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Re: Author Query to Editor PONE-D-20-31839 -

2 pesan

plosone <plosone@plos.org>

9 November 2020 15.11

Kepada: "purwati@fk.unair.ac.id" <purwati@fk.unair.ac.id>

Dear Dr. Purwati,

I can confirm that your manuscript is currently out for peer review and two reviewers have agreed to provide comments. After reading through reviewer comments, the Academic Editor will decide how best to proceed, either rendering a decision or inviting additional reviewers if they believe your paper would benefit from further review. Please be assured that we're keeping in contact with the editor to ensure that the review process runs smoothly.

If you have other questions, please don't hesitate to contact me again.

Best,
Sue Laborda
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Case Number: 06881411
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----- Original Message -----

From: - Purwati [em@editorialmanager.com]
Sent: 11/6/2020 11:05 PM
To: plosone@plos.org
Subject: Author Query to Editor PONE-D-20-31839 - [EMID:b1b4e7509f2a033c]

Manuscript information:

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PONE-D-20-31839

The in vitro anti-viral study of dual drug combinations of antiviral agent, antibiotics, and/or hydroxychloroquine against SARS-CoV-2 virus isolated from hospitalized patients in Surabaya, Indonesia

PLOS ONE

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Dear Editor,

Could you please inform us how long does the manuscript review will take time? many thanks

In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Use the following URL: <https://www.editorialmanager.com/pone/login.asp?a=r>). Please contact the publication office if you have any questions.

PURWATI PURWATI <purwati@fk.unair.ac.id>

11 November 2020 11.25

Kepada: plosone <plosone@plos.org>

Dear Dr. Laborda,
Many thanks for your information.

[Kutipan teks disembunyikan]

PLOS ONE: Response Requested Regarding PONE-D-20-31839

3 pesan

one_production <one_production@plos.org>
Kepada: "purwati@fk.unair.ac.id" <purwati@fk.unair.ac.id>

8 Juni 2021 06.08

Dear Dr. Purwati,

Our production team is currently in the process of preparing your paper, "An in vitro study of dual drug combinations of anti-viral agents, antibiotics, and/or hydroxychloroquine against the SARS-CoV-2 virus isolated from hospitalized patients in Surabaya, Indonesia" (PONE-D-20-31839), for publication. During our pre-composition checks, we have noted that references 19, 31, 32, and 33 are not cited in the manuscript text.

Before we can move forward with production, please add these citations to the attached manuscript file, ensuring that all references remain cited in ascending numeric order within the text.


Please note that any additional changes made to the attached manuscript beyond what we have outlined may require editorial approval and will result in a delay with your publication.

Thank you for your time and attention, and please let me know if you have any questions. I look forward to hearing from you.

Sincerely,
Glenn Jackson

Glenn Jackson | Senior Production Coordinator | he, him

Case Number: 07161214
ref:_00DU0Ifis._5004P1YGxcV:ref

 **pone.0252302_ForAuthors.docx**
92K

PURWATI PURWATI <purwati@fk.unair.ac.id>
Kepada: one_production <one_production@plos.org>

7 Juni 2021 17.07

Dear Sir,

Thank you for your email. We have do some corrections as the followings;

1. We have added reference number 19 into Line 79 as the following:
“..infections [19].”
2. We have added reference number 31 into Line 263 as the following:
“...contents of Fig 3, it is clear that, as previously reported [28–31], the stem cells were well”
3. We have corrected the reference citation in Line 540 :
“...agent against COVID-19 infection[21,22].”

and changed the reference into ref number 33 as the following:

“...agent against COVID-19 infection [33].”

4. We have added ref no. 32 in Line 521 as the following :

“...by inhibiting the protease activity of coronavirus [17,18,32].”

Please see the attachment. Many thanks.

[Kutipan teks disembunyikan]



pone.0252302_ForAuthors.docx

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one_production <one_production@plos.org>
Kepada: "purwati@fk.unair.ac.id" <purwati@fk.unair.ac.id>

11 Juni 2021 05.34

Dear Dr. Purwati,

Thank you for your prompt response. We will continue to prepare your article for publication and you can expect to receive a notification when the article has completed production and the publication date has been set.

In the meantime, please do not hesitate to contact us if you have any questions.

Sincerely,
Glenn Jackson

Glenn Jackson | Senior Production Coordinator | he, him

Case Number: 07161214
ref:_00DU0Ifis._5004P1YGxcV:ref

[Kutipan teks disembunyikan]

An in vitro study of dual drug combinations of anti-viral agents, antibiotics, and/or hydroxychloroquine against the SARS-CoV-2 virus isolated from hospitalized patients in Surabaya, Indonesia

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1 **Abstract**

2 A potent therapy for the infectious coronavirus disease COVID-19 is urgently required with, at
3 the time of writing, research in this area still ongoing. This study aims to evaluate the in vitro
4 anti-viral activities of combinations of certain commercially available drugs that have
5 recently formed part of COVID-19 therapy. Dual combinatory drugs, namely; Lopinavir-
6 Ritonavir (LOPIRITO)-Clarithromycin (CLA), LOPIRITO-Azithromycin (AZI), LOPIRITO-
7 Doxycycline (DOXY), Hydroxychloroquine (HCQ)-AZI, HCQ-DOXY, Favipiravir (FAVI)-
8 AZI, HCQ-FAVI, and HCQ-LOPIRITO, were prepared. These drugs were mixed at specific
9 ratios and evaluated for their safe use based on the cytotoxicity concentration (CC_{50}) values of
10 human umbilical cord mesenchymal stem cells. The anti-viral efficacy of these combinations
11 in relation to Vero cells infected with SARS-CoV-2 virus isolated from a patient in
12 Universitas Airlangga hospital, Surabaya, Indonesia and evaluated for IC_{50} 24, 48, and 72 hours
13 after viral inoculation was subsequently determined. Observation of the viral load in qRT-PCR
14 was undertaken, the results of which indicated the absence of high levels of cytotoxicity in any
15 samples and that dual combinatory drugs produced lower cytotoxicity than single drugs. In
16 addition, these combinations demonstrated considerable effectiveness in reducing the copy
17 number of the virus at 48 and 72 hours, while even at 24 hours, post-drug incubation resulted
18 in low IC_{50} values. Most combination drugs reduced pro-inflammatory markers, i.e. IL-6 and
19 $TNF-\alpha$, while increasing the anti-inflammatory response of IL-10. According to these results,
20 the descending order of effective dual combinatory drugs is one of LOPIRITO-
21 AZI>LOPIRITO-DOXY>HCQ-AZI>HCQ-FAVI>LOPIRITO-CLA>HCQ-DOX. It can be
22 suggested that dual combinatory drugs, e.g. LOPIRITO-AZI, can potentially be used in the
23 treatment of COVID-19 infectious diseases.

24

25 **Keywords:** antiviral; drugs combination; SARS-CoV-2; in vitro, infectious disease

26 **Introduction**

27 At the end of 2019, a case of pneumonia was diagnosed on the basis of a viral infection in
28 Wuhan, China [1]. The pathogen was identified as a novel enveloped RNA betacoronavirus2,
29 currently referred to as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2),
30 which has a phylogenetic similar to SARS-CoV. Since that time, it has developed into a global
31 pandemic due to Coronavirus SARS-CoV-2, also referred to as COVID-19 [2,3]. On March 2nd
32 2020, the Indonesian Ministry of Health reported the first confirmed domestic positive case of
33 SARS-CoV-2. By September 2020, more than 262,000 individuals had been infected with
34 10,105 cases culminating in death [4].

35 COVID-19 infection causes severe pneumonia with symptoms such as fever, a persistent
36 cough, and progressive breathing failure associated with respiratory complications. The high
37 hospitalization rate, risk of mortality and lack of a specific established treatment rendered
38 urgent the need for an effective therapy for COVID-19 to be developed. The main viral
39 proteinase has recently been considered positively as a suitable target for drug design against
40 COVID-19 infection due to its vital role in the poly-protein processing necessary for
41 coronavirus reproduction [5].

42 The term ‘antiviral agents’ refers to the medications prescribed to combat Middle East
43 Respiratory Syndrome (MERS) and SARS pandemics. Interferon α (IFN- α), lopinavir-
44 ritonavir, chloroquine phosphate, ribavirin, and Arbidol have been highlighted in the latest
45 version of the Guidelines for the Prevention, Diagnosis, and Treatment of Novel Coronavirus-
46 induced Pneumonia issued by the Republic of China’s National Health Commission (NHC) as
47 potential treatments for COVID-19 [6]. In addition to antiviral agents, antibiotics such as
48 amoxicillin, azithromycin or fluoroquinolones are also being employed [7] in an attempt to
49 eradicate the SARS-CoV-2 virus. However, given the continuing lack of data regarding their
50 efficacy as a form of COVID-19 therapy, this study aims to evaluate the use of dual combinatory

51 drugs as an antiviral therapy against the SARS-CoV-2 virus, specifically COVID-19, within
52 the Indonesian context.

53 During the present research, the respective in vitro antiviral activities of Lopinavir-
54 Ritonavir (LOPIRITO), Favipiravir (FAVI), Azithromycin (AZI), Clarithromycin (CLA),
55 Doxycycline (DOXY), and Hydroxychloroquine (HCQ) as dual combinatory drugs at
56 determined ratios were analyzed. These ratios were established based on the plasma
57 concentration of drugs administered at the usual dose during clinical therapy, (see Table 1).
58 However, in many cases, there were limited or even no reports regarding the pharmacokinetic
59 profiles in dual drug combinations.

60 **Table 1. Peak plasma concentration of Lopinavir/Ritonavir (LOPIRITO), Azithromycin**
61 **(AZI), Clarithromycin (CLA), Doxycycline (DOXY), Hydroxychloroquine (HCQ), and**
62 **Favipiravir (FAVI) after a single oral administration of the drug.**

Drugs	Dosage	Peak Plasma Concentration	Reference
Lopinavir/Ritonavir	Oral administration of Aluvia [®] tablet containing 400/100 mg Lopinavir/Ritonavir twice a day	Lopinavir: 6.9 to 17.7 µg/mL	[8]
Azithromycin	Single oral administration of 500 mg Azithromycin	0.35-0.45 mg/L after	[9]
Clarithromycin	oral administration of 250 and 500 mg Clarithromycin twice a day	1 and 2.41 µg/mL, respectively	[10]

Doxycycline	Single oral administration of 200 mg doxycycline	1.5 to 7.0 µg/ml after oral administration	[11]
Hydroxychloroquine	Single oral administration of 400 mg HCQ sulfate	0.28 to 0.54 µg/mL	[12]
Favipiravir	1600/600 mg twice a day	64.56 µg/mL	[13]

63

64 Lopinavir, Ritonavir, and Favipiravir have all been used as antiviral agents which act as
65 virus protease inhibitors [8,9]. Azithromycin is classified as a macrolide antibiotic which has
66 been used extensively in the treatment of severe respiratory lower tract infections such as
67 pneumonia. It can be employed for preventing secondary infection often resulting from viral
68 infection, thereby avoiding a severe prognosis. Azithromycin has been reported to be an
69 immune modulator and anti-inflammatory agent [10,11], while also inhibiting virus replication
70 and the cytopathic effect mediated by the Zika virus in Glial cell lines and astrocytes [17].
71 Moreover, the use of clarithromycin has been regarded in the same manner as that of
72 Azithromycin. Clarithromycin demonstrates a high affinity with the protein target of HIV-1
73 protease in the molecular docking study which is superior to that of doxycycline due to high
74 hydrophobicity and partition co-efficiency [18]. The combined application of Clarithromycin
75 and antiviral agents, i.e. Oseltamivir or Zanamivir, increased systemic immunity while reducing
76 rates of infection-related relapse in children infected with the influenza virus [16]. Doxycycline,
77 a tetracycline-derived drug, has an inhibitory effect on dengue fever viral replication and
78 reduces the proinflammatory marker IL-6 during viral infections. Consequently, it may prove
79 effective as a form of COVID-19 therapy [14,15]. Hydroxychloroquine is an aminoquinoline-
80 derivate compound producing fewer severe side effects than chloroquine [21]. It has been
81 employed as an antiviral agent [22,23] which impedes the viral pre-entry stage, inhibits both
82 viral replication mediated by acidic endocytosis and viral replication through modification of

83 post-translation virus protein, hinders virus maturation via pH modulation, and produces anti-
84 inflammatory effects by reducing IL-6 levels in serum [20].

85 In this present work, the efficacy of these drugs as a form of COVID-19 therapy was
86 evaluated on Vero cells as viral hosts cultured with SARS-CoV-2 virus isolated from
87 hospitalized patients in Universitas Airlangga Hospital, Surabaya, Indonesia. Furthermore, an
88 analysis of the structure-based computational modelling of ligand-receptor interactions
89 evaluated their potential use as the main protease of SARS-CoV-2 inhibitor [24].

90

91 **Material and Methods**

92 **Materials**

93 Lopinavir-Ritonavir (LOPIRITO) was produced by Abbott Laboratories (Aluvia®,
94 Chicago, USA); Favipiravir (FAVI) by Toyama Chemical (Fujifilm Group) (Avigan®, Japan);
95 Azithromycin (AZI) tablets by Gentec Pharmaceutical Group (Spain); Clarithromycin (CLA)
96 by Ind Swift Laboratories Limited (India); Doxycycline (DOXY) by Genero Pharmaceuticals
97 (Doxicor®, Indonesia); Hydroxychloroquine (HCQ) by Imedco Djaja (Hyloquin®, Indonesia);
98 and dimethyl sulfoxide by Sigma Aldrich (Singapore). All other reagents and solvents
99 employed in this study were of the highest quality available. Milli-Q water was used in all
100 experiments.

101

102 **Virus and cell collection**

103 Vero cells were used for virus inoculation against SARS-CoV-2 isolates in Indonesia. Cells
104 were seeded in a 12-well microplate at a cell density of 5×10^4 cells/well cultured in Dulbecco's
105 Modified Eagle's Medium (DMEM) (Gibco, USA) containing 10% foetal bovine serum
106 (Gibco, USA), 1% penicillin-streptomycin (Gibco, USA) and 1% amphotericin-B (Gibco,

107 USA). Cells were incubated in a CO₂ incubator at 37°C in a humidified atmosphere of 5% CO₂
108 for 24 hours and cultured to reach 80-90% confluence.

109 SARS-CoV-2 virus isolates were collected from PCR-positive confirmed patients in
110 Universitas Airlangga Hospital, Surabaya. Patient sputum sampling and clinical procedures
111 were performed in accordance with the ethical clearance issued by The Ethics Commission of
112 Universitas Airlangga Hospital (Certificate number 136/KEP/2020 dated April 20, 2020). The
113 sputum of conscious patients was collected in viral transport medium (VTM) containing
114 Gentamycin sulphate (100µg/ml) and Amphotericin B (0.5µg/ml). Further experiments were
115 conducted in the Biosafety Level (BSL)-3 Laboratory at The Institute of Tropical Disease,
116 Universitas Airlangga, Surabaya, Indonesia. In order to isolate the virus, the sputum samples
117 were inserted into a new conical tube, subsequently vortexed for five minutes, and centrifuged
118 at 13,000 rpm for ten minutes. After centrifugation, the supernatant of each sample was
119 extracted for the purposes of further experiments.

