



HAL
open science

Methylene blue inhibits replication of SARS-CoV-2 in vitro

Mathieu Gendrot, Julien Andreani, Isabelle Duflot, Manon Boxberger, Marion Le Bideau, Joel Mosnier, Priscilla Jardot, Isabelle Fonta, Clara Rolland, Hervé Bogreau, et al.

► To cite this version:

Mathieu Gendrot, Julien Andreani, Isabelle Duflot, Manon Boxberger, Marion Le Bideau, et al.. Methylene blue inhibits replication of SARS-CoV-2 in vitro. International Journal of Antimicrobial Agents, 2020, pp.106202. 10.1016/j.ijantimicag.2020.106202 . hal-02996278

HAL Id: hal-02996278

<https://hal.science/hal-02996278>

Submitted on 15 Dec 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 **Methylene blue inhibits the replication of SARS-Cov-2 *in vitro***

2

3 Mathieu Gendrot^{a,b,c,#}, Julien Andreani^{c,d,#}, Isabelle Duflot^{c,d}, Manon Boxberger^{c,d}, Marion Le

4 Bideau^{c,d}, Joel Mosnier^{a,b,c,e}, Priscilla Jardot^{c,d}, Isabelle Fonta^{a,b,c,e}, Clara Rolland^{c,d}, Hervé

5 Bogreau^{a,b,c,e}, Sébastien Hutter^{c,d}, Bernard La Scola^{c,d*}, Bruno Pradines^{a,c,b,e*}

6

7 *^aUnité Parasitologie et Entomologie, Département Microbiologie et Maladies Infectieuses,*

8 *Institut de Recherche Biomédicale des Armées, Marseille, France*

9 *^bAix Marseille Univ, IRD, SSA, AP-HM, VITROME, Marseille, France*

10 *^cIHU Méditerranée Infection, Marseille, France*

11 *^dAix Marseille Univ, IRD, AP-HM, MEPHI, Marseille, France*

12 *^eCentre National de Référence du Paludisme, Marseille, France*

13

14 #Authors contributed equally to this work.

15

16 *Co-corresponding author. Present address: Unité Parasitologie et Entomologie, Institut de

17 Recherche Biomédicale des Armées, IHU Méditerranée Infection, 19-21 Boulevard Jean

18 Moulin, 13005 Marseille, France. Tel.: +33 4 13 732 231.

19 E-mail address: bruno.pradines@gmail.com

20 MEPHI, IHU Méditerranée Infection, 19-21 Boulevard Jean Moulin, 13005 Marseille,

21 France.

22 E-mail address: bernard.la-scola@univ-amu.fr

23

24 Abstract

25 In December 2019, a new severe acute respiratory syndrome coronavirus (SARS-CoV-2)
26 causing coronavirus diseases 2019 (COVID-19) emerged in Wuhan, China. Currently, there is
27 no antiviral treatment recommended against SARS-CoV-2. Identifying effective antiviral
28 drugs is urgently needed. Methylene blue already demonstrated *in vitro* antiviral activity in
29 photodynamic therapy, and antibacterial, antifungal or antiparasitic activity in
30 nonphotodynamic assays. Non-photoactivated methylene blue showed *in vitro* activity at very
31 low-micromolar range with EC₅₀ of $0.3 \pm 0.03 \mu\text{M}$ and EC₉₀ of $0.75 \pm 0.21 \mu\text{M}$ at MOI of
32 0.25 against SARS-CoV-2. The EC₅₀ and EC₉₀ values for methylene blue are lower than those
33 obtained for hydroxychloroquine (1.5 and 3.0 μM) and azithromycin (20.1 and 41.9 μM). The
34 ratios $C_{\text{max}}/\text{EC}_{50}$ and $C_{\text{max}}/\text{EC}_{90}$ in blood for methylene blue after oral administration were
35 estimated at 10.1 and 4.0, respectively, and 33.3 and 13.3 after intravenous administration.
36 Methylene blue EC₅₀ and EC₉₀ are consistent with concentrations observed in human blood.
37 We propose that methylene blue is a promising drug for COVID-19 treatment. *In vivo* evaluation in
38 animal experimental models is now required to confirm its antiviral effects on SARS-CoV-2.
39 The potential interest of methylene blue to treat COVID-19 needs to be confirmed by
40 prospective comparative clinical studies.

