The combined treatment of Molnupiravir and Favipiravir results in a marked potentiation of antiviral efficacy in a SARS-CoV-2 hamster infection model

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Abstract

Favipiravir and Molnupiravir, orally available antivirals, have been reported to exert antiviral activity against SARS-CoV2. In recent days preliminary efficacy data have been reported in COVID-19 patients. We here studied the combined antiviral effect of the drugs in the SARS-CoV2 hamster infection model. We first demonstrate that Molnupiravir can reduce infectious virus titers in lungs of infected animals in a dose-dependent manner by up to 3.5 $\log_{10}$ which is associated with a marked improvement of virus-induced lung pathology. When animals are treated with a combination of suboptimal doses of Molnupiravir and Favipiravir (that each alone result in respectively a 1.3 $\log_{10}$ and 1.1 $\log_{10}$ reduction of infectious virus titers in the lungs), a marked combined potency is observed. Infectious virus titers in the lungs of animals treated with the combo are on average reduced by 4.5 $\log_{10}$ and infectious virus are no longer detected in the lungs of 60% of treated infected animals. Both drugs result in an increased mutation frequency of the remaining viral RNA recovered from the lungs. In the combo-treated hamsters an increased frequency of C-to-T and G-to-A mutations in the viral RNA is observed as compared to the single treatment groups which may explain the pronounced antiviral potency of the combination. Our findings may lay the basis for the design of clinical studies to test the efficacy of the combination of Molnupiravir and Favipiravir in the treatment of COVID-19.

Keywords

SARS-CoV-2; Antivirals; Molnupiravir; Favipiravir, hamsters, coronavirus

37 **Main text**

38 The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first identified in Wuhan, China
39 in December 2019 [1]. Since then, the virus rapidly spread around the globe with more than 115 million
40 cases and 2.5 million deaths reported until 3rd March 2021 [www.covid19.who.int]. Infection with
41 SARS-CoV-2 results in coronavirus-induced disease (COVID-19) which is characterized by a wide range
42 of symptoms including fever, dry cough, muscle and/or joint pain, headache, decreased sense of taste
43 and smell and diarrhea. The disease can progress into severe complications such as acute respiratory
44 distress syndrome (ARDS), respiratory failure, septic shock as well as multi-organ failure, which are
45 mainly attributed to a massive cytokine storm and exaggerated immune response [2].

46 To date, there are no approved, selective coronavirus antivirals to treat or prevent infections. Even
47 those vaccinated may not all be protected against infection and disease, in particular following
48 infection with variants that are less susceptible to the current vaccines. Antivirals against SARS-CoV-2
49 will, at least when given early enough after a positive test or after onset of symptoms, reduce the
50 chance to progress to (more) severe disease. In addition, such antiviral drugs will be useful to protect
51 for example healthcare workers and high-risk patients in a prophylactic setting. Such drugs are also
52 needed for the treatment of immunodeficient patients who do not mount a (sufficiently robust)
53 immune response following vaccination. Since the *de novo* development and approval of (a) specific,
54 highly potent antiviral(s) for SARS-CoV-2 may require years, the main focus for COVID-19 treatment in
55 the current pandemic is to repurpose drugs that have been approved or in clinical trials for other
56 diseases [3].

57 We recently demonstrated that the anti-influenza drug Favipiravir results in a pronounced antiviral
58 activity in SARS-CoV-2-infected hamsters [4]. The efficacy of the drug is currently being explored in
59 multiple phase II clinical studies. Interim data from a multicenter, open-labeled study of Avifavir
60 (Favipiravir) in patients with COVID-19, reveals a faster virological response, a shorter time to clinical
61 symptoms and reduced mortality rates compared to patients receiving the Standard of Care (Corritori

62 et al., Science Spotlight™, Conference on Retroviruses and Opportunistic Infections [CROI] 2021). The
63 ribonucleoside analogue, N4-hydroxycytidine (NHC, EIDD-1931), was initially developed as an influenza
64 inhibitor, but exerts also broader-spectrum antiviral activity against multiple viruses belonging to
65 different families of RNA viruses. Activity against SARS-CoV-2 has been reported in cell lines and
66 primary human airway epithelial cell cultures [5].