120

121 **Preparation of drugs solution**

122 Each tablet containing drugs was triturated and mixed until homogenous. Approximately
123 50 mg equivalent mass of drugs were weighed and added to dimethyl sulfoxide in order to
124 solubilize the drugs. The suspension was sonicated in a water bath for 15 minutes before being
125 added to Rosewell Park Memorial Institute (RPMI) media, sonicated again and vortexed to mix
126 it until homogenous. The suspension was then filtered through a polycarbonate membrane with
127 a pore size of 0.45 µm and then a pore size of 0.22 µm under aseptic conditions. The filtrate
128 was mixed with 10% foetal bovine serum and penicillin streptomycin before being vortexed to
129 produce a homogenous mixture to be used as a stock solution. The samples were prepared by
130 diluting the stock solution of each drug with RPMI complete media at an appropriate level of
131 dilution to produce a determined concentration. The dual combinatory drugs mixtures were
132 prepared by mixing appropriate amounts of two drug stock solutions in order to produce a final

133 concentration at the required level. The combinatory drugs were evaluated at both constant and
134 non-constant ratios to evaluate their effects on the cytotoxicity, including; antagonistic,
135 synergistic, or additive. A constant ratio of the mixture was achieved by adding drug solutions
136 at the same ratio, thereby increasing each drug concentration, to produce dose escalation. In
137 contrast, at a non-constant ratio, a fixed determined concentration of drug was added to
138 increased doses of other drug solution in order to produce different levels of drug concentration.

139

140 **Cytotoxicity assay for dual combinatory drugs**

141 The cytotoxic concentration (CC_{50}) of drugs was performed by means of MTT assay at the
142 Stem Cell Research and Development Center, Universitas Airlangga using human umbilical
143 cord mesenchymal stem cells which had been obtained from human placenta tissue as approved
144 by the Ethical Committee of Universitas Airlangga Hospital (Certificate number
145 101/KEH/2019 dated January 10, 2019). The cells were prepared as the primary cell culture
146 and used for the cytotoxicity assay because of their sensitivity to chemicals. Cells were seeded
147 into 96-well microplates at a concentration of 1×10^3 cells/well in 100 μ L Alpha Minimum
148 Essentials Medium (α -MEM, Gibco, USA) supplemented with 10% foetal bovine serum, 1%
149 penicillin-streptomycin and 1% amphotericin-B. The plates were then incubated in a CO_2
150 incubator at $37^\circ C$ with 5% CO_2 for 24 hours, at which point, the supernatant was replaced with
151 α -MEM containing drugs at each concentration and incubated for a further 48 hours.
152 Approximately 25 μ L of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium-bromide
153 (MTT) reagent at a concentration of 5mg/mL was subsequently added to each well and
154 incubated for four hours at $37^\circ C$ with 5% CO_2 . Purple formazan crystals were formed and
155 observed under an inverted microscope. Dimethyl sulfoxide was added to each well with the
156 complete solubilisation of formazan crystals subsequently being observed. The greater the
157 number of formazan crystals formed, the lower the toxicity of the samples which were read for
158 optical density of formazan using a multi reader at a measurement wavelength of 595 nm

159 (Promega Glomax, USA). The CC_{50} value was analyzed by CompuSyn software (the
160 ComboSyn Inc., accessed from www.combosyn.com).

161

162 **Virus inoculation and antiviral assay for dual combinatory drugs**

163 Vero cells obtained from Elabscience® (Catalog No. EP-CL-0242, USA) were seeded in a 12-
164 well plate and confirmed as reaching 80-90% confluence on the day of virus inoculation. The
165 culture medium was removed and the cells were then added to RPMI media containing SARS-
166 CoV-2 isolates, previously diluted with RPMI media at a ratio of 1:2. In this study, about 2,000
167 virus copies were added to 50,000 cells of Vero cells, with a multiplicity of infection (MoI)
168 degree of 0.04. The plate was gently agitated for 30 minutes and incubated at 37°C, 5% CO₂
169 for 24 hours. About 3 mL of complete culture medium were subsequently added to the plate
170 and incubated at 5% CO₂ 37°C for 24 hours, at which point 3 mL of RPMI media containing a
171 drug combination were introduced and incubated at 5% CO₂ 37°C for 24, 48, and 72 hours. The
172 drug mixtures were prepared at appropriate weight constant ratios selected on the basis of the
173 optimum safety profiles in the cytotoxicity study. The Vero cells were observed post-treatment
174 to observe the cytopathic effects, including; the rounding and detachment of cells. Moreover,
175 the IC_{50} values were determined in order to quantify antiviral activity by measuring the proviral
176 load in each well. The determination of the proviral load was performed by means of a Seegene
177 COVID-19 detection Kit (Beijing, China) which detected three target genes, i.e. N-gene, E-
178 gene and RdRP-gene. Amplification and data acquisition were carried out using the ABI Prism
179 7500 Sequence detector system (Applied Biosystems, USA). The IC_{50} value was further
180 analyzed using CompuSyn software (The ComboSyn Inc., accessed from
181 www.combosyn.com).

182

183 **Measurement of IL-6, IL-10 and TNF- α levels of virus-infected**

184 **Vero cells incubated with dual combinatory drugs**

185 To enable measurement of IL-6, IL-10 and TNF- α levels, the culture medium of the treated
186 cells was collected in sterile micro-tubes and centrifuged at 3,500 rpm for 20 minutes. The
187 supernatants were carefully collected and diluted with aquadest at a 1:5 volume ratio and
188 vortexed until homogenous. The samples were deposited onto a well-plate, added to ELISA
189 reagents (Bioassay Technology Laboratory, Shanghai, China), and incubated at 37°C for 60
190 minutes. Reagent substrate solution was then added to the well and incubated for ten minutes
191 at 37°C. The samples were measured for antigen concentration using the optical density (OD)
192 plotted into the standard curves of IL-6, IL-10, and TNF- α .

193

194 **Molecular docking study of drugs against main protease of SARS-**

195 **CoV-2 virus**

196 The molecular docking study was carried out by using Schrodinger Maestro 2019-2
197 Maestro software including protein preparation, ligand preparation, grid generation and
198 receptor-ligand docking. The Linux operating system was used for the computational study.
199 Ligands (Lopinavir, Ritonavir, Favipiravir, Azithromycin, Clarithromycin, Doxycycline, and
200 Hydroxychloroquine) were downloaded from the NCBI
201 (<http://www.ncbi.nlm.nih.gov/pccompound>). The crystal Structure of SARS-CoV-2 main
202 protease, PDB ID: ALU6 was retrieved from the Protein Data Bank (PDB)
203 (<https://www.rcsb.org/>).

204 The main protease protein was prepared for a docking study by using in Schrodinger
205 2019-2 Maestro software. All ligand compounds were prepared using LigPrep, which can
206 produce low energy isomer of the ligand in optimization by using the OPLS_2005 force field.
207 The OPLS_2005 force field was used for generating Grid on protein receptors. Schrodinger

208 2019-2 version was used to predict the binding affinity, ligand competence, and inhibitory
209 candidate to the protein by performing rigid, flexible docking. The ligands were docked with
210 generated Grid of receptor protein PDB ID: ALU6 The optimal ligand selection for the receptor
211 was done based on the docking score.

212 **Preparation of ligands and receptors**

213 Ligand-receptor complex. The complex in the form of a crystal structure consisting of native
214 ligands and receptors was downloaded from the Protein Data Bank (PDB) server at the web
215 address <https://www.rcsb.org> with ID 6LU7 [25]. 6LU7 protein structure consists of two chains
216 (A and C). The Main protease (M^{pro}) is in the A chain (shown in brown), while the native ligand
217 appears as blue in the C chain, as presented in Figure 1

218 **Figure 1. Image representation of Ligand-receptor Complex**

219 The receptors and ligands from the resulting crystalline structure did not undergo geometric
220 optimization treatment because they were obtained from the actual structure. For the purposes
221 of the docking procedure, the ligands of this crystal were given a partial charge of the atom
222 using the Austin Model 1 semi-empirical method with Bond Charge Correction (AM1-BCC)
223 [26], while the receptor partial charge was calculated by means of a molecular mechanics
224 approach with a force field of ff14SB [27].

225 Preparation of candidates as ligands. A sketch of the molecular structure of the ligand was
226 produced using the ChemDraw Professional version 17 program. This structural sketch was still
227 2-dimensional with the result that a 3-dimensional structure had to be made. This structure was
228 formed by calculations using the MM + molecular mechanics method to quickly obtain a 3-
229 dimensional structure. The calculations were performed using a HyperChem 6 program. The
230 structure of the calculation using the molecular mechanics method was then refined using a
231 semi-empirical Parametric Model number 3 (PM3) quantum mechanics calculation. The
232 calculations were completed using Gaussian 16 software. The partial atomic charge of each
233 ligand was calculated through application of the AM1-BCC semi-empirical method.

234 **Construction surface and receptor spheres**

235 The receptor surface (molecular surface, ms) consisting of a number of cluster spheres was
236 created and calculated using the dms module which is part of the Dock 6 program [28]. The
237 active side of the M^{pro} was determined based on the native ligand position in the cluster. This
238 active side location was used as the basis for the construction of the simulation box. The degree
239 of margin for the formation of the simulation box was 10 Å.

240 **Creating a simulation box**

241 Depending on the position of the native ligand, a simulation box was built around it in the shape
242 of a cube. The position of the simulation box, native ligand, and cluster of spheres relative to
243 the receptor can be seen in the Figure 2.

244 **Figure 2. The position of the simulation box, native ligand, and cluster of spheres**
245 **relative to the receptor.**

246

247 **Validation of docking parameters**

248 The parameters to be employed in docking the candidate to the receptor were validated by
249 redocking the native ligand to the receptor. An effective docking parameter must be able to
250 return the native ligand to its original position with a maximum root mean square deviation
251 (rmsd) tolerance of 2 Å [26]. The docking parameter validation resulted in an rmsd of 1.725 Å,
252 indicating that use of the docking parameters at the docking stage for candidate ligands was
253 feasible.

254

255 **Results**

256 **Characterization of Human umbilical cord mesenchymal stem cells**

257 For the cytotoxicity assay of combinatory drugs, the primary cell cultures of human
258 umbilical cord mesenchymal stem cells were used as the experimental cells. From the contents

259 of Fig 3, it is clear that, as previously reported [28–30], the stem cells were well differentiated
260 as indicated by immunocytochemistry assays conducted using CD45, CD90, and CD105
261 antibodies.

262 **Figure 3. Phase contrast and fluorescence images of human umbilical cord stem cells**
263 **stained with anti-CD45, CD90, and CD105 antibody and CF555-labelled secondary**
264 **antibody observed under a fluorescence microscope at a magnification of 100x.**

265

266 **Cytotoxicities of dual drug combination of LOPIRITO-AZI in** 267 **mesenchymal stem cells**

268 In this study, the cytotoxicity assay was evaluated for single and dual combinatory drugs
269 during a period of 48 hours of drug incubation. This assay was intended to evaluate the
270 toxicity of dual combinatory drugs on normal cells. The combination ratios were calculated
271 taking into consideration the usual therapeutic doses and plasma peak concentrations of the
272 drugs. To determine this cytotoxicity, the drugs were mixed at both constant and non-
273 constant ratios.

274 The evaluation of LOPIRITO and AZI in the stem cells showed that AZI had relatively
275 non-toxic properties compared to those of LOPIRITO, while the CC_{50} values were 1.3×10^{55}
276 $\mu\text{g/mL}$ for AZI and $4.29 \times 10^2 \mu\text{g/mL}$ for LOPIRITO, as shown in Fig 4. The combination
277 of LOPIRITO and AZI at constant weight ratios of 1:1 and 1:2 respectively, and non-
278 constant ratios resulted in decreases in the degree of cytotoxicity. These were much safer
279 than LOPIRITO as indicated by their higher CC_{50} values. These results indicate that a
280 combination of both drugs negates the side effects of each single one, possibly producing
281 an antagonist effect.’

282 **Figure 4. The cytotoxicity of Lopinavir-Ritonavir (LOPIRITO) and Azithromycin**
283 **(AZI) as a single drug (left) and dual drug combination at constant and non-constant**

284 ratios (right) analysed by CompuSyn Software (n=3). At non-constant ratios of
285 LOPIRITO 8 µg/mL + AZI , LOPIRITO was added at a concentration of 8 µg/mL to
286 each increased level of AZI, i.e. 0.2, 2, 10, 100, and 400 µg/mL. On the other hand,
287 AZI was then added at a concentration of 50 µg/mL to each increased level of
288 LOPIRITO, i.e. 0.2, 2, 10, 100, and 400 µg/mL to produce LOPIRITO + AZI 50
289 µg/mL.

290

291 **Cytotoxicities of dual drug combination of LOPIRITO-CLA in** 292 **mesenchymal stem cells**

293 The results of a cytotoxicity assay indicated that LOPIRITO was relatively more toxic to the
294 cells than CLA as indicated by their CC₅₀ values as a single drug which were 7.46x10² µg/mL
295 and 2.28x10³ µg/mL respectively, as shown in Fig 5. Moreover, the dual drug combination of
296 LOPIRITO:CLA at the weight ratio of 1:1 had a high CC₅₀ value of 1.22x10⁴ µg/mL, indicating
297 that this combination reduced the toxicity of both drugs in the stem cells.

298 **Figure 5. The cytotoxicities of Lopinavir-Ritonavir (LOPIRITO) and**
299 **Clarithromycin (CLA) as a single drug (left) and dual drug combination in constant**
300 **and non-constant ratios (right) analysed by using CompuSyn Software (n=3). At**
301 **non-constant ratios of LOPIRITO 8 µg/mL + CLA, LOPIRITO was added at a**
302 **concentration of 8 µg/mL to each increased levels of CLA i.e. 0.2, 2, 10, 100, and 400**
303 **µg/mL. On the other hand, CLA was then added at a concentration of 1 µg/mL to**
304 **each increased levels of LOPIRITO i.e. 0.2, 2, 10, 100, and 400 µg/mL to produce**
305 **LOPIRITO + CLA 1 µg/mL.**

306

307 **Cytotoxicities of dual drug combination of LOPIRITO-DOXY in**
308 **mesenchymal stem cells**

309 Further evaluation was conducted for the dual combination of LOPIRITO and DOXY. The
310 results showed that LOPIRITO has higher cytotoxicity than DOXY, as shown in Fig 6. The
311 dual combination of LOPIRITO and DOXY, at both constant and non-constant ratios, resulted
312 in significantly higher CC₅₀ values (until undetected) than those of single drugs which were
313 $3.45 \times 10^3 \mu\text{g/mL}$ and $1.65 \times 10^4 \mu\text{g/mL}$ respectively for LOPIRITO and DOXY. This indicated
314 that these combinations reduced drug toxicity in the stem cells.

