Nelfinavir markedly improves lung pathology in SARS-CoV-2-infected Syrian hamsters despite a lack of an antiviral effect

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Author Contributions

C.S.F., R.A., L.D., J.R.P. and J.N. designed research; C.S.F., R.A., S.J.F.K., S.T.H. R.M. and X.Z. performed research; P.A., L.D , J.R.P. and A.K.C. provided advice on the study design: C.S.F., R.A., and B.W. analyzed data; D.J., B.W. and J.N. provided advice on the interpretation of data; C.S.F., R.A.,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; C.S.F., R.A.,and J.N. supervised the study; L.V., J.N., L.C., P.L. and K.D. acquired funding.

Abstract

In response to the ongoing COVID-19 pandemic, repurposing of drugs for the treatment of SARS-CoV-2 infections is being explored. The FDA-approved HIV protease inhibitor Nelfinavir is one of the drugs that has been reported to inhibit *in vitro* SARS-CoV2 replication. We here report on the effect of Nelfinavir in the Syrian hamster SARS-CoV-2 infection model. Although treatment of infected hamsters with either 15 mg/kg BID or 50 mg/kg BID Nelfinavir [for four consecutive days, initiated on the day of infection] did not reduce viral RNA loads nor infectious virus titres in the lungs (as compared to the vehicle control at the end of treatment) the drug markedly improved virus-induced lung pathology at doses that were well tolerated. Yet, a massive interstitial infiltration of neutrophils was observed in the lungs of treated (infected and uninfected) animals. The protective effect of Nelfinavir on SARS-CoV-2-induced lung pathology that is unrelated to an antiviral effect warrants further exploration in the context of the treatment of COVID-19.

Keywords

COVID-19; SARS-CoV-2; Antivirals; Nelfinavir; preclinical model.

40 **Main text**

41 The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) resulted in more than 2.0 million
42 deaths within the time-span of a year after its discovery (1). SARS-CoV-2-induced COVID-19 is mainly
43 an inflammatory disease that displays a variety of symptoms including fever, dry cough, muscle
44 weakness, loss of sense of taste and smell, and diarrhea (2). In some patients, the disease is
45 accompanied with a massive cytokine storm and exaggerated immune response, resulting in severe
46 complications such as acute respiratory distress syndrome (ARDS), respiratory failure, septic shock and
47 multi-organ failure (2). There are no specific coronavirus antiviral drugs available to treat or prevent
48 infections with human coronaviruses. Since the *de novo* development of (a) potent specific antiviral(s)
49 for SARS-CoV-2 will take several years, repurposing of clinically approved/evaluated drugs is currently
50 adopted for rapid identification of treatment for COVID-19 (3).

51 Nelfinavir mesylate (Viracept) is an FDA-approved HIV-protease inhibitor (PI) (4). By inhibiting the HIV-
52 1 protease, it prevents the cleavage of viral Gag and Gag-Pol polyproteins, resulting in immature and
53 non-infectious virus particles (5,6). Nelfinavir has been reported to result in some *in vitro* inhibitory
54 activity against SARS-CoV-2. Additionally, inhibition of SARS-CoV-2 Spike glycoprotein mediated cell-
55 fusion has been reported (7–9) and *in silico* docking studies predict binding of Nelfinavir for the
56 catalytic site of the SARS-CoV-2 main protease, M^{pro} (10–12).

57 In this study, we wanted to assess whether Nelfinavir results in antiviral effect against SARS-CoV-2 in
58 Syrian hamsters, which are highly susceptible to infection with the virus. High viral titers are reached
59 in the lung upon infection and the animals develop of COVID-like lung pathology (13,14). The model is
60 well-suited to assess the impact of antiviral drugs as we and others have demonstrated. Notably, we
61 have demonstrated that Favipiravir and Molnupiravir (EIDD-2801) markedly reduce SARS-CoV-2
62 replication in hamsters, whereas hydroxychloroquine does not (14,15).

63 Six to eight weeks female Syrian Golden hamsters were treated with Nelfinavir (15 mg/kg/dose or 50
64 mg/kg/dose) or the vehicle control by intraperitoneal injection (IP) within one hour before intranasal