67 Both Favipiravir and NHC act through lethal mutagenesis. Incorporation into viral RNA results in the
68 accumulation of deleterious transition mutations beyond a permissible error threshold to sustain the
69 virus population, leading to error catastrophe [6,7]. The orally bioavailable, pro-drug counterpart of NHC
70 [8], Molnupiravir (EIDD-2801, MK-4482) was shown to result in an antiviral effect against SARS-CoV-2 in
71 a Syrian hamster model [9], in a mouse model [10] and in ferrets [11]. Data from a first-in-human, phase 1,
72 randomized, double-blind, placebo-controlled study in healthy volunteers indicate that the drug is well
73 tolerated and that plasma exposures exceed the expected efficacious doses based on scaling from
74 animal models [12]. The drug is currently being assessed for its potential as an antiviral treatment of
75 SARS-CoV-2 infection in Phase 2 clinical trials of infected patients (NCT04405570, NCT04405739). Very
76 recently interim data (on one secondary objective) were reported in 202 non-hospitalized adults who
77 had signs or symptoms of COVID-19 and active confirmed SARS-CoV2 infection. At day 5, a reduction
78 in positive viral culture from nasopharyngeal swabs was noted in subjects who received molnupiravir
79 (0/47) as compared to placebo (6/25). No safety signals were identified in the 202 participants (Painter
80 et al., Science Spotlight™, CROI 2021).

81 We here report on the pronounced combined activity of Favipiravir and Molnupiravir in the hamster
82 infection model.

83 **Results**

84 **Dose-response efficacy of Molnupiravir against SARS-CoV-2 in Syrian hamsters**

85 We first evaluated the (dose-response) effect of Molnupiravir (EIDD-2801) in SARS-CoV-2-infected
86 hamsters to select an appropriate dose for the combination study. Briefly, 6-8 weeks female SG

87 hamsters were treated orally with Molnupiravir (either 75, 150, or 200 mg/kg, BID) or the vehicle (i.e.
88 the control group) for four consecutive days starting one hour before intranasal infection with SARS-
89 CoV-2. At day four post-infection (pi), the animals were euthanized and lungs were collected for
90 quantification of viral RNA, infectious virus titers and lung histopathology as described before[4] (Fig.
91 1A). Molnupiravir treatment resulted in a dose-dependent reduction in the viral RNA copies per mg of
92 lung tissue with 1.3 (P=0.002), 1.9 (P<0.0001) and a 3.3 $\log_{10}$ (P<0.0001) reduction was noted in the
93 groups that had been treated BID with 75, 150 or 200 mg/kg, respectively (Fig. 1B). A similar pattern
94 was observed for the infectious virus load in the lungs whereas the intermediate and high doses, but
95 not the 75 mg/kg dose BID, significantly reduced infectious virus lung titers (Fig. 1C). The reduction in
96 infectious virus titers ($TCID_{50}$/mg tissue) in the lungs of hamsters treated BID with 150 and 200 mg/kg
97 was 1.3 (P=0.0002) and 3.5 (P<0.0001) $\log_{10}$, respectively (Fig. 1C). Treatment with 75, 150 and 200
98 mg/kg Molnupiravir BID significantly reduced the histological lung disease score (P=0.0025, P=0.005,
99 P<0.0001, respectively) (Fig. 1D). All the doses studied were well tolerated without significant weight
100 loss or any obvious adverse effects (Fig. 1E).

101 **The combined treatment of Molnupiravir and Favipiravir results in a marked potentiation of efficacy.**

102 Next, we studied what the combined efficacy is of suboptimal doses of Molnupiravir and Favipiravir
103 (Fig. 2A). Treatment with Favipiravir alone (300 mg/kg, BID) reduced viral RNA and infectious virus
104 loads in the lungs of infected animals by 0.7 (P=0.0009) and 1.2 (P=0.0002) $\log_{10}$/mg tissue, respectively
105 (Fig. 2B/C). Treatment with Molnupiravir (150 mg/kg BID) only resulted in 1.9 (P<0.0001) and 1.3
106 (P=0.0002) $\log_{10}$/mg reduction in viral RNA and infectious virus loads respectively. The combined
107 treatment resulted in a reduction of 2.7 $\log_{10}$ viral of lung viral RNA titers (Fig. 2B), but interestingly, in
108 a markedly enhanced reduction in infectious virus titers (4.5 $\log_{10}$ $TCID_{50}$ per mg lung, P=0.02, P=0.0005
109 as compared to Molnupiravir and Favipiravir alone, respectively) (Fig. 2C). Notably, there was no
110 detectable infectious virus in the lungs of six out of ten hamsters in the combined treatment group
111 (Fig. 2C). A marked improvement in the histological lung pathology scores was also observed in the

112 combined treatment group (Fig. 2D) and no significant weight loss or toxicity signs were observed
113 (Supplementary Fig. S1).

