

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

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### ▶ 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

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Version of Record: https://www.sciencedirect.com/science/article/pii/S0924857920304088 Manuscript bb43869a294158cee6375cde9896c4db

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

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24 Abstract

25 In December 2019, a new severe acute respiratory syndrome coronavirus (SARS-CoV-2) causing coronavirus diseases 2019 (COVID-19) emerged in Wuhan, China. Currently, there is 26 no antiviral treatment recommended against SARS-CoV-2. Identifying effective antiviral 27 drugs is urgently needed. Methylene blue already demonstrated *in vitro* antiviral activity in 28 photodynamic therapy, and antibacterial, antifungal or antiparasitic activity in 29 30 nonphotodynamic assays. Non-photoactivated methylene blue showed *in vitro* activity at very low-micromolar range with EC<sub>50</sub> of  $0.3 \pm 0.03 \mu$ M and EC<sub>90</sub> of  $0.75 \pm 0.21 \mu$ M at MOI of 31 0.25 against SARS-CoV-2. The EC<sub>50</sub> and EC<sub>90</sub> values for methylene blue are lower than those 32 33 obtained for hydroxychloroquine (1.5 and 3.0  $\mu$ M) and azithromycin (20.1 and 41.9  $\mu$ M). The ratios C<sub>max</sub>/EC<sub>50</sub> and C<sub>max</sub>/EC<sub>90</sub> in blood for methylene blue after oral administration were 34 estimated at 10.1 and 4.0, respectively, and 33.3 and 13.3 after intravenous administration. 35 36 Methylene blue  $EC_{50}$  and  $EC_{90}$  are consistent with concentrations observed in human blood. We propose that methylene blue is a promising drug for COVID-19 treatment. In vivo evaluation in 37 animal experimental models is now required to confirm its antiviral effects on SARS-CoV-2. 38 39 The potential interest of methylene blue to treat COVID-19 needs to be confirmed by prospective comparative clinical studies. 40 41

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

#### 44 **1. Introduction**

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

Plasma products can transmit a wide range of pathogens in transfusion. Methylene blue, a 52 53 synthesized thiazine dye, was known to be effective in photodynamic therapy against microbes and more especially virus. Methylene blue is able to intercalate into viral nucleic 54 acid when illuminated with visible light and prevents transmission of pathogens. The 55 56 illumination of methylene blue inactivated Zika, yellow fever, dengue, chikungunya, Ebola viruses and Middle East respiratory syndrome coronavirus in plasma [2-5]. Methylene blue 57 can also demonstrate antimicrobial activities without photoactivation. Methylene blue 58 inhibited in vitro colistin-resistant strains of Acinetobacter baumannii, Mycobacterium 59 ulcerans, Mycobacterium spp. and Candida albicans [6-8]. Methylene blue was also effective 60 in vivo against Buruli ulcer in experimental Mycobacterium ulcerans infection in mice [7]. 61 Additionally, methylene blue inactivated hepatis C virus in transplant organ perfused with 62 methylene blue [9]. The most studied effects are those on malaria. 63 In 1891, methylene blue was first used to treat effectively two patients with uncomplicated 64 malaria [10]. In the 2010s, methylene blue showed effective in vitro activity in the nanomolar 65 range against *Plasmodium falciparum* strains and isolates [11-14]. Methylene blue showed a 66

67 protective effect against cerebral malaria in a murine model infected with *P. berghei* [15-17].

Methylene blue showed several benefits when used as partner in triple combination with
artemisinin-based combination therapy in uncomplicated falciparum malaria in children [18].
Taken together, these reports suggest that methylene blue may have antiviral effects against
SARS-CoV-2. Therefore the activity of methylene blue was assessed *in vitro* against a
clinically isolated SARS-CoV-2 strain and compared with the activity of hydroxychloroquine
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,

USA) and methylene blue were prepared in water and azithromycin (Sigma) in methanol. All
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  $\mu$ M to 100  $\mu$ M. The

clinically isolated SARS-CoV-2 strain (IHUMI-3) [23] was maintained in production in Vero

E6 cells (American type culture collection ATCC® CRL-1586<sup>TM</sup>) in MEM with 4% of fetal

84 bovine serum and 1% glutamine (complete medium).

85

#### 86 2.2. Cytotoxicity assay

In vitro cell viability evaluation on the VERO E6 cell line was performed according to the method described by Mosmann with slight modifications [24]. Briefly,  $10^5$  cells in 200 µl of complete medium were added to each well of 96-well plates and incubated at 37 °C in a humidified 5% CO<sub>2</sub>. After 24 h incubation, 25 µl of complete medium and 25 µl of each concentration of methylene blue, hydroxychloroquine or azithromycin were added and the plates were incubated 48h at 37 °C. After removal of the surpernatant, 100 µL of MTT (3-

93	(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2 <i>H</i> -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 $\mu l$ 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 $^{\circ}$ 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 (CC <sub>50</sub> ) was calculated with the inhibitory sigmoid $E_{max}$ model, which estimated				
100	the $CC_{50}$ 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 $5.10^5$ 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]. EC <sub>50</sub> (median effective concentration) and EC <sub>90</sub> (90% effective concentration)				
112	were calculated with the inhibitory sigmoid $E_{max}$ model, which estimated the EC <sub>50</sub> and EC <sub>90</sub>				
113	through nonlinear regression by using a standard function of the R software (ICEstimator				
114	version 1.2). EC <sub>50</sub> and EC <sub>90</sub> values resulted in the mean of 6 different experimentations.				
115					

116 2.4. Data analysis and interpretation

Selectivity index (SI) as ratio of CC<sub>50</sub>/EC<sub>50</sub> was estimated for each drug. The expected
maximum blood concentration (C<sub>max</sub>) was estimated from literature for each drug at doses
commonly administered in oral malaria treatment, and for methylene blue at intravenous
doses used for FDA and MEA approved methemoglobinemia treatment. The ratios C<sub>max</sub>/EC<sub>50</sub>
and C<sub>max</sub>/EC<sub>90</sub> were estimated to find out if the effective concentration in plasma to cure
SARS-CoV-2 is achievable in human. If data on drug accumulation into lung was available,
the ratios C<sub>lung</sub>/EC<sub>50</sub> and C<sub>lung</sub>/EC<sub>90</sub> were calculated.