315 **Figure 6. The cytotoxicities of Lopinavir-Ritonavir (LOPIRITO) and Doxycycline**
316 **(DOXY) as a single drug (left) and dual drug combination in constant and non-constant**
317 **ratios (right) analysed by using CompuSyn Software (n=3). At non-constant ratios of**
318 **LOPIRITO 8 $\mu\text{g/mL}$ + DOXY, LOPIRITO was added at a concentration of 8 $\mu\text{g/mL}$ to**
319 **each increased levels of DOXY i.e. 0.2, 2, 10, 100, and 400 $\mu\text{g/mL}$. On the other hand,**
320 **DOXY was then added at a concentration of 2 $\mu\text{g/mL}$ to each increased levels of**
321 **LOPIRITO i.e. 0.2, 2, 10, 100, and 400 $\mu\text{g/mL}$ to produce LOPIRITO + DOXY 2 $\mu\text{g/mL}$.**

322

323 **Cytotoxicities of dual drug combination of HCQ-AZI in**
324 **mesenchymal stem cells**

325 The cytotoxicity assay was also evaluated for dual combination of HCQ and AZI. As shown in
326 Fig 7, HCQ produced higher cytotoxicity than AZI. Combining these drugs increased the CC₅₀
327 values resulting in a lower toxic effect than that of HCQ. The dual combination drug at a ratio
328 of 1:2 for HCQ and AZI produced the lowest cytotoxicity in the stem cells in which the CC₅₀
329 was $2.81 \times 10^4 \mu\text{g/mL}$, thus providing for its potential use in an anti-viral study of COVID-19.

330 **Figure 7. The cytotoxicities of Hydroxychloroquine (HCQ) and Azithromycin (AZI) as a**
331 **single drug (left) and dual drug combination in constant and non-constant ratios (right)**

332 analysed by using CompuSyn Software (n=3). At non-constant ratios of HCQ + AZI 50
333 µg/mL, AZI was added at a concentration of 50 µg/mL to each increased levels of HCQ
334 i.e. 0.2, 2, 10, 100, and 400 µg/mL. On the other hand, HCQ was then added at a
335 concentration of 6 µg/mL to each increased levels of AZI i.e. 0.2, 2, 10, 100, and 400
336 µg/mL to produce HCQ 6 µg/mL + AZI.

337

338 **Cytotoxicities of dual drug combination of HCQ-DOXY in** 339 **mesenchymal stem cells**

340 The use of HCQ was combined with DOXY to evaluate its safety when used during antiviral
341 studies. As can be seen in Fig 8, HCQ had higher cytotoxicity than DOXY. Furthermore, the
342 results showed that the dual drug combination produced lower toxicity in the stem cells than
343 that of a single HCQ-based treatment. The CC_{50} values of a combination of HCQ-DOXY at
344 respective weight ratios of 1:1 and 1:2 were 4.37×10^3 µg/mL and 1.77×10^5 µg/mL, while the
345 HCQ was 1.50×10^3 µg/mL.

346 **Figure 8. The cytotoxicities of Hydroxychloroquine (HCQ) and Doxycycline (DOXY) as**
347 **a single drug (left) and dual drug combination in constant and non-constant ratios**
348 **(right) analysed by using CompuSyn Software (n=3). At non-constant ratios of HCQ +**
349 **DOXY 2 µg/mL, DOXY was added at a concentration of 2 µg/mL to each increased**
350 **levels of HCQ i.e. 0.2, 2, 10, 100, and 400 µg/mL. On the other hand, HCQ was then**
351 **added at a concentration of 6 µg/mL to each increased levels of DOXY i.e. 0.2, 2, 10, 100,**
352 **and 400 µg/mL to produce HCQ 6 µg/mL + DOXY.**

353

354 **Cytotoxicities of dual drug combination of FAVI-AZI in**
355 **mesenchymal stem cells**

356 The use of FAVI and AZI in an antiviral study of COVID-19 was initially evaluated for
357 cytotoxicity against primary cultured stem cells. As shown in Fig 9, the results indicated that
358 both FAVI and AZI, administered either as a single drug or in dual combination, produced very
359 low cytotoxicity effects. It could be confirmed that FAVI and AZI were considered drugs not
360 harmful to mesenchymal stem cells.

361 **Figure 9. The cytotoxicities of Favipiravir (FAVI) and Azithromycin (AZI) as a single**
362 **drug (left) and dual drug combination in constant and non-constant ratios (right)**
363 **analysed by using CompuSyn Software (n=3). At non-constant ratios of FAVI + AZI 50**
364 **µg/mL, AZI was added at a concentration of 50 µg/mL to each increased levels of FAVI**
365 **i.e. 0.2, 2, 10, 100, and 400 µg/mL. On the other hand, FAVI was then added at a**
366 **concentration of 66 µg/mL to each increased levels of AZI i.e. 0.2, 2, 10, 100, and 400**
367 **µg/mL to produce FAVI 66 µg/mL + AZI.**

368

369 **Cytotoxicities of dual drug combination of HCQ-FAVI in**
370 **mesenchymal stem cells**

371 The HCQ was also evaluated for its combination with FAVI. As presented in Fig 10, as a single
372 drug, HCQ produced more intense cytotoxic effects in the mesenchymal stem cells than did
373 FAVI whose CC₅₀ value of HCQ was 11.75 µg/mL. Combining HCQ with FAVI reduced the
374 toxicity resulting in higher CC₅₀ values of the HCQ-FAVI combination which were 343 µg/mL
375 and 954 µg/mL for HCQ-FAV mixed at the ratios of 1:5 and 1:10 respectively.

376 **Figure 10. The cytotoxicities of Hydroxychloroquine (HCQ) and Favipiravir (FAVI) as a**
377 **single drug (left) and dual drug combination in constant and non-constant ratios (right)**

378 analysed by using CompuSyn Software (n=3). At non-constant ratios of HCQ 6 µg/mL +
379 FAVI, HCQ was added at a concentration of 66 µg/mL to each increased levels of FAVI
380 i.e. 0.2, 2, 10, 100, and 400 µg/mL. On the other hand, FAVI was then added at a
381 concentration of 66 µg/mL to each increased levels of HCQ i.e. 0.2, 2, 10, 100, and 400
382 µg/mL to produce HCQ + FAVI 66 µg/mL.

383

384 **Cytotoxicities of dual drug combination of HCQ-LOPIRITO in** 385 **mesenchymal stem cells**

386 HCQ was dually combined with LOPIRITO and evaluated for its safe use against
387 mesenchymal stem cells. In this assay, HCQ and LOPIRITO produced relatively low CC₅₀
388 values of 2.51 and 58.55 µg/mL and were considered potentially toxic drugs and combinations
389 as shown in Fig 11. The dual combination of HCQ and LOPIRITO produced higher CC₅₀ values
390 than single HCQ, i.e. 9.38 µg/mL and 8.45 µg/mL, for HCQ:LOPIRITO combined at weight
391 ratios of 1:1 and 1:2. respectively. However, they were still more toxic than LOPIRITO.

392 **Figure 11. The cytotoxicities of Hydroxychloroquine (HCQ) and Lopinavir-Ritonavir**
393 **(LOPIRITO) as a single drug (left) and dual drug combination in constant and non-**
394 **constant ratios (right) analysed by using CompuSyn Software (n=3). At non-constant**
395 **ratios of HCQ + LOPIRITO 8 µg/mL, LOPIRITO was added at a concentration of 8**
396 **µg/mL to each increased levels of HCQ i.e. 0.2, 2, 10, 100, and 400 µg/mL. On the other**
397 **hand, HCQ was then added at a concentration of 6 µg/mL to each increased levels of**
398 **LOPIRITO i.e. 0.2, 2, 10, 100, and 400 µg/mL to produce HCQ 6 µg/mL + LOPIRITO.**

399

400 **Antiviral activity in Vero cells infected with SARS-CoV-2-isolated**
401 **human virus**

402 After cytotoxic evaluation of dual drug combination in mesenchymal stem cells, the drugs
403 were subsequently assessed for antiviral activities against the SARS-CoV-2 virus isolated from
404 patients in Universitas Airlangga Hospital. The Vero cells were inoculated with the virus which
405 led to certain changes in their morphology indicating that the virus had successfully infected
406 them. Fig 12 contains the typical formations of virus-infected cells observed at 24, 48, and 72
407 hours post-inoculation. At 24 hours post-inoculation, the presence of groups or colonies of
408 detached cells indicated that they were dead. Furthermore, the formation of giant cells was
409 observed in the 48 hours followed by a cytopathic effect clearly evident in the cells at 72 hours
410 after the virus inoculation.

411 **Figure 12. The photomicrographs of morphology changes of Vero cells before virus**
412 **inoculation (A), at 24-h (B), 48-h (C), and 72-h (D) after virus inoculation observed at a**
413 **magnification of 100x. The black arrow shows a giant cell formation and the white arrow**
414 **indicates a cytopathic effect.**

415 In addition to the photomicrographs of cell morphological changes, pro-viral load
416 determination indicated that virus copy numbers had increased during the incubation period, as
417 shown in Table 2.

418

419 **Table 2. Virus titer of Vero cells infected with the SARS-CoV-2 virus isolates at a**
420 **multiplicity of infection (MoI) of 0.04 at 24, 48, and 72 hours post infection.**

Incubation period of viral infection	Virus Titer per μL
24 hours	12.10
48 hours	14.29
72 hours	38.19

421

422 The single drug and dual drug combination were added to the infected Vero cells and incubated
423 for 24, 48 and 72 hours. The virus challenge test (IC_{50} in ppm) of single drug and drug
424 combination against Vero cells infected with SARS-CoV-2 isolate, with a multiplicity of
425 infection (MoI) value of 0.04, showed that combining drugs resulted in lower IC_{50} of each single
426 drug than those of single drug uses. As can be seen in Table 3 and Fig 13-14, LOPIRITO + AZI
427 (1:2) resulted in an IC_{50} of less than 8.33 ppm for 24-hour incubation which was lower than
428 those of single use LOPIRITO and AZI which were 12.10 and 51.90 $\mu\text{g/mL}$ respectively.
429 LOPIRITO + CLA (1:1) also produced a similar result at 24 hours post-incubation with a lower
430 IC_{50} value, at 6.90 $\mu\text{g/mL}$, than those of single LOPIRITO and CLA at 12.10 and 4.60 $\mu\text{g/mL}$.
431 A drug combination of LOPIRITO + DOXY (1:1) lowered the IC_{50} of DOXY at 24 hours after
432 drug incubation, which was reduced from 18 $\mu\text{g/mL}$ as a single drug to 13.94 $\mu\text{g/mL}$ as a
433 dual drug combination. On the other hand, the combination of HCQ with AZI, DOXY, FAVI,
434 and LOPIRITO increased the IC_{50} values against their single drug uses, as well as the
435 combination of FAVI + AZI (2:1).

436

437 **Table 3. The summary of antiviral activity (IC_{50}) of single and combination drugs against**
438 **Vero cells infected with SARS-CoV-2 at an multiplicity of infection (MoI) value of 0.04.**

Drugs	IC_{50} ($\mu\text{g/mL}$)		
	24h	48h	72h
Lopinavir/Ritonavir (LOPIRITO)	12.10	<1.00	0.90
Azithromycin (AZI)	51.90	19.60	<10.00
Clarithromycin (CLA)	4.60	0.60	0.90
Doxycycline (DOXY)	18.00	4.70	0.40
Hydroxychloroquine (HCQ)	9.50	4.70	1.40

Favipiravir (FAVI)	9.60	18.60	<10.00
Lopinavir/Ritonavir + Azithromycin (LOPIRITO:AZI, 1:2)	<8.33	48.09	<8.33
Lopinavir/Ritonavir + Clarithromycin (LOPIRITO:CLA, 1:1)	6.90	3.90	<0.50
Lopinavir/Ritonavir + Doxycycline (LOPIRITO:DOXY, 1:1)	13.94	4.79	<2.50
Hydroxychloroquine + Azithromycin (HCQ:AZI, 1:2)	39.68	39.68	<16.66
Hydroxychloroquine + Doxycycline (HCQ:DOXY, 1:2)	30.80	<6.67	30.80
Favipiravir + Azithromycin (FAVI:AZI, 2:1)	48.46	14.53	86.99
Hydroxychloroquine + Favipiravir (HCQ:FAVI, 1:10)	57.72	74.77	<31.82
Hydroxychloroquine + Lopinavir/Ritonavir (HCQ:LOPIRITO, 1:2)	24.90	23.49	25.61

439

440 **Figure 13. The efficacy (IC₅₀) evaluation of Lopinavir-Ritonavir (LOPIRITO),**
441 **Favipiravir (FAVI), Azithromycin (AZI), Clarithromycin (CLA), Doxycycline (DOXY),**
442 **and Hydroxychloroquine (HCQ) as a single drug in Vero cells infected with SARS-CoV-**
443 **2 virus isolates for 24 hours (A), 48 hours (B), and 72 hours (C) analysed using**
444 **CompuSyn Software at a multiplicity of infection (MoI) value of 0.04.**

445

446

447 **Figure 14. The efficacy (IC₅₀) evaluation of dual combination of Lopinavir-Ritonavir**
448 **(LOPIRITO), Azithromycin (AZI), Doxycycline (DOXY), Favipiravir (FAVI),**

449 **Clarithromycin (CLA), and Hydroxychloroquine (HCQ) as a single drug in Vero cells**
 450 **infected with SARS-CoV-2 virus isolates for 24 hours (A), 48 hours (B), and 72 hours**
 451 **(C) analysed using CompuSyn Software at a multiplicity of infection (MoI) value of 0.04.**

452
 453 On the other hand, the evaluation of each concentration of drug combination at a determined
 454 drug incubation period reveals that the use of drug combinations resulted in a lower drug
 455 concentration required for producing undetected virus numbers than the single drug uses, as
 456 evident from Table 4. The combination of LOPIRITO + AZI (1:2) composed of 13.4 µg/mL
 457 LOPIRITO and 33.6 µg/mL AZI had produced undetected virus numbers at 24, 48, and 72
 458 hours post-incubation at a concentration of 50 µg/mL which were lower than the concentrations
 459 of each single drug required for generating a similar result, namely; 37.5 and 125 µg/mL for
 460 LOPIRITO and AZI respectively. This was also observed for a drug combination of LOPIRITO
 461 + CLA(1:1), LOPIRITO + DOXY (1:1), and HCQ + LOPIRITO (1:2). However, the
 462 combination of HCQ + AZI (1:2), HCQ + DOXY (1:2), FAVI + AZI (2:1), and HCQ + FAVI
 463 (1:10) produced no higher efficacy in respect of virus eradication than their single drugs.