114 Molnupiravir is known to increase the mutation frequency of MERS-CoV viral RNA in infected mice [5].
115 To test whether this is also the case in SARS-CoV-2-infected hamsters, we used Illumina deep
116 sequencing to determine the SARS-CoV-2 mutations rate in remaining viral RNA in lung samples of
117 hamsters after treatment. A dose-dependent increase in the mutation count (in particular, C-to-T and
118 G-to-A transitions) in samples from Molnupiravir treated hamsters was observed as compared to the
119 vehicle control group (Supplementary Fig. S2). The Molnupiravir (150mg/kg) + Favipiravir (300 mg/kg)
120 combination resulted in a markedly higher number of C-to-T and G-to-A mutations (68 and 50,
121 respectively), as compared to the single dose groups [150 mg/kg Molnupiravir group (33 and 31,
122 respectively) and 300 mg/kg Favipiravir (14 and 21, respectively)] (Fig. 2E). The C-to-T and G-to-A
123 mutation count in the combination group was also markedly higher than in the highest dose group of
124 Molnupiravir (45 and 39, respectively) (Supplementary Fig. S2). These results may at least partially
125 explain the markedly enhanced reduction in infectious viral loads observed in the combination
126 treatment group.

127 **Discussion**

128 Remdesivir (Veklury), is the first drug to have received FDA approval for use in hospitalised COVID19
129 patients, although the World Health Organisation has issued a conditional recommendation against its
130 use on 20 Nov 2020 (www.who.int). Remdesivir needs to be administrated intravenously which
131 precludes its use in the early stages of the infection/disease or even a prophylactic use. We previously
132 demonstrated that treatment of SARS-CoV-2-infected hamsters with a high doses of Favipiravir largely
133 reduces infectious virus titers in the animals and results as a consequence in a markedly improved lung
134 pathology [4]. Favipiravir is currently being studied for the treatment of COVID-19 in clinical trials in
135 several countries and was very recently reported to result in COVID-19 patients in a faster virological
136 response, a shorter time to clinical symptoms and reduced mortality rates (Corritori et al., Science

137 SpotlightTM, CROI 2021). Also Molnupiravir has been reported to exert therapeutic and prophylactic
138 activity against SARS-CoV-2 in several animal models [9–11]. Importantly very recent interim data from
139 phase II clinical studies in COVID-19 patients, revealed a reduction in the time required to reach
140 negative isolation of infectious virus from the nasopharyngeal swabs from participants with
141 symptomatic SARS-CoV-2 infection (Painter et al., Science Spotlight^{TM}, CROI 2021). Both Molnupiravir
142 and Favipiravir have a high barrier to resistance, resistant variants have a loss in fitness and induce
143 lethal viral mutagenesis [6,7].

144 We here demonstrate that the combination of suboptimal doses of Molnupiravir and Favipiravir results
145 in a marked antiviral activity in the hamster model. Infectious virus titers were reduced to undetectable
146 levels in 6 out of 10 treated animals. A median reduction of 4.5 $\log_{10}$ $TCID_{50}$/mg lung tissue was
147 achieved, which is markedly more pronounced than what could be expected from an additive activity
148 of either Molnupiravir (1.3 $\log_{10}$) or Favipiravir (1.1 $\log_{10}$) when dosed alone. This pronounced efficacy
149 of the combination may partially or even entirely be explained by the increased total mutation count
150 in viral RNA collected from the lungs of combo-treated hamsters as compared to the single treatment
151 groups.

152 In conclusion, the combination of Molnupiravir and Favipiravir (two oral drugs with a high barrier to
153 resistance for which there is very recent initial evidence that they exert antiviral activity in COVID-19
154 patients), is particularly effective in the treatment of SARS-CoV2 infections in hamsters. This efficacy
155 may be explained by an enhanced accumulation of mutations as compared to monotherapy. Our
156 findings may lay the basis for the design of clinical studies to test the efficacy of the combination of
157 Molnupiravir and Favipiravir in the treatment of COVID-19.

