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Multidrug treatment with nelfinavir and cepharanthine against COVID-19

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45 **Summary**

46 Antiviral treatments targeting the emerging coronavirus disease 2019 (COVID-19) are urgently required.
47 We screened a panel of already-approved drugs in a cell culture model of severe acute respiratory
48 syndrome coronavirus 2 (SARS-CoV-2) and identified two new antiviral agents: the HIV protease inhibitor
49 Nelfinavir and the anti-inflammatory drug Cepharranthine. *In silico* modeling shows Nelfinavir binds the
50 SARS-CoV-2 main protease consistent with its inhibition of viral replication, whilst Cepharranthine inhibits
51 viral attachment and entry into cells. Consistent with their different modes of action, *in vitro* assays
52 highlight a synergistic effect of this combined treatment to limit SARS-CoV-2 proliferation. Mathematical
53 modeling *in vitro* antiviral activity coupled with the known pharmacokinetics for these drugs predicts that
54 Nelfinavir will facilitate viral clearance. Combining Nelfinavir/Cepharranthine enhanced their predicted
55 efficacy to control viral proliferation, to ameliorate both the progression of disease and risk of transmission.
56 In summary, this study identifies a new multidrug combination treatment for COVID-19.

57

58

59 **Introduction**

60 The novel coronavirus infectious disease 2019 (COVID-19), caused by the severe acute respiratory
61 syndrome coronavirus 2 (SARS-CoV-2), is a global public health problem that is impacting social and
62 economic damage worldwide (Huang et al., 2020; Zhou et al., 2020; Zhu et al., 2020). As of April 12,
63 2020, 1,696,588 confirmed cases with 105,952 deaths were reported across 213
64 countries/areas/territories (WHO). COVID-19 was characterized as a pandemic by the World Health
65 Organization (WHO), however, there is no approved treatment. Several drugs have been evaluated in
66 COVID-19 patients in clinical trials: including Lopinavir (LPV) and Ritonavir, Chloroquine (CLQ),
67 Favipiravir (FPV), and Interferon, all repurposed FDA-approved drugs, together with Remdesivir (RDV),
68 an antiviral agent that awaits clinical approval (Cao et al., 2020; Dong et al., 2020; Touret and de
69 Lamballerie, 2020). The clinical efficacies of these drugs are expected shortly, however, additional
70 treatment options are urgently needed.

71 In this study, we screened a panel of FDA/EMA/PMDA-approved drugs in a SARS-CoV-2 infection cell
72 culture assay and identified two, Nelfinavir (NFV) and Cepharranthine (CEP), that show more potent
73 antiviral activity in this *in vitro* screen compared to drugs currently being trialed. Our screen shows that
74 both NFV and CEP inhibit SARS-CoV-2 at concentrations that can be achieved in the clinic and their
75 different modes of action provide an exciting opportunity for combined multidrug treatment against
76 COVID-19.

77
78

79 **Results**

80 **Anti-SARS-CoV-2 activity of Nelfinavir and Cepharranthine.**

81 We established a cell-based drug screening system to identify compounds that protect cells from
82 SARS-CoV-2-induced cytopathology (Fig. 1A): VeroE6/TMPRSS2 cells were treated with compounds for
83 1 h during inoculation with a clinical isolate of SARS-CoV-2 (Matsuyama et al., 2020) at a multiplicity of
84 infection (MOI) of 0.01. Unbound virus was removed by washing and the cells treated with compounds
85 for 48 h to assess cell viability (Fig. 1A) (Methods). SARS-CoV-2 replication in VeroE6/TMPRSS2
86 induced a cytopathic effect and to validate our assay we show that two compounds, LPV and CLQ, that
87 were reported to inhibit SARS-CoV-2 infection (Wang, M. et al., 2020), reduced virus-induced
88 cytopathicity (Fig. 1B, compare b and c, d).

89 After screening 306 FDA/EMA/PMDA-approved drugs, we identified compounds that protected cell
90 viability by 20-fold compared with a DMSO solvent control (Methods) (Supplementary Table S1). Among
91 these, we selected to study NFV and CEP as candidates showing the greatest anti-cytopathic activity
92 (Fig. 1B, f and g). NFV targets human immunodeficiency virus (HIV) protease and CEP is a Stephania-
93 derived alkaloid extract having anti-inflammatory and anti-oxidative activities (Bailly, 2019; Kao et al.,
94 2015; Markowitz et al., 1998). To confirm and extend these observations we assessed SARS-CoV-2
95 encoded N protein expression 24 h post-inoculation by immunofluorescence (Fig. 1C, red) and

