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Article

Evaluation of the Efficacy of Methylene Blue Administration in SARS-CoV-2-Affected Patients: A Proof-of-Concept Phase 2, Randomized, Placebo-Controlled, Single-Blind Clinical Trial

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Abstract: The SARS-CoV-2 pandemic has revolutionized the scientific and medical world in recent years. Methylene blue (MB) is a well-known molecule. The aim of our study was to assess the efficacy of MB against early-phase SARS-CoV-2 infections. All patients with a positive swab for SARS-CoV-2 were eligible for the trial. The intervention was a starting dose of 200 mg MB or placebo in the morning and 100 mg in the evening on the first day and afterwards the standard daily dose of 200 mg. Patients were followed up for safety and efficacy until day 84. We analyzed 21 patients for the safety profile and 19 for the efficacy objective: of these, there were 11 in the MB group and 8 in the placebo one. In both groups, patients had undetectable RNA from day 3 and 10 out of 11 subjects in the MB group were virus free by day 12 vs. 6 out of 8 in the placebo one. None of the patients experienced serious adverse events. MB has proved to be a safe and well-tolerated drug. We did not find superiority of efficacy or viral clearance of MB compared to the placebo. Given the good in vitro efficacy, larger studies are needed to assess MB efficacy against COVID-19 in vivo.

Keywords: methylene blue 2; COVID-19 3 SARS-CoV-2

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1. Introduction

The 2020 SARS-CoV-2 epidemic has destroyed and reinvented the medical world as we used to know and handle it. The virus forced the scientific world to make an enormous effort to study, understand and cure a new virus in an extremely brief time. Moreover, the urgent need for a vaccine pushed pharmaceutical companies to produce one in the shortest time possible, and it is currently the fastest- and most-distributed vaccine worldwide [1–3]. The repurposing of well-known, widely available drugs represents an attractive approach to speed up the availability of active treatments. Substances such as hydroxychloroquine and others are already under investigation and in widespread off-label use [4–6]. The new antiviral drug nemaltremavir has shown a good efficacy in preventing severe forms of COVID-19 [7,8]. Probably the biggest change in the disease evolution was provided by the vaccine; people who are vaccinated have less severe pneumonia and more favorable outcomes if infected [9]. These factors, together with the mutation of the virus itself, have dramatically changed the COVID-19 scenario and its impact on public health.

Methylene blue (MB) is a water-soluble thiazine dye that acts as a non-enzymatic oxidation–reduction agent. MB has been known in medicine for more than 100 years and was the first synthetic drug. It was and is still used in combination with UV light to protect blood products against infective agents, including SARS-Co-V [10]; since the last decades

of the XIXth century, it has been used against malaria, both in therapy and prophylaxis. A review on its efficacy and safety has been recently published [10]. MB is registered as a treatment for acquired methemoglobinemia under the trade name Proveblue® as an i.v. formulation for pediatric and adult use [10].

Recently, a large French study of more than 2000 cancer patients treated with 225 mg MB p.o. daily showed that none of them developed influenza-like illness during the first wave of the pandemic [10]. They hypothesize a prophylactic effect of MB against SARS-CoV-2 infection. Lobo et al. demonstrated that MB photo-inactivated SARS-CoV-2 in samples collected from COVID-19 patients. Nasal photo-disinfection with MB proved efficacious in the inactivation of multidrug-resistant bacteria colonizing the nose of surgical patients [11].

The oral formulation of MB 200 mg was registered in the EU for the detection of patients with intestinal carcinoma or adenomas during colonoscopy [10,12].

Regarding safety, MB is generally well tolerated with a broad therapeutic index and can be used as an intravenous and as an oral drug. The most common side effects described for the oral formulation are a transient increase in hepatic enzymes, dysuria and gastrointestinal disorders [13].

Given these interesting results [10], we designed a controlled, randomized, phase 2 clinical trial to assess the efficacy of MB in SARS-CoV-2 patients.

2. Materials and Methods

2.1. Design of the Study

This study was a phase 2, proof-of-concept, controlled, randomized clinical trial.

