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Original Article

The rationale for methylene blue utility against SARS-CoV-2 infection complications

[**F**undamentación de la utilidad del azul de metileno contra las complicaciones de la infección por SARS-CoV-2]

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Abstract

Context: Almost one year after the onset of COVID-19 pandemic in Wuhan, China and still no specific therapy has emerged, counting millions of dead worldwide. The association of an uncontrolled SARS-CoV-2 replication and host-dependent mechanisms in COVID-19 pathogenesis suggest that any therapeutic strategy must combine antiviral drugs and adjuvant therapy to modulate the host's responses. Owing to the multiplicity of mechanisms involved in COVID-19 pathogenic expressions, such as severe hypoxia, excessive inflammatory reaction and impaired immune response, an emerging therapeutic paradigm is the searching for agents acting as multifunctional drugs. Methylene blue (MB), the antique medication, seems to meet the above criterion.

Aims: To summarize the probable beneficial effects of MB against COVID-19 supported by a discussion of the drug mechanisms of action counteracting the pathogenic mechanisms of the disease.

Methods: PubMed, Google Scholar, and Scopus databases were used to collect the biomedical research on MB, and the discussed dataset finally included 150 published articles. Those COVID-19 pathogenic pathways possibly targeted by MB were critically appraised.

Results: It was found that MB may act as multimodal agent by targeting simultaneously several pathogenic mechanisms of COVID-19 as hypoxic damage, hyper-inflammatory reaction and death signaling activation. It also may act as a virucidal agent by preventing virus-induced metabolic re-orientation. Its high safety profile, low cost, along with the mechanisms discussed herein might be essential criteria to test MB as an adjuvant therapy against COVID-19.

Conclusions: Overall, this critical review provides theoretical grounds for MB clinical evaluation in the therapeutic management of SARS-CoV-2 infection.

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

Resumen

Contexto: Casi un año después del inicio de la pandemia de COVID-19 en Wuhan, China, y aún no ha surgido una terapia específica, contando millones de muertos en todo el mundo. La asociación de una replicación no controlada del SARS-CoV-2 y los mecanismos dependientes del hospedero en la patogénesis del COVID-19 sugieren que cualquier estrategia terapéutica debe combinar fármacos antivirales y terapia adyuvante para modular las respuestas del hospedero. Debido a la multiplicidad de mecanismos involucrados en las expresiones patogénicas de la COVID-19, como la hipoxia severa, la reacción inflamatoria excesiva y la respuesta inmune deteriorada, un paradigma terapéutico emergente es la búsqueda de agentes que actúen como fármacos multifuncionales. El azul de metileno (AM), un antiguo medicamento, parece cumplir con el criterio anterior.

Objetivos: Resumir los probables efectos beneficiosos del AM contra la COVID-19 apoyados por una discusión de los mecanismos de acción del fármaco que pudieran contrarrestar los mecanismos patogénicos de la enfermedad.

Métodos: Se utilizaron las bases de datos PubMed, Google Scholar y Scopus para recopilar las investigaciones biomédicas sobre el AM, y el conjunto de datos que se discutió finalmente incluyó 150 artículos publicados. Se evaluaron críticamente aquellos mecanismos patogénicos de la COVID-19 posibles blancos farmacológicos del AM.

Resultados: Se encontró que el AM puede actuar como un agente multimodal al actuar simultáneamente sobre varios mecanismos patogénicos de la COVID-19 como el daño hipóxico, la reacción hiperinflamatoria y la activación de señalizaciones de muerte. También puede actuar como agente virucida al prevenir la reorientación metabólica del hospedero inducida por el virus. Su elevado perfil de seguridad, bajo costo, junto con los mecanismos discutidos en este documento, podrían ser criterios esenciales para probar el AM como terapia adyuvante contra la COVID-19.

Conclusiones En general, esta revisión crítica proporciona las bases teóricas para la evaluación clínica del AM en el manejo terapéutico de la infección por SARS-CoV-2.

Palabras Clave: azul de metileno; COVID-19; SARS-CoV-2.