96 immunoblotting (Fig. 1D). Both NFV and CEP significantly reduced N protein expression, confirming
97 these compounds inhibit SARS-CoV-2 proliferation. To quantify their anti-SARS-CoV-2 activity, we
98 treated cells with a range of drug concentrations and measured secreted viral RNA 24 h post-infection.
99 NFV and CEP, together with CLQ and LPV, significantly reduced viral RNA levels in a dose-dependent
100 manner to 0.001 ~ 0.01% of the untreated control infections (Fig. 1E). FPV showed negligible antiviral
101 activity against SARS-CoV-2, consistent with previous reports (Choy et al., 2020; Wang, M. et al., 2020).
102 In parallel we assessed cell viability and noted cell death at high drug concentrations up to 64 μ M (Fig.
103 1F). The concentration of drugs required to inhibit 50% (IC₅₀) or 90% (IC₉₀) of virus replication along
104 with their 50% cytotoxicity (CC₅₀) are listed in Fig. 1E and F. These experiments highlight a > 70-fold
105 window (CC₅₀/IC₅₀) where NFV and CEP can inhibit SARS-CoV-2 proliferation with minimal toxicity.

106 We previously reported a method to quantify instantaneous inhibitory potential (IIP) (Koizumi et al.,
107 2017) and imply that NFV and CEP will have higher antiviral potentials than LPV and CLQ, respectively
108 (Supplementary Note, Supplementary Table S2).

109

110 **Modes of action of Nelfinavir and Cepharranthine.**

111 To define how these compounds impact on the viral replicative life cycle, we performed a time of
112 addition assay (Fig. 2A). We measured the antiviral activity of drugs added at different times: (a) present
113 during the 1 h virus inoculation step and maintained throughout the 24 h infection period (**whole life**
114 **cycle**); (b) present during the 1 h virus inoculation step and for an additional 2 h and then removed
115 (**entry**); or (c) added after the inoculation step and present for the remaining 22 h of infection (**post-entry**).
116 CLQ, a known modulator of intracellular pH that non-specifically inhibits virus entry (Akpovwa, 2016), was
117 recently reported to inhibit SARS-CoV-2 (Liu, J. et al., 2020; Wang, M. et al., 2020) and we confirmed its
118 activity in the early stages of infection (Fig. 2B, lane 8). RDV was previously reported to inhibit the
119 process for intracellular viral replication (Wang, M. et al., 2020) and we confirmed this mode of action
120 showing a reduction in viral RNA levels with a negligible effect on virus entry (Fig. 2B, lane 5).

121 This assay identified NFV as a replication inhibitor whilst CEP targeted the virus entry phase (Fig. 2B,
122 lanes 10-15). These data are consistent with reports that LPV and NFV inhibited the replication of other
123 coronaviruses, SARS-CoV (Liu et al., 2005; Wu et al., 2004; Yamamoto et al., 2004), and that CEP
124 reduced the entry of human coronavirus OC43 (Kim et al., 2019).

125 Since NFV binds the HIV-1 protease we used an *in silico* docking simulation to assess its potential
126 interaction with the SARS-CoV-2 encoded main protease (Fig. 2C). NFV was ranked in the top 1.5% of
127 compounds following an *in silico* screen of the SARS-CoV-2 encoded main protease (see Methods) (Fig.
128 2C, cyan stick: NFV, green: main protease). Our docking model predicts that NFV interacts with the
129 SARS-CoV-2 protease active site pocket and would block the recruitment of substrates.

130 To investigate whether CEP inhibits SARS-CoV-2 particle attachment or internalization into cells, we
131 established an assay to measure viral attachment to cells by pre-chilling to inhibit particle endocytosis.
132 Cell-bound virus particles are measured by qPCR of viral RNA. Viruses frequently exploit cellular

133 heparan sulfate proteoglycans to initiate low affinity attachment and heparin shows broad-spectrum
134 inhibition of virus-cell attachment (De Clercq, 1998; Lang et al., 2011). Unsurprisingly, heparin blocked
135 SARS-CoV-2 particle attachment to VeroE6/TMPRSS2 cells (Fig. 2D). We demonstrate that CEP
136 significantly inhibited SARS-CoV-2 attachment to cells, whereas CLQ that targets intracellular trafficking
137 pathways (Liu, J. et al., 2020) had no effect (Fig. 2D). *In silico* docking simulation confirms that CEP
138 molecule (a major component of the pharmaceutical preparation of CEP) can bind to SARS-CoV-2 Spike
139 protein and interfere with the Spike engagement to its receptor, angiotensin-converting enzyme 2 (ACE2)
140 (Lan et al., 2020; Walls et al., 2020; Wang, Q. et al., 2020) (Fig. 2E, green stick: CEP molecule, orange:
141 Spike, semi-transparent cyan: ACE2). These data highlight a new role for CEP to inhibit SARS-CoV-2
142 particle attachment to cells.

