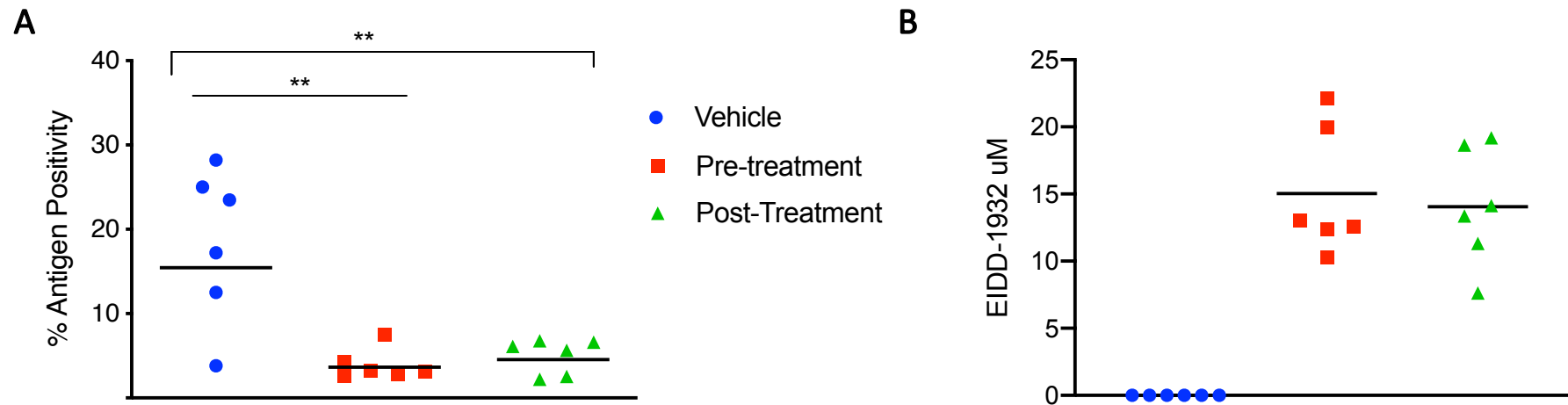


Figure 4: Morphometric analysis of viral antigen and drug concentration in the lungs. (A) A longitudinal cross section of the right lung was stained for viral antigen and scanned to measure the total amount of viral antigen present in the lung section. (B) EIDD-1931 concentrations in the lungs. (A and B) Blue circle, vehicle control; red square, pre-infection treatment; green triangle, post-infection treatment. **Summary of results:** (A) The area of lung staining positive for viral antigen showed a statistically significant difference between both of the MK-4482 treatment groups, compared to vehicle controls. No difference between individual treatment groups was present. For A and B, means are shown. ANOVA followed by Kruskal-Wallis analysis and a pairwise Wilcox test was used to analyze differences among groups. **p<0.008

		EIDD-1931 Lung Concentration						
		Vehicle control						Avg ± Std
nmol/gram		0	0	0	0	0	0	0
Estimated concentration	μM	0	0	0	0	0	0	0
		Pre-treatment						Avg ± Std
nmol/gram		16.29	15.43	27.62	12.82	24.93	15.69	18.80 ± 5.97
Estimated concentration	μM	13.03	12.34	22.1	10.25	19.95	12.55	15.04 ± 4.78
		Post-treatment						Avg + Std
nmol/gram		23.98	9.54	23.3	17.67	14.15	16.72	17.56 ± 5.49
Estimated concentration	μM	19.18	7.63	18.64	14.14	11.32	13.37	14.05 ± 4.39

Table 1: Lung concentrations of EIDD-1931

Molecule	MRM pair	DP (V)	EP (V)	CE (V)	CXP (V)
MK-4482	330.0/128.0*	70	10	20	15
MK-4482	330.0/110.0	60	10	40	15
EIDD-1931	260.0/128.0*	90	10	20	15
EIDD-1931	260.0/110.0	110	10	50	15

*Signal used for quantification

Table 2: LCMS/MS MRM source conditions for the quantification of MK-4482 and EIDD-1931

Key: MRM: multiple reaction monitoring; DP: declustering potential; EP: entrance potential;
CE: collision cell entrance potential; CXP: collision cell exit potential

