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International Journal of Antimicrobial Agents 56 (2020) 106202

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

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

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ARTICLE INFO

Keywords:
SARS-CoV-2
Coronavirus
COVID-19
Antiviral
Methylene blue
In vitro

ABSTRACT

In December 2019, a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), causing coronavirus diseases 2019 (COVID-19) emerged in Wuhan, China. Currently there is no antiviral treatment recommended against SARS-CoV-2. Identifying effective antiviral drugs is urgently required. Methylene blue has already demonstrated in vitro antiviral activity in photodynamic therapy as well as antibacterial, antifungal and antiparasitic activities in non-photodynamic assays. In this study, non-photoactivated methylene blue showed in vitro activity at very low micromolar range with an EC_{50} (median effective concentration) of 0.30 ± 0.03 μM and an EC_{90} (90% effective concentration) of 0.75 ± 0.21 μM at a multiplicity of infection (MOI) of 0.25 against SARS-CoV-2 (strain IHUMI-3). The EC_{50} and EC_{90} values for methylene blue are lower than those obtained for hydroxychloroquine (1.5 μM and 3.0 μM) and azithromycin (20.1 μM and 41.9 μM). The ratios C_{max}/EC_{50} and C_{max}/EC_{90} in blood for methylene blue were estimated at 10.1 and 4.0, respectively, following oral administration and 33.3 and 13.3 following intravenous administration. Methylene blue EC_{50} and EC_{90} values are consistent with concentrations observed in human blood. We propose that methylene blue is a promising drug for treatment of COVID-19. In vivo evaluation in animal 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 prospective comparative clinical studies.

1. Introduction

In December 2019, a novel coronavirus, 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. Presently there is no antiviral treatment recommended against SARS-CoV-2. Different drugs or combinations 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 by transfusion. Methylene blue, a synthesised thiazine dye, is known to be effective in photodynamic therapy against microbes and particularly viruses. Methylene blue is able to intercalate into viral nucleic acid when illuminated with visible light and prevents transmission of pathogens. Illumination of methylene blue inactivated Zika, yellow fever, dengue, chikungunya and Ebola viruses and Middle East respiratory syndrome coronavirus in plasma [2–5]. Methylene blue also demonstrates antimicrobial activities without photoactivation. Methylene blue inhibited in vitro colistin-resistant strains of *Acinetobacter baumannii, Mycobacterium ulcerans, Mycobacterium* spp. and *Candida albicans* [6–8]. Methylene blue was also effective in vivo against Buruli ulcer in experimental *M. ulcerans* infection in mice [7]. Additionally, methylene blue inacti-

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https://doi.org/10.1016/j.ijantimicag.2020.106202
0924-8579/

vated hepatis C virus in transplant organ perfused with methylene blue [9]. The most studied effects of methylene blue are those on malaria.

In 1891, methylene blue was first used to effectively treat two patients with uncomplicated malaria [10]. In the 2010s, methylene blue showed effective in vitro activity in the nanomolar range against *Plasmodium falciparum* strains [11–14]. Methylene blue showed a protective effect against cerebral malaria in a murine model infected with *Plasmodium berghei* [15–17]. Methylene blue showed several benefits when used as a 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, in this study the activity of methylene blue was assessed in vitro against a clinically isolated SARS-CoV-2 strain and was compared with the activity of hydroxychloroquine and azithromycin, which have already been evaluated in vitro and in vivo in humans [19–22].

2. Materials and methods

2.1. Antimalarial drugs, virus and cells

Methylene blue (methylthioninium chloride; Proveblue®) was provided by Provepharm SAS (Marseille, France). Stocks solutions of hydroxychloroquine (Sigma, St Louis, MO, USA) and methylene blue were prepared in water, and azithromycin (Sigma) was prepared in methanol. All stock solutions were then diluted in Minimum Essential Medium (MEM) (Gibco, Thermo Fisher) to achieve seven final concentrations ranging from 0.1–100 μM. A clinically isolated SARS-CoV-2 strain (IHUMI-3) [23] was maintained in production in Vero E6 cells (American Type Culture Collection ATCC® CRL-1586™) in MEM with 4% of fetal bovine serum (FBS) and 1% glutamine (complete medium).

2.2. Cytotoxicity assay

In vitro cell viability evaluation using 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 were incubated at 37 °C in a humidified 5% CO_2 atmosphere. After 24 h of 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 for 48 h at 37 °C. After removal of the supernatant, 100 μL of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] (Sigma-Aldrich, France) solution (0.5 mg/mL in MEM without FBS) were then added to each well. Cells were incubated for 2 h at 37 °C. Following incubation, MTT solution was removed and 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Plates were then shaken at 700 rpm for 10 min at 37 °C. The absorbance was measured at 570 nm using a Tecan Infinite F200 Microplate Reader. DMSO was used as a blank. The 50% cytotoxic concentration (CC_{50}) was calculated with an inhibitory sigmoid E_{max} model, which estimated the CC_{50} through non-linear regression by using a standard function of the R software (ICEstimator v.1.2; http://www.antimalarial-icestimator.net). The CC_{50} value was the mean of six different experimentations.