143

144 **Nelfinavir and Cepharranthine show synergistic antiviral activity.**

145 NFV showed a modest increase in antiviral activity (IC_{90} of 1.18 μ M) compared to LPV (IC_{90} of 3.61
146 μ M), similarly CEP (IC_{90} of 0.91 μ M) showed greater antiviral activity than CLQ (IC_{90} of 3.97 μ M).
147 Importantly, both NFV and CEP show anti-SARS-CoV-2 activity within the concentration ranges achieved
148 in patients, where the C_{max} of both drugs are 6.9 and 2.3 μ M (by administration of 500 mg NFV orally and
149 of 100 mg CEP by intravenous injection) respectively (Markowitz et al., 1998; Yasuda et al., 1989).
150 Since NFV and CEP have different mode of actions, we examined their potential for synergistic effects.
151 Antiviral activity and cell viability were determined by qPCR enumeration of viral RNA and MTT activity,
152 respectively, following treatment with each compound alone or in combination (Fig. 3). For these
153 experiments, we infected cells with lower amounts of SARS-CoV-2 (MOI=0.001) than used in our earlier
154 drug screen. Single treatment with NFV or CEP reduced viral RNA in a dose-dependent manner and
155 co-treatment further reduced viral RNA levels (Fig. 3A): e.g. NFV (2.24 μ M) or CEP (3.20 μ M) alone
156 reduced viral RNA to 5.8% and 6.3% of untreated control, respectively, however, their combination
157 reduced viral RNA level to 0.068%. Higher doses of the NFV/CEP combined treatment (4 μ M each)
158 reduced the viral RNA to undetectable levels. We compared the observed experimental antiviral activity
159 (Fig. 3A, Supplementary Fig. S1A) with theoretical predictions calculated using a classical Bliss
160 independence method that assumes the drugs act independently (Supplementary Note, Supplementary
161 Fig. S1B) (Greco et al., 1995; Koizumi and Iwami, 2014). The difference between the observed values
162 and theoretical predictions suggest that NFV and CEP exhibit a synergistic activity over a broad range of
163 concentrations (Fig. 3C red: synergistic effect).

164

165 **Modeling the impact of Nelfinavir and Cepharranthine on SARS-CoV-2 dynamics.**

166 Combining the published clinical pharmacokinetics information for these drugs with our observed
167 dose-dependent antiviral activities, we can predict the time-dependent antiviral activity (Fig. 4A: left, NFV
168 oral; center, CEP intravenous drip; right: CEP oral) and the resultant viral load dynamics after drug
169 administration (Fig. 4B, Supplementary Note, Supplementary Fig. S2). From such viral dynamics shown
170 in Fig. 4B, we calculated the cumulative viral RNA burden (i.e., area under the curve of viral load) (Fig.
171 4C, upper) and the time period to reduce the viral load to undetectable levels (Fig. 4C, lower). Our

172 modeling analysis predict that NFV would reduce the cumulative viral load by 91.4% (Fig. 4C, upper, red)
173 and would require 11.9 days to eliminate virus (Fig. 4B, upper left, red), 3.98 days shorter than non-
174 treatment condition (Fig. 4C, lower, red). In contrast, treatment with CEP alone showed a limited effect
175 on the viral load [Fig. 4B, upper right or lower left, green], most likely reflecting the low concentration of
176 the drug when administered orally or by intravenous drip (see Discussion). However, co-administering
177 NFV (oral) and CEP (intravenous drip) resulted in a more rapid decline in viral RNA, with undetectable
178 levels 5.5 days earlier than non-treatment and 1.5 days earlier than NFV alone (Fig. 4C). Another
179 advantage of combination treatment is discussed in Discussion. In summary, NFV is likely to show
180 antiviral activity at clinically achievable drug concentration and combination treatment with CEP will
181 facilitate virus elimination.