124

#### 125 **3. Results**

126 CC<sub>50</sub>, EC<sub>50</sub>, EC<sub>90</sub> and SI for each drug are presented in Table 1. Methylene blue and 127 hydroxychloroquine showed EC<sub>50</sub> and EC<sub>90</sub> at low micromolar range (Table 1). The EC<sub>50</sub> and 128 EC<sub>90</sub> 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<sub>50</sub> of  $0.3 \pm 0.03$  $\mu$ M and EC<sub>90</sub> of 0.75 ± 0.21  $\mu$ M at MOI of 0.25 (SI > 333) (Table 1). The EC<sub>50</sub> and EC<sub>90</sub> 135 values for methylene blue are lower than those obtained for hydroxychloroquine and 136 azithromycin. Azithromycin demonstrated low in vitro efficacy against SARS-CoV-2 used 137 alone but potentiated the effects of hydroxychloroquine in combination [20]. An oral uptake 138 of 325 mg of methylene blue led to a  $C_{max}$  (maximum blood concentration) value of 0.97 139  $\mu$ g/ml (around 3  $\mu$ M) and t<sub>1/2</sub> (elimination half-life) of 14.9 h [26]. Methylene blue dose of 2 140 mg/kg intravenous showed a C<sub>max</sub> of 2.917 µg/ml (around 10 µM) [27]. The ratios C<sub>max</sub>/EC<sub>50</sub> 141

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 $\mu$ M [29]. Hydroxychloroquine accumulated 30 times more in lungs than
148	in blood [30]. The azithromycin $C_{max}$ ranged from 0.18 to 0.4 $\mu$ g/ml of blood (around 0.22 to
149	$0.51\mu\text{M})$ 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 µg/g
151	(around 10 to 12 $\mu$ M) [31,32]. The C <sub>max</sub> expected in lung was below the EC <sub>50</sub> and EC <sub>90</sub> .
152	However, due to potentiation of the antiviral effects when azithromycin is combined with
153	hydroxychloroquine, azithromycin can be used <i>in vitro</i> at lower concentrations (5 and 10 $\mu$ M)
154	[20]. These concentrations are compatible with expected concentrations in lungs.
155	Methylene blue showed a low cytotoxicity <i>in vitro</i> against Vero E6 cells with $CC_{50} > 100 \mu M$ .
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 <sub>50</sub> , ranging from > 50 $\mu$ M to 250 $\mu$ M against Vero E6 cells [19,34] or above
159	500µM in Felis catus whole fetus-4 cells [35]. Azithromycin showed also a low cytotoxicity
160	against Vero E6 cells with CC <sub>50</sub> > 100 $\mu$ M and SI > 5. CC <sub>50</sub> 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

2016, for the treatment of acquired methemoglobinemia, based upon a confirmed positivebenefit/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 IC<sub>50</sub> (0.3  $\mu$ M) and IC<sub>90</sub> (0.75  $\mu$ M) compatible with oral uptake and intravenous

administrations. This *in vitro* activity is higher than those obtained with drugs which are

evaluated in clinical trials worldwide like hydroxychloroquine (1.5 μM), azithromycin (20.1

 $\mu$ M), remdisivir (23  $\mu$ M), lopinavir (26.6  $\mu$ M) or ritonavir (> 100  $\mu$ M) [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

potential interest of methylene blue to treat COVID-19 needs to be confirmed by prospectivecomparative clinical studies.

180

#### 181 Declarations

**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 **Competiing 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,
- 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.

- Acknowledgments: The authors thank Provepharm for providing the methylene blue(Proveblue®)
- 195
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- Table 1: Median effective concentration (EC<sub>50</sub>), 90% effective concentration (EC<sub>90</sub>) against
- 312 SARS-CoV-2, 50% cytotoxicity concentration (CC<sub>50</sub>) and selectivity index (SI) for methylene
- 313 blue, hydroxychloroquine and azithromycin
- 314

Drug	$EC_{50}$ in $\mu M$	$EC_{90}$ in $\mu M$	$CC_{50}$ in $\mu M$	SI
Methylene blue	$0.30 \pm 0.03$	$0.75 \pm 0.21$	> 100	> 333
Hydroxychloroquine	$1.5 \pm 0.3$	3.0 ±1.9	$20.4 \pm 1.4$	13.6
Azithromycin	$20.1 \pm 4.5$	$41.9 \pm 18.0$	> 100	> 5

315

Figure 1: Bar chart displaying C<sub>max</sub>/EC<sub>50</sub> (in black) and C<sub>max</sub>/EC<sub>90</sub> (in grey) for methylene
blue, hydroxychloroquine and azithromycin for *in vitro* activity against SARS-CoV-2