158

159 **Methods**

160 **SARS-CoV-2**

161 The SARS-CoV-2 strain used in this study, BetaCov/Belgium/GHB-03021/2020 (EPI ISL 109
162 407976|2020-02-03), was recovered from a nasopharyngeal swab taken from an RT-qPCR confirmed
163 asymptomatic patient who returned from Wuhan, China in the beginning of February 2020. A close
164 relation with the prototypic Wuhan-Hu-1 2019-nCoV (GenBank accession 112 number MN908947.3)
165 strain was confirmed by phylogenetic analysis. Infectious virus was isolated by serial passaging on
166 HuH7 and Vero E6 cells [4]; passage 6 virus was used for the study described here. Live virus-related
167 work was conducted in the high-containment A3 and BSL3+ facilities of the KU Leuven Rega Institute
168 (3CAPS) under licenses AMV 30112018 SBB 219 2018 0892 and AMV 23102017 SBB 219 20170589
169 according to institutional guidelines.

170 **Cells**

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

175 **Compounds**

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

181 **SARS-CoV-2 infection model in hamsters**

182 The hamster infection model of SARS-CoV-2 has been described before [4,13]. Housing conditions and
183 experimental procedures were approved by the ethics committee of animal experimentation of KU
184 Leuven (license P065-2020). For infection, female hamsters of 6-8 weeks old were anesthetized with
185 ketamine/xylazine/atropine and inoculated intranasally with 50 µL containing 2×10^6 TCID50 SARS-CoV-
186 2 (day 0).

187 **Treatment regimen**

188 For dose-response treatment, animals were treated twice daily with 75, 150 or 200 mg/kg of EIDD-
189 2801 by oral gavage just before infection with SARS-CoV-2. For combination therapy, hamsters were
190 treated from day0 with 150 mg/kg EIDD-2801 (oral gavage) and 300 mg/kg Favipiravir (intraperitoneal,
191 i.p.) twice daily. All the treatments continued until day 3 pi. Hamsters were monitored for appearance,
192 behavior and weight. At day 4 pi, hamsters were euthanized by i.p. injection of 500 µL Dolethal
193 (200mg/mL sodium pentobarbital, Vétoquinol SA). Lungs were collected and viral RNA and infectious
194 virus were quantified by RT-qPCR and end-point virus titration, respectively.

195 **SARS-CoV-2 RT-qPCR**

196 Hamster lung tissues were collected after sacrifice and were homogenized using bead disruption
197 (Precellys) in 350 µL TRK lysis buffer (E.Z.N.A.® Total RNA Kit, Omega Bio-tek) and centrifuged (10.000
198 rpm, 5 min) to pellet the cell debris. RNA was extracted according to the manufacturer's instructions.
199 RT-qPCR was performed on a LightCycler96 platform (Roche) as described before [4].

200 **End-point virus titrations**

201 Lung tissues were homogenized using bead disruption (Precellys) in 350 µL minimal essential medium
202 and centrifuged (10,000 rpm, 5min, 4°C) to pellet the cell debris. To quantify infectious SARS-CoV-2
203 particles, endpoint titrations were performed as described before [4].

204 **Histology**

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

211 **Deep sequencing and analysis of whole genome sequences**

212 Genomic sequences from all samples were obtained using SureSelect^{XT} target enrichment and Illumina
213 sequencing. Reads generated were trimmed with Trim Galore
214 (https://github.com/FelixKrueger/TrimGalore). Duplicated reads were removed using Picard
215 (http://broadinstitute.github.io/picard). Reads from the inoculation sample were mapped to the SARS-
216 CoV-2 reference genome (NC_045512) from GenBank using BWA-MEM [14]. The mapping quality was
217 checked using Qualimap and the consensus whole genome sequence was generated using QUASR [15,16].
218 Reads from the lung samples were mapped to this unique reference sequence. Genomes with less than
219 less than a 100 read depth were excluded. Variants above 1% and with a minimum of 2 supporting
220 reads per strand were identified at sites with a read depth of ≥ 10 using VarScan [17].

221 **Statistics**

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

225 **Data Availability**

226 All of the data generated or analysed during this study are included in this published article.

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Author Contributions

R.A., C.S.F. and J.N. designed the studies; R.A., C.S.F., S.J.F.K., X.Z., J.B., J.P. and L.L. performed the studies ; R.A., C.S.F., J.B, J.P. and B.W. analyzed data; D.J. and 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., R.W. and J.N. supervised the study; L.V., L.C., P.L., J.N. and K.D. acquired funding.