65 infection with 50 µl containing $2x10^6$ $TCID_{50}$ of SARS-CoV-2 [BetaCov/Belgium/GHB-03021/2020 (EPI
66 ISL 109 72 407976|2020-02-03)] (Fig. 1A). The drug as well as the vehicle control were administered
67 twice daily for four consecutive days. At day four post-infection, lungs were collected at end-point
68 sacrifice (day 4 p.i) for viral RNA load and infectious virus titres quantification as well as lung
69 histopathology as previously described (14). Groups treated with either 15 mg/kg BID or 50 mg/kg BID
70 Nelfinavir had comparable levels of lung viral RNA load and infectious virus titres as the vehicle control
71 group (Fig. 1B, C), indicating a lack of *in vivo* antiviral efficacy of Nelfinavir in this model. Hamsters in
72 the control and 15 mg/kg BID Nelfinavir-treated groups maintained relatively stable weights
73 throughout the study, whereas a general weight loss of up to 10% was observed in the 50 mg/kg BID
74 Nelfinavir-treated group as compared to the vehicle group at 0 dpi (Fig. 1D). This weight loss can be
75 attributed to the combination of SARS-CoV-2 infection and 50 mg/kg BID Nelfinavir treatment, as this
76 dose alone did not affect the weight of two uninfected hamsters (data not shown). No other obvious
77 adverse effects were observed in either of the treatment groups. Remarkedly and intriguingly, in
78 infected animals treated with the 50 mg/kg BID Nelfinavir dose, a significant three-fold reduction in
79 the overall histological disease score compared to the placebo control was noted. In fact, the
80 histopathology score in the treated group was more or less comparable to the baseline score in
81 untreated non-infected hamsters (median score of 1.25) (Fig. 2A). Yet, despite this remarkable
82 beneficial effect on lung pathology, an unusual interstitial infiltration of neutrophils was noted in the
83 treated animals (Fig. 2A, 2B). Two uninfected hamsters treated with 50 mg/kg Nelfinavir had
84 histopathology scores at the baseline of 1 but presented as well with neutrophil infiltration (data not
85 shown), confirming that these intriguing observations are indeed attributed to Nelfinavir treatment.

86 To confirm the exposure of treated hamsters to Nelfinavir in the group treated with 50 mg/kg BID,
87 plasma levels of Nelfinavir were determined at the time of sacrifice (16h after the last treatment),
88 whereby average plasma concentrations of 174 nM were observed.

89 Taken together, although Nelfinavir does not reduce viral load in the lungs of SARS-CoV-2-infected
90 hamsters, it markedly improves lung pathology despite a massive infiltration of neutrophils.

91 The lack of antiviral efficacy observed here is consistent with preliminary data reported in another
92 study in which Nelfinavir was dosed *per os* at a dose of 250 mg/kg/day BID (boosted with Ritonavir) for
93 two consecutive days followed by sacrifice on day 3 post-infection (Nougairede et al., 2020 – interim
94 report). No significant reduction of viral RNA or infectious virus titers was observed in the lungs of
95 hamsters in this study, but no histopathological analysis was reported.

96 An improvement of CD4+ T cell counts and clinical status has been reported in HIV patients treated
97 with PIs (such as indinavir, ritonavir, indinavir, saquinavir, nelfinavir) that did not result in a reduction
98 of viral loads (16–21). This has been attributed to the fact that apart from their antiviral activity, PIs
99 exert multiple effects on various cellular processes, including apoptosis and immune cell functions (22–
100 24). In particular, Nelfinavir was reported to markedly inhibit apoptosis of neutrophils and neutrophil
101 function through inhibition of calpain (25). These findings, made in the context of HIV, are consistent
102 with our observations in 50 mg/kg Nelfinavir-treated SARS-CoV-2-infected hamsters. As part of Highly
103 Active Antiretroviral Therapy (HAART) treatment, Nelfinavir is dosed at 1250 mg BID or 750 mg TID,
104 resulting in a C_{trough} of about 370 nM measured after 12 h in patients (26). The Nelfinavir C_{trough} plasma
105 concentrations that are obtained here in hamsters with a 50 mg/kg dose are therefore in a clinically
106 relevant range.

107 Neutrophils extracellular traps (NETs) and matrix metalloproteinase-9 (MMP-9), which is a neutrophil-
108 released enzyme, are significantly increased in COVID-19 patients (27). Moreover, the activation of
109 NETs in COVID-19 patients was reported to correlate with disease severity and to be associated with
110 thrombo-inflammation events (27,28). Consequently, the inhibition of neutrophil function by
111 Nelfinavir and hence downstream immune signalling cascades may contribute to the observed
112 reduction of SARS-CoV-2-induced pathology as observed in hamsters. Furthermore, the reduction of

113 neutrophil apoptosis by the compound may account for the unusually large neutrophil infiltration
114 observed in the lung interstitium in treated hamsters.

115 Akin to its effect in HIV-infected patients, Nelfinavir may possibly result in a protective effect that is
116 unrelated an antiviral effect. It is proposed that the potential beneficial effect of Nelfinavir, in particular
117 in cases where "cytokine storms" develop, be explored. In such a case that the drug would also protect
118 patients against lung damage, combination therapy with directly-acting antivirals against SARS-CoV-2
119 may also be considered.