41

42 Keywords: SARS-CoV-2; COVID-19; Antiviral; Methylene blue; *in vitro*

43

44 **1. Introduction**

45 In December 2019, a new severe acute respiratory syndrome coronavirus (SARS-CoV-2)
46 causing coronavirus diseases 2019 (COVID-19) emerged in Wuhan, China [1]. Despite
47 containment measures, SARS-CoV-2 spread in Asia, Southern Europe, then in America and
48 currently in Africa. Currently, there is no antiviral treatment recommended against SARS-
49 CoV-2. Different drugs or combination have been evaluated worldwide. Identifying effective
50 low cost antiviral drugs with limited side effects affordable immediately is urgently needed,
51 especially for emerging countries.

52 Plasma products can transmit a wide range of pathogens in transfusion. Methylene blue, a
53 synthesized thiazine dye, was known to be effective in photodynamic therapy against
54 microbes and more especially virus. Methylene blue is able to intercalate into viral nucleic
55 acid when illuminated with visible light and prevents transmission of pathogens. The
56 illumination of methylene blue inactivated Zika, yellow fever, dengue, chikungunya, Ebola
57 viruses and Middle East respiratory syndrome coronavirus in plasma [2-5]. Methylene blue
58 can also demonstrate antimicrobial activities without photoactivation. Methylene blue
59 inhibited *in vitro* colistin-resistant strains of *Acinetobacter baumannii*, *Mycobacterium*
60 *ulcerans*, *Mycobacterium spp.* and *Candida albicans* [6-8]. Methylene blue was also effective
61 *in vivo* against Buruli ulcer in experimental *Mycobacterium ulcerans* infection in mice [7].
62 Additionally, methylene blue inactivated hepatitis C virus in transplant organ perfused with
63 methylene blue [9]. The most studied effects are those on malaria.

64 In 1891, methylene blue was first used to treat effectively two patients with uncomplicated
65 malaria [10]. In the 2010s, methylene blue showed effective *in vitro* activity in the nanomolar
66 range against *Plasmodium falciparum* strains and isolates [11-14]. Methylene blue showed a
67 protective effect against cerebral malaria in a murine model infected with *P. berghei* [15-17].

68 Methylene blue showed several benefits when used as partner in triple combination with
69 artemisinin-based combination therapy in uncomplicated falciparum malaria in children [18].
70 Taken together, these reports suggest that methylene blue may have antiviral effects against
71 SARS-CoV-2. Therefore the activity of methylene blue was assessed *in vitro* against a
72 clinically isolated SARS-CoV-2 strain and compared with the activity of hydroxychloroquine
73 and azithromycin, which have been already evaluated *in vitro* and *in vivo* in human [19-22].
74

75 **2. Material and methods**

76 2.1. Antimalarial drugs, virus and cells

77 Methylene blue (methylthioninium chloride; Proveblue®) was provided from Provepharm
78 SAS (Marseille, France). Stocks solutions of hydroxychloroquine (Sigma, Saint Louis, MO,
79 USA) and methylene blue were prepared in water and azithromycin (Sigma) in methanol. All
80 the stock solutions were then diluted in Minimum Essential Media (MEM, Gibco,
81 ThermoFischer) in order to have 7 final concentrations ranging from 0.1 μM to 100 μM . The
82 clinically isolated SARS-CoV-2 strain (IHUMI-3) [23] was maintained in production in Vero
83 E6 cells (American type culture collection ATCC® CRL-1586™) in MEM with 4% of fetal
84 bovine serum and 1% glutamine (complete medium).
85

86 2.2. Cytotoxicity assay

87 *In vitro* cell viability evaluation on the VERO E6 cell line was performed according to the
88 method described by Mosmann with slight modifications [24]. Briefly, 10^5 cells in 200 μl of
89 complete medium were added to each well of 96-well plates and incubated at 37 °C in a
90 humidified 5% CO_2 . After 24 h incubation, 25 μl of complete medium and 25 μl of each
91 concentration of methylene blue, hydroxychloroquine or azithromycin were added and the
92 plates were incubated 48h at 37 °C. After removal of the supernatant, 100 μL of MTT (3-

93 (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide, Sigma Aldrich, France)
94 solution (0.5 mg/ml in MEM without FBS) were then added to each well. Cells were
95 incubated for 2 h at 37 °C. After incubation, the MTT solution was removed and 100 µl of
96 dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Then, plates were
97 shaken at 700 rpm for 10 min at 37 °C. The absorbance was measured at 570 nm using a
98 TECAN Infinite F200 Microplate Reader. DMSO was used as blank. The 50% cytotoxicity
99 concentration (CC₅₀) was calculated with the inhibitory sigmoid E_{max} model, which estimated
100 the CC₅₀ through nonlinear regression by using a standard function of the R software
101 (ICEstimator version 1.2, <http://www.antimalarial-icestimator.net>). CC₅₀ value resulted in the
102 mean of 6 different experimentations.

