Molnupiravir (EIDD-2801) inhibits SARS-CoV-2 replication and enhances the efficacy of favipiravir in a Syrian hamster infection model

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Author Contributions

R.A., C.S.F. and J.N. designed research; R.A., C.S.F., S.J.F.K., X.Z. and L.L. performed research; R.A., C.S.F. and B.W. analyzed data; J.N. provided advice on the interpretation of data; R.A., C.S.F. and J.N. wrote the paper with input from co-authors; A.K.C. and S.D.J provided essential reagents; V.G. and E.H. provided and facilitated access to essential infrastructure; R.A., C.S.F. and J.N. supervised the study; L.V., L.C., P.L., J.N. and K.D. acquired funding.

Abstract

Since its emergence in Wuhan, China in December 2019, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread worldwide resulting in a global pandemic with >2 million deaths within a year of the emergence of the virus. In the search for small molecule inhibitors of SARS-CoV-2 Molnupiravir (EIDD-2801), an orally bioavailable nucleoside analog that was originally developed as an antiviral against influenza viruses but that exerts also activity against a number of other RNA viruses, including SARS-CoV2 and other coronaviruses. We here report on the effect of EIDD-2801 in a well-established Syrian hamster SARS-CoV-2 infection model. Oral treatment of SARS-CoV-2-infected hamsters with EIDD-2801 for four consecutive days, starting from the day of infection, significantly reduced infectious virus titers and viral RNA loads in the lungs and markedly improved lung histopathology in a dose-dependent manner when assessed at 4 dpi. When onset of treatment with 500 mg/kg/dose was delayed until 24h post-infection, a modest but significant antiviral effect was observed. When suboptimal doses of both favipiravir (300 mg/kg, BID) and EIDD-2801 (150 mg/kg, BID) were combined, a complete reduction (~5 $\log_{10}$) of infectious virus titers was observed in the lungs of most of the combo-treated animals whereas either compound alone resulted in a reduction of respectively 1.2 and 1.3 $\log_{10}$. The potential of EIDD-2801 for the treatment and/or prevention of SARS-CoV-2 alone or in combination with favipiravir deserves further attention.

Keywords

SARS-CoV-2; Antivirals; EIDD-2801; MK-4482; Molnupiravir; preclinical model.

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a β-coronavirus that was first identified in Wuhan, China in December 2019 (1). Since then, the virus rapidly spread around the globe with more than 96 million cases reported by the end of 2020 and 2 million deaths one year after the emergence of the virus [https://covid19.who.int/]. Infection with SARS-CoV-2 results in coronavirus-induced disease (COVID-19) which is characterized by a wide range of symptoms including fever, dry cough, muscle and/or joint pain, headache, decreased sense of taste and smell and diarrhea. The disease can also progress into severe complications such as acute respiratory distress syndrome (ARDS), respiratory failure, septic shock and multi-organ failure, which are mainly attributed to a massive cytokine storm and exaggerated immune response (2).

To date, there are no approved, selective coronavirus antivirals to treat or prevent infections. The use of potent antivirals against SARS-CoV-2 will reduce viral loads and may hence reduce the chance to progress to a severe disease. In addition, such antiviral drugs could be useful to protect for example health care workers and high-risk groups in a prophylactic setting. Since the *de novo* development and approval of (a) specific, highly potent antiviral(s) for SARS-CoV-2 requires years, the main focus for COVID-19 treatment in the current pandemic is to repurpose drugs that have been approved or in clinical trials for other diseases (3).

The ribonucleoside analogue, N4-hydroxycytidine (NHC, EIDD-1931), was initially developed as an influenza inhibitor, but exerts also roader-spectrum antiviral activity against multiple viruses belonging to different families of RNA viruses. Activity against SARS-CoV and SARS-CoV-2 has been reported in cell lines and primary human airway epithelial cell cultures (4). Acting through lethal mutagenesis, its incorporation into viral RNA results in the accumulation of deleterious transition mutations beyond a permissible error threshold to sustain the virus population, leading to error catastrophe (5). The orally bioavailable, pro-drug counterpart of NHC (6), Molnupiravir (EIDD-2801, MK-4482) is currently being assessed for its potential as an antiviral treatment of SARS-CoV-2 infection in Phase 2 clinical trials of

infected patients (NCT04405570, NCT04405739). To our knowledge, three recent studies reported on the activity of orally dosed EIDD-2801 in SARS-CoV-2 infected animals. Oral treatment of SARS-CoV-2 infected Syrian hamsters with high doses of EIDD-2801 was reported to result in marked reduction (1 to 2 $\log_{10}$) of viral loads when administered either in a pre-exposure (12h before infection) or post-exposure (start of treatment 12h post-infection, pi) settings (7). In a ferret model infection model, EIDD-2801 was reported to significantly reduce virus loads in the lungs when start of treatment was delayed until 12 or 36h pi and to block also SARS-CoV-2 contact transmission (8). In a humanized mouse model i.e. implanted with human lung tissues, EIDD-2801 prevented SARS-CoV-2 infection in a pre-exposure prophylaxis setting (9).