182

183

184 **Discussion**

185 Screening a panel of approved drugs identified two agents, NFV and CEP, with potent antiviral activity
186 against SARS-CoV-2. NFV inhibits SARS-CoV-2 replication and our modeling data suggests this is
187 mediated via a direct interaction with the viral encoded main protease (Fig. 2B and C). A recent study
188 reported that CEP exhibited anti-SARS-CoV-2 activity (Fan et al., 2020), these authors speculated that
189 CEP targeted both the entry and viral replication phase. However, our time of addition experiments
190 suggest that CEP predominantly inhibits viral entry (Fig. 2B, lane 14). Furthermore, virus-cell
191 attachment assays and docking simulations confirm that CEP inhibits virus attachment to target cells (Fig.
192 2D and E). There is a significant global effort to generate a COVID-19 vaccine that will target the SARS-
193 CoV-2 encoded Spike glycoprotein (Thanh Le et al., 2020) that is required for particle engagement of the
194 receptor ACE2 for infecting cells. We predict that CEP may work synergistically with vaccine induced
195 anti-S antibody responses and such experiments are worthy of future investigation. Further mechanistic
196 studies will be required to confirm the proposed mechanisms of action of these compounds. However,
197 our observation that NFV and CEP target different steps in the viral life cycle support the development of
198 multidrug combination therapies for treating COVID-19.

199 Our mathematical modeling studies assess how anti-SARS-CoV-2 drug candidates can suppress virus
200 proliferation and facilitate virus elimination (Fig. 4). At clinical doses NFV can maintain strong antiviral
201 effect over time and thus can reduce SARS-CoV-2 RNA burden that results in shortening the time required
202 to eliminate infection. In contrast, CEP monotherapy is predicted to have a modest antiviral effect
203 because of a low concentration *in vivo* when administered by oral or intravenous drip. However, higher
204 doses of CEP, based on its relatively safe toxicity profile (Rogosnitzky and Danks, 2011), may increase
205 drug efficacy in a clinical setting. It is noteworthy that combining CEP with NFV further reduced the
206 cumulative viral load and facilitated virus elimination. As the cumulative viral load in patients is likely to
207 be closely related with the progression of disease and the risk for new transmission (Liu, Y. et al., 2020),
208 such multidrug treatment will be of benefit to improve clinical outcome and to control epidemic. In
209 addition to potentiating antiviral effects, multidrug treatment can limit the emergence of viral drug-

210 resistance which is frequently reported for RNA viruses such as coronavirus.

211 One limitation of our modeling of drug efficacy is the use of *in vitro* data derived cell culture infection
212 systems without confirmation using *in vivo* infection models. Recently, a SARS-CoV-2 infection system
213 was reported using ferrets, but as yet there is no evidence on the usefulness of this model for evaluating
214 anti-SARS-CoV-2 drugs (Kim et al., 2020). Given the urgency of the problem, this lack of *in vivo* testing
215 should not prevent the assessment of new antiviral agents. Our screening of approved drugs has
216 identified NFV and CEP as potential anti-SARS-CoV-2 agents. As both NFV and CEP show superior
217 antiviral activities compared to many current drug candidates, these agents offer a promising new
218 multidrug treatment to combat COVID-19.

219

220

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232

233

234 **Author Contributions**

235 Conceptualization, K.W.; Investigation, H.O., K.W., W.S., K.S., S.Iwanami, T.H., T.S., S.K., Y.I., K.S.K.,
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237 authors; Writing and editing, K.W., T.H., K.A., S.Iwami, J.A.M; Funding Acquisition, K.W., M.T.;
238 Supervision, K.W.

239

240

241 **Competing Interests**

242 No interests

243

244

245 **References**

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346

347 **Figure Legends**

348 **Fig. 1. Nelfinavir (NFV) and Cepharranthine (CEP) inhibit SARS-CoV-2 infection.** (A) Schematic of
349 the SARS-CoV-2 infection assay. VeroE6/TMPRSS2 cells were inoculated with SARS-CoV-2 at an
350 MOI=0.01 in the presence of compounds. After washing out unbound virus, the cells were incubated
351 with compounds for 24-48 h. Cells were harvested for immunofluorescence (IFA) or immunoblot
352 analyses of viral N protein at 24 h and cytopathic effects (CPE) at 48 h post-infection. Solid and dashed
353 boxes indicate the periods with and without treatment, respectively. (B) Virus-induced CPE following
354 drug treatment was recorded at 48 h post-infection. Immunofluorescence (C) and immunoblot (D)
355 detection of viral N protein expression in the infected cells at 24 h post-infection, where the red and blue
356 signals show N and DAPI, respectively. Dimethyl sulfoxide (DMSO), 0.4%; Lopinavir (LPV), 16 μ M;
357 Chloroquine (CLQ), 16 μ M; Favipiravir (FPV), 32 μ M; NFV, 4 μ M; CEP, 8 μ M. (E, F) Dose-response
358 curves for compounds. In (E), secreted viral RNA at 24 h post-inoculation was quantified and plotted
359 against drug concentration and chemical structures shown below each graph (for CEP, the structure of a
360 major component is shown). In (F), viability of cells treated with the compounds was quantified by MTT
361 assay. Inferred IC₅₀, IC₉₀, and CC₅₀ values are shown. (G) The antiviral activity for each drug is
362 determined and Instantaneous inhibitory potential (IIP) shown.