2.3. Antiviral activity assay

Briefly, 96-well plates were prepared with 5×10^5 cells/mL of Vero E6 cells (200 μL per well) as previously described [20]. Methylene blue, hydroxychloroquine or azithromycin concentrations were added 4 h before infection. Vero E6 cells were infected with SARS-CoV-2 strain IHUMI-3 at a multiplicity of infection (MOI) of 0.25. At 48 h post-infection, replication was estimated by RT-PCR using a SuperScript™ III Platinum™ One-Step Kit w/ROX (Invitrogen) after extraction with a BIoExtract® SuperBall® Kit (Biosellal, Dardilly, France). The primers used have been described previously [25]. The EC_{50} (median effective concentration) and EC_{90} (90% effective concentration) were calculated with an inhibitory sigmoid E_{max} model, which estimated the EC_{50} and EC_{90} through non-linear regression using a standard function of the R software (ICEstimator v.1.2). EC_{50} and EC_{90} values were the mean of six different experimentations.

Table 1
EC_{50} and EC_{90} values against SARS-CoV-2, CC_{50} and selectivity index (SI) for methylene blue, hydroxychloroquine and azithromycin

Drug	EC_{50} (μM)	EC_{90} (μM)	CC_{50} (μM) [a]	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

EC_{50}, median effective concentration; EC_{90}, 90% effective concentration; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; CC_{50}, 50% cytotoxic concentration.
[a] In Vero E6 cells.

2.4. Data analysis and interpretation

The selectivity index (SI) was estimated for each drug as the ratio of CC_{50}/EC_{50}. The expected maximum blood concentration (C_{max}) was estimated from the literature for each drug at doses commonly administered in oral malaria treatment and for methylene blue at intravenous (i.v.) doses used for US Food and Drug Administration (FDA) and European Medicines Agency (EMA)-approved methemoglobinemia treatment. The ratios C_{max}/EC_{50} and C_{max}/EC_{90} were estimated to determine whether the effective concentration in plasma to cure SARS-CoV-2 is achievable in humans. If data on drug accumulation in the lung were available, the ratios C_{lung}/EC_{50} and C_{lung}/EC_{90} were calculated.

3. Results

The CC_{50}, EC_{50}, EC_{90} and SI for each drug are presented in Table 1. Methylene blue and hydroxychloroquine showed EC_{50} and EC_{90} values in the low micromolar range (Table 1). The EC_{50} and EC_{90} values for methylene blue were lower than those obtained for hydroxychloroquine and azithromycin. The ratios C_{max}/EC_{50} and C_{max}/EC_{90} in blood for methylene blue were estimated at 10.1 and 4.0, respectively, following oral administration and at 33.3 and 13.3 following i.v. administration (Fig. 1).

4. Discussion

Methylene blue showed in vitro activity at very low micromolar range with an EC_{50} of 0.30 ± 0.03 μM and an EC_{90} of 0.75 ± 0.21 μM at a MOI of 0.25 (SI > 333) (Table 1). The EC_{50} and EC_{90} values for methylene blue are lower than those obtained for hydroxychloroquine and azithromycin. Azithromycin demonstrated low in vitro efficacy against SARS-CoV-2 when used alone but potentiated the effects of hydroxychloroquine in combination [20]. Oral uptake of 325 mg of methylene blue led to a C_{max} in blood of 0.97 μg/mL (~3 µM) and an elimination half-life ($t_{1/2}$) of 14.9 h [26]. A methylene blue dose of 2 mg/kg i.v. showed a C_{max} of 2.917 μg/mL (~10 μM) [27]. The ratios C_{max}/EC_{50} and C_{max}/EC_{90} for methylene blue were estimated at 10.1 and 4.0 for the oral route and 33.3 and 13.3 for the i.v. route, respectively. Methylene blue EC_{50} and EC_{90} values are consistent with concentrations observed in human blood. Approximately 3–5% of methylene blue per gram of lung was found