464
 465 **Table 4. The concentration of single and combination drugs (at a mass ratio) that**
 466 **produced an undetected virus copy number in the in vitro antiviral study against Vero**
 467 **cells infected with SARS-CoV-2 at a multiplicity of infection (MoI) value of 0.04 at 24, 48,**
 468 **and/or 72 hours' incubation.**

Drugs	Drug concentration (µg/mL)	Results
Lopinavir/Ritonavir (LOPIRITO)	37.5	24, 48, 72h virus undetected

Azithromycin (AZI)	125	24, 48, 72h virus undetected
Clarithromycin (CLA)	8	24, 48, 72h virus undetected
Doxycycline (DOXY)	37.5	24, 48, 72h virus undetected
Hydroxychloroquine (HCQ)	37.5	48, 72h virus undetected
Favipiravir (FAVI)	37.5	24, 48, 72h virus still detected with decreasing number
Lopinavir/Ritonavir + Azithromycin (LOPIRITO:AZI, 1:2)	50	24, 48, 72h virus undetected
Lopinavir/Ritonavir + Clarithromycin (LOPIRITO:CLA, 1:1)	30	48, 72h virus undetected
Lopinavir/Ritonavir + Doxycycline (LOPIRITO:DOXY, 1:1)	25	24, 48, 72h virus undetected
Hydroxychloroquine + Azithromycin (HCQ:AZI, 1:2)	100	24, 48, 72h virus undetected
Hydroxychloroquine + Doxycycline (HCQ:DOXY, 1:2)	25	48, 72h virus undetected
Favipiravir + Azithromycin (FAVI:AZI, 2:1)	200	24, 48, 72h virus still detected with decreasing number

Hydroxychloroquine + Favipiravir (HCQ:FAVI, 1:10)	150	24, 48, 72h virus undetected
Hydroxychloroquine + Lopinavir/Ritonavir (HCQ:LOPIRITO, 1:2)	50	24, 48, 72h virus still detected with decreasing number

469

470 **IL-6, IL-10 and TNF- α levels of virus-infected Vero cells incubated**
471 **with dual combinatory drugs**

472 An analysis of pro-inflammatory and anti-inflammatory responses was further conducted
473 included Interleukin-10 (IL-10), Interleukin-6 (IL-6), and Tumor Necrosis Factor- α (TNF- α).
474 As shown in Table 5, the administration of LOPIRITO, AZI, CLA, and HCQ increased IL-10
475 levels and reduced the efficacy of IL-6 as a pro-inflammatory marker, but had no effects on
476 TNF- α levels. However, for the most part, the use of dual drug administration increased IL-10
477 levels as an anti-inflammatory marker and reduced IL-6 and TNF- α levels as pro-inflammatory
478 markers, but there were no noticeable effects on these interleukin levels for the FAVI + AZI
479 (2:1) combination.

480

481 **Table 5. The summary of the cytokine levels of Vero cells infected with SARS-CoV-2**
482 **isolates an multiplicity of infection (MoI) value of 0.04 at 24, 48, and 72 hours incubated**
483 **with single and drug combinations. The data were in duplicates.**

Drugs	IL-10	IL-6	TNF- α
Lopinavir/Ritonavir (LOPIRITO)	\nearrow (37.5 μ g/mL; 72h)	\searrow (15 μ g/mL; 24, 48h)	No effects
Azithromycin (AZI)	\nearrow	\searrow	No effects

	(15 µg/mL; 24h)	(to 125 µg/mL; 24, 48, 72h)	
Clarithromycin (CLA)	↗↗ (8 µg/mL; 48h)	↘↘ (1, 4, 8 µg/mL; 24, 48, 72h)	No effects
Doxycycline (DOXY)	↗↗ (1 µg/mL; 48, 72h)	↘↘ (1 µg/mL; 24h)	↘↘ (1 µg/mL; 24h)
Hydroxychloroquine (HCQ)	↗↗ (15 µg/mL; 48h)	↘↘ (1 µg/mL; 24h)	No effects
Favipiravir (FAVI)	↗↗ (10, 15 µg/mL; 48, 72h)	↘↘ (to 100 µg/mL; 48h)	↘↘ (10 ppm; 24h)
Lopinavir/Ritonavir + Azithromycin (LOPIRITO:AZI, 1:2)	↗↗ (25, 50, 100 µg/mL; 48,72h) → strong	↘↘ (and IL-2) (25, 50, 100 µg/mL; 24, 48, 72h) → strong IL-2: ↘↘ (100 µg/mL; 24, 48h)	↘↘ (25 ppm; 24h)
Lopinavir/Ritonavir + Clarithromycin (LOPIRITO:CLA, 1:1)	↗↗ (1, 10 µg/mL; 24, 48, 72h)	↘↘ (1 µg/mL; 24, 48h)	↘↘ (30 µg/mL; 24, 48, 72h)
Lopinavir/Ritonavir + Doxycycline	↗↗ (5, 10 µg/mL; 48, 72h)	↘↘ (and IL-2)	↘↘

(LOPIRITO:DOXY, 1:1)		(10, 25 µg/mL; 48h) → strong IL-2: √√ (5, 10 µg/mL; 48, 72 h)	(5, 10, 25 µg/mL; 24, 48, 72h) → strong
Hydroxychloroquine + Azithromycin (HCQ:AZI, 1:2)	↗↗ (25,50 µg/mL; 48,72h)	√√ (and IL-2) (25, 50, 100 µg/mL; 24, 48, 72h) → strong	√√ (25 µg/mL; 24h)
Hydroxychloroquine + Doxycycline (HCQ:DOXY, 1:2)	↗↗ (25 µg/mL; 24, 48, 72h)	No effects	√√ (10, 25, 50 µg/mL; 24, 48, 72h)
Favipiravir + Azithromycin (FAVI:AZI, 2:1)	No effects	No effects	No effects
Hydroxychloroquine + Favipiravir (HCQ:FAVI, 1:10)	No effects	√√ (35, 75 µg/mL; 24h)	No effects
Hydroxychloroquine + Lopinavir/Ritonavir (HCQ:LOPIRITO, 1:2)	↗↗ (25, 50 µg/mL; 48h)	√√ (25, 50 µg/mL; 48h)	No effects

484 **Note:**

485 (25, 50 µg/mL; 48h) means that at concentration of 25 and 50 µg/mL of drug combination, the
486 changes in interleukin levels were observed at 48 hours post incubation. ↗↗: increased, ↘↘:
487 decreased

488

489 **Molecular docking study of drugs against main protease of SARS-** 490 **CoV-2 virus**

491 By using an in silico method as shown in Figure 15, it can be seen that all the ligands
492 including LOPIRITO, FAVI, AZI, CLA, DOXY, and HCQ can interact with the virus main
493 protease with high docking scores ranging from -37.46 to -22.01 (see Table 6). DOXY recorded
494 the lowest docking score, -37.46 kcal/mol and had a potency higher than Ritonavir (RITO). In
495 contrast, AZI had the highest docking score of approximately -22.01 kcal/mol.

496 **Figure 15. The molecular structures of native ligand binding to receptor in SARS-CoV-2**

497 The parameters to validate the docking parameters were employed to perform the docking of
498 each candidate ligand. From the docking results, the binding energy was obtained in the form
499 of a grid score (kcal / mol) for each ligand to the receptor as presented in Table 6.

500 **Table 6. The docking scores of potential SARS-CoV-2 main protease inhibitor drug.**

No	Chemical Name	Molecular Weight (g/mol)	Docking Score (kcal/mol)
1	Lopinavir (LOPI, C ₃₇ H ₄₈ N ₄ O ₅)	628.8	-28.56
2	Ritonavir (RITO, C ₃₇ H ₄₈ N ₆ O ₅ S ₂)	720.9	-30.47
3	Favipiravir	157.1	-23.11

	(FAVI, C ₅ H ₄ FN ₃ O ₂)		
4	Azithromycin (AZI, C ₃₈ H ₇₂ N ₂ O ₁₂)	749	-22.01
5	Clarithromycin (CLA, C ₃₈ H ₆₉ NO ₁₃)	748	-25.48
6	Doxycycline (DOXY, C ₂₂ H ₂₄ N ₂ O ₈)	444.4	-37.46
7	Hydroxychloroquine (HCQ, C ₁₈ H ₂₆ ClN ₃ O)	335.9	-29.59

501

502

503 Discussion

504 The in vitro antiviral activities of dual combinatory drugs consisting of antiviral agents, i.e.
505 LOPIRITO, FAVI, antibiotics such as AZI, CLA, DOXY, and HCQ against Vero cells infected
506 with SARS-CoV-2 virus isolated from hospitalized patients in Surabaya, Indonesia were
507 evaluated. These drugs have recently become the subject of interest for use in clinical trials,
508 thereby providing information about their therapeutic effects as combinatory drugs within a
509 highly effective strategy of providing pre-clinical evidence supporting their clinical use for
510 combating pandemic COVID-19.

511 LOPIRITO is a protease inhibitor commonly employed in the treatment of HIV that,
512 interestingly, has also been shown to have an antiviral effect on SARS-CoV and MERS-CoV
513 by inhibiting the protease activity of coronavirus [17,18]. Within this study, its combined use
514 with other drugs was evaluated. Significantly, most of these drug combinations demonstrated
515 greater in vitro antiviral potency against the SARS-CoV-2 virus with lower cytotoxicity
516 observed in mesenchymal stem cells than the single drug itself.

517 The drug combinations were prepared in two ratio types, i.e. constant and non-constant
518 weight ratios, due to the lack of data regarding the growth inhibition curves of these drugs in
519 mesenchymal stem cells in addition to their IC₅₀ values. Moreover, there is a paucity of
520 information about which drug is more toxic to the cells and drug use in combination as
521 evaluated in this study. This study aimed to identify the profile of drug interaction, whether
522 synergistic, additional, or antagonistic, in order to establish their cytotoxic effect on
523 mesenchymal stem cells. In principal, to obtain the appropriate ratio for clinical use, drug
524 combinations were prepared at both constant and non-constant ratios, with their IC₅₀ values
525 being subsequently determined. After the profiles had been obtained, the constant ratio with
526 low cytotoxicity was selected for further antiviral evaluation, while the non-constant ratio was
527 not considered further. This was because the use of commercial products at a largely general
528 dosage represents a more practical therapeutic application of COVID-19, not involving a
529 customized dose or Fixed Dose Combination products.

530 LOPIRITO was combined with AZI, primarily used in the treatment of respiratory,
531 enteric and genitourinary infection, which had also been recently employed as a therapeutic
532 agent against COVID-19 infection [21,22]. In this study, the dual combination of LOPIRITO
533 and AZI at respective ratios of 1:1 and 1:2 reduced the cytotoxicity of each single drug on
534 mesenchymal stem cells. Moreover, their combination produced higher efficacy in reducing
535 virus numbers, while also increasing IL-10 and reducing IL-6 and TNF- α levels.

536 LOPIRITO was also combined with CLA. Instead of monotherapy using only
537 LOPIRITO, several hospitalized patients received CLA, a macrolide antibiotic, which inhibits
538 protein synthesis in susceptible organisms (e.g. bacteria) by binding to the 50S ribosomal sub-
539 unit [34]. The same results were also achieved by combining LOPIRITO and CLA at a weight
540 ratio of 1:1. There was a decrease in cytotoxicity in normal cells and an increase of antiviral
541 activity against SARS-CoV-2 virus compared with each single drug.

542 FAVI is an antiviral medication used to treat influenza in Japan which is also being
543 evaluated for its effectiveness against other viral infections [35]. However, there is evidence
544 that FAVI is teratogenic, with the result that considerable care needs to be exercised in avoiding
545 its extensive use during pregnancy [36,37]. AZI is a broad-spectrum macrolide antibiotic with
546 a long half-life, excellent tissue penetration and a large distribution volume [21,9]. DOXY is a
547 broad-spectrum tetracycline-class antibiotic used in the treatment of infections caused by
548 bacteria and certain parasites. It is used to treat bacterial pneumonia, acne, chlamydia infections,
549 early-stage Lyme disease, cholera, typhus, and syphilis [38]. HCQ is a medication used to
550 prevent and treat malaria in areas where the disease remains resistant to chloroquine. Other
551 applications include the treatment of rheumatoid arthritis, lupus, and porphyria cutanea tarda.
552 HCQ is currently being studied to establish its efficacy in the prevention and treatment of
553 COVID-19 [39].

554 The same results are also obtained by use of a combination of LOPIRITO + CLA (Fig 5),
555 LOPIRITO + DOXY (Fig 6), HCQ + AZI (Fig 7), and HCQ + DOXY (Fig 8). These
556 combinations showed the absence of cytotoxic effect in cells and viability exceeding 90%. The
557 use of this combination provides a potential opportunity for antiviral testing due to its minimal
558 toxic effects on mesenchymal cells.

559 Both FAVI and AZI, when administered as single drugs, and their combination (FAVI +
560 AZI) produce extremely low cytotoxicity since they are relatively non-toxic to mesenchymal
561 cells, as indicated by the high CC_{50} value, (see Fig 9). On the other hand, a drug combination
562 of FAVI + HCQ has a higher CC_{50} value than HCQ as a single drug, which is relatively more
563 toxic than FAVI, as can be seen from the contents of Fig 10. A combination of LOPIRITO +
564 HCQ also has a higher CC_{50} value than HCQ as a single drug which is relatively more toxic
565 than LOPIRITO, (see Fig 11).

566 Based on the CC_{50} value data obtained, the application of a combination of LOPIRITO,
567 AZI, CLA, DOXY, FAVI, and HCQ has the potential to reduce the degree of toxicity of the

568 drug administered. Most drug combinations exhibit antagonistic effects-which negate the side
569 effects of other drugs. Thus, when viewed from the perspective of safety and toxicity, the
570 potential use of a combination of therapeutic drugs, especially the treatment of COVID-19, is
571 extremely high and can be considered effective. Furthermore, a virus challenge test was
572 performed on a combination of drugs which was declared to be relatively safe.

573 Antiviral activity was assessed using Vero cells previously infected with SARS-CoV-2
574 isolates obtained from Universitas Airlangga Hospital. A summary of results can be seen in
575 Table 3. It can be noted that the use of a single drug has the ability to reduce the amount of
576 virus. The analysis involving the use of software can be seen in Fig 13. With a single drug, there
577 was a decrease in the number of copies of the virus ($Fa = \text{number of copies of virus samples} /$
578 positive controls) in accordance with the duration of drug incubation in the sample, whereby at
579 72 hours, almost all viruses in the test group had died. The antiviral activities of drug
580 combinations can be seen in Fig 14 with a summary of the results contained in Table 4. The
581 results indicate that drug combinations demonstrated greater effectiveness in reducing the
582 amount of virus where IC_{50} values decreased after 24, 48 and 72 hours of the incubating of cells
583 infected with the drug. As a combination drug, there was a decrease in the number of copies of
584 the virus in some samples whereas, depending on the incubation time of the drug in the sample,
585 there was a significant reduction in the amount of virus in the test group.

586 An analysis of pro-inflammatory and anti-inflammatory responses was conducted,
587 including Interleukin-10 (IL-10), Interleukin-6 (IL-6), and Tumor Necrosis Factor- α (TNF- α).
588 From the results presented in Table 5, the majority of drug administration increased IL-10 levels
589 as an anti-inflammatory marker and reduced IL-6 and TNF- α levels as pro-inflammatory
590 markers. Only in the combination of FAVI + AZI (2:1) was the effect negligible. The
591 interactions observed in this study can be physical or chemical and affect the ability of the drugs
592 to infiltrate the cell to cause further toxic effects and inhibit or reduce the rate of viral infectivity
593 in host cells.