Figures

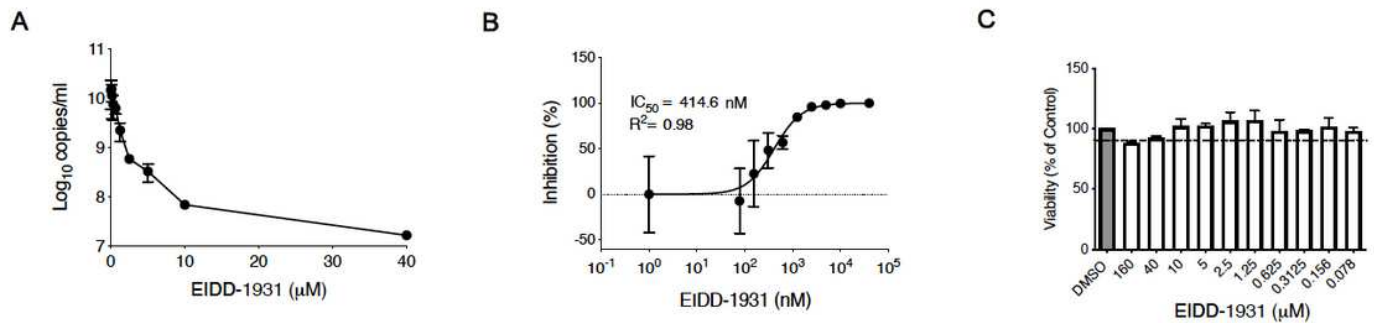


Figure 1

EIDD-1931 inhibits SARS-CoV-2 replication in human lung epithelial Calu-3 cells. Cells were pretreated for 1 hour with differing EIDD-1931 concentrations, followed by infection with SARS-CoV-2 at a MOI of 0.01 for 1 hour. After 1 hour, media was replaced, and cells were cultured in the presence of drug for 24 hours at 37°C in a 5% CO₂ incubator. (A) Virus yield in the cell supernatant was measured by quantitative RT-PCR of clarified culture supernatant by using primer and probe sets to quantify total viral RNA (N gene; genomic and subgenomic RNA). (B) IC₅₀ values were determined using results from the RT-PCR following log-based transformation of drug concentrations and normalization to percentage inhibition based on diluent alone controls by fitting to drug-dose response curves using Prism software. (C) Absence of toxicity (>90% viability; shown by dotted line) at highest EIDD-1931 concentration used for analysis of SARS-CoV-2 replication (40µM) was confirmed using CellTiter-Glo® 2.0 Assay (Promega, Corp., Madison, WI, USA) as per manufacturer’s protocol. For A to C, means are shown ± standard deviation.

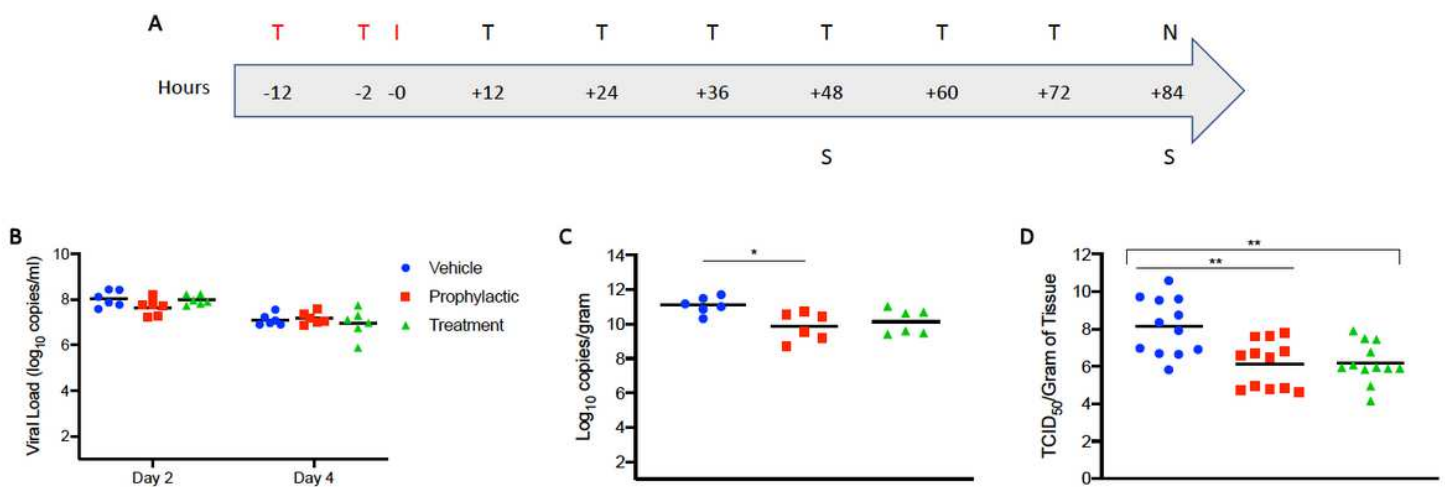


Figure 2

Syrian hamster model – Study design, viral shedding, viral load, infectious titers and viral antigen. (A) Study design. Hamsters were infected with SARS-CoV-2 by the intranasal route. MK-4482 was