103

104 2.3. Antiviral activity assay

105 Briefly, 96-well plates were prepared with 5.10⁵ cells/mL of Vero E6 (200µL per well), as
106 previously described [20]. Methylene blue, hydroxychloroquine or azithromycin
107 concentrations were added 4 h before infection. Vero E Cells were infected with IHUMI-3
108 strain at an MOI of 0.25. After 48h post-infection, the replication was estimated by RT-PCR
109 using the Superscrit III platinum one step with Rox kit (Invitrogene) after extraction with the
110 BIoExtract SuperBall kit (Biosellal, Dardilly, France). The primers used were previously
111 described [25]. EC₅₀ (median effective concentration) and EC₉₀ (90% effective concentration)
112 were calculated with the inhibitory sigmoid E_{max} model, which estimated the EC₅₀ and EC₉₀
113 through nonlinear regression by using a standard function of the R software (ICEstimator
114 version 1.2). EC₅₀ and EC₉₀ values resulted in the mean of 6 different experimentations.

115

116 2.4. Data analysis and interpretation

117 Selectivity index (SI) as ratio of CC_{50}/EC_{50} was estimated for each drug. The expected
118 maximum blood concentration (C_{max}) was estimated from literature for each drug at doses
119 commonly administered in oral malaria treatment, and for methylene blue at intravenous
120 doses used for FDA and MEA approved methemoglobinemia treatment. The ratios C_{max}/EC_{50}
121 and C_{max}/EC_{90} were estimated to find out if the effective concentration in plasma to cure
122 SARS-CoV-2 is achievable in human. If data on drug accumulation into lung was available,
123 the ratios C_{lung}/EC_{50} and C_{lung}/EC_{90} were calculated.

124

125 **3. Results**

126 CC_{50} , EC_{50} , EC_{90} and SI for each drug are presented in Table 1. Methylene blue and
127 hydroxychloroquine showed EC_{50} and EC_{90} at low micromolar range (Table 1). The EC_{50} and
128 EC_{90} values for methylene blue are lower than those obtained for hydroxychloroquine and
129 azithromycin. The ratios C_{max}/EC_{50} and C_{max}/EC_{90} in blood for methylene blue were estimated
130 at 10.1 and 4.0, respectively after oral administration and at 33.3 and 13.3 after intravenous
131 administration (Figure 1).

132

133 **4. Discussion**

134 Methylene blue showed *in vitro* activity at very low-micromolar range with EC_{50} of 0.3 ± 0.03
135 μM and EC_{90} of $0.75 \pm 0.21 \mu\text{M}$ at MOI of 0.25 ($SI > 333$) (Table 1). The EC_{50} and EC_{90}
136 values for methylene blue are lower than those obtained for hydroxychloroquine and
137 azithromycin. Azithromycin demonstrated low *in vitro* efficacy against SARS-CoV-2 used
138 alone but potentiated the effects of hydroxychloroquine in combination [20]. An oral uptake
139 of 325 mg of methylene blue led to a C_{max} (maximum blood concentration) value of 0.97
140 $\mu\text{g/ml}$ (around $3 \mu\text{M}$) and $t_{1/2}$ (elimination half-life) of 14.9 h [26]. Methylene blue dose of 2
141 mg/kg intravenous showed a C_{max} of 2.917 $\mu\text{g/ml}$ (around $10 \mu\text{M}$) [27]. The ratios C_{max}/EC_{50}