594 Molecular docking was employed to predict interactions between ligands and protein.
595 The interaction can be seen from the binding site of the macromolecular target. The docking
596 process consists of two interrelated stages, docking algorithm and scoring function. The
597 docking algorithm obtains the most stable conformation of the ligand-protein complex formed.
598 Molecular bonds will be formed from functional groups of ligands that interact with residues
599 of amino acid receptor proteins. The scoring function is intended to evaluate conformation by
600 calculating the strength of the affinity between ligand and protein and then directing the
601 exploration of the ligand conformation to a position with a stronger affinity [40]. The affinity
602 value obtained was in the form of Gibbs free energy. A low Gibbs free energy value indicates
603 that the conformation formed is stable, while a high one indicates the formation of a less stable
604 complex. The more negative the value produced, the stronger the affinity of the ligand-protein
605 complex, with the result that its activity is expected to be of even higher quality [41,42].

606 The SARS-CoV-2 main protease (PDB ID: ALU6) is a ~306 amino acid long main protease
607 whose crystal structure with a resolution of 1.93 Å has been elucidated. The main protease
608 enzyme is the optimum target for inhibiting the SARS-CoV-2 virus. This protease breaks the
609 spikes and is further established by penetration. This study was undertaken to identify possible
610 compounds that can bind to the main protease which may be used as a potential drug for SARS-
611 CoV-2. The results indicated that all the ligands, i.e. LOPI, RITO, FAVI, AZI, CLA, DOXY,
612 and HCQ, can bind with the main protease with a high docking score of -37.46 to -22.01
613 kcal/mol (see Table 6). It is probable that the compounds inhibit the process of viral replication
614 and translation and may have an extremely significant impact on controlling the viral load in
615 infected individuals.

616

617 **Conclusion**

618 Using a combination of drugs would reduce the degree of cytotoxicity compared to a single
619 drug, increase antiviral activity, and produce a lower effect on pro-inflammatory markers and
620 intensify anti-inflammatory response. Hence, it can reduce the toxic potency in cells and
621 increase the effectiveness with regard to reducing the number of copies of the SARS-CoV-2
622 virus. Based on the degree of therapeutic effectiveness, toxicity in vitro, and response to
623 inflammatory markers, the activity of a single drug from the highest to the lowest is as follows:
624 $CLA > LOPIRITO > DOXY > AZI > HCQ$.

625 Based on the degree of therapeutic effectiveness, toxicity in vitro, and the response to
626 inflammatory markers, the activity of a drug combination ranging from the highest to lowest is
627 the following: $LOPIRITO + AZI > LOPIRITO + AZI > HCQ + AZI > HCQ + FAVI >$
628 $LOPIRITO + CLA > HCQ + DOXY$. However, further studies are required regarding the
629 possible interactions.

630

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637

638 **Declarations**

639

640 - *Consent to publication*

641 Not applicable.

642

643

644 **Supporting Data**

645 The supporting data have been uploaded as the supplementary files

646

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An in vitro study of dual drug combinations of anti-viral agents, antibiotics, and/or hydroxychloroquine against the SARS-CoV-2 virus isolated from hospitalized patients in Surabaya, Indonesia

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1 **Abstract**

2 A potent therapy for the infectious coronavirus disease COVID-19 is urgently required with,
3 at the time of writing, research in this area still ongoing. This study aims to evaluate the in
4 vitro anti-viral activities of combinations of certain commercially available drugs that have
5 recently formed part of COVID-19 therapy. Dual combinatory drugs, namely; Lopinavir-
6 Ritonavir (LOPIRITO)-Clarithromycin (CLA), LOPIRITO-Azithromycin (AZI), LOPIRITO-
7 Doxycycline (DOXY), Hydroxychloroquine (HCQ)-AZI, HCQ-DOXY, Favipiravir (FAVI)-
8 AZI, HCQ-FAVI, and HCQ-LOPIRITO, were prepared. These drugs were mixed at specific
9 ratios and evaluated for their safe use based on the cytotoxicity concentration (CC₅₀) values of
10 human umbilical cord mesenchymal stem cells. The anti-viral efficacy of these
11 combinations in relation to Vero cells infected with SARS-CoV-2 virus isolated from a
12 patient in Universitas Airlangga hospital, Surabaya, Indonesia and evaluated for IC₅₀ 24, 48,
13 and 72 hours after viral inoculation was subsequently determined. Observation of the viral
14 load in qRT-PCR was undertaken, the results of which indicated the absence of high levels of
15 cytotoxicity in any samples and that dual combinatory drugs produced lower cytotoxicity than
16 single drugs. In addition, these combinations demonstrated considerable effectiveness in
17 reducing the copy number of the virus at 48 and 72 hours, while even at 24 hours, post-drug
18 incubation resulted in low IC₅₀ values. Most combination drugs reduced pro-inflammatory
19 markers, i.e. IL-6 and TNF- α , while increasing the anti-inflammatory response of IL-10.
20 According to these results, the descending order of effective dual combinatory drugs is one of
21 LOPIRITO-AZI>LOPIRITO-DOXY>HCQ-AZI>HCQ-FAVI>LOPIRITO-CLA>HCQ-DOX.
22 It can be suggested that dual combinatory drugs, e.g. LOPIRITO-AZI, can potentially be used
23 in the treatment of COVID-19 infectious diseases.

24

25 **Keywords:** antiviral; drugs combination; SARS-CoV-2; in vitro, infectious disease

26 **Introduction**

27 At the end of 2019, a case of pneumonia was diagnosed on the basis of a viral infection in
28 Wuhan, China [1]. The pathogen was identified as a novel enveloped RNA betacoronavirus2,
29 currently referred to as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2),
30 which has a phylogenetic similar to SARS-CoV. Since that time, it has developed into a
31 global pandemic due to Coronavirus SARS-CoV-2, also referred to as COVID-19 [2,3]. On
32 March 2nd 2020, the Indonesian Ministry of Health reported the first confirmed domestic
33 positive case of SARS-CoV-2. By September 2020, more than 262,000 individuals had been
34 infected with 10,105 cases culminating in death [4].

35 COVID-19 infection causes severe pneumonia with symptoms such as fever, a persistent
36 cough, and progressive breathing failure associated with respiratory complications. The high
37 hospitalization rate, risk of mortality and lack of a specific established treatment rendered
38 urgent the need for an effective therapy for COVID-19 to be developed. The main viral
39 proteinase has recently been considered positively as a suitable target for drug design against
40 COVID-19 infection due to its vital role in the poly-protein processing necessary for
41 coronavirus reproduction [5].

42 The term ‘antiviral agents’ refers to the medications prescribed to combat Middle East
43 Respiratory Syndrome (MERS) and SARS pandemics. Interferon α (IFN- α), lopinavir-
44 ritonavir, chloroquine phosphate, ribavirin, and Arbidol have been highlighted in the latest
45 version of the Guidelines for the Prevention, Diagnosis, and Treatment of Novel Coronavirus-
46 induced Pneumonia issued by the Republic of China’s National Health Commission (NHC) as
47 potential treatments for COVID-19 [6]. In addition to antiviral agents, antibiotics such as
48 amoxicillin, azithromycin or fluoroquinolones are also being employed [7] in an attempt to
49 eradicate the SARS-CoV-2 virus. However, given the continuing lack of data regarding their
50 efficacy as a form of COVID-19 therapy, this study aims to evaluate the use of dual

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51 combinatory drugs as an antiviral therapy against the SARS-CoV-2 virus, specifically
52 COVID-19, within the Indonesian context.

53 During the present research, the respective in vitro antiviral activities of Lopinavir-
54 Ritonavir (LOPIRITO), Favipiravir (FAVI), Azithromycin (AZI), Clarithromycin (CLA),
55 Doxycycline (DOXY), and Hydroxychloroquine (HCQ) as dual combinatory drugs at
56 determined ratios were analyzed. These ratios were established based on the plasma
57 concentration of drugs administered at the usual dose during clinical therapy, (see Table 1).
58 However, in many cases, there were limited or even no reports regarding the pharmacokinetic
59 profiles in dual drug combinations.

60 **Table 1. Peak plasma concentration of Lopinavir/Ritonavir (LOPIRITO), Azithromycin**
61 **(AZI), Clarithromycin (CLA), Doxycycline (DOXY), Hydroxychloroquine (HCQ), and**
62 **Favipiravir (FAVI) after a single oral administration of the drug.**

Drugs	Dosage	Peak Plasma Concentration	Reference
Lopinavir/Ritonavir	Oral administration of Aluvia® tablet containing 400/100 mg Lopinavir/Ritonavir twice a day	Lopinavir: 6.9 to 17.7 µg/mL	[8]
Azithromycin	Single oral administration of 500 mg Azithromycin	0.35-0.45 mg/L after	[9]
Clarithromycin	oral administration of 250 and 500 mg Clarithromycin twice a day	1 and 2.41 µg/mL, respectively	[10]
Doxycycline	Single oral administration of 200 mg doxycycline	1.5 to 7.0 µg/ml after oral administration	[11]
Hydroxychloroquine	Single oral administration of	0.28 to 0.54 µg/mL	[12]

	400 mg HCQ sulfate		
Favipiravir	1600/600 mg twice a day	64.56 µg/mL	[13]

63

64 Lopinavir, Ritonavir, and Favipiravir have all been used as antiviral agents which act as
65 virus protease inhibitors [8,9]. Azithromycin is classified as a macrolide antibiotic which has
66 been used extensively in the treatment of severe respiratory lower tract infections such as
67 pneumonia. It can be employed for preventing secondary infection often resulting from viral
68 infection, thereby avoiding a severe prognosis. Azithromycin has been reported to be an
69 immune modulator and anti-inflammatory agent [10,11], while also inhibiting virus
70 replication and the cytopathic effect mediated by the Zika virus in Glial cell lines and
71 astrocytes [17]. Moreover, the use of clarithromycin has been regarded in the same manner as
72 that of Azithromycin. Clarithromycin demonstrates a high affinity with the protein target of
73 HIV-1 protease in the molecular docking study which is superior to that of doxycycline due to
74 high hydrophobicity and partition co-efficiency [18]. The combined application of
75 Clarithromycin and antiviral agents, i.e. Oseltamivir or Zanamivir, increased systemic
76 immunity while reducing rates of infection-related relapse in children infected with the
77 influenza virus [16]. Doxycycline, a tetracycline-derived drug, has an inhibitory effect on
78 dengue fever viral replication and reduces the proinflammatory marker IL-6 during viral
79 infections [19]. Consequently, it may prove effective as a form of COVID-19 therapy [14,15].

80 Hydroxychloroquine is an aminoquinoline-derivate compound producing fewer severe side
81 effects than chloroquine [20]. It has been employed as an antiviral agent [21,22] which
82 impedes the viral pre-entry stage, inhibits both viral replication mediated by acidic
83 endocytosis and viral replication through modification of post-translation virus protein,
84 hinders virus maturation via pH modulation, and produces anti-inflammatory effects by
85 reducing IL-6 levels in serum [23].

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86 In this present work, the efficacy of these drugs as a form of COVID-19 therapy was
87 evaluated on Vero cells as viral hosts cultured with SARS-CoV-2 virus isolated from
88 hospitalized patients in Universitas Airlangga Hospital, Surabaya, Indonesia. Furthermore, an
89 analysis of the structure-based computational modelling of ligand-receptor interactions
90 evaluated their potential use as the main protease of SARS-CoV-2 inhibitor [24].

91

92 **Material and Methods**

93 **Materials**

94 Lopinavir-Ritonavir (LOPIRITO) was produced by Abbott Laboratories (Aluvia®,
95 Chicago, USA); Favipiravir (FAVI) by Toyama Chemical (Fujifilm Group) (Avigan®,
96 Japan); Azithromycin (AZI) tablets by Gentec Pharmaceutical Group (Spain); Clarithromycin
97 (CLA) by Ind Swift Laboratories Limited (India); Doxycycline (DOXY) by Genero
98 Pharmaceuticals (Doxicor®, Indonesia); Hydroxychloroquine (HCQ) by Imedco Djaja
99 (Hyloquin®, Indonesia); and dimethyl sulfoxide by Sigma Aldrich (Singapore). All other
100 reagents and solvents employed in this study were of the highest quality available. Milli-Q
101 water was used in all experiments.

102

103 **Virus and cell collection**

104 Vero cells were used for virus inoculation against SARS-CoV-2 isolates in Indonesia.
105 Cells were seeded in a 12-well microplate at a cell density of 5×10^4 cells/well cultured in
106 Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) containing 10% foetal bovine
107 serum (Gibco, USA), 1% penicillin-streptomycin (Gibco, USA) and 1% amphotericin-B
108 (Gibco, USA). Cells were incubated in a CO₂ incubator at 37°C in a humidified atmosphere
109 of 5% CO₂ for 24 hours and cultured to reach 80-90% confluence.

110 SARS-CoV-2 virus isolates were collected from PCR-positive confirmed patients in
111 Universitas Airlangga Hospital, Surabaya. Patient sputum sampling and clinical procedures
112 were performed in accordance with the ethical clearance issued by The Ethics Commission of
113 Universitas Airlangga Hospital (Certificate number 136/KEP/2020 dated April 20, 2020). The
114 sputum of conscious patients was collected in viral transport medium (VTM) containing
115 Gentamycin sulphate (100µg/ml) and Amphotericin B (0.5µg/ml). Further experiments were
116 conducted in the Biosafety Level (BSL)-3 Laboratory at The Institute of Tropical Disease,
117 Universitas Airlangga, Surabaya, Indonesia. In order to isolate the virus, the sputum samples
118 were inserted into a new conical tube, subsequently vortexed for five minutes, and centrifuged
119 at 13,000 rpm for ten minutes. After centrifugation, the supernatant of each sample was
120 extracted for the purposes of further experiments.

121

122 **Preparation of drugs solution**

123 Each tablet containing drugs was triturated and mixed until homogenous. Approximately
124 50 mg equivalent mass of drugs were weighed and added to dimethyl sulfoxide in order to
125 solubilize the drugs. The suspension was sonicated in a water bath for 15 minutes before
126 being added to Rosewell Park Memorial Institute (RPMI) media, sonicated again and
127 vortexed to mix it until homogenous. The suspension was then filtered through a
128 polycarbonate membrane with a pore size of 0.45 µm and then a pore size of 0.22 µm under
129 aseptic conditions. The filtrate was mixed with 10% foetal bovine serum and penicillin
130 streptomycin before being vortexed to produce a homogenous mixture to be used as a stock
131 solution. The samples were prepared by diluting the stock solution of each drug with RPMI
132 complete media at an appropriate level of dilution to produce a determined concentration. The
133 dual combinatory drugs mixtures were prepared by mixing appropriate amounts of two drug
134 stock solutions in order to produce a final concentration at the required level. The
135 combinatory drugs were evaluated at both constant and non-constant ratios to evaluate their

136 effects on the cytotoxicity, including; antagonistic, synergistic, or additive. A constant ratio of
137 the mixture was achieved by adding drug solutions at the same ratio, thereby increasing each
138 drug concentration, to produce dose escalation. In contrast, at a non-constant ratio, a fixed
139 determined concentration of drug was added to increased doses of other drug solution in order
140 to produce different levels of drug concentration.