This study was conducted in accordance with the Declaration of Helsinki and registered with a ClinicalTrials.gov Identifier with the number NCT04635605.

We enrolled patients who tested positive for a SARS-CoV-2 infection at Ticino Checkpoints (CPs) or at family doctors' practices. The CPs were dedicated structures established in March 2020 by the Ordine dei Medici del Canton Ticino to face the SARS-CoV-2 pandemic. Symptomatic patients were evaluated by a physician and had a nasopharyngeal swab performed for the diagnosis of SARS-CoV-2 infection.

Oral consent was obtained from each patient by the CP physician; they were given a closed envelope to open only in case of confirmed infection.

2.2. Clinical Trial Schedule

The trial started on the 3rd of December 2020 and lasted until the 20th of May 2021. It was conducted in Ticino, Switzerland.

All patients willing to participate who had a mild SARS-CoV-2 infection and were handled at home,, above 18 years old and without a known G-6-Phosphatase deficiency, were eligible. The first trial visit was scheduled within 24 h from the diagnosis.

The trial consisted of two treatment arms, either MB 200 mg in the morning and 100 mg in the evening on the first day and afterwards 100 mg twice a day for another four consecutive days, or placebo capsules 100 mg twice a day for five consecutive days.

Neither the patient nor the physician knew in which arm the patient was enrolled.

At the first visit after the diagnosis, blood samples were taken to rule out the laboratory (hemoglobin: <10 g/dL for females, <12 g/dL for males; leucocytes: <3000 /µL, thrombocytes: $<125 \times 10^3/\mu L$) and clinical (clinically relevant renal or hepatic insufficiency, a resting SaO_2 of <94% or a resting respiratory rate of >20/min or any comorbidity that might compromise the patient's safety) exclusion criteria.

Once included, the patients were randomized to one treatment arm and visits were planned at 3, 6, 9, 12, 15 and 21 days for the assessment of the disease and safety; compliance was assessed by counting the remaining pills at each visit and asking the patients for confirmation. At each scheduled visit, we performed a nose–pharyngeal swab and blood exam, and the physician performed a clinical evaluation of the patients. Safety was assessed at each visit through a questionnaire; compliance was assessed by counting the capsules at each visit.

MB and placebo capsules were produced, blinded, labeled and delivered by Les Hôpitaux Universitaires de Genève.

An ad interim analysis was planned after the enrollment of 28 patients (14 in each arm) to assess the efficacy of the drug and possibly proceed with an early interruption of the trial.

2.3. Statistical Analysis

The primary outcome was the viral load kinetics (measured as the area under the curve [AUC] of the quantitative PCR, day 1 to day 21, of SARS-CoV-2) in the enrolled patients with a SARS-CoV-2-positive nasopharyngeal swab demonstrating a reduction in the AUC between day 0 and day 21 of at least 25%. Viral load was quantified as 2ˆdeltaCt. For the analysis of the primary endpoint, a log10 transformation was applied to these values.

Secondary endpoints of the study were the number of patients clearing SARS-CoV-2 by 3, 6, 9, 12, 15 and 21 days after diagnosis and the number of patients with a reduction in viral load of > 2 log by day 3.

The sample size was estimated considering the data published on the effect of Lopinavir/Ritonavir on the quantitative PCR of SARS-CoV-2,. Anticipating a 10% difference in the AUC between groups and an estimated standard deviation of 2.6, the use of the group sequential t-test (assuming a two-sided Type I error of 5% and a power of 95%) led to a sample size estimate of 32 patients per arm (64 patients in total).

We conducted an interim analysis for efficacy and futility (two-sided, symmetric, O'Brien–Fleming Analog Boundary) after 14 patients were recruited in each arm (28 total).

Statistical significance thresholds were set at 10%, 5%, 1% and 0.1%. All statistical analyses were conducted using Stata/IC 16.1 (StataCorp, 4905 Lakeway Drive, College Station, TX, USA).

