In vitro Evaluation of the Antiviral Activity of Repurposable Drugs against SARS-CoV-2: Methylene Blue

Running title: Antiviral activity of methylene blue

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**Background.** A new severe acute respiratory syndrome coronavirus (SARS-CoV-2) causing coronavirus diseases 2019 (COVID-19), which emerged in Wuhan, China in December 2019, has spread worldwide. Currently, there is no antiviral treatment recommended against SARS-CoV-2. Identifying effective low cost antiviral drugs with limited side effects affordable immediately is urgently needed. Methylene blue, a synthesized thiazine dye, may be a potential antiviral drug.

**Methods.** Antiviral activity of methylene blue used alone or in combination with several antimalarial drug or remdesivir were assessed against infected Vero E6 cells with two clinically isolated SARS-CoV-2 strains (IHUMI-3 and IHUMI-6). Effects both on viral entry in the cell and on post-entry were also investigated. After 48h post-infection, the viral replication was estimated by RT-PCR.

**Results.** The median effective concentration (EC$_{50}$) and 90% effective concentration (EC$_{90}$) of methylene blue against IHUMI-3 were 0.41 ± 0.34 µM and 1.85 ± 1.41 µM, respectively; and 1.06 ± 0.46 µM and 5.68 ± 1.83 µM against IHUMI-6. Methylene blue interacted at both entry and post-entry stages of SARS-CoV-2 infection in Vero E6 cells as retrieved for hydroxychloroquine. The effects of methylene blue were additive with those of quinine, mefloquine and pyronaridine. The combinations of methylene blue with chloroquine, hydroxychloroquine, desethylamodiaquine, piperaquine, lumefantrine, ferroquine, dihydroartemisinin and remdesivir were antagonist.

**Conclusions.** These results support in vivo evaluation in animal experimental models to confirm methylene blue antiviral effects on SARS-CoV-2.

**Keywords.** COVID-19; SARS-CoV-2; antiviral; *in vitro*; methylene blue
In December 2019, a new severe acute respiratory syndrome coronavirus (SARS-CoV-2) causing coronavirus diseases 2019 (COVID-19) emerged in Wuhan, China [1]. Currently, there is no antiviral treatment recommended against SARS-CoV-2. Identifying effective low cost antiviral drugs with limited side effects affordable immediately is urgently needed, especially for emerging countries. An efficient approach to drug discovery is drug repurposing that consists in evaluating whether existing approved drugs can be efficient against SARS-CoV-2. Several compounds have been already evaluated at least in vitro including antimalarial drugs (chloroquine, mefloquine, quinine, pyronaridine, piperaquine, lumefantrine, artemisinin) [2-5], antibiotics (azithromycin, doxycycline) [6,7], antiparasitic drugs (ivermectin) [8] or antiviral agents (remdesivir, ritonavir, lopinavir, favipiravir) [4,5,9,10].

Methylene blue, a synthesized thiazine dye, is able to intercalate into viral nucleic acid when illuminated with visible light and can inactivate Zika, yellow fever, dengue, chikungunya, Ebola viruses and Middle East respiratory syndrome coronavirus in plasma [11-14]. Methylene blue was also shown to exert in vitro and in vivo antimicrobial effects without photoactivation, and more particularly against Plasmodium spp. [15-19]. Methylene blue may have a role in the treatment of COVID-19 [20]. SARS-CoV-2 was inactivated by photoactivation [21,22]. Moreover, methylene blue was found to inhibit SARS-CoV-2 in vitro at concentrations achievable after oral or intravenous administration [21,23].

The aim of this study was to confirm the antiviral activity of methylene blue against SARS-CoV-2, to investigate its effects on viral entry in the cell and on post-entry and its activity in combination with other potential drugs.

MATERIAL AND METHODS

Drugs, virus and cells
Methylene blue (methylthioninium chloride; Proveblue®) was provided by Provepharm SAS (Marseille, France). Chloroquine diphosphate (Sigma Aldrich, St Quentin Fallavier, France) and remdesivir (Apollo Scientific, Manchester, UK) were used as comparators. Stock solutions of methylene blue and hydroxychloroquine were prepared in water and remdesivir in DMSO/water 10%. All the stock solutions were then diluted in Minimum Essential Media (MEM, Gibco, ThermoFischer) in order to have 7 final concentrations ranging from 0.1 µM to 100 µM. Two clinically-isolated SARS-CoV-2 strains (IHUMI-3 and IHUMI-6) [24] were maintained in production in Vero E6 cells (American type culture collection ATCC® CRL-1586™) in MEM with 4% of fetal bovine serum and 1% of glutamine (complete medium).