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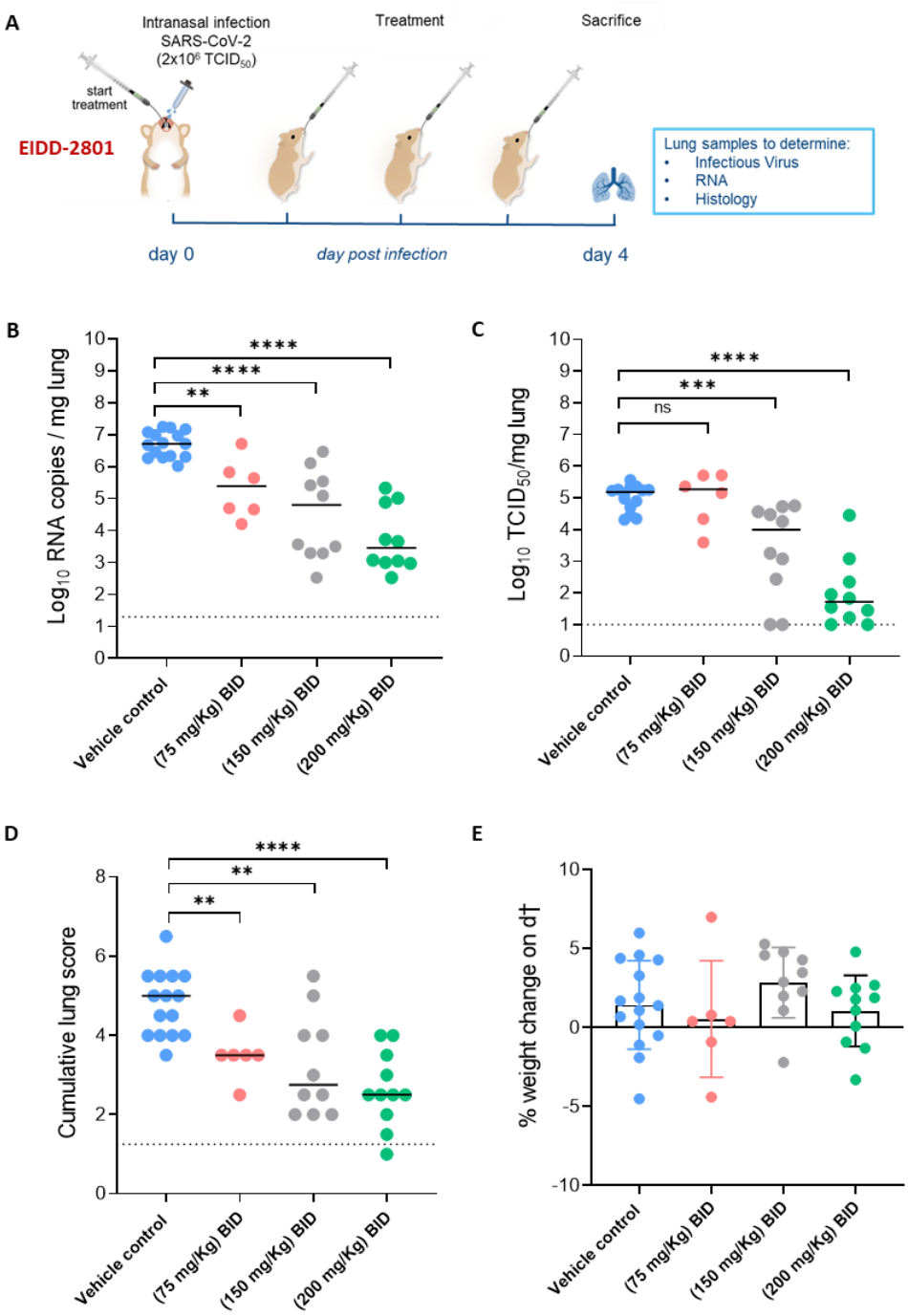


Fig.1. Dose-response efficacy of Molnupiravir (EIDD-2801) against SARS-CoV-2 in a hamster infection model. (A) Set-up of the study. (B) Viral RNA levels in the lungs of control (vehicle-treated) and EIDD-2801-treated (75, 150 or 200 mg/kg, BID) SARS-CoV-2–infected hamsters at day 4 post-infection (pi) 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 mg/kg group. The number of animals were 15, 6, 10, and 10 for respectively the vehicle 75, 150, and 200 mg/kg condition.