142 and C_{\max}/EC_{90} for methylene blue were estimated at 10.1 and 4.0 for oral route and 33.3 and
143 13.3 for IV, respectively. Methylene blue EC_{50} and EC_{90} are consistent with concentrations
144 observed in human blood. Around 3 to 5% of methylene blue per g of lung was found after
145 intravenous methylene blue injection but methylene blue concentration decreased rapidly
146 under 0.1% after 10 h [28]. In comparison, an oral uptake of 400 mg of hydroxychloroquine
147 led to a C_{\max} of 1.22 μM [29]. Hydroxychloroquine accumulated 30 times more in lungs than
148 in blood [30]. The azithromycin C_{\max} ranged from 0.18 to 0.4 $\mu\text{g}/\text{ml}$ of blood (around 0.22 to
149 0.51 μM) after the last dose of oral administration of 500 mg once daily for 3 days or after a
150 single dose of 500 mg [31-33]. These doses led to C_{\max} in lung ranging from 8 to 9 $\mu\text{g}/\text{g}$
151 (around 10 to 12 μM) [31,32]. The C_{\max} expected in lung was below the EC_{50} and EC_{90} .
152 However, due to potentiation of the antiviral effects when azithromycin is combined with
153 hydroxychloroquine, azithromycin can be used *in vitro* at lower concentrations (5 and 10 μM)
154 [20]. These concentrations are compatible with expected concentrations in lungs.
155 Methylene blue showed a low cytotoxicity *in vitro* against Vero E6 cells with $CC_{50} > 100 \mu\text{M}$.
156 The selectivity index (SI) as ratio of CC_{50}/EC_{50} was estimated above 333. The present CC_{50} of
157 hydroxychloroquine with SI around 13 against Vero E6 cells was higher than previous
158 reported CC_{50} , ranging from $> 50 \mu\text{M}$ to 250 μM against Vero E6 cells [19,34] or above
159 500 μM in *Felis catus* whole fetus-4 cells [35]. Azithromycin showed also a low cytotoxicity
160 against Vero E6 cells with $CC_{50} > 100 \mu\text{M}$ and $SI > 5$. CC_{50} for azithromycin was consistent
161 with previous data (> 130) [34]. Methylene blue showed a low cytotoxicity but
162 predominatingly the higher SI.
163 Although methylene blue is on the list of drugs potentially dangerous for patients with
164 glucose-6-phosphate dehydrogenase (G6PD), no association between methylene blue and
165 severe hemolysis has been detected after oral administration [36]. Additionally, IV route
166 methylene blue has been granted a Market authorization in Europe in 2011 and in the US in

167 2016, for the treatment of acquired methemoglobinemia, based upon a confirmed positive
168 benefit/risk ratio in this pathology.

169

170 **5. Conclusion**

171 Methylene blue showed high *in vitro* antiviral effective activity against SARS-CoV-2 with
172 IC₅₀ (0.3 μM) and IC₉₀ (0.75 μM) compatible with oral uptake and intravenous
173 administrations. This *in vitro* activity is higher than those obtained with drugs which are
174 evaluated in clinical trials worldwide like hydroxychloroquine (1.5 μM), azithromycin (20.1
175 μM), remdisivir (23 μM), lopinavir (26.6 μM) or ritonavir (> 100 μM) [37]. We propose that
176 methylene blue is a promising drug for COVID-19 treatment. *In vivo* evaluation in animal
177 experimental models is now required to confirm its antiviral effects on SARS-CoV-2. The
178 potential interest of methylene blue to treat COVID-19 needs to be confirmed by prospective
179 comparative clinical studies.

180

181 **Declarations**

182 **Funding:** This study was supported by the Institut Hospitalo-Universitaire (IHU)
183 Méditerranée Infection, the National Research Agency under the program « Investissements
184 d'avenir », reference ANR-10-IAHU-03.

185 **Competing Interests:** Bernard La Scola and Bruno Pradines are associated as co-inventors
186 with ProvePharm in the patent EP 20305425.9 (30/04/2020) but have no financial interest
187 with the subject matter. Manon Boxberger received a PhD grant supported by L'Occitane
188 Society. ProvePharm or funders had no role in the design of the study; in the collection,
189 analyses, or interpretation of data; in the writing of the manuscript, or in the decision to
190 publish the results. The other authors have no conflict of interest to declare.

191 **Ethical approval:** Not required.

192

193 **Acknowledgments:** The authors thank Provepharm for providing the methylene blue
194 (Proveblue®)

195

196 **References**

197 [1] Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, et al. A new coronavirus associated
198 with human respiratory disease in China. *Nature* 2020;579:365-9.