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INTRODUCTION

Coronavirus infectious disease (COVID-19), the ongoing pandemic infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has generated significant socio-economic disruption, overwhelming national's health systems worldwide. At the time this review was being written, more than 90 million reported cases and over 2 million deaths have been already reported globally (WHO, 2021). Approximately 10-20% of confirmed cases progress to critical illness, with a higher mortality rate than less severe patients (Huang et al., 2020; Zhou et al., 2020). Indeed, mortality of critically ill patients is around 50%, by contrast with 2.3% for overall COVID-19 patients (Guan et al., 2020; Huang et al., 2020; Wu and McGoogan, 2020). This phenomenon unravels particularities of pathogenesis mechanisms and risk factors interactions leading to a critical illness state, characterized by severe pneumonia, acute respiratory distress syndrome (ARDS), septic shock and/or multiple organ failure (Huang et al., 2020; Xu et al., 2020). Currently, there are no clinically approved vaccines or specific therapeutic drugs available for COVID-19, being the symptomatic management and oxygen supply the main clinical treatment options.

Although vaccines seem to be the ultimate solution against COVID-19, therapeutics targeting the abovementioned pathogenic mechanisms, and related to the host responses, are needed. However, drug development is a costly and timely process with a high attrition rate (Lythgoe and Middleton, 2020). Drugs repurposing seems to be the expedite way to deliver a medication to the bedside with the minimum bench time. Along these lines, several proposals have emerged since COVID-19 has spread (Jean et al., 2020; Kandeel and Al-Nazawi, 2020; Li and De Clercq, 2020; Serafin et al., 2020), and more than 600 clinical trials have launched, including repurposed antivirals, antibiotics, and host-targeted agents like immunomodulators, anti-inflammatory drugs, and antioxidants (Lythgoe and Middleton, 2020; Ottaviani and Stebbing, 2020). Yet, nonstandard/specific treatment has emerged against COVID-19, which keeps active the quest for other anti-COVID-19 compounds (Huang et al., 2020).

Methylene Blue (MB) was first synthesized as a dye in 1876, and soon after Paul Erlich demonstrated its antimalarial effects (Guttmann and Ehrlich, 1891). As early as in the 1930s, MB began to be used for the treatment of methemoglobinemia (Mansouri and Lurie, 1993), while it proved to be an effective antidote for carbon monoxide and cyanide (CN) poisoning as well (Brooks, 1933; Draize, 1933). MB is also a recommended treatment for vasoplegic syndrome in critically ill cardiac surgical patients (Evora et al., 1997; Evora, 2000; McCartney et al., 2018), and in septic shock, if administered early (Puntillo et al., 2020). It is currently being utilized as antimalarial agent (Dicko et al., 2018; Mendes et al., 2019) and for the decontamination of plasma by various European blood collection/treatment agencies (Wainwright 2000; 2002). Recently, a phase I clinical trials (NCT04370288; April 19, 2020) reported the beneficial effects of MB administration to five critical COVID-19 patients: four patients surmounted the disease by this intervention (Alamdari et al., 2020).

This review is intended to provide mechanistic evidence supporting the findings cited above. At the same time, we suggest that MB acting multifunctionally against key pathogenic components of COVID-19 might have supportive adjuvant usefulness in treating the infection, particularly its complications, including severe hypoxemia, hyper-inflammatory reactions, and apoptotic-mediated lymphopenia. The long period of safe use of MB in humans makes it much easier therapeutically to develop and it is one of the reasons why there is so much interest in it. Bearing in mind that the pathogenesis of COVID-19 has been recently extensively reviewed (Chu et al., 2020; Domingo et al., 2020; Li et al., 2020), only those pathogenic pathways possibly targeted by MB will be discussed here.

MATERIAL AND METHODS

This study was conducted as a systematic review in which was explored the biomedical literature on methylene blue (1891-2020) using PubMed, Google Scholar, and Scopus databases, and first included in the search terms the words "methylene blue". The search yielded 20837 articles, of which 1263 were selected for a preliminary review based mainly on their potential relation to the pathogenic mechanisms of COVID-19. For such selection, we combined "methylene blue" with the following words: "antiviral" (195 articles), "inflammation" (353 articles), "apoptosis" (196 articles), "ischemia-reperfusion" (105 articles), "hypoxia/anoxia" (221-227 articles), "substrate-level phosphorylation (5 articles), "sepsis" (156 articles), and "COVID-19" (15 articles). MB pharmacokinetics, usual dosages, safety, and contraindications (11 articles), were also reviewed. The current dataset finally included 150 published articles that were selected based on the following criteria: relevance of the pharmacological activity concerning the pathogenesis and complications of COVID-19, the robustness of the scientific finding and scientific quality of the journal, and the timeframe of publication. A group of classic papers on COVID-19 pathogenesis and its clinical manifestation and complications was included in the revision to help the discussion on MB mechanisms of action counteracting the pathogenic mechanisms of the disease. Collectively, the data collected here provide grounds for MB clinical evaluation in the therapeutic management of SARS-CoV-2 infection.