We recently demonstrated that the influenza drug Favipiravir results in a pronounced antiviral activity in SARS-CoV-2 infected hamsters, whereas hydroxychloroquine lacks antiviral activity in this model. Here, we use the same hamster model to obtain further information on the antiviral activity of EIDD-2801 either when used alone or in combination with favipiravir.

Results

In vivo efficacy of EIDD-2801 against SARS-CoV-2 at the time of infection

First, we evaluated the dose-response effect of EIDD-2801 in SARS-CoV-2-infected hamsters. Briefly, 6-8 weeks female SG hamsters were treated orally with EIDD-2801 (either 75, 150, 200 or 500 mg/kg, BID) or the vehicle (i.e. the control group) for four consecutive days starting one hour before intranasal infection with SARS-CoV-2 [BetaCov/Belgium/GHB-03021/2020 (EPI ISL 109 407976|2020-02-03)]. At day four post-infection (pi), the animals were euthanized and lungs were collected for quantification of viral RNA, infectious virus titers and lung histopathology as described previously (10) (Fig. 1A). EIDD-2801 treatment resulted in a dose-dependent reduction in the viral RNA copies per mg of lung tissue with 1.3 ($P=0.002$), 1.9 ($P<0.0001$), 3.3 ($P<0.0001$) and 2.8 ($P=0.01$) $\log_{10}$ reduction was noted in the groups that had been treated BID with 75, 150, 200 and 500 mg/kg, respectively (Fig. 1B). A similar pattern was observed for the infectious virus load in the lungs whereas the high doses, but not the 75

mg/kg dose BID, significantly reduced infectious virus lung titers (Fig. 1C). The reduction in infectious virus titers ($TCID_{50}$ / mg tissue) in the lungs of hamsters treated BID with 150, 200 and 500 mg/kg was 1.3 (P=0.0002), 3.5 (P<0.0001) and 1 (P=0.0002) log_{10}, respectively (Fig. 1C). However, some variations in viral loads reduction was observed in the group treated with the highest dose.

Treatment with 75 and 200 mg/kg EIDD-2801 BID significantly reduced the histological lung disease score (P=0.0025; P<0.0001), because of a large variation no such inhibition was observed at 150 mg/kg and likewise and surprisingly no significant protective activity was noted at the highest dose used (Fig. 1D). All the doses studied were well tolerated without significant weight loss or any obvious adverse effects (Fig. 1E).

In vivo efficacy of EIDD-2801 against SARS-CoV-2 in a post-exposure setting

We next explored whether delayed EIDD-2801 treatment (started at 24 h after infection) (Fig. 2A) has an impact on the infection. Delaying the start of treatment with EIDD-2801 (200 mg/kg or 500 mg/kg BID) by 1 day resulted in 0.4 (P=0.03) and 1 (P=0.05, ns) log_{10} reduction of viral RNA copies/mg lung, respectively (Fig. 2B). Likewise no substantial reduction of infectious virus load in the 200 mg/kg Day 1 group was noted, whereas a modest but significant reduction [1 log_{10} reduction in TCID50/mg lung tissue (P=0.0003)] in the 500 mg/kg Day 1 group was observed (Fig. 2C). A modest reduction of the histological lung disease score was observed in the 200 mg/kg (P=0.0007) and 500 mg/kg (P=0.27, ns) Day 1 treatment groups (Fig. 2D). Thus even if the delayed start of treatment with EIDD-2801 is not sufficient to efficiently stop viral replication, the drug may still able to delay to some extend disease progression.