199 [2] Fryk JJ, Marks DC, Hobson-Peters J, Prow NA, Watterson D, Hall RA, et al. Dengue and
200 chikungunya viruses in plasma are effectively inactivated after treatment with methylene blue
201 and visible light. *Transfusion* 2016;56:2278-85.

202 [3] Faddy HM, Fryk JJ, Hal RA, Young PR, Reichenberg S, Tolksdorf F, et al. Inactivation of
203 yellow fever virus in plasma after treatment with methylene blue and visible light and in
204 platelet concentrates following treatment with ultraviolet C light. *Transfusion* 2019;59:2223-
205 7.

206 [4] Wang Y, Ren K, Liao X, Luo G, Kumthip K, Leetrakool N, et al. Inactivation of Zika
207 virus in plasma and derivatives by four different methods. *J Med Virol* 2019;91:2059-65.

208 [5] Eickmann M, Gravemann U, Handke W, Tolksdorf F, Reichenberg S, Müller TH, et al.
209 Inactivation of Ebola virus and Middle East respiratory syndrome coronavirus in platelet
210 concentrates and plasma by ultraviolet C light and methylene blue plus visible light,
211 respectively. *Transfusion* 2018;58:2202-7.

212 [6] Gazel D, Tatman Otkun M, Akçali A. In vitro activity of methylene blue and eosin
213 methylene blue agar on colistin-resistant *A. baumannii*: an experimental study. *J Med*
214 *Microbiol* 2019;68:1607-13.

215 [7] Tian RBD, Asmar S, Napez C, Lépidi H, Drancourt M. Effectiveness of purified
216 methylene blue in an experimental model of *Mycobacterium ulcerans* infection. Int J
217 Antimicrob Agents 2017;49:290-5.

218 [8] Pal R, Ansari MA, Saibabu V, Das S, Fatima Z, Hameed S. Nonphotodynamic roles of
219 methylene blue: Display of distinct antimycobacterial and anticandidal mode of actions. J
220 Pathog 2018;2018:3759704.

221 [9] Helfritz FA, Bojkova D, Wanders V, Kuklinski N, Westhaus S, von Horn C, et al.
222 Methylene blue treatment of grafts during cold ischemia time reduces the risk of hepatitis C
223 virus transmission. J Infect Dis 2018;218:1711-21.

224 [10] Guttman P, Ehrlich P. Ueber die Wirkung des Methylenblau bei Malaria. Berl Klin
225 Wochenschr 1891;28:953-6.

226 [11] Pascual A, Henry M, Briolant S, Charras S, Baret E, Amalvict R, et al. *In vitro* activity of
227 Proveblue (methylene blue) on *Plasmodium falciparum* strains resistant to standard
228 antimalarial drugs. Antimicrob Agents Chemother 2011;55:2472-4.

229 [12] Fall B, Camara C, Fall M, Nakoulima A, Dionne P, Diatta B, et al. *Plasmodium*
230 *falciparum* susceptibility to standard and potential anti-malarial drugs in Dakar, Senegal,
231 during the 2013-2014 malaria season. Malar J 2015;14:60.

232 [13] Fall B, Madamet M, Diawara S, Briolant S, Wade KA, Lo G, et al. *Ex vivo* activity of
233 Proveblue, a methylene blue, against field isolates of *Plasmodium falciparum* in Dakar,
234 Senegal from 2013 to 2015. Int J Antimicrob Agents 2017;50:155-8.

235 [14] Gendrot M, Madamet M, Mosnier J, Fonta I, Amalvict R, Benoit N, et al. Baseline and
236 multinormal distribution of ex vivo susceptibilities of *Plasmodium falciparum* to methylene
237 blue in Africa, 2013-18. J Antimicrob Agents 2020, in press.

238 [15] Dormoi J, Pradines B. Dose responses of Proveblue methylene blue in an experimental
239 murine cerebral malaria model. Antimicrob Agents Chemother 2013;57:4080-1.

240 [16] Dormoi J, Briolant S, Desgrouas C, Pradines B. Efficacy of Proveblue (methylene blue)
241 in an experimental cerebral malaria murine model. *Antimicrob Agents Chemother*
242 2013;57:3412-4.

243 [17] Dormoi J, Briolant S, Desgrouas C, Pradines B. Impact of methylene blue and
244 atorvastatin combination therapy on the apparition of cerebral malaria in a murine model.
245 *Malar J* 2013;12:127.