RESULTS AND DISCUSSION

Chemical features of MB that could justify antiviral effects against SARS CoV-2

MB (3,7-bis (dimethyl amino) phenothiazine-5-ium chloride) is a planar tricyclic heteroaromatic compound (Fig. 1). This feature would support the potential for intercalation between base pairs in nucleic acid (Tuite and Kelly, 1993), which in turn could inhibit viral replication. Indeed, MB in the presence of light potently inactivates RNA viruses like VIH-1 and West Nile Virus (Floyd et al., 2004). Studies with model viruses indicate that MB-photomediated viral RNA-protein cross linkage is a crucial lethal lesion, most likely promoted by singlet oxygen as a key intermediate (Foote, 1976; Floyd et al., 2004). The positive charge of MB increases its affinity to the negatively charged RNA and guarantees the proximity of target to the singlet oxygen generation, and therefore, antiviral effectiveness (Kovacs, 1960; Schneider et al., 1993; Jockusch et al., 1996; Floyd et al., 2004).

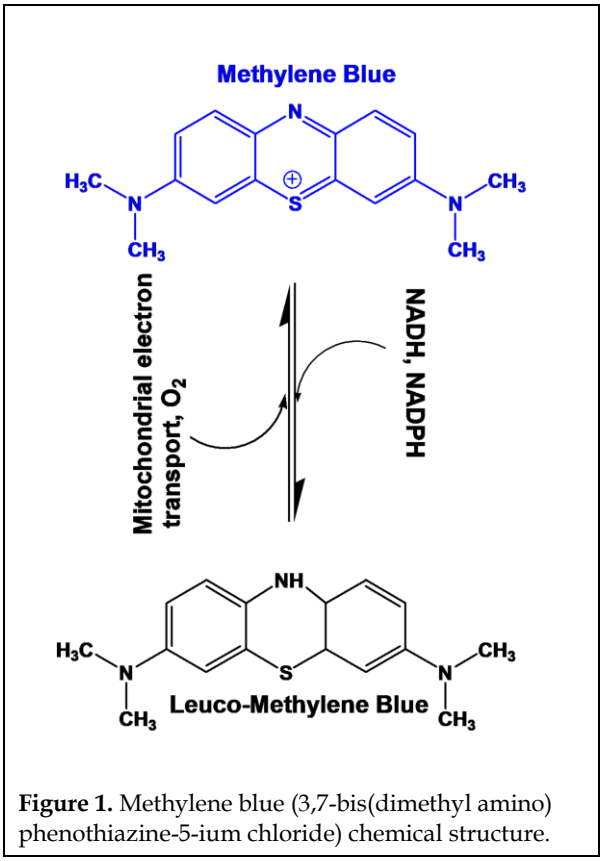

Figure 1. Methylene blue (3,7-bis(dimethyl amino) phenothiazine-5-ium chloride) chemical structure.

The alkalization of intracellular pH of endosomes and lysosomes could also contribute to the viral decontamination by MB. Its reduced and uncharged derivative (leuco-MB) (Fig. 1) could easily penetrate lysosomal membranes and protonate it, thus favoring a pH increase (Wainwright and Amaral, 2005). Accordingly, it could be presumed that endosome maturation might be blocked at intermediate stages of endocytosis, resulting in impairment of further import of virions into the cytosol This effect has also been reported for chloroquine, the anti-malarial drug structurally derived

from MB, a fact currently used as an argument to justify its use as off-label therapy against COVID-19 (Liu et al., 2020a; Wang et al., 2020a). Indeed, some authors reported that MB was mainly localized in the lysosomes of murine fibrosarcoma cells RIF-2, after 2 h incubation (Walker et al., 2004; Mellish et al., 2002). Interesting, on exposure to light, this molecule re-localized to the nucleus, were could interfere with the virus interaction with the host genome (Walker et al., 2004).