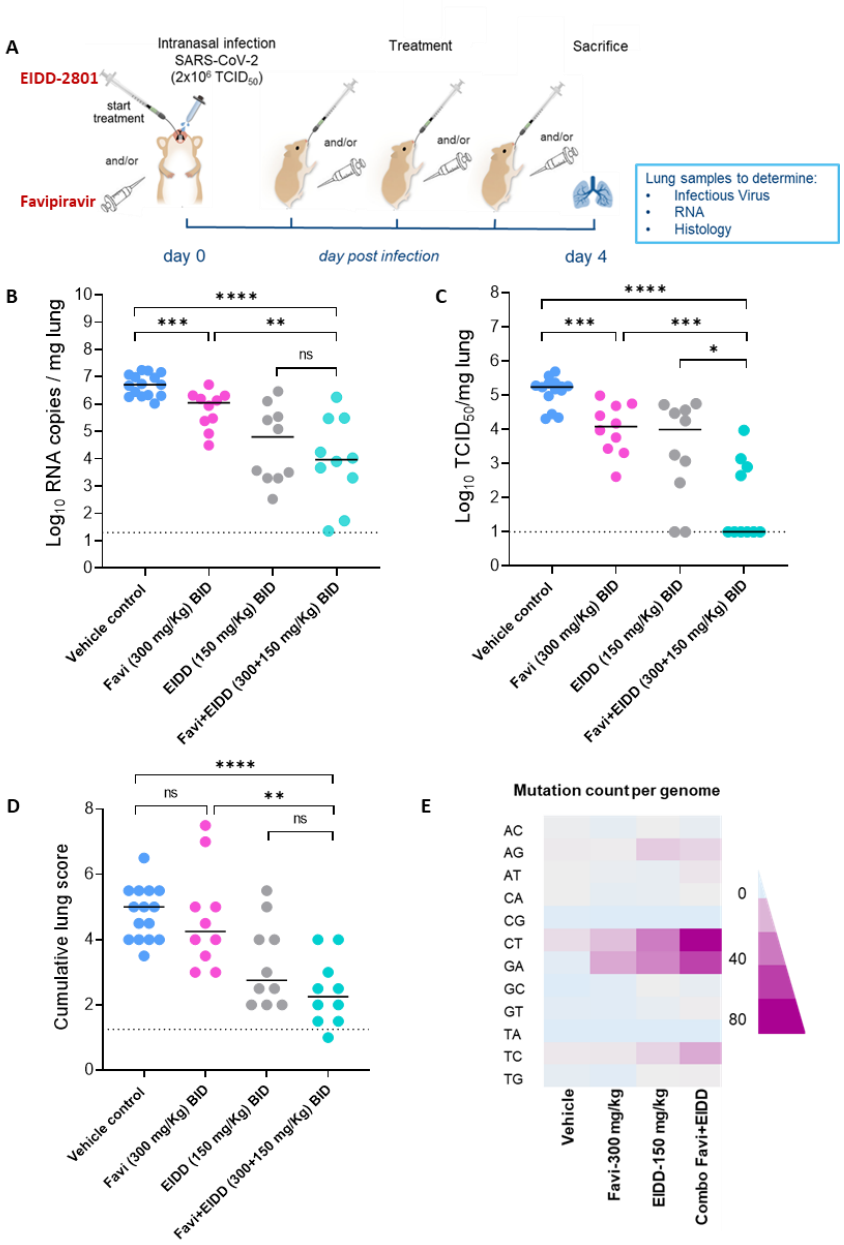
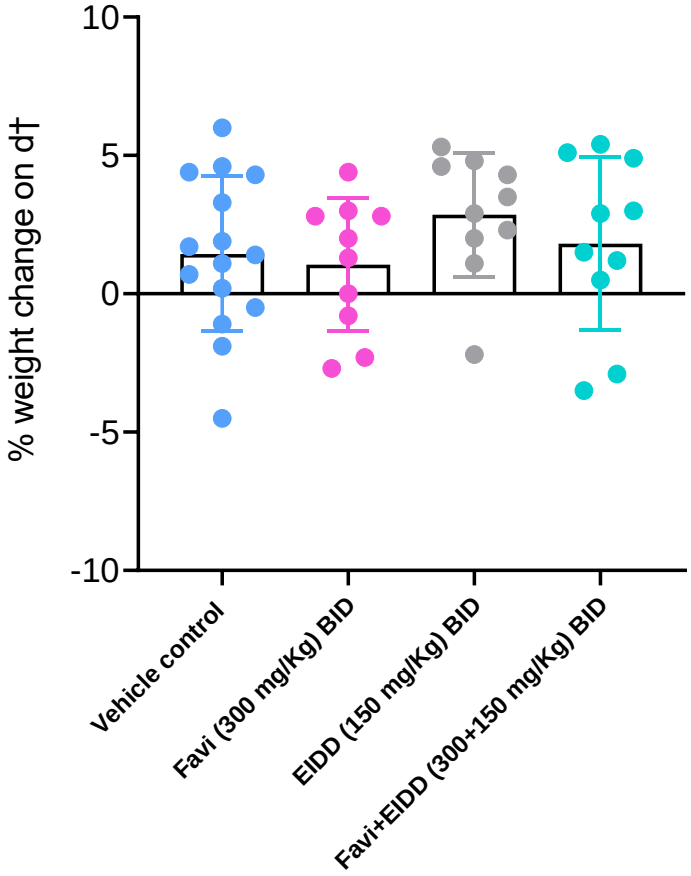