246 [18] Mendes Jorge M, Ouermi L, Meissner P, Compaoré G, Coulibaly B, Nebie E, et al.
247 Safety and efficacy of artesunate-amodiaquine combined with either methylene blue or
248 primaquine in children with falciparum malaria in Burkina Faso: A randomized controlled
249 trial. *PLoS One* 2019;14:e0222993.

250 [19] Liu J, Cao R, Xu M, Wang X, Zhang H, Hu H, et al. Hydroxychloroquine, a less toxic
251 derivative of chloroquine, is effective in inhibiting SARS-CoV-2 infection in vitro. *Cell*
252 *Discov* 2020;6:16.

253 [20] Andreani J, Le Bideau M, Dufлот I, Jardot P, Rolland C, Boxberger M, et al. In vitro
254 testing of hydroxychloroquine and azithromycin on SARS-CoV-2 shows synergistic effect.
255 *Microb Pathog* 2020;145:104228.

256 [21] Gautret P, Lagier JC, Parola P, Hoang VT, Meddeb L, Sevestre J, et al. Clinical and
257 microbiological effect of a combination of hydroxychloroquine and azithromycin in 80
258 COVID-19 patients with at least a six-day follow up: an observational study. *Trav Med Infect*
259 *Dis* 2020;34:101663.

260 [22] Million M, Lagier JC, Gautret P, Colson P, Fournier PE, Amrane S, et al. Early treatment
261 of 1061 COVID-19 patients with hydroxychloroquine and azithromycin, Marseille, France.
262 *Travel Med Dis* 2020;35:101738.

263 [23] Gautret P, Lagier JC, Parola P, Hoang VT, Meddeb L, Mailhe M, et al.
264 Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label
265 non-randomized clinical trial. *Int J. Antimicrob Agents* 2020;56:105949.

266 [24] Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to
267 proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55-63.

268 [25] Amrane S, Tissot-Dupont H, Doudier B, Eldin C, Hocquart M, Mailhe M, et al. Rapid
269 viral diagnosis and ambulatory management of suspected COVID-19 cases presenting at the
270 infection diseases referral hospital in Marseille, France, -January 31st to March 1st, 2020: A
271 respiratory virus snapshot. *Travel Med Infect Dis* 2020;36:101632.

272 [26] Anh CX, Chavchich M, Birrell GW, van Breda K, Travers T, Rowcliffe K, et al.
273 Pharmacokinetics and ex vivo antimalarial activity of artesunate-amodiaquine plus methylene
274 blue in healthy volunteers. *Antimicrob Agents Chemother* 2020;64:e01441-19.

275 [27] Center for drug evaluation and research. Clinical pharmacology and biopharmaceutics
276 review(s): Application number 204630Orig1s000.
277 https://www.accessdata.fda.gov/drugsatfda_docs/nda/2016/204630Orig1s000ClinPharmR.pdf

278 [28] Link EM, Costa DC, Lui D, Ell PJ, Lower PJ, Spittle MF. Targeting disseminated
279 melanoma with radiolabelled methylene blue. *Acta Oncolog* 1996;35:331-41.

280 [29] Rainsford KD, Parke AL, Clifford-Rashotte M, Kean WF. Therapy and pharmacological
281 properties of hydroxychloroquine and chloroquine in the treatment of systemic lupus
282 erythematosus, rheumatoid arthritis and related diseases. *Inflammatopharmacol* 2015;23:231-
283 69.

284 [30] Chhonker YS, Sleightholm RL, Li J, Oupicky D, Murry DJ. Simultaneous quantification
285 of hydroxychloroquine and its metabolites in mouse blood and tissues using LC-ESI-MS/MS:
286 An application for pharmacokinetic studies. *J Chromatogr Analyt Technol Biomed Life Sci*
287 2018;1072:320-7.

288 [31] Danesi R, Lupetti A, Barbara C, Ghelardi E, Chella A, Malizia T, et al. Comparative
289 distribution of azithromycin in lung tissue of patient given oral daily doses of 500 and 1000
290 mg. *J Antimicrob Chemother* 2003;51:939-45.

291 [32] Lucchi M, Damle B, Fang A, de Caprariis PJ, Mussi A, Sanchez SP, et al.
292 Pharmacokinetics of azithromycin in serum, bronchial washings, alveolar macrophages and
293 lung tissue following a single oral dose of extended or immediate release formulations of
294 azithromycin. *J Antimicrob Chemother* 2008;61:884-91.