3. Results

A flowchart of this study is presented in Figure 1. We recruited 29 patients in total before running the ad interim analysis. Ten patients (four in the MB group and six in the placebo group) were excluded from the analysis for different reasons. Briefly, seven patients were found negative at the first SARS-CoV-2 nose–pharyngeal swab performed 24 h after the diagnosis, one patient missed the treatment on day 1 in the placebo group and one patient in each group was lost to follow-up.

Nineteen patients were analyzed for efficacy and twenty-nine for safety. Of the 19 patients, 11 were in the MB arm and 8 in the placebo one.

The demographic characteristics are described in Table 1. Fifteen patients were randomized in the MB treatment arm and fourteen in the placebo arm. We found no significant difference in the baseline characteristics between the treatment and control groups.

Table 1. Patient characteristics at baseline.

	Methylene Blue Group (*n* = 15)	**Placebo Group (*n* = 14)**	**Statistical Test Result**
Gender Woman Man	 8 7	 8 9	$\chi^2(1) = 0.909$
Age Median (IQR)	 50.0 (21.0)	 46.5 (18.3)	z = 0.481
Body mass index Median (IQR)	 26.8 (6.5)	 26.0 (9.1)	z = 0.349
Ethnicity White Asian	 14 1	 14 0	$\chi^2(1) = 0.007$