Effect of the combined treatment of EIDD-2801 and favipiravir

We earlier demonstrated that a high dose of favipiravir (i.e. 500 mg/kg, BID) can reduce infectious virus loads of SARS-CoV2 in the lungs of hamsters to undetectable levels. We here set out to explore whether a similar potency can be achieved by combined treatment of SARS-CoV-2-infected hamsters with suboptimal doses of either favipiravir and EIDD-2801 (Fig. 3A). Single treatment with favipiravir

(300 mg/kg, BID, intraperitoneal injection) reduced viral RNA and infectious virus loads in the lungs of infected hamsters by 0.7 (P=0.0009) and 1.2 (P=0.0002) log_{10}/ mg lung tissue, respectively (Fig. 3B/C). When combined with EIDD-2801 (150 mg/kg, BID), additional reduction in viral RNA loads in the lungs (2.7 log_{10} per mg lung, P<0.0001) was noted compared to the hamsters treated solely with either compound (Fig. 3B). Interestingly, a significant enhanced reduction in infectious virus titers (4.5 log_{10} $TCID_{50}$ per mg lung, P<0.0001) was observed in the combination group as compared to the groups treated with either compound alone (Fig. 3C). Notably, six out of ten hamsters in the combined treatment group had no detectable infectious virus in their lungs (Fig. 3C). A modest but significant (P=0.03) improvement in the histological lung pathology scores was also observed in the combined treatment group (Fig. 3D). No significant weight loss or toxicity signs were observed in the combined treatment group (Fig. 3E).

Discussion

Using a SARS-CoV2 hamster model (10, 11), we here show that EIDD-2801 can markedly reduce, albeit at a relatively high dose, SARS-CoV-2 infection and virus induced pathology in particular when treatment is started at the time of Infection. In another study (7) a dose of 250 mg/kg of EIDD-2801, (given orally every 12 hours starting 12 hours pre-infection) was less effective (1 log_{10} reduction in viral RNA and 2 log_{10} reduction in infectious virus titers) than the 200 mg/kg BID dose in our study, despite the fact that the authors used a much lower virus inoculum (5×10^2 $TCID_{50}$) than was the case in our study.

EIDD-2801 has also been reported to be effective in SARS-CoV infected C57/BL6 mice either when administered prophylactically (start of treatment 2 h before infection) or therapeutically (start of treatment delayed until 48 hpi) at a dose of 500 mg/kg twice daily (4). In a humanized mouse model of SARS-CoV-2 infection, pre-exposure (12 h before infection) with 500 mg/kg of EIDD-2801 twice daily was efficacious in preventing SARS-CoV-2 infection, with a ~6 log_{10} reduction in virus lung titers (9). Recently, EIDD-2801 (15 mg/kg, BID) was found to markedly reduce SARS-CoV-2 viral titers in the upper

140 respiratory tract of ferrets when start of treatment was delayed until 12 hpi to 36 hpi. EIDD-2801, when
141 treatment was initiated at 12 hpi, also prevented contact transmission when treated SARS-CoV-2-
142 infected animals were co-housed with non-infected untreated sentinels (8).

143 Our results are consistent with other recent studies (in hamster, mouse and ferret models) showing
144 that pre-emptive and early intervention with high doses of orally given EIDD-2801 results in antiviral
145 activity. In our Syrian hamster model, the delayed treatment with a dose of 500 mg/kg resulted 1 $\log_{10}$
146 reduction of viral RNA and infectious virus loads in the lung when treatment was initiated 24 hpi.
147 However, a slight improvement in lung pathology was observed in this group.

148 The antiviral drug, Remdesivir (Veklury), is the first drug to receive FDA approval for use in hospitalised
149 COVID19 patients, although the World Health Organisation has recently recommended against its use
150 (https://www.fda.gov/news-events/press-announcements/fda-approves-first-treatment-covid-19;
151 https://www.who.int/news-room/feature-stories/detail/who-recommends-against-the-use-of-
152 remdesivir-in-covid-19-patients). Both Remdesivir and EIDD-2801 are nucleoside analogues acting on
153 the viral RNA replication pathway, with Remdesivir resulting in chain termination and EIDD-2801 in
154 lethal mutagenesis (5, 11). Additionally, both have a high barrier to resistance and resistant variants
155 have a loss in fitness (6, 12). Remdesivir needs to be administrated intravenously which precludes its
156 use in the early stages of the infection/disease or even prophylactic use. On the other hand, EIDD-2801
157 can be dosed via the oral route.

158 Favipiravir is another broad-spectrum antiviral drug that can be dosed orally, which is currently being
159 studied in clinical trials against SARS-CoV-2 in several countries (13). Similar to EIDD-2801, favipiravir
160 has been reported to interact with the viral polymerase of several viruses and to induce lethal
161 mutagenesis in small animal models (14). We previously showed that treatment of SARS-CoV-2-
162 infected hamsters with a high doses of favipiravir can reduce infectious titers in the lungs to
163 undetectable and markedly improves lung histopathology (10). Interestingly, combination treatment
164 of SARS-CoV-2-infected hamsters with moderate doses of both favipiravir (300 mg/kg, BID) and EIDD-

2801 (150 mg/kg, BID) resulted in a complete reduction of infectious virus titers in 6 out of 10 treated animals, with a median reduction of 4.5 $\log_{10}$ TCID50/mg lung tissue, which could not be achieved by single treatment with either compound. This reduction in the lung viral loads in the hamsters treated with combined therapy is comparable to the one achieved using a favipiravir dose of 500 mg/kg, BID (with a loading dose of 600 mg/kg, BID in the first day) in our previous study (10). These results suggest that EIDD-2801 may enhance the efficacy of favipiravir in COVID-19 patients and therefore lower doses of both compounds could be administered.