Fig.2. Combined efficacy of Favipiravir and Molnupiravir (EIDD-2801) against SARS-CoV-2 in a hamster infection model. (A) Set-up of the study. (B) Viral RNA levels in the lungs of control (vehicle-treated), Favipiravir-treated (300 mg/kg, BID), EIDD-2801-treated (150 mg/kg, BID) and combination-treated (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) Mean mutation count (per the whole genome) in the viral RNA isolated from 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). 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 and 10 animals for respectively the vehicle, Favipiravir 300 mg/kg, EIDD-2801 150 mg/kg and Favipiravir+EIDD-2801 condition.



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323 **Fig. S1. Tolerability of combined treatment with Favipiravir and EIDD-2801 in SARS-CoV-2-infected hamsters.**
324 Weight change at day 4 post-infection in percentage, normalized to the body weight at the time of infection.
325 Bars represent means ± SD.

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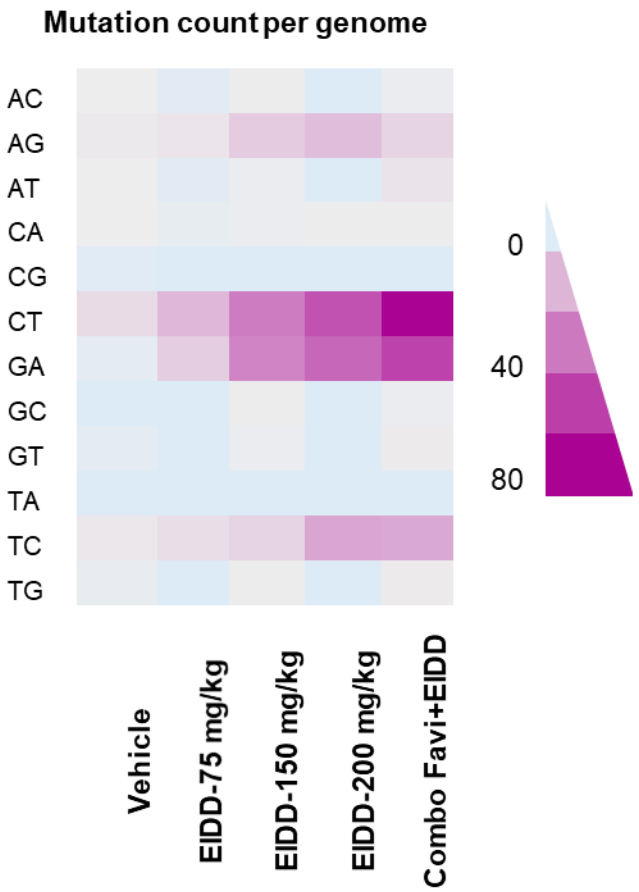

Fig. S2. Dose-dependent increase in mutation count in SARS-CoV-2 viral RNA by Molnupiravir (EIDD-2801). Mean mutation count (per the whole genome) in the viral RNA isolated from the lungs of control (vehicle-treated), EIDD-2801-treated (75, 150 or 200 mg/kg, BID) and combination-treated (Favipiravir+EIDD-2801 at 300+150 mg/kg, BID, respectively) SARS-CoV-2–infected hamsters at day 4 post-infection (pi).