363

364 **Fig. 2. Antiviral modes of action for NFV and CEP.** (A, B) Time of addition analysis to examine steps
365 in SARS-CoV-2 life cycle. (A) shows the schematic of the time of addition analysis. Compounds were
366 added at different times (a, whole; b, entry; or c, post-entry): (a): presentation during the 1h virus
367 inoculation step and maintained throughout the 24 h infection period (**whole life cycle**); (b) present during
368 the 1 h virus inoculation step and for an additional 2 h and then removed (**entry**); or (c): added after the
369 inoculation step and present for the remaining 22 h of infection (**post-entry**). Solid and dashed boxes
370 indicate the periods with and without treatment, respectively. In (B), the antiviral activities of each
371 compound under the various protocols are estimated by quantifying the levels of secreted viral RNA at
372 24 h post-inoculation. (C) Predicted binding of NFV to SARS-CoV-2 main protease. Representation
373 of SARS-CoV-2 main protease (green), NFV molecule (cyan stick) and protease binding site residues
374 around NFV within 4 Å (surface representation) are shown. (D) Virus-cell attachment assay.
375 VeroE6/TMPRSS2 cells were incubated with virus (MOI=0.001) in the presence of the indicated
376 compounds for 5 min at 4°C to allow virus-cell attachment with no internalization. After extensive
377 washing, viral RNA on the cell surface was quantified, where the background depicts residual viral inocula
378 in the absence of cells. (E) Predicted binding of CEP molecule to SARS-CoV-2 Spike protein. Spike
379 protein, CEP molecule and protein binding site residues around CEP within 4 Å are shown in cartoon
380 representation colored in orange, green stick and surface representation, respectively. An Overlapping
381 view of the ACE2 with CEP is shown in semi-transparent cartoon representation colored in cyan.

382

383 **Fig. 3. Combination treatment with NFV and CEP.** (A) Dose-response curve of NFV/CEP co-
384 treatment in the infection experiment (MOI=0.001). Extracellular viral RNA levels at 24 h post-infection

385 were quantified and plotted against concentrations of NFV (1.08, 1.30, 1.56, 1.87, and 2.24 μM) and CEP
386 (0.78, 1.25, 2.00, 3.20, and 5.12 μM). **(B)** Cell viability upon co-treatment with compounds. **(C)** The
387 three-dimensional interaction landscapes of NFV and CEP were evaluated based on the Bliss
388 independence. Red and blue colors on the contour plot indicate synergy and antagonism, respectively.
389

390 **Fig. 4. Mathematical prediction of the impact of NFV and CEP therapy on viral dynamics.** **(A)** The
391 time-dependent antiviral effects of NFV (500 mg, TID, oral) and CEP [100 mg, intravenous drip or 120
392 mg, oral] predicted by pharmacokinetics/pharmacodynamics (PK/PD) model are shown with enlarged
393 views of the gray zones in upper panels. **(B)** Viral load dynamics in the presence or absence of NFV
394 (oral), CEP (intravenous), CEP (oral), and NFV (oral)/CEP (intravenous) combined therapies predicted
395 by pharmacokinetics/pharmacodynamics/viral-dynamics (PK/PD/VD) models are shown. **(C)** The
396 cumulative antiviral load [area under the curve in (B)] (upper) and the reduction time (days) for virus
397 elimination (lower) with drug single or combined treatments are shown.
398