By demonstrating the antiviral effect of orally-dosed EIDD-2801 in the SARS-CoV-2 hamster infection model, either alone or in combination with favipiravir, we contribute to the pre-clinical profiling of EIDD-2801, and provide further evidence in support of the ongoing clinical trials. Our data lend support to plan clinical studies in which the combined efficacy of Molnupiravir and favipiravir is explored.

Materials and methods

SARS-CoV-2

The SARS-CoV-2 strain used in this study, BetaCov/Belgium/GHB-03021/2020 (EPI ISL 109 407976|2020-02-03), was recovered from a nasopharyngeal swab taken from an RT-qPCR confirmed asymptomatic patient who returned from Wuhan, China in the beginning of February 2020. A close relation with the prototypic Wuhan-Hu-1 2019-nCoV (GenBank accession 112 number MN908947.3) strain was confirmed by phylogenetic analysis. Infectious virus was isolated by serial passaging on HuH7 and Vero E6 cells (10); passage 6 virus was used for the study described here. The titer of the virus stock was determined by end-point dilution on Vero E6 cells by the Reed and Muench method (15). Live virus-related work was conducted in the high-containment A3 and BSL3+ facilities of the KU Leuven Rega Institute (3CAPS) under licenses AMV 30112018 SBB 219 2018 0892 and AMV 23102017 SBB 219 20170589 according to institutional guidelines.

Cells

Vero E6 cells (African green monkey kidney, ATCC CRL-1586) were cultured in minimal essential medium (Gibco) supplemented with 10% fetal bovine serum (Integro), 1% L- glutamine (Gibco) and 1% bicarbonate (Gibco). End-point titrations were performed with medium containing 2% fetal bovine serum instead of 10%.

Compounds

For the first pilot experiment, EIDD-2801 was kindly provided by Calibr at Scripps Research (USA). For further studies, EIDD-2801 was purchased from Excenen Pharmatech Co., Ltd (China) and was formulated as 50 or 100 mg/ml (for groups with the highest dose) stocks in a vehicle containing 10%PEG400 and 2.5% Kolliphor-EL in water. Favipiravir was purchased from BOC Sciences (USA) and was formulated as a 50 mg/mL stock in 3% sodium bicarbonate.

SARS-CoV-2 infection model in hamsters

The hamster infection model of SARS-CoV-2 has been described before (10). In brief, wild-type Syrian Golden hamsters (Mesocricetus auratus) were purchased from Janvier Laboratories and were housed per two in ventilated isolator cages (IsoCage N Biocontainment System, Tecniplast) with ad libitum access to food and water and cage enrichment (wood block). The animals were acclimated for 4 days prior to study start. Housing conditions and experimental procedures were approved by the ethics committee of animal experimentation of KU Leuven (license P065-2020). Female hamsters of 6-8 weeks old were anesthetized with ketamine/xylazine/atropine and inoculated intranasally with 50 µL containing 2×10^6 TCID50 SARS-CoV-2 (day 0).

Treatment regimen

For D0 treatment, animals were treated twice daily with 75, 150, 200 or 500 mg/kg of EIDD-2801 by oral gavage just before infection with SARS-CoV-2. For delayed treatment groups, animals were treated with either, 200 or 500 mg/kg of EIDD-2801 starting from day1 post-infection (pi) by oral gavage. For combination therapy, hamsters were treated from day0 with 150 mg/kg EIDD-2801 (oral gavage) and 300 mg/kg favipiravir (intraperitoneal, i.p.) twice daily. All the treatments continued until day 3 pi. Hamsters were monitored for appearance, behavior and weight. At day 4 pi, hamsters were euthanized by i.p. injection of 500 µL Dolethal (200mg/mL sodium pentobarbital, Vétoquinol SA). Lungs were collected and viral RNA and infectious virus were quantified by RT-qPCR and end-point virus titration, respectively.