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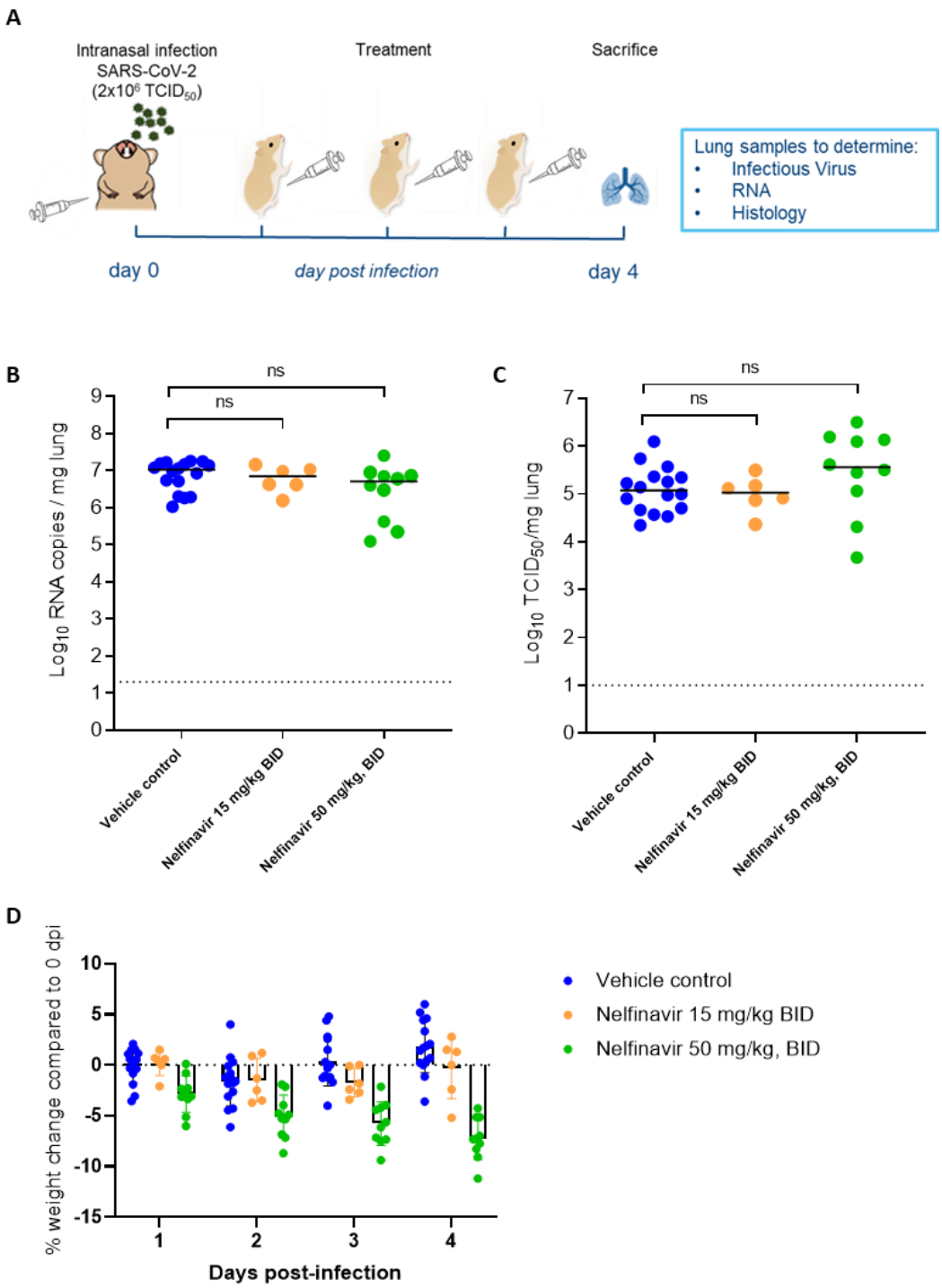
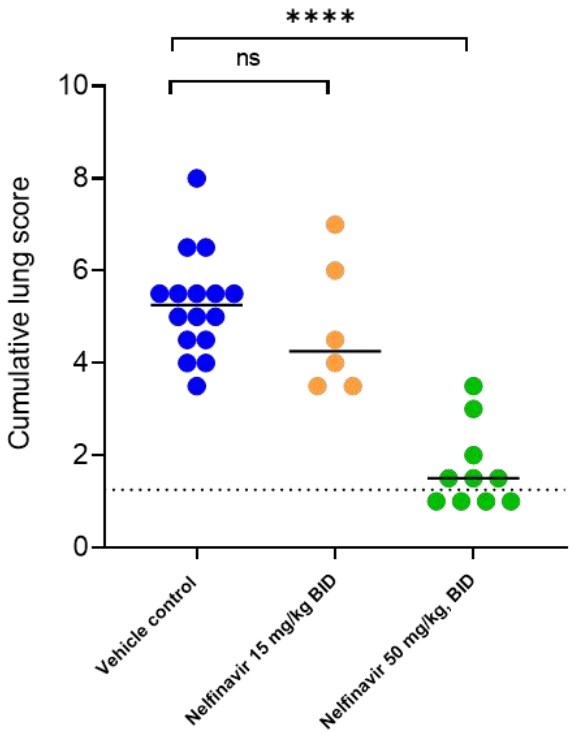