141

142 **Cytotoxicity assay for dual combinatory drugs**

143 The cytotoxic concentration (CC_{50}) of drugs was performed by means of MTT assay at
144 the Stem Cell Research and Development Center, Universitas Airlangga using human
145 umbilical cord mesenchymal stem cells which had been obtained from human placenta tissue
146 as approved by the Ethical Committee of Universitas Airlangga Hospital (Certificate number
147 101/KEH/2019 dated January 10, 2019). The cells were prepared as the primary cell culture
148 and used for the cytotoxicity assay because of their sensitivity to chemicals. Cells were seeded
149 into 96-well microplates at a concentration of 1×10^3 cells/well in 100 μ L Alpha Minimum
150 Essentials Medium (α -MEM, Gibco, USA) supplemented with 10% foetal bovine serum, 1%
151 penicillin-streptomycin and 1% amphotericin-B. The plates were then incubated in a CO_2
152 incubator at $37^\circ C$ with 5% CO_2 for 24 hours, at which point, the supernatant was replaced
153 with α -MEM containing drugs at each concentration and incubated for a further 48 hours.
154 Approximately 25 μ L of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium-bromide
155 (MTT) reagent at a concentration of 5mg/mL was subsequently added to each well and
156 incubated for four hours at $37^\circ C$ with 5% CO_2 . Purple formazan crystals were formed and
157 observed under an inverted microscope. Dimethyl sulfoxide was added to each well with the
158 complete solubilisation of formazan crystals subsequently being observed. The greater the
159 number of formazan crystals formed, the lower the toxicity of the samples which were read
160 for optical density of formazan using a multi reader at a measurement wavelength of 595 nm

161 (Promega Glomax, USA). The CC_{50} value was analyzed by CompuSyn software (the
162 ComboSyn Inc., accessed from www.combosyn.com).

163

164 **Virus inoculation and antiviral assay for dual combinatory drugs**

165 Vero cells obtained from Elabscience® (Catalog No. EP-CL-0242, USA) were seeded in a
166 12-well plate and confirmed as reaching 80-90% confluence on the day of virus inoculation.

167 The culture medium was removed and the cells were then added to RPMI media containing

168 SARS-CoV-2 isolates, previously diluted with RPMI media at a ratio of 1:2. In this study,

169 about 2,000 virus copies were added to 50,000 cells of Vero cells, with a multiplicity of

170 infection (MoI) degree of 0.04. The plate was gently agitated for 30 minutes and incubated at

171 37°C, 5% CO₂ for 24 hours. About 3 mL of complete culture medium were subsequently

172 added to the plate and incubated at 5% CO₂ 37°C for 24 hours, at which point 3 mL of RPMI

173 media containing a drug combination were introduced and incubated at 5% CO₂ 37°C for 24,

174 48, and 72 hours. The drug mixtures were prepared at appropriate weight constant ratios

175 selected on the basis of the optimum safety profiles in the cytotoxicity study. The Vero cells

176 were observed post-treatment to observe the cytopathic effects, including; the rounding and

177 detachment of cells. Moreover, the IC_{50} values were determined in order to quantify antiviral

178 activity by measuring the proviral load in each well. The determination of the proviral load

179 was performed by means of a Seegene COVID-19 detection Kit (Beijing, China) which

180 detected three target genes, i.e. N-gene, E-gene and RdRP-gene. Amplification and data

181 acquisition were carried out using the ABI Prism 7500 Sequence detector system (Applied

182 Biosystems, USA). The IC_{50} value was further analyzed using CompuSyn software (The

183 ComboSyn Inc., accessed from www.combosyn.com).

184

185 **Measurement of IL-6, IL-10 and TNF- α levels of virus-infected**

186 **Vero cells incubated with dual combinatory drugs**

187 To enable measurement of IL-6, IL-10 and TNF- α levels, the culture medium of the treated
188 cells was collected in sterile micro-tubes and centrifuged at 3,500 rpm for 20 minutes. The
189 supernatants were carefully collected and diluted with aquadest at a 1:5 volume ratio and
190 vortexed until homogenous. The samples were deposited onto a well-plate, added to ELISA
191 reagents (Bioassay Technology Laboratory, Shanghai, China), and incubated at 37°C for 60
192 minutes. Reagent substrate solution was then added to the well and incubated for ten minutes
193 at 37°C. The samples were measured for antigen concentration using the optical density (OD)
194 plotted into the standard curves of IL-6, IL-10, and TNF- α .

195

196 **Molecular docking study of drugs against main protease of SARS-**
197 **CoV-2 virus**

198 The molecular docking study was carried out by using Schrodinger Maestro 2019-2
199 Maestro software including protein preparation, ligand preparation, grid generation and
200 receptor-ligand docking. The Linux operating system was used for the computational study.
201 Ligands (Lopinavir, Ritonavir, Favipiravir, Azithromycin, Clarithromycin, Doxycycline, and
202 Hydroxychloroquine) were downloaded from the NCBI
203 (<http://www.ncbi.nlm.nih.gov/pccompound>). The crystal Structure of SARS-CoV-2 main
204 protease, PDB ID: ALU6 was retrieved from the Protein Data Bank (PDB)
205 (<https://www.rcsb.org/>).

206 The main protease protein was prepared for a docking study by using in Schrodinger
207 2019-2 Maestro software. All ligand compounds were prepared using LigPrep, which can
208 produce low energy isomer of the ligand in optimization by using the OPLS_2005 force field.
209 The OPLS_2005 force field was used for generating Grid on protein receptors. Schrodinger

210 2019-2 version was used to predict the binding affinity, ligand competence, and inhibitory
211 candidate to the protein by performing rigid, flexible docking. The ligands were docked with
212 generated Grid of receptor protein PDB ID: ALU6 The optimal ligand selection for the
213 receptor was done based on the docking score.

214 **Preparation of ligands and receptors**

215 Ligand-receptor complex. The complex in the form of a crystal structure consisting of native
216 ligands and receptors was downloaded from the Protein Data Bank (PDB) server at the web
217 address <https://www.rcsb.org> with ID 6LU7 [25]. 6LU7 protein structure consists of two
218 chains (A and C). The Main protease (M^{Pro}) is in the A chain (shown in brown), while the
219 native ligand appears as blue in the C chain, as presented in Figure 1

220 **Figure 1. Image representation of Ligand-receptor Complex**

221 The receptors and ligands from the resulting crystalline structure did not undergo geometric
222 optimization treatment because they were obtained from the actual structure. For the purposes
223 of the docking procedure, the ligands of this crystal were given a partial charge of the atom
224 using the Austin Model 1 semi-empirical method with Bond Charge Correction (AM1-BCC)
225 [26], while the receptor partial charge was calculated by means of a molecular mechanics
226 approach with a force field of ff14SB [27].

227 Preparation of candidates as ligands. A sketch of the molecular structure of the ligand was
228 produced using the ChemDraw Professional version 17 program. This structural sketch was
229 still 2-dimensional with the result that a 3-dimensional structure had to be made. This
230 structure was formed by calculations using the MM + molecular mechanics method to quickly
231 obtain a 3-dimensional structure. The calculations were performed using a HyperChem 6
232 program. The structure of the calculation using the molecular mechanics method was then
233 refined using a semi-empirical Parametric Model number 3 (PM3) quantum mechanics
234 calculation. The calculations were completed using Gaussian 16 software. The partial atomic

235 charge of each ligand was calculated through application of the AM1-BCC semi-empirical
236 method.

237 **Construction surface and receptor spheres**

238 The receptor surface (molecular surface, ms) consisting of a number of cluster spheres was
239 created and calculated using the dms module which is part of the Dock 6 program [28]. The
240 active side of the M^{pro} was determined based on the native ligand position in the cluster. This
241 active side location was used as the basis for the construction of the simulation box. The
242 degree of margin for the formation of the simulation box was 10 Å.

243 **Creating a simulation box**

244 Depending on the position of the native ligand, a simulation box was built around it in the
245 shape of a cube. The position of the simulation box, native ligand, and cluster of spheres
246 relative to the receptor can be seen in the Figure 2.

247 **Figure 2. The position of the simulation box, native ligand, and cluster of spheres**
248 **relative to the receptor.**

249

250 **Validation of docking parameters**

251 The parameters to be employed in docking the candidate to the receptor were validated by
252 redocking the native ligand to the receptor. An effective docking parameter must be able to
253 return the native ligand to its original position with a maximum root mean square deviation
254 (rmsd) tolerance of 2 Å [26]. The docking parameter validation resulted in an rmsd of 1.725
255 Å, indicating that use of the docking parameters at the docking stage for candidate ligands
256 was feasible.

257

258 **Results**

259 **Characterization of Human umbilical cord mesenchymal stem** 260 **cells**

261 For the cytotoxicity assay of combinatory drugs, the primary cell cultures of human
262 umbilical cord mesenchymal stem cells were used as the experimental cells. From the
263 contents of Fig 3, it is clear that, as previously reported [28–31(4)], the stem cells were well
264 differentiated as indicated by immunocytochemistry assays conducted using CD45, CD90,
265 and CD105 antibodies.

266 **Figure 3. Phase contrast and fluorescence images of human umbilical cord stem**
267 **cells stained with anti-CD45, CD90, and CD105 antibody and CF555-labelled secondary**
268 **antibody observed under a fluorescence microscope at a magnification of 100x.**

270 **Cytotoxicities of dual drug combination of LOPIRITO-AZI in** 271 **mesenchymal stem cells**

272 In this study, the cytotoxicity assay was evaluated for single and dual combinatory drugs
273 during a period of 48 hours of drug incubation. This assay was intended to evaluate the
274 toxicity of dual combinatory drugs on normal cells. The combination ratios were
275 calculated taking into consideration the usual therapeutic doses and plasma peak
276 concentrations of the drugs. To determine this cytotoxicity, the drugs were mixed at both
277 constant and non-constant ratios.

278 The evaluation of LOPIRITO and AZI in the stem cells showed that AZI had
279 relatively non-toxic properties compared to those of LOPIRITO, while the CC_{50} values
280 were 1.3×10^{55} $\mu\text{g/mL}$ for AZI and 4.29×10^2 $\mu\text{g/mL}$ for LOPIRITO, as shown in Fig 4. The
281 combination of LOPIRITO and AZI at constant weight ratios of 1:1 and 1:2 respectively,

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282 and non-constant ratios resulted in decreases in the degree of cytotoxicity. These were
283 much safer than LOPIRITO as indicated by their higher CC_{50} values. These results
284 indicate that a combination of both drugs negates the side effects of each single one,
285 possibly producing an antagonist effect.’

286 **Figure 4. The cytotoxicity of Lopinavir-Ritonavir (LOPIRITO) and Azithromycin**
287 **(AZI) as a single drug (left) and dual drug combination at constant and non-constant**
288 **ratios (right) analysed by CompuSyn Software (n=3). At non-constant ratios of**
289 **LOPIRITO 8 µg/mL + AZI , LOPIRITO was added at a concentration of 8 µg/mL to**
290 **each increased level of AZI, i.e. 0.2, 2, 10, 100, and 400 µg/mL. On the other hand,**
291 **AZI was then added at a concentration of 50 µg/mL to each increased level of**
292 **LOPIRITO, i.e. 0.2, 2, 10, 100, and 400 µg/mL to produce LOPIRITO + AZI 50**
293 **µg/mL.**

294

295 **Cytotoxicities of dual drug combination of LOPIRITO-CLA in** 296 **mesenchymal stem cells**

297 The results of a cytotoxicity assay indicated that LOPIRITO was relatively more toxic to the
298 cells than CLA as indicated by their CC_{50} values as a single drug which were 7.46×10^2 µg/mL
299 and 2.28×10^3 µg/mL respectively, as shown in Fig 5. Moreover, the dual drug combination of
300 LOPIRITO:CLA at the weight ratio of 1:1 had a high CC_{50} value of 1.22×10^4 µg/mL,
301 indicating that this combination reduced the toxicity of both drugs in the stem cells.

302 **Figure 5. The cytotoxicities of Lopinavir-Ritonavir (LOPIRITO) and**
303 **Clarithromycin (CLA) as a single drug (left) and dual drug combination in constant**
304 **and non-constant ratios (right) analysed by using CompuSyn Software (n=3). At**
305 **non-constant ratios of LOPIRITO 8 µg/mL + CLA, LOPIRITO was added at a**
306 **concentration of 8 µg/mL to each increased levels of CLA i.e. 0.2, 2, 10, 100, and 400**

332 ratio of 1:2 for HCQ and AZI produced the lowest cytotoxicity in the stem cells in which the
333 CC_{50} was 2.81×10^4 $\mu\text{g/mL}$, thus providing for its potential use in an anti-viral study of
334 COVID-19.

335 **Figure 7. The cytotoxicities of Hydroxychloroquine (HCQ) and Azithromycin (AZI) as a**
336 **single drug (left) and dual drug combination in constant and non-constant ratios (right)**
337 **analysed by using CompuSyn Software (n=3). At non-constant ratios of HCQ + AZI 50**
338 **$\mu\text{g/mL}$, AZI was added at a concentration of 50 $\mu\text{g/mL}$ to each increased levels of HCQ**
339 **i.e. 0.2, 2, 10, 100, and 400 $\mu\text{g/mL}$. On the other hand, HCQ was then added at a**
340 **concentration of 6 $\mu\text{g/mL}$ to each increased levels of AZI i.e. 0.2, 2, 10, 100, and 400**
341 **$\mu\text{g/mL}$ to produce HCQ 6 $\mu\text{g/mL}$ + AZI.**

342

343 **Cytotoxicities of dual drug combination of HCQ-DOXY in**
344 **mesenchymal stem cells**

345 The use of HCQ was combined with DOXY to evaluate its safety when used during antiviral
346 studies. As can be seen in Fig 8, HCQ had higher cytotoxicity than DOXY. Furthermore, the
347 results showed that the dual drug combination produced lower toxicity in the stem cells than
348 that of a single HCQ-based treatment. The CC_{50} values of a combination of HCQ-DOXY at
349 respective weight ratios of 1:1 and 1:2 were 4.37×10^3 $\mu\text{g/mL}$ and 1.77×10^5 $\mu\text{g/mL}$, while the
350 HCQ was 1.50×10^3 $\mu\text{g/mL}$.