SARS-CoV-2 RT-qPCR

Hamster lung tissues were collected after sacrifice and were homogenized using bead disruption (Precellys) in 350 µL RLT buffer (RNeasy Mini kit, Qiagen) and centrifuged (10.000 rpm, 5 min) to pellet the cell debris. RNA was extracted according to the manufacturer's instructions. Of 50 µL eluate, 4 µL was used as a template in RT-qPCR reactions. RT-qPCR was performed on a LightCycler96 platform (Roche) using the iTaq Universal Probes One-Step RT-qPCR kit (BioRad) with N2 primers and probes

225 targeting the nucleocapsid (10). Standards of SARS-CoV-2 cDNA (IDT) were used to express viral
226 genome copies per mg tissue or per mL serum.

227 **End-point virus titrations**

228 Lung tissues were homogenized using bead disruption (Precellys) in 350 µL minimal essential medium
229 and centrifuged (10,000 rpm, 5min, 4°C) to pellet the cell debris. To quantify infectious SARS-CoV-2
230 particles, endpoint titrations were performed on confluent Vero E6 cells in 96- well plates. Viral titers
231 were calculated by the Reed and Muench method (15) using the Lindenbach calculator and were
232 expressed as 50% tissue culture infectious dose (TCID50) per mg tissue.

233 **Histology**

234 For histological examination, the lungs were fixed overnight in 4% formaldehyde and embedded in
235 paraffin. Tissue sections (5 µm) were analyzed after staining with hematoxylin and eosin and scored
236 blindly for lung damage by an expert pathologist. The scored parameters, to which a cumulative score
237 of 1 to 3 was attributed, were the following: congestion, intra-alveolar hemorrhagic, apoptotic bodies
238 in bronchus wall, necrotizing bronchiolitis, perivascular edema, bronchopneumonia, perivascular
239 inflammation, peribronchial inflammation and vasculitis.

240 **Statistics**

241 GraphPad Prism (GraphPad Software, Inc.) was used to perform statistical analysis. Statistical
242 significance was determined using the non-parametric Mann Whitney U-test. P-values of ≤0.05 were
243 considered significant.

244

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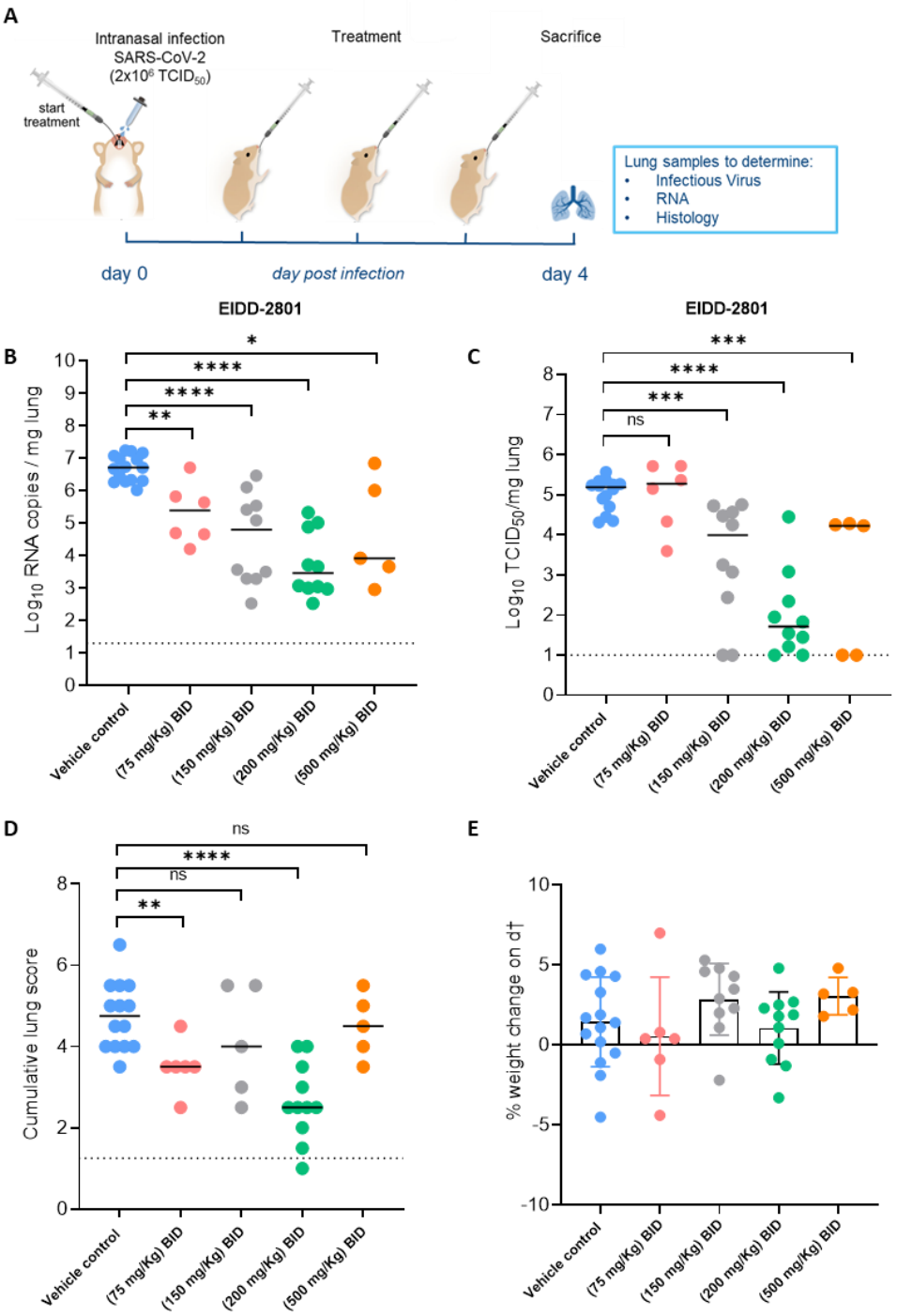