**** $p < 0.001$, *** $p < 0.01$, ** $p < 0.05$, * $p < 0.1$.

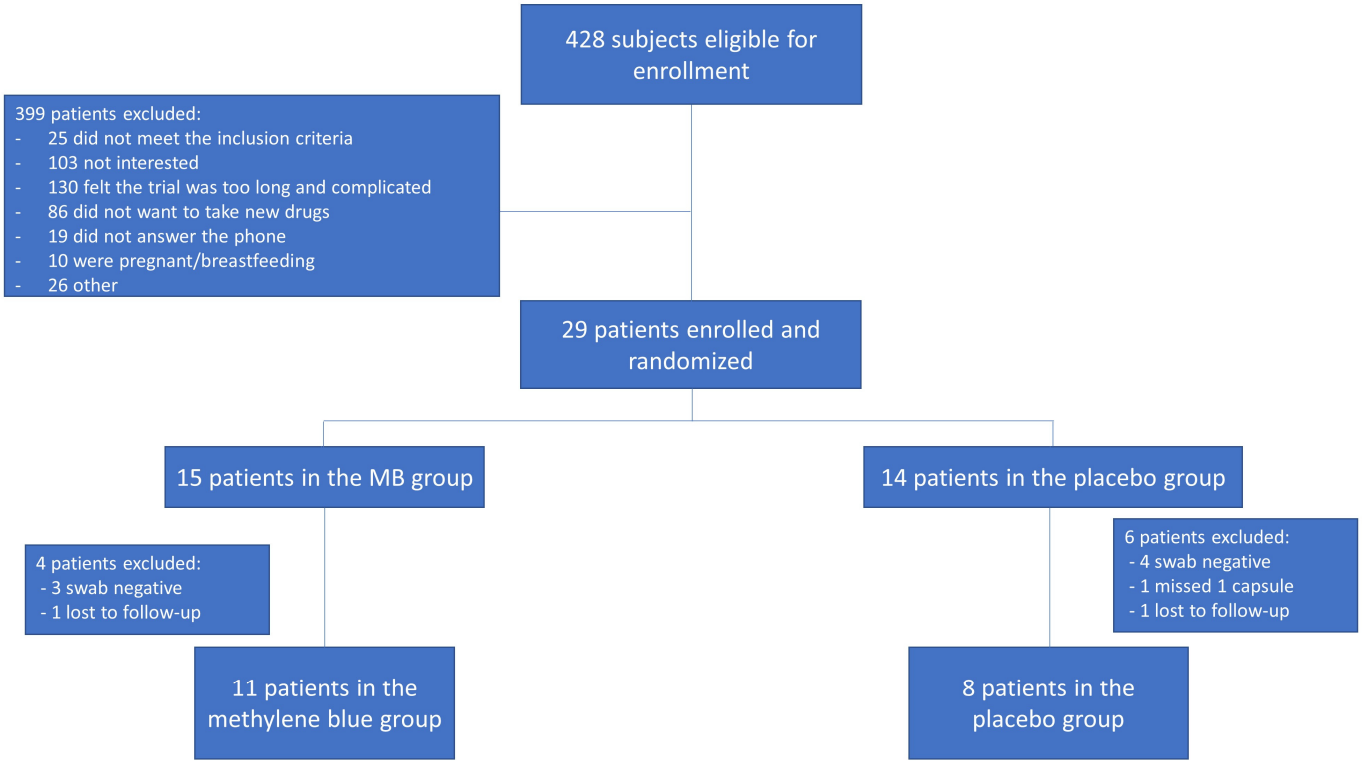

Figure 1. Flowchart of this study.

In Table 2, we report the baseline symptoms assessed during the enrollment visit; the treatment group included slightly more patients reporting nasal congestion (11 vs. 6 in the control group, *p*-value = 0.096) and fever (9 vs. 4 in the control group, *p*-value = 0.089); no other statistically significant differences were found.

Table 2. SARS-CoV-2-related symptoms at enrollment.

Symptom	Methylene Blue Group (*n* = 11)	Placebo Group (*n* = 8)	Statistical Test Result
Fever	9	4	$\chi^2(1) = 2.89$ *
Cough	9	10	$\chi^2(1) = 0.41$
Fatigue	13	9	$\chi^2(1) = 1.981$
Nasal congestion	11	6	$\chi^2(1) = 2.77$ *
Smell alteration	5	3	$\chi^2(1) = 0.51$
Taste alteration	7	4	$\chi^2(1) = 1.00$
Dyspnoea	3	4	$\chi^2(1) = 0.29$
Conjunctivitis	0	1	$\chi^2(1) = 1.11$
Diarrhea	4	4	$\chi^2(1) = 0.01$
Muscular exertion	8	6	$\chi^2(1) = 0.31$

**** $p < 0.001$, *** $p < 0.01$, ** $p < 0.05$, * $p < 0.1$.

Efficacy analysis was performed on the 21 included patients who completed the trial. The results of the ad interim analysis of the primary outcome are presented in Table 3. The parameters reported indicate a higher AUC for the methylene blue group; however, the difference is not statistically significant.

The results (Table 4) obtained for the secondary efficacy endpoints showed no significant difference between the two groups. Nonetheless, one patient in the placebo arm met the viral load reduction of at least 2 log by day 3 and none in the MB arm met this reduction. Viral clearance at scheduled timepoints was not significantly different in the two arms; by day 21, all patients cleared the virus.

Table 3. Comparison between groups of the area under the curve (AUC) of the viral load kinetics in the enrolled patients with a SARS-CoV-2-positive nasopharyngeal swab, demonstrating a reduction in the area under the curve from day 0 to day 21 of at least 25%.

Area under the Curve (AUC)	Group		Difference	95% Confidence Interval of Difference	t-Statistic
	Methylene Blue	Placebo			
AUC Mean (SD [1])	5.1 (5.2)	3.4 (3.0)	1.70 [2]	(−2.10,5.50)	0.94
AUC Median (Min;Max)	3.5 (0.0;12.6)	2.6 (0.0;6.8)	0.88 [3]	(−2.59,6.10) [3]	-
AUC for 2^DCt expressed as % from baseline [Median (Min;Max)]	271 (121;820)	125 (100;436)	-	-	-

**** $p < 0.001$, *** $p < 0.01$, ** $p < 0.05$, * $p < 0.1$. [1] Standard deviation. [2] Standard error of the difference is 1.95. [3] Hodges–Lehmann estimator for difference with 95% Confidence Interval.

Table 4. Percentage of patients clearing SARS-CoV-2 at days 3, 6, 12, 15 and 21.