MB could also promote H_2O_2 production upon re-generation from its reduced form. MB acting as an alternative electron acceptor in mitochondria that takes electrons from complex I, complex II, and α-glycero phosphate dehydrogenase, is transformed to MBH_2 (Fig. 1), which in turn may reduce not only cytochrome c but also O_2, thus generating H_2O_2 and recycling back to MB (Atamna et al., 2008; Tretter et al., 2014). MB has a redox potential of 11 mV (Kamat et al., 1987) and it is very efficient in cycling between oxidized and reduced forms by suitable redox centers and reducing agents such as those in the mitochondria. Both hypoxia by increasing the reductive sources for MB (NADH, NADPH, $FADH_2$), and re-oxygenation by supplying O_2 for MBH_2 oxidation, would favor the H_2O_2 generation. The increased concentrations of MB-derived H_2O_2, particularly in phagocytes and neutrophils could facilitate their biocidal actions against SARS-CoV-2 due to increased phagosomal HOCl formation mediated by the action of myeloperoxidase (Chesney et al., 1996; Ramalingam et al., 2018). Overall, these mechanisms may justify the potent *in vitro* virucidal effects of MB recently described (Gendrot et al., 2020).

MB restores the cellular energetic balance after CN intoxication, which could be beneficial against hypoxic- mediated energetic failure in COVID-19

The uncontrolled SARS-CoV-2 replication primarily in type II pneumocytes, provokes their apoptosis/pyroptosis and the release of large amounts of pro-inflammatory factors that ultimately led to lungs malfunction and deficient blood oxygenation. COVID-19 severe patients often have dyspnea and/or hypoxemia, after which septic shock, ARDS, and metabolic acidosis develop rapidly (Huang et al., 2020; Liu et al., 2020b; Singh et al., 2020a).

Cyanide (CN) intoxication mimics those clinical symptoms observed in hypoxia/anoxia, consisting of lactic acidosis, coma, and seizures with an early depression in medullary neurons producing apnea and gasping (Haouzi et al., 2018). Thus, it appears to share some similar pathophysiological pathways with COVID-19. Indeed, CN has been used as a surrogate for anoxia in experimental settings as the inhibition by CN of the mitochondrial respiratory complexes, particularly cytochrome oxidase would mimic the acute effects of a reduction in O_2 supply (Cooper and Brown, 2008). The interaction of CN with cytochrome oxidase blocks electron transfer to O_2, inhibiting both respiration and ATP synthesis (Fig. 2) (Petersen, 1977). As a consequence, the NADH/NAD^+ ratio increases, as NADH is not oxidized anymore by the fully reduced NADH-ubiquinone oxidoreductase (complex I). The limited amount of NAD^+ hinders the TCA cycle by negative feedback (LaNoue et al., 1972; Haouzi et al., 2019), which in turn suppresses the synthesis of molecules of ATP via the mitochondrial substrate-level phosphorylation (Fig. 2).

In the cytoplasm, the NAD^+ dependent substrate level phosphorylation catalyzed by the glyceraldehyde 3-phosphate dehydrogenase is also halted, and the increase in NADH/NAD^+ ratio catalyzes the transformation of pyruvate into lactate, resulting in severe lactic acidosis (Burgner and Ray, 1984; Haouzi et al., 2019). Both conditions, CN intoxication and COVID-19 severe infection provoke a metabolism impairment driven by mitochondrial inability to reduce O_2. MB acts effectively against CN- induced cardiac and brain damage (Cheung et al., 2018; Haouzi et al., 2018; 2019; 2020). MB antidotal effect against mitochondrial toxics is based mainly on the ability to target the organelle, thus restoring the TCA cycle and the glycolytic activity by oxidizing NADH and decreasing the NADH/NAD^+ ratio (Komlodi and Tretter, 2017) (Fig. 2). The thiazine heterocyclic aromatic ring gives it enough lipophilicity, and jointly with the positive charge, secures mitochondrial accumulation (Gabrielli et al., 2004).