Fig.1. *In vivo* efficacy of EIDD-2801 against SARS-CoV-2 at the time of infection. (A) Set-up of the study. (B) Viral RNA levels in the lungs of control (vehicle-treated) and EIDD-2801-treated (75, 150, 200 or 500 mg/kg, BID) SARS-CoV-2–infected hamsters at day 4 post-infection (pi) are expressed as log_{10} SARS-CoV-2 RNA copies per mg lung tissue. Individual data and median values are presented. (C) Infectious viral loads in the lungs of control (vehicle-treated) and EIDD-2801-treated SARS-CoV-2–infected hamsters at day 4 pi are expressed as log_{10} $TCID_{50}$ per mg lung tissue Individual data and median values are presented. (D) Cumulative severity score from H&E stained slides of lungs from control (vehicle-treated) and EIDD-2801-treated SARS-CoV-2–infected hamsters. Individual data and median values are presented and the dotted line represents the median score of untreated non-infected hamsters. (E) Weight change at day 4 pi in percentage, normalized to the body weight at the time of infection. Bars represent means ± SD. Data were analyzed with the Mann–Whitney U test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns=non-significant. All data (panels B, C, D, E) are from two independent experiments except for the 75 and 500 mg/kg groups with 15, 6, 10, 10, 5 animals for respectively the vehicle, 75, 150, 200 and 500 mg/kg condition.