351 **Figure 8. The cytotoxicities of Hydroxychloroquine (HCQ) and Doxycycline (DOXY) as**
352 **a single drug (left) and dual drug combination in constant and non-constant ratios**
353 **(right) analysed by using CompuSyn Software (n=3). At non-constant ratios of HCQ +**
354 **DOXY 2 $\mu\text{g/mL}$, DOXY was added at a concentration of 2 $\mu\text{g/mL}$ to each increased**
355 **levels of HCQ i.e. 0.2, 2, 10, 100, and 400 $\mu\text{g/mL}$. On the other hand, HCQ was then**

356 added at a concentration of 6 µg/mL to each increased levels of DOXY i.e. 0.2, 2, 10, 100,
357 and 400 µg/mL to produce HCQ 6 µg/mL + DOXY.

358

359 **Cytotoxicities of dual drug combination of FAVI-AZI in** 360 **mesenchymal stem cells**

361 The use of FAVI and AZI in an antiviral study of COVID-19 was initially evaluated for
362 cytotoxicity against primary cultured stem cells. As shown in Fig 9, the results indicated that
363 both FAVI and AZI, administered either as a single drug or in dual combination, produced
364 very low cytotoxicity effects. It could be confirmed that FAVI and AZI were considered drugs
365 not harmful to mesenchymal stem cells.

366 **Figure 9. The cytotoxicities of Favipiravir (FAVI) and Azithromycin (AZI) as a single**
367 **drug (left) and dual drug combination in constant and non-constant ratios (right)**
368 **analysed by using CompuSyn Software (n=3). At non-constant ratios of FAVI + AZI 50**
369 **µg/mL, AZI was added at a concentration of 50 µg/mL to each increased levels of FAVI**
370 **i.e. 0.2, 2, 10, 100, and 400 µg/mL. On the other hand, FAVI was then added at a**
371 **concentration of 66 µg/mL to each increased levels of AZI i.e. 0.2, 2, 10, 100, and 400**
372 **µg/mL to produce FAVI 66 µg/mL + AZI.**

373

374 **Cytotoxicities of dual drug combination of HCQ-FAVI in** 375 **mesenchymal stem cells**

376 The HCQ was also evaluated for its combination with FAVI. As presented in Fig 10, as a
377 single drug, HCQ produced more intense cytotoxic effects in the mesenchymal stem cells than
378 did FAVI whose CC₅₀ value of HCQ was 11.75 µg/mL. Combining HCQ with FAVI reduced

379 the toxicity resulting in higher CC₅₀ values of the HCQ-FAVI combination which were 343
380 µg/mL and 954 µg/mL for HCQ-FAV mixed at the ratios of 1:5 and 1:10 respectively.

381 **Figure 10. The cytotoxicities of Hydroxychloroquine (HCQ) and Favipiravir (FAVI) as a**
382 **single drug (left) and dual drug combination in constant and non-constant ratios (right)**
383 **analysed by using CompuSyn Software (n=3). At non-constant ratios of HCQ 6 µg/mL +**
384 **FAVI, HCQ was added at a concentration of 66 µg/mL to each increased levels of FAVI**
385 **i.e. 0.2, 2, 10, 100, and 400 µg/mL. On the other hand, FAVI was then added at a**
386 **concentration of 66 µg/mL to each increased levels of HCQ i.e. 0.2, 2, 10, 100, and 400**
387 **µg/mL to produce HCQ + FAVI 66 µg/mL.**

388

389 **Cytotoxicities of dual drug combination of HCQ-LOPIRITO in** 390 **mesenchymal stem cells**

391 HCQ was dually combined with LOPIRITO and evaluated for its safe use against
392 mesenchymal stem cells. In this assay, HCQ and LOPIRITO produced relatively low CC₅₀
393 values of 2.51 and 58.55 µg/mL and were considered potentially toxic drugs and
394 combinations as shown in Fig 11. The dual combination of HCQ and LOPIRITO produced
395 higher CC₅₀ values than single HCQ, i.e. 9.38 µg/mL and 8.45 µg/mL, for HCQ:LOPIRITO
396 combined at weight ratios of 1:1 and 1:2, respectively. However, they were still more toxic
397 than LOPIRITO.

398 **Figure 11. The cytotoxicities of Hydroxychloroquine (HCQ) and Lopinavir-Ritonavir**
399 **(LOPIRITO) as a single drug (left) and dual drug combination in constant and non-**
400 **constant ratios (right) analysed by using CompuSyn Software (n=3). At non-constant**
401 **ratios of HCQ + LOPIRITO 8 µg/mL, LOPIRITO was added at a concentration of 8**
402 **µg/mL to each increased levels of HCQ i.e. 0.2, 2, 10, 100, and 400 µg/mL. On the other**

403 hand, HCQ was then added at a concentration of 6 µg/mL to each increased levels of
404 LOPIRITO i.e. 0.2, 2, 10, 100, and 400 µg/mL to produce HCQ 6 µg/mL + LOPIRITO.

405
406 **Antiviral activity in Vero cells infected with SARS-CoV-2-isolated**
407 **human virus**

408 After cytotoxic evaluation of dual drug combination in mesenchymal stem cells, the
409 drugs were subsequently assessed for antiviral activities against the SARS-CoV-2 virus
410 isolated from patients in Universitas Airlangga Hospital. The Vero cells were inoculated with
411 the virus which led to certain changes in their morphology indicating that the virus had
412 successfully infected them. Fig 12 contains the typical formations of virus-infected cells
413 observed at 24, 48, and 72 hours post-inoculation. At 24 hours post-inoculation, the presence
414 of groups or colonies of detached cells indicated that they were dead. Furthermore, the
415 formation of giant cells was observed in the 48 hours followed by a cytopathic effect clearly
416 evident in the cells at 72 hours after the virus inoculation.

417 **Figure 12. The photomicrographs of morphology changes of Vero cells before**
418 **virus inoculation (A), at 24-h (B), 48-h (C), and 72-h (D) after virus inoculation observed**
419 **at a magnification of 100x. The black arrow shows a giant cell formation and the white**
420 **arrow indicates a cytopathic effect.**

421 In addition to the photomicrographs of cell morphological changes, pro-viral load
422 determination indicated that virus copy numbers had increased during the incubation period,
423 as shown in Table 2.

424
425 **Table 2. Virus titer of Vero cells infected with the SARS-CoV-2 virus isolates at a**
426 **multiplicity of infection (MoI) of 0.04 at 24, 48, and 72 hours post infection.**

Incubation period of viral infection	Virus Titer per µL
--------------------------------------	--------------------

24 hours	12.10
48 hours	14.29
72 hours	38.19

427

428 The single drug and dual drug combination were added to the infected Vero cells and
429 incubated for 24, 48 and 72 hours. The virus challenge test (IC₅₀ in ppm) of single drug and
430 drug combination against Vero cells infected with SARS-CoV-2 isolate, with a multiplicity of
431 infection (MoI) value of 0.04, showed that combining drugs resulted in lower IC₅₀ of each
432 single drug than those of single drug uses. As can be seen in Table 3 and Fig 13-14,
433 LOPIRITO + AZI (1:2) resulted in an IC₅₀ of less than 8.33 ppm for 24-hour incubation which
434 was lower than those of single use LOPIRITO and AZI which were 12.10 and 51.90 µg/mL
435 respectively. LOPIRITO + CLA (1:1) also produced a similar result at 24 hours post-
436 incubation with a lower IC₅₀ value, at 6.90 µg/mL, than those of single LOPIRITO and CLA
437 at 12.10 and 4.60 µg/mL. A drug combination of LOPIRITO + DOXY (1:1) lowered the IC₅₀
438 of DOXY at 24 hours after drug incubation, which was reduced from 18 µg/mL as a
439 single drug to 13.94 µg/mL as a dual drug combination. On the other hand, the combination of
440 HCQ with AZI, DOXY, FAVI, and LOPIRITO increased the IC₅₀ values against their single
441 drug uses, as well as the combination of FAVI + AZI (2:1).

442

443 **Table 3. The summary of antiviral activity (IC₅₀) of single and combination drugs**
444 **against Vero cells infected with SARS-CoV-2 at an multiplicity of infection (MoI) value**
445 **of 0.04.**

Drugs	IC ₅₀ (µg/mL)		
	24h	48h	72h
Lopinavir/Ritonavir (LOPIRITO)	12.10	<1.00	0.90

Azithromycin (AZI)	51.90	19.60	<10.00
Clarithromycin (CLA)	4.60	0.60	0.90
Doxycycline (DOXY)	18.00	4.70	0.40
Hydroxychloroquine (HCQ)	9.50	4.70	1.40
Favipiravir (FAVI)	9.60	18.60	<10.00
Lopinavir/Ritonavir + Azithromycin (LOPIRITO:AZI, 1:2)	<8.33	48.09	<8.33
Lopinavir/Ritonavir + Clarithromycin (LOPIRITO:CLA, 1:1)	6.90	3.90	<0.50
Lopinavir/Ritonavir + Doxycycline (LOPIRITO:DOXY, 1:1)	13.94	4.79	<2.50
Hydroxychloroquine + Azithromycin (HCQ:AZI, 1:2)	39.68	39.68	<16.66
Hydroxychloroquine + Doxycycline (HCQ:DOXY, 1:2)	30.80	<6.67	30.80
Favipiravir + Azithromycin (FAVI:AZI, 2:1)	48.46	14.53	86.99
Hydroxychloroquine + Favipiravir (HCQ:FAVI, 1:10)	57.72	74.77	<31.82
Hydroxychloroquine + Lopinavir/Ritonavir (HCQ:LOPIRITO, 1:2)	24.90	23.49	25.61

446
447 **Figure 13. The efficacy (IC₅₀) evaluation of Lopinavir-Ritonavir (LOPIRITO),**
448 **Favipiravir (FAVI), Azithromycin (AZI), Clarithromycin (CLA), Doxycycline (DOXY),**
449 **and Hydroxychloroquine (HCQ) as a single drug in Vero cells infected with SARS-CoV-**
450 **2 virus isolates for 24 hours (A), 48 hours (B), and 72 hours (C) analysed using**
451 **CompuSyn Software at a multiplicity of infection (MoI) value of 0.04.**

452

453

454 **Figure 14. The efficacy (IC₅₀) evaluation of dual combination of Lopinavir-Ritonavir**
455 **(LOPIRITO), Azithromycin (AZI), Doxycycline (DOXY), Favipiravir (FAVI),**
456 **Clarithromycin (CLA), and Hydroxychloroquine (HCQ) as a single drug in Vero cells**
457 **infected with SARS-CoV-2 virus isolates for 24 hours (A), 48 hours (B), and 72 hours**
458 **(C) analysed using CompuSyn Software at a multiplicity of infection (MoI) value of 0.04.**

459

460 On the other hand, the evaluation of each concentration of drug combination at a determined
461 drug incubation period reveals that the use of drug combinations resulted in a lower drug
462 concentration required for producing undetected virus numbers than the single drug uses, as
463 evident from Table 4. The combination of LOPIRITO + AZI (1:2) composed of 13.4 µg/mL
464 LOPIRITO and 33.6 µg/mL AZI had produced undetected virus numbers at 24, 48, and 72
465 hours post-incubation at a concentration of 50 µg/mL which were lower than the
466 concentrations of each single drug required for generating a similar result, namely; 37.5 and
467 125 µg/mL for LOPIRITO and AZI respectively. This was also observed for a drug
468 combination of LOPIRITO + CLA(1:1), LOPIRITO + DOXY (1:1), and HCQ + LOPIRITO
469 (1:2). However, the combination of HCQ + AZI (1:2), HCQ + DOXY (1:2), FAVI + AZI
470 (2:1), and HCQ + FAVI (1:10) produced no higher efficacy in respect of virus eradication
471 than their single drugs.

472

473 **Table 4. The concentration of single and combination drugs (at a mass ratio) that**
474 **produced an undetected virus copy number in the in vitro antiviral study against Vero**
475 **cells infected with SARS-CoV-2 at a multiplicity of infection (MoI) value of 0.04 at 24,**
476 **48, and/or 72 hours' incubation.**

Drugs	Drug concentration (µg/mL)	Results
Lopinavir/Ritonavir (LOPIRITO)	37.5	24, 48, 72h virus undetected
Azithromycin (AZI)	125	24, 48, 72h virus undetected
Clarithromycin (CLA)	8	24, 48, 72h virus undetected
Doxycycline (DOXY)	37.5	24, 48, 72h virus undetected
Hydroxychloroquine (HCQ)	37.5	48, 72h virus undetected
Favipiravir (FAVI)	37.5	24, 48, 72h virus still detected with decreasing number
Lopinavir/Ritonavir + Azithromycin (LOPIRITO:AZI, 1:2)	50	24, 48, 72h virus undetected
Lopinavir/Ritonavir + Clarithromycin (LOPIRITO:CLA, 1:1)	30	48, 72h virus undetected
Lopinavir/Ritonavir + Doxycycline (LOPIRITO:DOXY, 1:1)	25	24, 48, 72h virus undetected
Hydroxychloroquine + Azithromycin (HCQ:AZI, 1:2)	100	24, 48, 72h virus undetected
Hydroxychloroquine +	25	48, 72h virus undetected

Doxycycline (HCQ:DOXY, 1:2)		
Favipiravir + Azithromycin (FAVI:AZI, 2:1)	200	24, 48, 72h virus still detected with decreasing number
Hydroxychloroquine + Favipiravir (HCQ:FAVI, 1:10)	150	24, 48, 72h virus undetected
Hydroxychloroquine + Lopinavir/Ritonavir (HCQ:LOPIRITO, 1:2)	50	24, 48, 72h virus still detected with decreasing number

477

478 **IL-6, IL-10 and TNF- α levels of virus-infected Vero cells**
479 **incubated with dual combinatory drugs**

480 An analysis of pro-inflammatory and anti-inflammatory responses was further conducted
481 included Interleukin-10 (IL-10), Interleukin-6 (IL-6), and Tumor Necrosis Factor- α (TNF- α).
482 As shown in Table 5, the administration of LOPIRITO, AZI, CLA, and HCQ increased IL-10
483 levels and reduced the efficacy of IL-6 as a pro-inflammatory marker, but had no effects on
484 TNF- α levels. However, for the most part, the use of dual drug administration increased IL-10
485 levels as an anti-inflammatory marker and reduced IL-6 and TNF- α levels as pro-
486 inflammatory markers, but there were no noticeable effects on these interleukin levels for the
487 FAVI + AZI (2:1) combination.