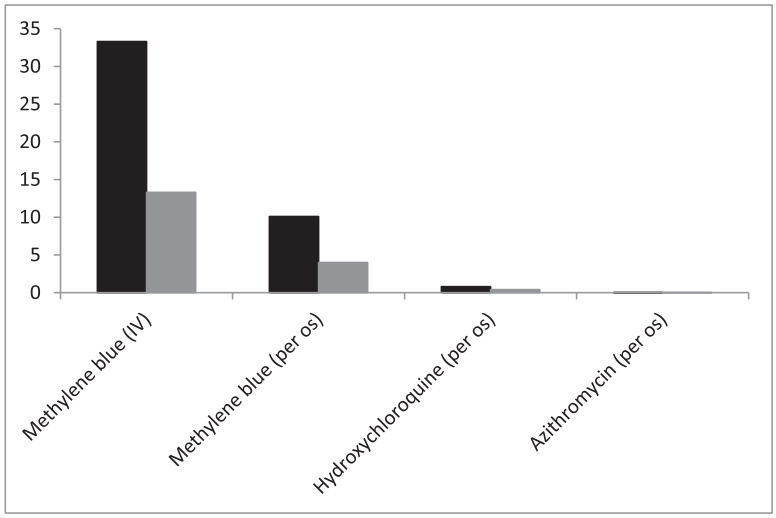

Fig. 1. Bar chart displaying the ratios C_{max}/EC_{50} (black) and C_{max}/EC_{90} (grey) for methylene blue, hydroxychloroquine and azithromycin for in vitro activity against SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2). C_{max}, maximum concentration in blood; EC_{50}, median effective concentration; EC_{90}, 90% effective concentration.

after i.v. methylene blue injection but the methylene blue concentration decreased rapidly below 0.1% after 10 h [28]. In comparison, oral uptake of 400 mg of hydroxychloroquine led to a C_{max} of 1.22 μM [29]. Hydroxychloroquine accumulated 30 times more in the lungs than in blood [30]. The azithromycin C_{max} ranged from 0.18–0.4 μg/mL of blood (~0.22–0.51 μM) after the last dose of oral administration of 500 mg once daily for 3 days or after a single dose of 500 mg [31–33]. These doses led to a C_{max} in the lung ranging from 8–9 μg/g (~10–12 μM) [31,32]. The C_{max} expected in the lung was below the EC_{50} and EC_{90}. However, due to potentiation of the antiviral effects when azithromycin is combined with hydroxychloroquine, azithromycin can be used in vitro at lower concentrations (5 μM and 10 μM) [20]. These concentrations are compatible with expected concentrations in the lungs.

Methylene blue showed low cytotoxicity in vitro against Vero E6 cells with CC_{50} > 100 μM. The SI as a ratio of CC_{50}/EC_{50} was estimated to be >333. The present CC_{50} of hydroxychloroquine with an SI of ~13 against Vero E6 cells was higher than previously reported CC_{50} values, ranging from >50 μM to 250 μM against Vero E6 cells [19,34] or >500 μM in *Felis catus* whole fetus-4 cells [35]. Azithromycin also showed low cytotoxicity against Vero E6 cells with CC_{50} > 100 μM and SI > 5. The CC_{50} for azithromycin was consistent with previous data (>130 μM) [34]. Methylene blue showed low cytotoxicity but predominantly the higher SI.

Although methylene blue is on the list of drugs potentially dangerous for patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency, no association between methylene blue and severe haemolysis has been detected after oral administration [36]. Additionally, the i.v. route for methylene blue has been granted a marketing authorisation in Europe in 2011 and in the USA in 2016 for the treatment of acquired methemoglobinemia based upon a confirmed positive benefit/risk ratio in this pathology.

5. Conclusion

Methylene blue showed high in vitro antiviral effective activity against SARS-CoV-2 with an IC_{50} (0.3 μM) and IC_{90} (0.75 μM) compatible with oral uptake and i.v. administration. This in vitro activity is higher than those obtained with drugs that have been evaluated in clinical trials worldwide such as hydroxychloroquine (1.5 μM), azithromycin (20.1 μM), remdesivir (23 μM), lopinavir (26.6 μM) or ritonavir (>100 μM) [37]. We propose that methylene blue is a promising drug for the treatment of COVID-19. In vivo evaluation in animal experimental models is now required to confirm its antiviral effects against SARS-CoV-2. The potential interest of methylene blue to treat COVID-19 needs to be confirmed by prospective comparative clinical studies.

Acknowledgment

The authors thank Provepharm for providing the methylene blue (Proveblue®).

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

Competing interests: BLS and BP are associated as co-inventors with Provepharm in the patent EP 20305425.9 (30/04/2020) but have no financial interest with the subject matter; MB received a PhD grant supported by L'Occitane Society. Provepharm or the funders had no role in the design of the study, in the collection, analysis or interpretation of the data, in the writing of the manuscript or in the decision to publish the results. All other authors declare no competing interests.

Ethical approval: Not required.

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