administered either pre-infection at 12 and 2 hours prior to infection, or post-infection with treatment started 12 hours post-infection. Treatment was then continued in both treatment groups every 12 hours for 3 consecutive days until end of the experiment. Animals were euthanized on day 4 and lungs were harvested for pathology and virology. 'T' denotes treatment (red: pre-infection and black: post-infection treatments); 'I' denotes infection; 'S' denotes swab samples and 'N' indicates necropsy. (B) Viral shedding. Oral swabs were collected on days 2 and 4 post-infection to measure viral shedding, determined by RT-PCR (N gene: genomic and subgenomic) (C) Viral load in lung tissue. Lung viral loads based on RT-PCR (N gene: genomic and subgenomic) were determined as a correlate for lower respiratory tract infection. (D) Infectious virus in lung tissue. Lung samples were homogenized and titered for infectious on Vero E6 cells. Infectious titers were determined as TCID₅₀ equivalents using the Reed-Muench method³⁶. Two independent lung samples were measured from each animal. (B-D) Blue circle, vehicle control; red square, pre-infection treatment; green triangle, post-infection treatment. Summary of Results: (B) No statistical significance in virus shedding was found between either of the two MK-4482 treatment groups and vehicle controls. (C) There was a significant difference in lung viral loads between the pre-infection group compared to the vehicle control. Although the post-infection group trended towards lower levels, there was no significant difference between this group and vehicle control. (D) Infectious titers in the lungs were significantly different between both pre-infection and post-infection groups, compared to vehicle control group, but no significance was found between treatment groups from each other. For B to D geometric means are shown. ANOVA followed by Kruskal-Wallis analysis and a pairwise Wilcoxon test was used to analyze differences among groups. * $p < 0.05$, ** $p < 0.008$