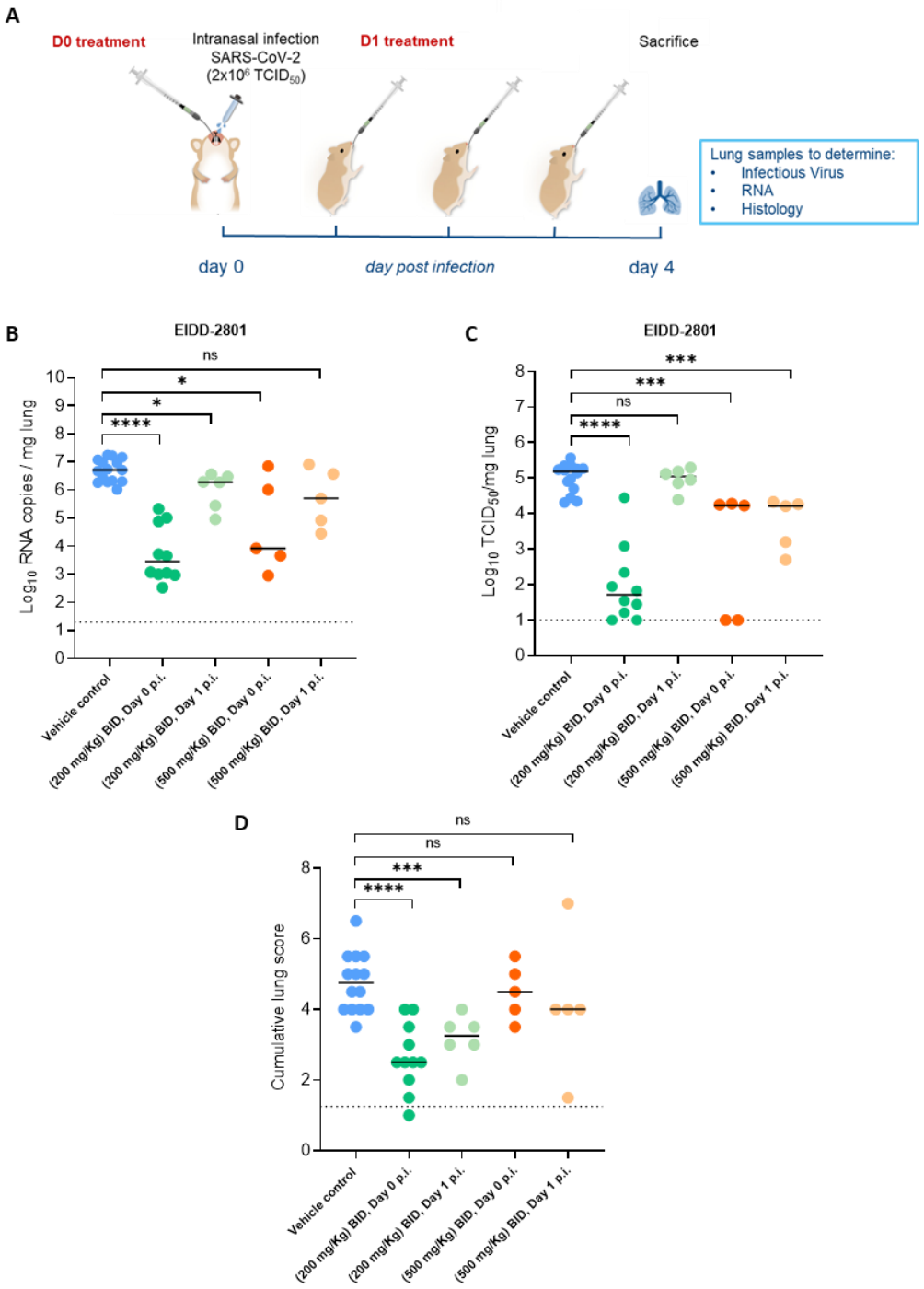

Fig.2. *In vivo* efficacy of EIDD-2801 against SARS-CoV-2 in a post-exposure setting. (A) Set-up of the study. (B) Viral RNA levels in the lungs of control (vehicle-treated) and EIDD-2801-treated (200 or 500 mg/kg, BID starting from day 0 or day 1 post-infection, p.i.) SARS-CoV-2–infected hamsters at day 4 post-infection (pi) are expressed as log10 SARS-CoV-2 RNA copies per mg lung tissue. Individual data and median values are presented. (C) Infectious viral loads in the lungs of control (vehicle-treated) and EIDD-2801-treated SARS-CoV-2–infected hamsters at day 4 pi are expressed as log10 $TCID_{50}$ per mg lung tissue. Individual data and median values are presented. (D) Cumulative severity score from H&E stained slides of lungs from control (vehicle-treated) and EIDD-2801-treated SARS-CoV-2–infected hamsters. Individual data and median values are presented and the dotted line represents the median score of untreated non-infected hamsters. Data were analyzed with the Mann–Whitney U test. *P < 0.05, ***P < 0.001, ****P < 0.0001, ns=non-significant. All data (panels B, C, D) are from one experiment except for the vehicle and 200 mg/kg day 0 p.i. groups with 15, 10, 6, 5, 5 animals for respectively the vehicle, 200 mg/kg day 0 p.i., 200 mg/kg day 1 p.i, 500 mg/kg day 0 p.i and 500 mg/kg day1 p.i. condition.