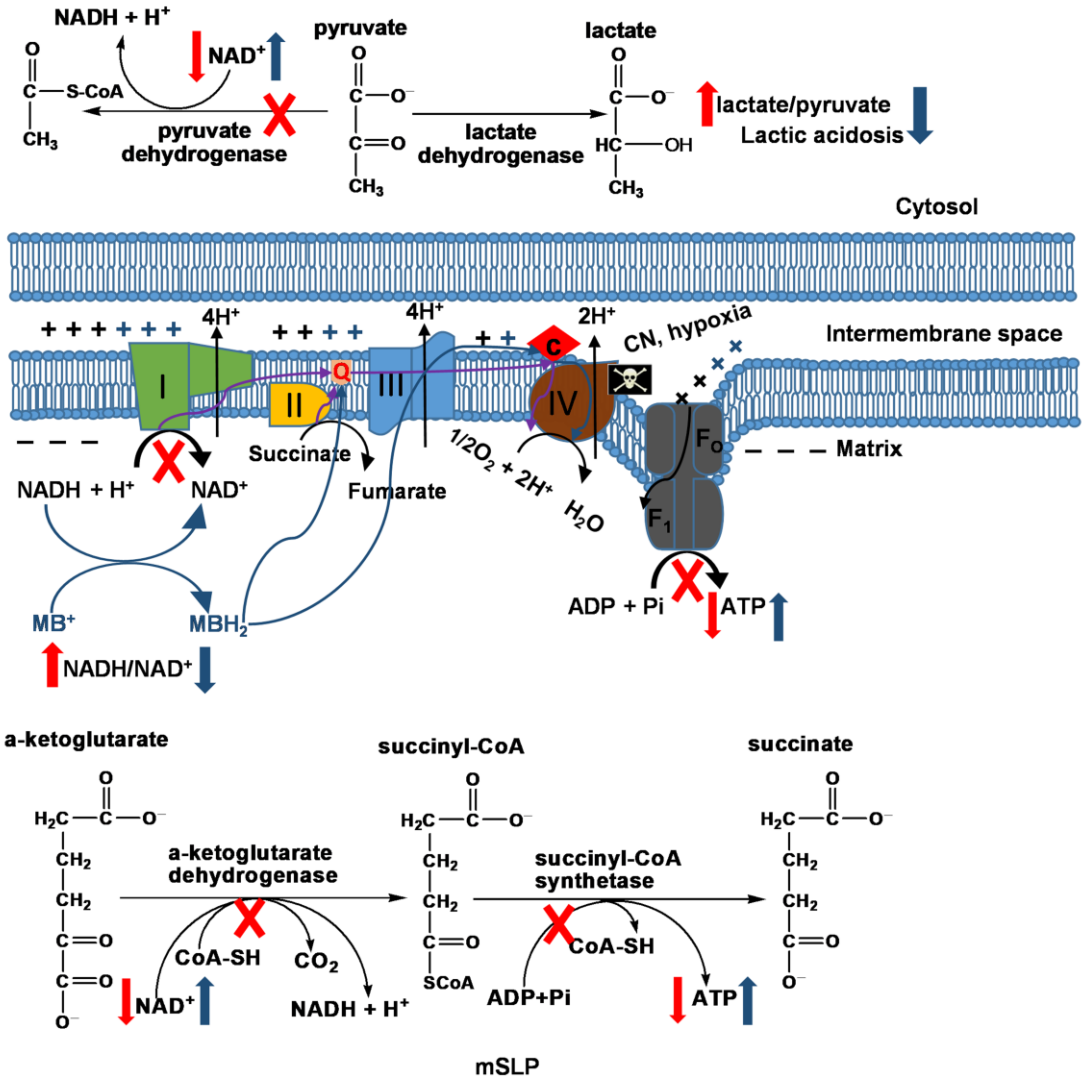

Figure 2. Methylene blue (MB) stimulates mitochondrial substrate level phosphorylation (mSLP) in impaired mitochondria.