Fig.1 Nelfinavir does not reduce viral replication in SARS-CoV-2 infected hamsters (A) Set-up of the study. (B) Viral RNA levels in the lungs of hamsters treated with vehicle (control), 15 mg/kg Nelfinavir BID, or 50 mg/kg Nelfinavir BID at 4 dpi expressed as $\log_{10}$ SARS-CoV-2 RNA copies per mg lung tissue. Individual data and median values are presented. (C) Infectious virus titers in the lungs of hamsters treated with vehicle (control), 15 mg/kg Nelfinavir BID, or 50 mg/kg Nelfinavir BID at 4 dpi expressed as $\log_{10}$ $TCID_{50}$ per mg lung tissue. Individual data and median values are presented. (D) Daily weight change in percentage, normalized to the body weight at the time of infection. Bars represent average ± SD. All data (panels B, C, D) are from one to two independent experiments with a total of 16, 6, and 10 animals for the placebo, 15 mg/kg, and 50 mg/kg conditions respectively. Data were analyzed with the Mann-Whitney U test. ns, non-significant.

218

A



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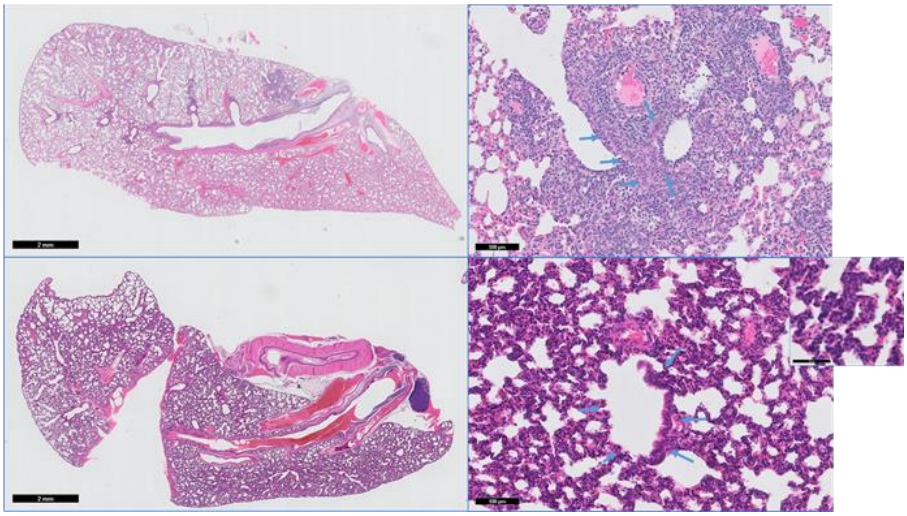

219

220 **Fig.2 Nelfinavir markedly improves lung pathology of SARS-CoV-2-infected Syrian hamsters despite**
221 **interstitial neutrophil infiltration.** (A) Cumulative severity score from H&E-stained slides of lungs from
222 vehicle control and Nelfinavir (50 mg/kg BID)-treated SARS-CoV-2-infected hamsters at 4 dpi.
223 Individual datapoints and lines indicating median values are presented. The dotted line represents the
224 median lung score in healthy untreated non-infected animals. Data were analyzed using the Mann-
225 Whitney U test. ****P<0.0001. (B) Representative H&E-stained slides of lungs from vehicle control
226 (top panels) and Nelfinavir-treated (bottom panels) SARS-CoV-2-infected hamsters at 4 dpi. Top, left:
227 peri-bronchial inflammation and bronchopneumonia in vehicle-treated hamsters. Top, right: higher
228 magnification of bronchopneumonia centred on a bronchiol (blue arrows) in vehicle-treated hamsters.
229 Bottom, left: dark blue-stained interstitium and bronchial structures appearing normal without
230 inflammation in Nelfinavir-treated hamsters. Bottom, right: severe interstitial inflammation with
231 mostly PMN and congestion. Blue arrows indicate bronchiol (Insert at higher magnification).