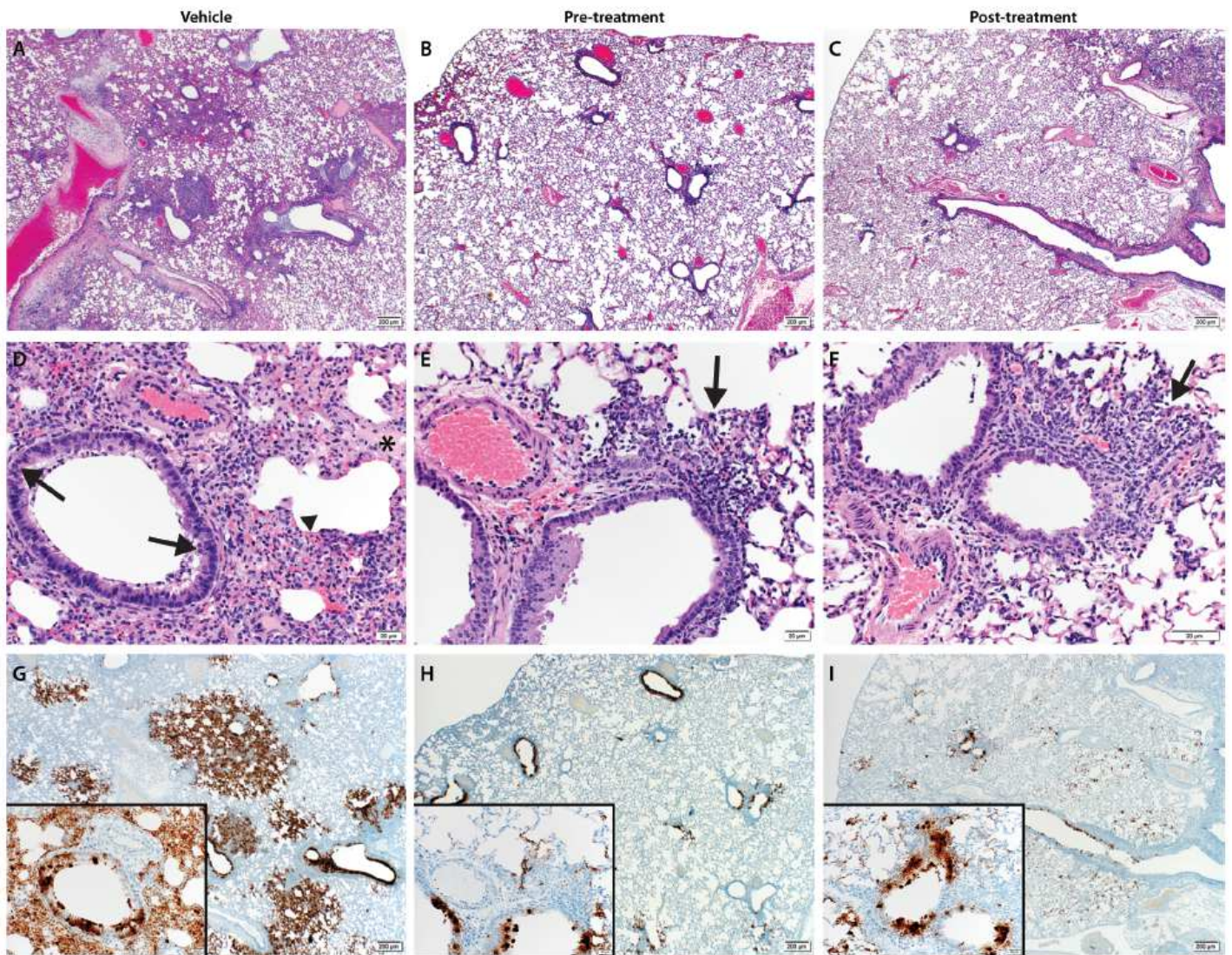


Figure 3

Pathological analysis of the lung tissue. Hematoxylin and eosin (H&E) staining was used on lung sections to examine lung pathology post-inoculation. Immunohistochemistry (IHC) was used to detect viral antigen in the same lung sections. (A, D and G) untreated vehicle control, (B, E and H) pre-infection treatment with antiviral drug MK-4482 and (C, F and I) post-infection treatment with MK-4482. (A-F) H&E stain (G, H and I) IHC for SARS-CoV-2 nucleocapsid antibody. (A) Lung 20X: multifocal, moderate broncho-interstitial pneumonia. (B and C) Lung 20X: minimal peribronchial interstitial pneumonia. (D) Lung 200X epithelial cell necrosis (arrow), edema (asterisk), interstitial pneumonia (arrowhead). (E and F) peribronchial and interstitial infiltrates (arrow). (G) Lung 20X; insert 200X: numerous immunoreactive bronchiolar epithelial cells, type I and II pneumocytes and fewer macrophages. (H and I) Lung 20X; insert 200X: scattered to moderate numbers of immunoreactive bronchiolar epithelial cells, type I and II pneumocytes and macrophages.

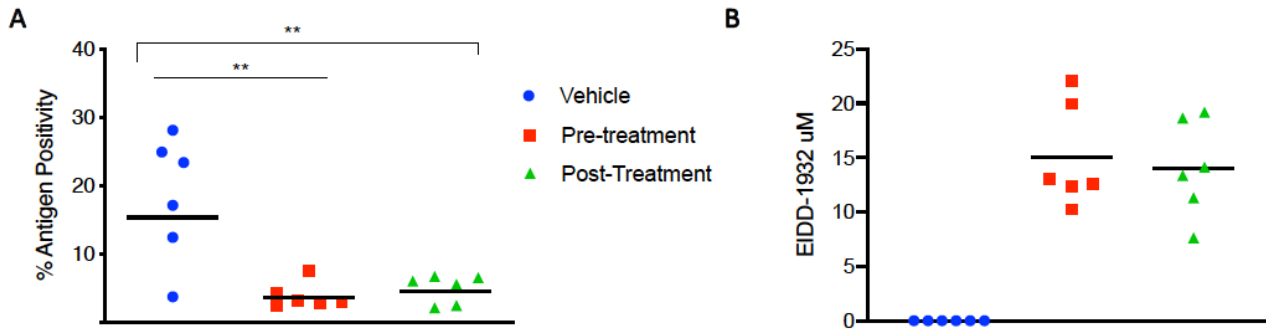


Figure 4

Morphometric analysis of viral antigen and drug concentration in the lungs. (A) A longitudinal cross section of the right lung was stained for viral antigen and scanned to measure the total amount of viral antigen present in the lung section. (B) EIDD-1931 concentrations in the lungs. (A and B) Blue circle, vehicle control; red square, pre-infection treatment; green triangle, post-infection treatment. Summary of results: (A) The area of lung staining positive for viral antigen showed a statistically significant difference between both of the MK-4482 treatment groups, compared to vehicle controls. No difference between individual treatment groups was present. For A and B, means are shown. ANOVA followed by Kruskal-Wallis analysis and a pairwise Wilcoxon test was used to analyze differences among groups. $**p < 0.008$