**Antiviral activity assay**

Briefly, 96-well plates were prepared with 5.10^5 cells/mL of Vero E6 (200µL per well), as previously described [6]. Methylene blue, hydroxychloroquine or remdesivir concentrations were added 4 h before infection. Vero E6 Cells were infected with IHUMI-3 or IHUMI-6 strains at an MOI of 0.01. After 48h post-infection, the replication was estimated by RT-PCR using the Superscrit III platinum one step with Rox kit (Invitrogene) after extraction with the BIoExtract SuperBall kit ( Biosellal, Dardilly, France). The primers used were previously described [25]. EC_{50} (median effective concentration) and EC_{90} (90% effective concentration) were calculated with the inhibitory sigmoid E_{max} model, which estimated the EC_{50} and EC_{90} through nonlinear regression by using a standard function of the R software (ICEstimator version 1.2). EC_{50} and EC_{90} values resulted in the mean of 6 to 12 independent experimentations.

**Determination of the inhibition stage**
Determining in vitro at what stage methylene blue, hydroxychloroquine or remdesivir is acting against the SARS-CoV-2 IHUMI-003 strain was assessed at a concentration of 10 µM. For “full-time treatment”, Vero E6 cells were pre-treated with one of the three drugs for 4 h and virus was then added for 48 h. For “entry” treatment, the drug was added to Vero E6 cells 4 h before viral infection and the virus-drug mixture was replaced with fresh medium after 2 h post-infection and was maintained for 46 h. For “post-entry” treatment, the drug was added after 2 h post-infection and was maintained for 46 h. The percentage of inhibition of SARS-CoV-2 replication by 10 µM of drug was estimated for each drug concentration as following: 

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\frac{\text{mean CT}_{\text{drug concentration}} - \text{mean CT}_{\text{control 0\%}}}{\text{mean CT}_{\text{control 100\%}} - \text{mean CT}_{\text{control 0\%}}} \times 100.
\]

The result was the mean of 6 to 9 independent experiments.

**RESULTS**

The antiviral activity of methylene blue against the clinically-isolated SARS-CoV-2 strains IHUMI-3 and IHUMI-6 was concentration-dependent (Figure 1). The median effective concentration (EC\(_{50}\)) and 90% effective concentration (EC\(_{90}\)) of methylene blue against IHUMI-3 were 0.41 ± 0.34 µM and 1.85 ± 1.41 µM (n=12), respectively; and 1.06 ± 0.46 µM and 5.68 ± 1.83 against IHUMI-6 (n=6). The difference between EC\(_{50}\) against the two was significant (p = 0.015, Welch two sample t-test).

In comparison, EC\(_{50}\) and EC\(_{90}\) of remdesivir against IHUMI-6 were 1.00 ± 0.41 µM and 3.2 ± 2.9 µM, respectively (n=6). There was no significant difference between methylene blue and remdesivir EC\(_{50}\) or EC\(_{90}\) (p = 0.786 and p = 0.113, Welch two sample t-test).

EC\(_{50}\) and EC\(_{90}\) of hydroxychloroquine against IHUMI-6 were 6.25 ± 2.20 µM and 12.32 ± 2.82 µM, respectively (n=6). Methylene blue was significantly more effective than hydroxychloroquine against IHUMI-6 (p = 0.005 for EC\(_{50}\) and p = 0.003 for EC\(_{90}\); Welch two sample t-test).
Methylene blue interacted at both entry and post-entry stages of SARS-CoV-2 infection in Vero E6 cells, as hydroxychloroquine did (Figure 2). Contrariwise, remdesivir, which is an antiviral drug, interacted only at post-entry stage.

The effects of methylene blue were additive with those of quinine (Figure 3), mefloquine (Figure 4) and pyronaridine (Figure 5). The combinations of methylene blue with chloroquine (Figure 6), hydroxychloroquine, desethylamodiaquine, piperaquine, lumefantrine, ferroquine, dihydroartemisinin and remdesivir (data not shown) were antagonist.

**DISCUSSION**

Our data confirmed the \textit{in vitro} activity of methylene blue at very low-micromolar range with EC$_{50}$ between 0.41 and 1.06 and EC$_{90}$ between 1.85 ± 1.41 µM and 5.68 ± 1.83 µM against two strains of SARS-Cov 2 IHUMI-3 and IHUMI-6 [23,26,27]. Methylene blue was effective as antiviral remdesivir against IHUMI-6 strain and more effective than hydroxychloroquine \textit{in vitro}. These effective concentrations are compatible with blood concentrations after usual oral intake or intravenous injection of methylene blue. An oral uptake of 325 mg of methylene blue led to a C$_{\text{max}}$ (maximum blood concentration) value of 0.97 µg/mL (around 3 µM) [28] and a dose of 2 mg/kg intravenous showed a C$_{\text{max}}$ of 2.917 µg/mL (around 10 µM) [29]. In another study, blood concentrations of 6-7 µM were obtained after three oral daily doses of 69 mg (207 mg/day) [30]. Methylene blue EC$_{50}$ and EC$_{90}$ are consistent with concentrations observed in human blood. Moreover, methylene blue is accumulated in lungs tissue. Around 3 to 5% of methylene blue per g of lung was found after intravenous methylene blue [31].