Efficacy Endpoints		Group		Statistical Test Result
		Methylene Blue (n = 11)	Placebo (n = 8)	
Virus clearance after 3 days	Yes	2	1	$\chi^2(1) = 0.115$
Virus clearance after 6 days	Yes	4	2	$\chi^2(1) = 0.281$
Virus clearance after 9 days	Yes	9	4	$\chi^2(1) = 2.170$
Virus clearance after 12 days	Yes	10	6	$\chi^2(1) = 0.882$
Virus clearance after 15 days	Yes	8	6	$\chi^2(1) = 0.012$
Virus clearance after 21 days	Yes	11	8	No test performed
Viral load reduction of at least 2 log by day 3	Yes No VCW2LR [2]	0 9 2	1 7 0	$\chi^2(2) = 2.847$

**** $p < 0.001$, *** $p < 0.01$, ** $p < 0.05$, * $p < 0.1$. [2] Virus clearance without a 2 log reduction.

No safety concerns attributable to methylene blue were reported. Most adverse events were observed in both treatment arms and considered related to the SARS-CoV-2 viral infection.

4. Discussion

To our knowledge, this was the first clinical trial to test the efficacy of MB in vivo against SARS-CoV-2 infection. The promising in vitro results gave us the input to test it in this pandemic to tackle the new virus.

We noticed good kinetics in the resolution of the infection in both groups. However, despite the lack of statistical significance in the difference between the two groups, the average of the AUC was higher for the MB group, indicating a probable higher severity of the infection, a finding that may be corroborated by the significantly higher number of patients with fever and nasal congestion in the MB group. In such a setting, the results presented in Table 4 showing a comparable virus clearing time (or even a slightly quicker resolution in the MB group, since after 9 days we had 9 virus clearances out of 11 patients in the MB group vs 4 out of 8 in the placebo group) seem to indicate that MB may actually be effective for a faster virus clearance, which could lead to a better and quicker resolution of the infection and might avoid the complications of long COVID-19 and a possible worse clinical course [14]. As is widely known, complications due to COVID-19 are secondary to

the immune system response and the inflammation caused by a sustained viral load. It is easy to understand that this process would be impossible with the halving of the duration of the virus presence.

In our trial, in the MB group the majority (8/11) had undetectable RNA by day 15, but we could see that already from day 9/11 patients had cleared the virus. In the placebo group, only four out of eight patients had cleared the virus by day 9. We noticed a non-significant variation in the RNA levels across the trial, which were interpreted as PCR blips that do not actually have clinical relevance. The time schedule of the trial was designed with safety and efficacy in mind, but in clinical practice the test would not have been repeated at such short intervals.

This result in the frame of COVID-19 is relevant. Our patients were mostly infected by the omicron virus variant, which is characterized by a milder clinical course in healthy, immunocompetent patients. According to protocol, we only enrolled patients who did not need hospitalization or did not experience severe symptoms; the aim of our study was in fact to find a drug for out-patients and to help them to recover faster from the disease.

Almost all patients complied with the program; only one patient in the placebo group missed one administration (excluded from analysis).

The trial has a few main limitations: First, we discontinued it at the ad interim analysis. We had assessed participants while the epidemic curve was decreasing in the numbers of infected people, and by the time we had our results, only a few subjects were infected.

Between enrollment and the first viral load determination, 7 out of 29 patients had already cleared the virus and thus reduced the number of evaluable patients. Also, the recruitment for this study, which recruited the first patients on the 3rd of December 2020 and the last patient on the 20th of May 2021, fell into the final phase of the second wave of the COVID-19 epidemic in Ticino, which was accompanied by the progressive dismantling of the "Checkpoints" and a dramatic fall in eligible patients.

Secondly, the disease itself changed its course and clinical features; luckily for the patients, they were burdened by fewer cases of severe respiratory distress or pneumonia and the majority only had flu-like symptoms. In this scenario, people were more interested in a medicine that could help them recover sooner and avoid the idea of hospitalization and, hence, recruitment was harder and less effective.

Last but not least, the ad interim analysis did not show a striking effect of MB against the infection that would have pushed the scientific community into continuing the trial and postponing the scheduled enrollment plan.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in this study.

Conflicts of Interest: The authors declare no conflicts of interest.

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