488

489 **Table 5. The summary of the cytokine levels of Vero cells infected with SARS-CoV-2**
 490 **isolates an multiplicity of infection (MoI) value of 0.04 at 24, 48, and 72 hours incubated**
 491 **with single and drug combinations. The data were in duplicates.**

Drugs	IL-10	IL-6	TNF- α
Lopinavir/Ritonavir (LOPIRITO)	↗↗ (37.5 μ g/mL; 72h)	↘↘ (15 μ g/mL; 24, 48h)	No effects
Azithromycin (AZI)	↗↗ (15 μ g/mL; 24h)	↘↘ (to 125 μ g/mL; 24, 48, 72h)	No effects
Clarithromycin (CLA)	↗↗ (8 μ g/mL; 48h)	↘↘ (1, 4, 8 μ g/mL; 24, 48, 72h)	No effects
Doxycycline (DOXY)	↗↗ (1 μ g/mL; 48, 72h)	↘↘ (1 μ g/mL; 24h)	↘↘ (1 μ g/mL; 24h)
Hydroxychloroquine (HCQ)	↗↗ (15 μ g/mL; 48h)	↘↘ (1 μ g/mL; 24h)	No effects
Favipiravir (FAVI)	↗↗ (10, 15 μ g/mL; 48, 72h)	↘↘ (to 100 μ g/mL; 48h)	↘↘ (10 ppm; 24h)
Lopinavir/Ritonavir + Azithromycin (LOPIRITO:AZI, 1:2)	↗↗ (25, 50, 100 μ g/mL; 48,72h) \rightarrow strong	↘↘ (and IL-2) (25, 50, 100 μ g/mL; 24, 48, 72h) \rightarrow strong IL-2: ↘↘ (100 μ g/mL; 24, 48h)	↘↘ (25 ppm; 24h)

Lopinavir/Ritonavir + Clarithromycin (LOPIRITO:CLA, 1:1)	↗↗ (1, 10 µg/mL; 24, 48, 72h)	↘↘ (1 µg/mL; 24, 48h)	↘↘ (30 µg/mL; 24, 48, 72h)
Lopinavir/Ritonavir + Doxycycline (LOPIRITO:DOXY, 1:1)	↗↗ (5, 10 µg/mL; 48, 72h)	↘↘ (and IL-2) (10, 25 µg/mL; 48h) → strong IL-2: ↘↘ (5, 10 µg/mL; 48, 72 h)	↘↘ (5, 10, 25 µg/mL; 24, 48, 72h) → strong
Hydroxychloroquine + Azithromycin (HCQ:AZI, 1:2)	↗↗ (25,50 µg/mL; 48,72h)	↘↘ (and IL-2) (25, 50, 100 µg/mL; 24, 48, 72h) → strong	↘↘ (25 µg/mL; 24h)
Hydroxychloroquine + Doxycycline (HCQ:DOXY, 1:2)	↗↗ (25 µg/mL; 24, 48, 72h)	No effects	↘↘ (10, 25, 50 µg/mL; 24, 48, 72h)
Favipiravir + Azithromycin (FAVI:AZI, 2:1)	No effects	No effects	No effects
Hydroxychloroquine + Favipiravir (HCQ:FAVI, 1:10)	No effects	↘↘ (35, 75 µg/mL; 24h)	No effects
Hydroxychloroquine	↗↗	↘↘	No effects

+ Lopinavir/Ritonavir (HCQ:LOPIRITO, 1:2)	(25, 50 µg/mL; 48h)	(25, 50 µg/mL; 48h)	
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492 **Note:**

493 (25, 50 µg/mL; 48h) means that at concentration of 25 and 50 µg/mL of drug combination, the
494 changes in interleukin levels were observed at 48 hours post incubation. ↗↗: increased, ↘↘:
495 decreased

496

497 **Molecular docking study of drugs against main protease of SARS-** 498 **CoV-2 virus**

499 By using an in silico method as shown in Figure 15, it can be seen that all the ligands
500 including LOPIRITO, FAVI, AZI, CLA, DOXY, and HCQ can interact with the virus main
501 protease with high docking scores ranging from -37.46 to -22.01 (see Table 6). DOXY
502 recorded the lowest docking score, -37.46 kcal/mol and had a potency higher than Ritonavir
503 (RITO). In contrast, AZI had the highest docking score of approximately -22.01 kcal/mol.

504 **Figure 15. The molecular structures of native ligand binding to receptor in SARS-CoV-2**

505 The parameters to validate the docking parameters were employed to perform the docking of
506 each candidate ligand. From the docking results, the binding energy was obtained in the form
507 of a grid score (kcal / mol) for each ligand to the receptor as presented in Table 6.

508 **Table 6. The docking scores of potential SARS-CoV-2 main protease inhibitor drug.**

No	Chemical Name	Molecular Weight (g/mol)	Docking Score (kcal/mol)
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1	Lopinavir (LOPI, C ₃₇ H ₄₈ N ₄ O ₅)	628.8	-28.56
2	Ritonavir (RITO, C ₃₇ H ₄₈ N ₆ O ₅ S ₂)	720.9	-30.47
3	Favipiravir (FAVI, C ₅ H ₄ FN ₃ O ₂)	157.1	-23.11
4	Azithromycin (AZI, C ₃₈ H ₇₂ N ₂ O ₁₂)	749	-22.01
5	Clarithromycin (CLA, C ₃₈ H ₆₉ NO ₁₃)	748	-25.48
6	Doxycycline (DOXY, C ₂₂ H ₂₄ N ₂ O ₈)	444.4	-37.46
7	Hydroxychloroquine (HCQ, C ₁₈ H ₂₆ ClN ₃ O)	335.9	-29.59

509

510

511 Discussion

512 The in vitro antiviral activities of dual combinatory drugs consisting of antiviral agents, i.e.
513 LOPIRITO, FAVI, antibiotics such as AZI, CLA, DOXY, and HCQ against Vero cells
514 infected with SARS-CoV-2 virus isolated from hospitalized patients in Surabaya, Indonesia
515 were evaluated. These drugs have recently become the subject of interest for use in clinical
516 trials, thereby providing information about their therapeutic effects as combinatory drugs
517 within a highly effective strategy of providing pre-clinical evidence supporting their clinical
518 use for combating pandemic COVID-19.

519 LOPIRITO is a protease inhibitor commonly employed in the treatment of HIV that,
520 interestingly, has also been shown to have an antiviral effect on SARS-CoV and MERS-CoV
521 by inhibiting the protease activity of coronavirus [17,18,32]. Within this study, its combined
522 use with other drugs was evaluated. Significantly, most of these drug combinations
523 demonstrated greater in vitro antiviral potency against the SARS-CoV-2 virus with lower
524 cytotoxicity observed in mesenchymal stem cells than the single drug itself.

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525 The drug combinations were prepared in two ratio types, i.e. constant and non-constant
526 weight ratios, due to the lack of data regarding the growth inhibition curves of these drugs in
527 mesenchymal stem cells in addition to their IC₅₀ values. Moreover, there is a paucity of
528 information about which drug is more toxic to the cells and drug use in combination as
529 evaluated in this study. This study aimed to identify the profile of drug interaction, whether
530 synergistic, additional, or antagonistic, in order to establish their cytotoxic effect on
531 mesenchymal stem cells. In principal, to obtain the appropriate ratio for clinical use, drug
532 combinations were prepared at both constant and non-constant ratios, with their IC₅₀ values
533 being subsequently determined. After the profiles had been obtained, the constant ratio with
534 low cytotoxicity was selected for further antiviral evaluation, while the non-constant ratio was
535 not considered further. This was because the use of commercial products at a largely general
536 dosage represents a more practical therapeutic application of COVID-19, not involving a
537 customized dose or Fixed Dose Combination products.

538 LOPIRITO was combined with AZI, primarily used in the treatment of respiratory,
539 enteric and genitourinary infection, which had also been recently employed as a therapeutic
540 agent against COVID-19 infection [33][21,22]. In this study, the dual combination of
541 LOPIRITO and AZI at respective ratios of 1:1 and 1:2 reduced the cytotoxicity of each single
542 drug on mesenchymal stem cells. Moreover, their combination produced higher efficacy in
543 reducing virus numbers, while also increasing IL-10 and reducing IL-6 and TNF- α levels.

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544 LOPIRITO was also combined with CLA. Instead of monotherapy using only
545 LOPIRITO, several hospitalized patients received CLA, a macrolide antibiotic, which inhibits
546 protein synthesis in susceptible organisms (e.g. bacteria) by binding to the 50S ribosomal sub-
547 unit [34]. The same results were also achieved by combining LOPIRITO and CLA at a weight
548 ratio of 1:1. There was a decrease in cytotoxicity in normal cells and an increase of antiviral
549 activity against SARS-CoV-2 virus compared with each single drug.

550 FAVI is an antiviral medication used to treat influenza in Japan which is also being
551 evaluated for its effectiveness against other viral infections [35]. However, there is evidence
552 that FAVI is teratogenic, with the result that considerable care needs to be exercised in
553 avoiding its extensive use during pregnancy [36,37]. AZI is a broad-spectrum macrolide
554 antibiotic with a long half-life, excellent tissue penetration and a large distribution volume
555 [21,9]. DOXY is a broad-spectrum tetracycline-class antibiotic used in the treatment of
556 infections caused by bacteria and certain parasites. It is used to treat bacterial pneumonia,
557 acne, chlamydia infections, early-stage Lyme disease, cholera, typhus, and syphilis [38]. HCQ
558 is a medication used to prevent and treat malaria in areas where the disease remains resistant
559 to chloroquine. Other applications include the treatment of rheumatoid arthritis, lupus, and
560 porphyria cutanea tarda. HCQ is currently being studied to establish its efficacy in the
561 prevention and treatment of COVID-19 [39].

562 The same results are also obtained by use of a combination of LOPIRITO + CLA (Fig
563 5), LOPIRITO + DOXY (Fig 6), HCQ + AZI (Fig 7), and HCQ + DOXY (Fig 8). These
564 combinations showed the absence of cytotoxic effect in cells and viability exceeding 90%.
565 The use of this combination provides a potential opportunity for antiviral testing due to its
566 minimal toxic effects on mesenchymal cells.

567 Both FAVI and AZI, when administered as single drugs, and their combination (FAVI +
568 AZI) produce extremely low cytotoxicity since they are relatively non-toxic to mesenchymal
569 cells, as indicated by the high CC_{50} value, (see Fig 9). On the other hand, a drug combination

570 of FAVI + HCQ has a higher CC_{50} value than HCQ as a single drug, which is relatively more
571 toxic than FAVI, as can be seen from the contents of Fig 10. A combination of LOPIRITO +
572 HCQ also has a higher CC_{50} value than HCQ as a single drug which is relatively more toxic
573 than LOPIRITO, (see Fig 11).

574 Based on the CC_{50} value data obtained, the application of a combination of LOPIRITO,
575 AZI, CLA, DOXY, FAVI, and HCQ has the potential to reduce the degree of toxicity of the
576 drug administered. Most drug combinations exhibit antagonistic effects-which negate the side
577 effects of other drugs. Thus, when viewed from the perspective of safety and toxicity, the
578 potential use of a combination of therapeutic drugs, especially the treatment of COVID-19, is
579 extremely high and can be considered effective. Furthermore, a virus challenge test was
580 performed on a combination of drugs which was declared to be relatively safe.

581 Antiviral activity was assessed using Vero cells previously infected with SARS-CoV-2
582 isolates obtained from Universitas Airlangga Hospital. A summary of results can be seen in
583 Table 3. It can be noted that the use of a single drug has the ability to reduce the amount of
584 virus. The analysis involving the use of software can be seen in Fig 13. With a single drug,
585 there was a decrease in the number of copies of the virus (F_a = number of copies of virus
586 samples / positive controls) in accordance with the duration of drug incubation in the sample,
587 whereby at 72 hours, almost all viruses in the test group had died. The antiviral activities of
588 drug combinations can be seen in Fig 14 with a summary of the results contained in Table 4.
589 The results indicate that drug combinations demonstrated greater effectiveness in reducing the
590 amount of virus where IC_{50} values decreased after 24, 48 and 72 hours of the incubating of
591 cells infected with the drug. As a combination drug, there was a decrease in the number of
592 copies of the virus in some samples whereas, depending on the incubation time of the drug in
593 the sample, there was a significant reduction in the amount of virus in the test group.

594 An analysis of pro-inflammatory and anti-inflammatory responses was conducted,
595 including Interleukin-10 (IL-10), Interleukin-6 (IL-6), and Tumor Necrosis Factor- α (TNF- α).

596 From the results presented in Table 5, the majority of drug administration increased IL-10
597 levels as an anti-inflammatory marker and reduced IL-6 and TNF- α levels as pro-
598 inflammatory markers. Only in the combination of FAVI + AZI (2:1) was the effect
599 negligible. The interactions observed in this study can be physical or chemical and affect the
600 ability of the drugs to infiltrate the cell to cause further toxic effects and inhibit or reduce the
601 rate of viral infectivity in host cells.

602 Molecular docking was employed to predict interactions between ligands and protein.
603 The interaction can be seen from the binding site of the macromolecular target. The docking
604 process consists of two interrelated stages, docking algorithm and scoring function. The
605 docking algorithm obtains the most stable conformation of the ligand-protein complex
606 formed. Molecular bonds will be formed from functional groups of ligands that interact with
607 residues of amino acid receptor proteins. The scoring function is intended to evaluate
608 conformation by calculating the strength of the affinity between ligand and protein and then
609 directing the exploration of the ligand conformation to a position with a stronger affinity [40].
610 The affinity value obtained was in the form of Gibbs free energy. A low Gibbs free energy
611 value indicates that the conformation formed is stable, while a high one indicates the
612 formation of a less stable complex. The more negative the value produced, the stronger the
613 affinity of the ligand-protein complex, with the result that its activity is expected to be of even
614 higher quality [41,42].

615 The SARS-CoV-2 main protease (PDB ID: ALU6) is a ~306 amino acid long main
616 protease whose crystal structure with a resolution of 1.93 Å has been elucidated. The main
617 protease enzyme is the optimum target for inhibiting the SARS-CoV-2 virus. This protease
618 breaks the spikes and is further established by penetration. This study was undertaken to
619 identify possible compounds that can bind to the main protease which may be used as a
620 potential drug for SARS-CoV-2. The results indicated that all the ligands, i.e. LOPI, RITO,
621 FAVI, AZI, CLA, DOXY, and HCQ, can bind with the main protease with a high docking

622 score of -37.46 to -22.01 kcal/mol (see Table 6). It is probable that the compounds inhibit the
623 process of viral replication and translation and may have an extremely significant impact on
624 controlling the viral load in infected individuals.

625

626 **Conclusion**

627 Using a combination of drugs would reduce the degree of cytotoxicity compared to a single
628 drug, increase antiviral activity, and produce a lower effect on pro-inflammatory markers and
629 intensify anti-inflammatory response. Hence, it can reduce the toxic potency in cells and
630 increase the effectiveness with regard to reducing the number of copies of the SARS-CoV-2
631 virus. Based on the degree of therapeutic effectiveness, toxicity in vitro, and response to
632 inflammatory markers, the activity of a single drug from the highest to the lowest is as
633 follows: CLA > LOPIRITO > DOXY > AZI > HCQ.

634 Based on the degree of therapeutic effectiveness, toxicity in vitro, and the response to
635 inflammatory markers, the activity of a drug combination ranging from the highest to lowest
636 is the following: LOPIRITO + AZI > LOPIRITO + AZI > HCQ + AZI > HCQ + FAVI >
637 LOPIRITO + CLA > HCQ + DOXY. However, further studies are required regarding the
638 possible interactions.

639

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646

647 **Declarations**

648

649 - ***Consent to publication***

650 Not applicable.

651

652

653 **Supporting Data**

654 The supporting data have been uploaded as the supplementary files

655

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