Methylene blue could be association with antimalarial drugs such as quinine, mefloquine or pyronaridine to improve its antiviral activity. Mefloquine concentrations are 10 times higher in the lung than in the blood (a concentration which can go up to 180 mg/kg in the lung) [32]. A single oral dose of 2 mg (10 mg/kg) of pyronaridine in rats led to a blood C$_{\text{max}}$
of 223 ng/mL and a lung $C_{\text{max}}$ of 36.4 µg/g of tissue (165 more concentrated) [33]. In rat, after intravenous dose of 10 mg/kg of quinine, the observed concentration lung/blood ratio was at 246 [34]. These three drugs accumulate in lungs and could be potent partners for methylene blue for COVID-19 treatment.

Methylene blue interacted at both entry and post-entry stages of SARS-CoV-2 infection in Vero E6 cells. The inhibition of the viral entry is consistent with the results interaction between the spike protein (S) and the angiotensin converting enzyme 2 (ACE2) via its receptor binding domain (RBD), binding required for SARS-CoV-2 cell entry. Methylene blue inhibits the binding of SARS-CoV-2 spike S protein to ACE2 at micro-molar range [27]. Moreover, the inhibition of both entry and viral replication after SARS-CoV-2 entry is consistent with the results from combinatorial computational approaches. Docking analysis showed that methylene blue could bind the spike protein S of SARS-CoV-2, but lesser than hydroxychloroquine, and the main protease (M), but lesser than remdesivir [35]. This protein, also called 3C-like protease, is essential to conduct the replication cycle of SARS-CoV-2 by leading to the formation of non-structural proteins (NSPs) [36].

Besides its antiviral activity, methylene blue is reduced into leukomethylene blue which reduces the methemoglobin to hemoglobin. Methylene blue could reduce hypoxia, one of the main complications in COVID-19 patients, by decreasing methemoglobin. Moreover, methylene blue decreases inflammation and oxidative stress [37,38]. Pro-inflammatory cytokines and nitric oxide were considerably increased in the cytokine storm due to COVID-19 [39].

These results support in vivo evaluation in animal experimental models to confirm methylene blue 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. Methylene
blue has been assessed in combination with vitamin C and N-acetyl cysteine in severe COVID-19 [40,41].

Notes

Disclaimer. The findings and conclusion in this report are those of the authors and do not represent the views of the Ministère des Armées and Ministère de l’Enseignement supérieur, de la Recherche et de l’Innovation.

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**Figure 1.** Anti-SARS-CoV-2 activity of methylene blue in % of antiviral inhibition on IHUMI-3 (mean of 12 independent experiments) and IHUMI-6 (mean of 6 independent experiments) clinically-isolated strains (error bar represents standard deviation)

**Figure 2.** Antiviral activities of methylene blue, hydroxychloroquine and remdesivir at 10 µM against the SARS-CoV-2 IHUMI-006 strain *in vitro*. For ‘full-time’ treatment, Vero E6 cells were pre-treated with one of the three drugs for 4h and virus was then added for 48h. For ‘entry’ treatment, the drug was added to Vero E6 cells 4h before viral infection and virus-drug mixture was replaced with fresh medium after 2h post-infection and maintained for 46h. For ‘post-entry’ treatment, the drug was added after 2h post-infection and maintained for 46h. Error bars represent standard deviation of 6 to 9 independent experiments

**Figure 3.** Antiviral activities of methylene blue (MB) at 0.1 and 0.5 µM in combination with quinine (QN) at 1, 5, 10 and 25 µM (error bars represent standard deviation of 13 independent experiments)

**Figure 4.** Antiviral activities of methylene blue (MB) at 0.1 and 0.5 µM in combination with mefloquine (MQ) at 0.5, 1, 5 and 10 µM (error bars represent standard deviation of 13 independent experiments)

**Figure 5.** Antiviral activities of methylene blue (MB) at 0.1 and 0.5 µM in combination with pyronaridine (PND) at 0.1, 0.5, 1 and 5 µM (error bars represent standard deviation of 9 independent experiments)
**Figure 6.** Antiviral activities of methylene blue (MB) at 0.1 and 0.5 µM in combination with chloroquine (CQ) at 0.5, 1, 5 and 10 µM (error bars represent standard deviation of 9 independent experiments)
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Figure 2. Antiviral activities of methylene blue, hydroxychloroquine and remdesivir at 10 µM against the SARS-CoV-2 IHUMI-006 strain in vitro. For ‘full-time’ treatment, Vero E6 cells were pre-treated with one of the three drugs for 4h and virus was then added for 48h. For ‘entry’ treatment, the drug was added to Vero E6 cells 4h before viral infection and virus-drug mixture was replaced with fresh medium after 2h post-infection and maintained for 46h. For ‘post-entry’ treatment, the drug was added after 2h post-infection and maintained for 46h. Error bars represent standard deviation of 6 to 9 independent experiments.
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