Fig. 1

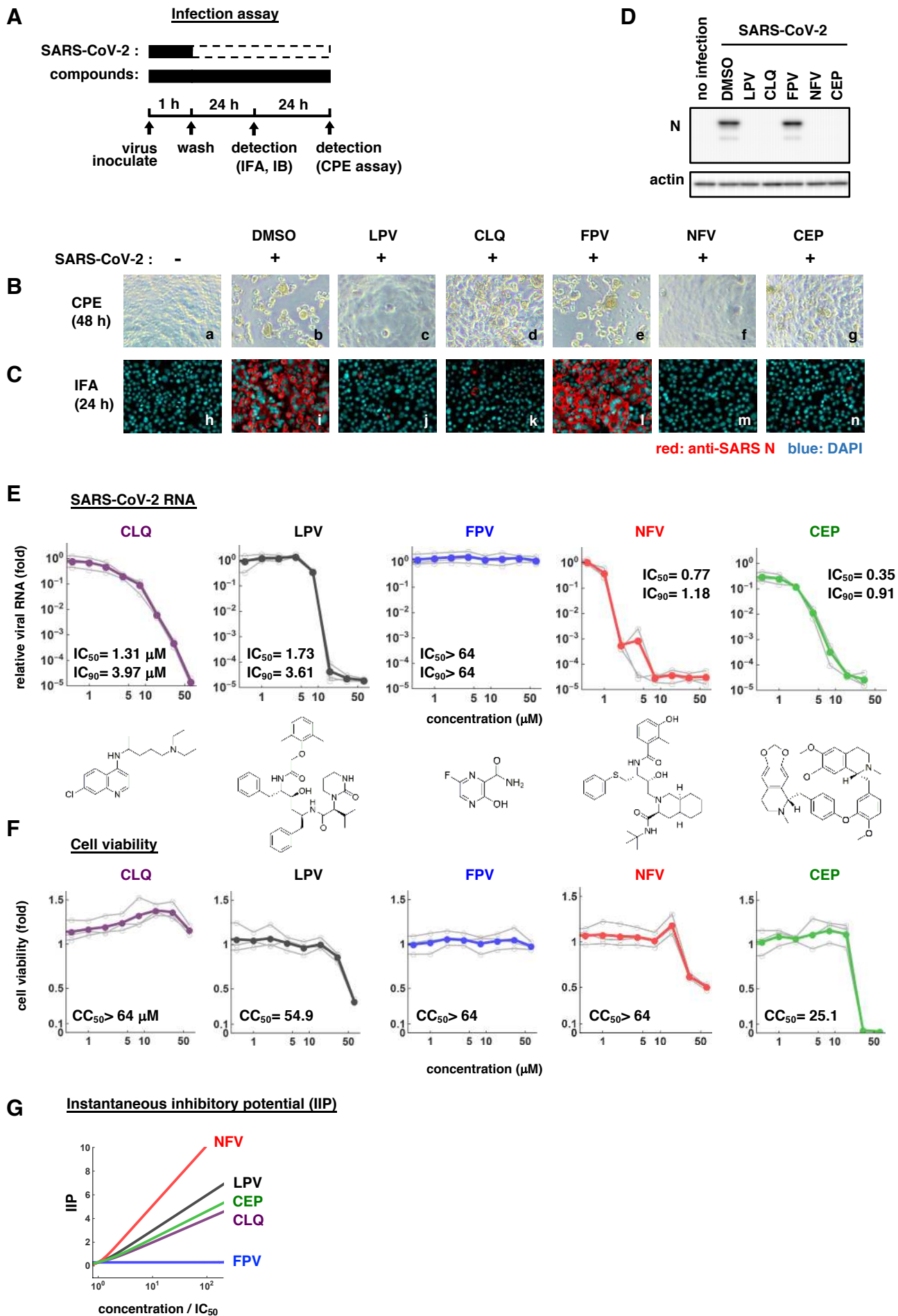


Fig. 2

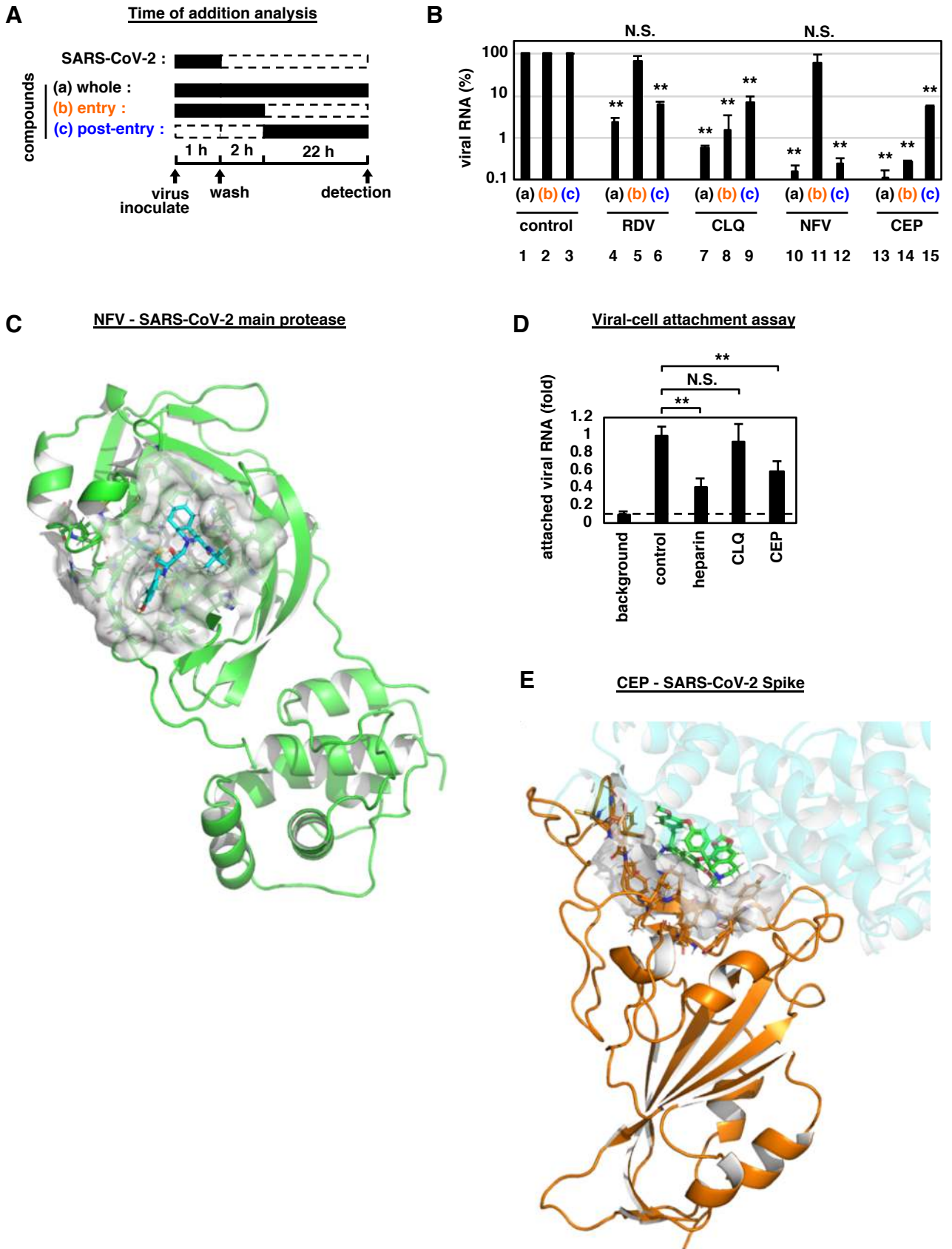


Fig. 3