295 [33] Davidson RJ. In vitro activity and pharmacodynamic/pharmacokinetic parameters of
296 clarithromycin and azithromycin: why they matter in the treatment of respiratory tract
297 infections. *Infect Drug Resist* 2019;12:585-96.

298 [34] Madrid PB, Panchal RG, Warren TK, Shurleff AC, Endsley AN, Green CE, et al.
299 Evaluation of Ebola virus inhibitors for drug repurposing. *ACS Infect Dis* 2015;1:317-26.

300 [35] Takano T, Satoh K, Doki T, Tanabe T, Hohdatsu T. Antiviral effects of
301 hydroxychloroquine and type I interferon on in vitro fatal feline coronavirus infection.
302 *Viruses* 2020;12:576.

303 [36] Lu G, Nagbanshi M, Goldau N, Mendes Jorge M, Meissner P, Jahn A, et al. Efficacy and
304 safety of methylene blue in the treatment of malaria: a systemic review. *BMC Med*
305 2018;16:59.

306 [37] Choy KT, Wong AYL, Kaewpreedee P, Sia SF, Chen D, Hui KPY, et al. Remdesivir,
307 lopinavir, emetine, and homoharringtonine inhibits SARS-CoV-2 replication in vitro.
308 *Antiviral Res* 2020;178:104786.

309
310

311 Table 1: Median effective concentration (EC₅₀), 90% effective concentration (EC₉₀) against
312 SARS-CoV-2, 50% cytotoxicity concentration (CC₅₀) and selectivity index (SI) for methylene
313 blue, hydroxychloroquine and azithromycin

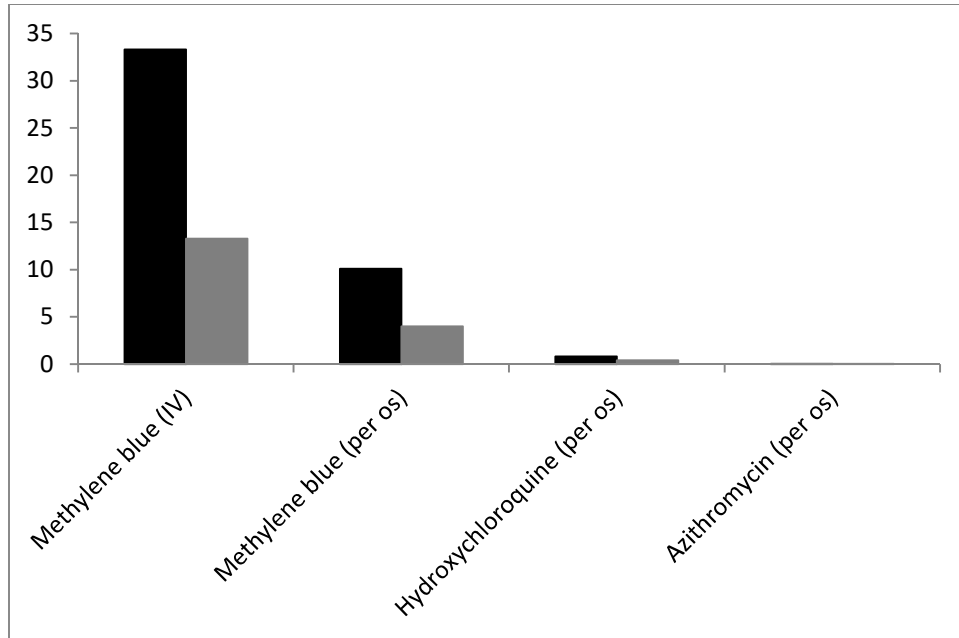
314

Drug	EC ₅₀ in μM	EC ₉₀ in μM	CC ₅₀ in μM	SI
Methylene blue	0.30 ± 0.03	0.75 ± 0.21	> 100	> 333
Hydroxychloroquine	1.5 ± 0.3	3.0 ± 1.9	20.4 ± 1.4	13.6
Azithromycin	20.1 ± 4.5	41.9 ± 18.0	> 100	> 5

315

316

317 Figure 1: Bar chart displaying C_{max}/EC_{50} (in black) and C_{max}/EC_{90} (in grey) for methylene
318 blue, hydroxychloroquine and azithromycin for *in vitro* activity against SARS-CoV-2
319



320

321