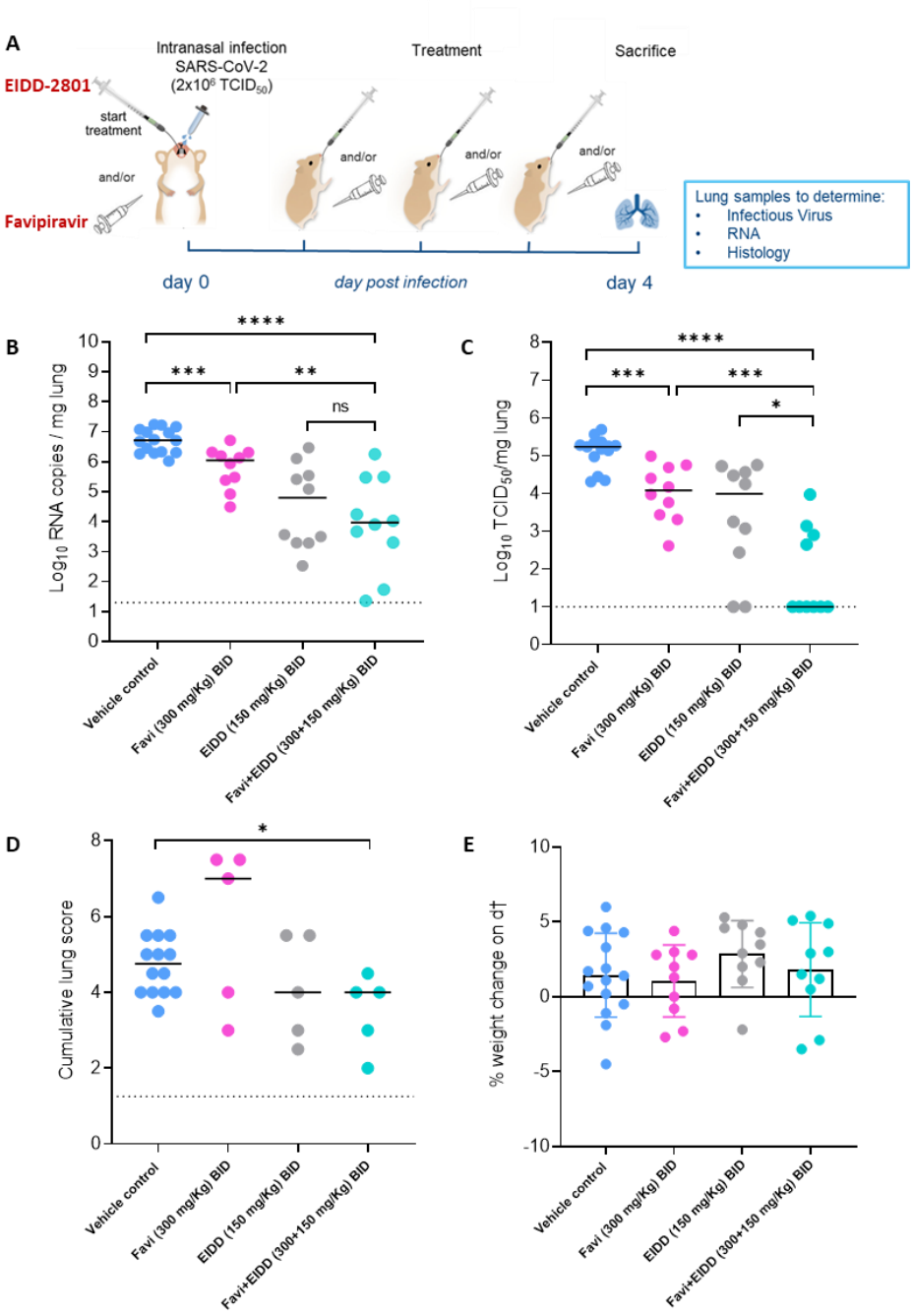

Fig.3. *In vivo* efficacy of combined treatment with favipiravir and EIDD-2801 against SARS-CoV-2. (A) Set-up of the study. (B) Viral RNA levels in the lungs of control (vehicle-treated), favipiravir-treated (300 mg/kg, BID), EIDD-2801-treated (150 mg/kg, BID) and combination-treated (favipiravir+EIDD-2801 at 300+150 mg/kg, BID, respectively) SARS-CoV-2–infected hamsters at day 4 post-infection (pi) are expressed as log_{10} SARS-CoV-2 RNA copies per mg lung tissue. Individual data and median values are presented. (C) Infectious viral loads in the lungs of control (vehicle-treated), favipiravir-treated, EIDD-2801-treated and combination-treated (favipiravir+EIDD-2801) SARS-CoV-2–infected hamsters at day 4 pi are expressed as log_{10} $TCID_{50}$ per mg lung tissue. Individual data and median values are presented. (D) Cumulative severity score from H&E stained slides of lungs from control (vehicle-treated), favipiravir-treated, EIDD-2801-treated and combination-treated (favipiravir+EIDD-2801) SARS-CoV-2–infected hamsters. Individual data and median values are presented and the dotted line represents the median score of untreated non-infected hamsters. (E) Weight change at day 4 pi in percentage, normalized to the body weight at the time of infection. Bars represent means ± SD. Data were analyzed with the Mann–Whitney U test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns=non-significant. Favi=favipiravir, EIDD=EIDD-2801. All data (panels B, C, D) are from two independent experiments with 15, 10, 10, 10 animals for respectively the vehicle, Favipiravir 300 mg/kg, EIDD-2801 150 mg/kg and Favipiravir+EIDD-2801 condition.