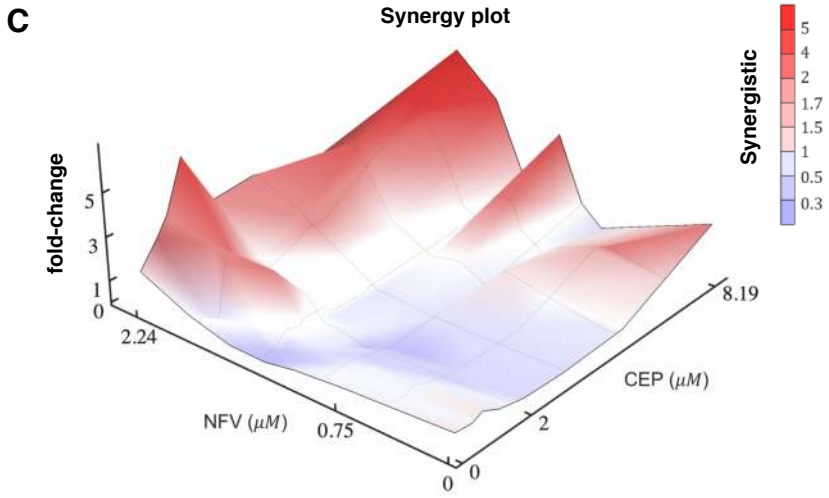
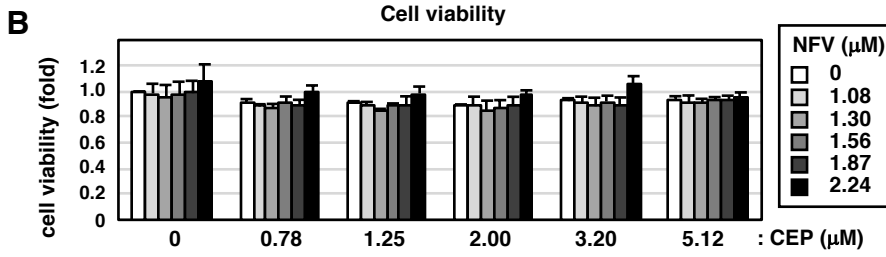
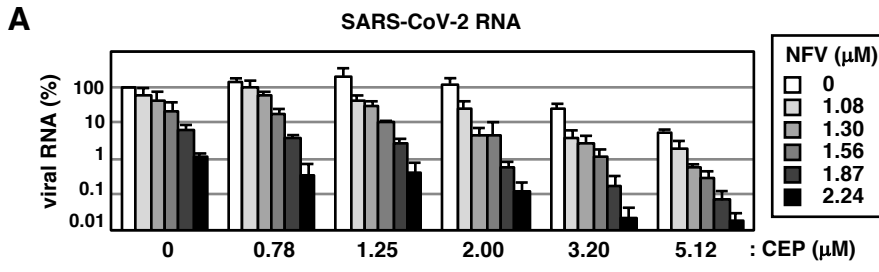