Under normal condition, the mitochondrial electron transport chain (ETC) guarantees the electron flow (purple arrows) from NADH or $FADH_2$ to Oxygen in a chemiosmotic process that push protons from the matrix to the intermembrane space (black straight arrows). The proton gradient is used by the F_1F_0 ATPase to synthetize ATP reflowing protons back to the matrix. This process is known as oxidative phosphorylation and the oxygen reduction to water is tightly coupled to the proton gradient generation and ATP synthesis. Under hypoxic condition or after CN poisoning, mitochondrial complex IV (cytochrome oxidase) stop functioning, which also blocks the rest of the ETC complexes; the proton gradient is dissipated and the F_1F_0 ATPase stop synthetizing ATP. The high reductive state of the ETC boosts the $NADH/NAD^+$ ratio that in turn interrupts the mitochondrial substrate level phosphorylation (mSLP) that depends on NAD^+. In cytosol, the acetyl CoA formation from pyruvate is hindered by the blockade of pyruvate dehydrogenase activity. Instead, pyruvate is reduced to lactic acid by the lactate dehydrogenase activity, lessening cytosolic pH. Because of low redox potential (10 mV), MB can be reduced by NADH, thus functioning as an alternative electron acceptor under an impaired mitochondrial electron transport. This effect re-generates NAD^+, re-starting the mSLP catalyzed by succinyl CoA synthetase and increasing ATP (blue arrows). MBH_2, the reduced derivative of MB, could transfer electrons to the redox centers at the ETC like coenzyme Q, complex III and cytochrome oxidase, re-generating MB^+. These redox processes might fuel electrons to the ETC, promoting the re-building of the transmembrane membrane potential and the ATP synthesis by the classical way. By resuming the Krebs cycle, MB^+ promotes pyruvate conversion to acetyl CoA instead of lactate, thus reducing the lactate to pyruvate ratio and the lactic academia.

NAD^+ supply to glycolytic and TCA cycle pathways, efficiently stimulate substrate level phosphorylation-mediated ATP synthesis, which lessens the energetic insufficiency generated by the lack of oxidative phosphorylation (Tretter et al., 2014; Komlodi and Tretter, 2017). After functioning as an alternative electron acceptor, the reduced form of MB could further re-oxidize the mitochondrial electron carriers, thus generating enough H^+ gradient to drive ATP synthesis by F_1F_0 ATP synthase (Fig. 2). Along these lines, MB treatment has proven to restore the mitochondrial membrane potential in left ventricular myocytes exposed to 100 μM CN (Cheung et al., 2018; Haouzi et al., 2018). The repolarizing effects of MB could also avoid the reverse or hydrolytic action of F_1F_0 ATP synthase, one of the largest ATP consumers under ischemic conditions (Christophe and Nicolas, 2006; Grover et al., 2008). In the cytoplasm, the restoration of NADH/NAD^+ ratio, and the reestablishment of TCA cycle inhibit the conversion of pyruvate to lactate, therefore preventing lactic acidosis (Fig. 2) (Tranquada et al., 1964; Levine, 1977). In this way, antidotal action of MB against mitochondrial poisoning could account for its protective role against Acute Lung Injury or Acute Respiratory Distress Syndrome-mediated hypoxemia in severe cases of SARS-CoV-2 infection. This protection might be linked to the restoration of the cellular energetic balance, which in turn could facilitate the activation of energy-consuming endogenous survival pathways.

It is also well established that viruses use intracellular compartments such as mitochondria for their save replication and dissemination in a toxic residency that destroy the organelles (Hagemeijer et al., 2012; Singh et al., 2020b). The interaction between SARS-CoV2 and host mitochondria may disturb both the membrane integrity and functional aspects of the mitochondria like the intermembrane potential (Gordon et al., 2020). Dysfunctional mitochondria can be selectively eliminated via mitophagy (Youle and Narendra, 2011). In this process, injured organelles are enclosed by the autophagosomes, which are then delivered to lysosomes for degradation (Kim and Lemasters, 2011). It has been observed that MB induced mitophagy both *in vitro* and *in vivo*, preserving the organelle structure and increasing the membrane potential (Di et al., 2015). Besides contributing to the mitochondrial quality control selection with the associated improvement in energetic balance, MB may reduce the host viral load by promoting its elimination throughout the induction of virus-loaded organelles degradation.

MB anti-inflammatory effects might protect against COVID-19 associated hyper inflammatory reaction

Patients with severe COVID-19 exhibit considerably elevated blood levels of pro- inflammatory cytokines counting IL-1β, as well as IL-2, IL-6, IL-8, IL-17, G- CSF, GM- CSF, IP10, MCP1, MIP1α (also known as CCL3) and TNF-α. This phenomenon has been called cytokine release syndrome or "cytokine storm" (Domingo et al., 2020; Chu et al. 2020; Huang et al., 2020; Li et al., 2020; Moore and June, 2020; Xu et al., 2020). This hyper-inflammatory condition, the leading cause of morbidity in patients infected with SARS-CoV and MERS-CoV (Channappanavar and Perlman, 2017) may result in immune-mediated damage of tissues and organs.