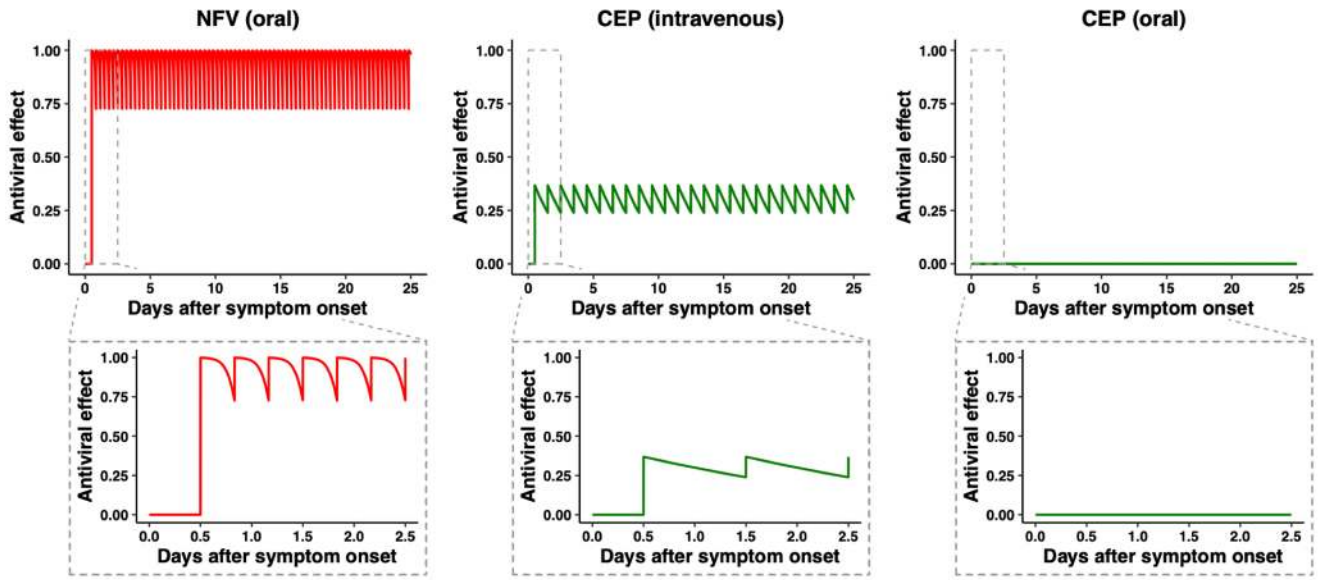
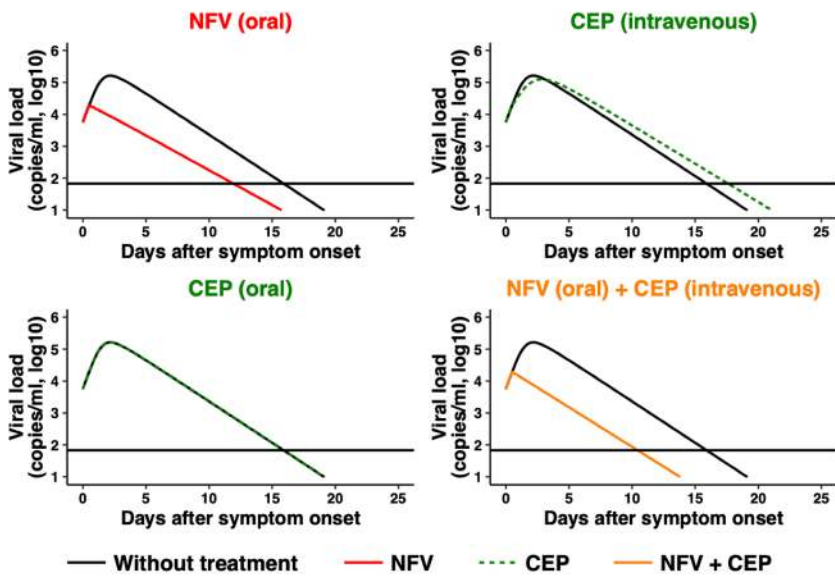


Fig. 4

A



B



C