The NOD-like receptor protein 3 (NLRP3) inflammasome is a multiprotein complex integrated by the NLRP3 protein scaffold, procaspase-1, and an adaptor apoptosis speck-like protein (ASC), which plays a central role in regulating inflammation (Wang et al., 2020b). It is commonly involved in the immune response to bacteria, viruses, fungi, and parasites (Franchi et al., 2012). Its sustained and abnormal signaling underlies many degenerative and chronic diseases, including lupus, periodic auto-inflammatory syndromes, Crohn's disease, osteoarthritis, Alzheimer's disease, type 2 diabetes, atherosclerosis, macular degeneration and cancer (Lamkanfi and Dixit, 2012; Heneka et al., 2014). Fatal inflammation in mammalian host as a result of the H7N9 influenza A virus infection, occurs via NLRP3 inflammasome assembly and activation (Ren et al., 2017). In SARS-CoV infection, viroporin 3a triggers the activation NLRP3 inflammasome and the secretion of IL-1-β by macrophages (Chen et al., 2019). So, a persistent and aberrant NLRP3

inflammasome signaling because of uncontrolled SARS-CoV-2 replication could explain the hyper-inflammatory response in severe COVID-19 patients. Its inhibition, instead of blocking specific cytokines, may be a good choice for protecting against the cytokine storm (Freeman and Swartz, 2020; Ratajczak and Kucia, 2020; van den Berg and Te Velde, 2020).

A recent study showed that MB inhibited assembly of the NLRP3 inflammasome induced by nigericin, ATP, or MSU crystals in LPS-primed bone marrow-derived macrophages (BMDMs) and the human monocyte-like cell line, THP-1 (Ahn et al., 2017). As the result, MB also attenuated secretion of IL-1β and caspase-1 as well as aggregation of Asc, characteristic readouts of inflammasome activation (Ahn et al., 2017). MB also curbed the mRNA expression up-regulation of other cytokines such as IL-1α, IL-6, IL-10, IL-12β, and TNF-α in BMDMs treated with LPS. Such findings suggest that MB attenuates the LPS-TLR4 signaling pathway, which is essential for NLRP3 inflammasome activation (Zhou et al., 2011). The molecular pathway involved in the anti-NLRP3 inflammasome effect of MB included the diminution of mitochondrial ROS production, phagocytosis, caspase 1 activity, and NLRP3 promoter activity (Ahn et al., 2017). Noticeably, MB showed high efficacy against two inflammasome-mediated disease models, LPS-induced lethality and Listeria peritonitis (Ahn et al., 2017). MB inhibitory action on NLRP3 inflammasome activation and the inflammatory response was also confirmed in microglia after spinal cord injury in rats (Lin et al., 2017), and rats' retinas after streptozotocin-induced diabetes (Hao et al., 2018). The importance of targeting inflammasome to control COVID-19 was recently unveiled by ongoing clinical trials with Tranilast, the antiallergic analogue of a tryptophan metabolite, which is a NLRP3 inflammasome inhibitor (Lythgoe and Middleton, 2020).

Nitric oxide (NO) plays a prominent role in virus- induced neumonia (Akaike et al., 1996; Perrone et al., 2013). The cytokines IFN-ɣ, IL-1β, IL-2, IL-6, TNF-α, all released during COVID-19-associated hyper-inflammation, activate NO synthesis (Hibbs et al., 1992; Akaike et al., 1996; Vaz et al., 2011). Blood nitrites and nitrates levels, which may reflect NO status (Shiva et al., 2006) have been found significantly elevated in COVID-19 patients (Alamdari et al., 2020). Worth mentioning is that NO inhibits mitochondrial respiration by targeting cytochrome oxidase (Cleeter et al., 1994), and NADH ubiquinone oxidoreductase (Riobo et al., 2001), which in turn might potentiate the SARS-CoV-2 infection-associated hypoxic condition. MB has been shown to inhibit the nitric oxide (NO) action on vasculature by different mechanisms: *i*) by hindering the signal transduction of NO through deactivating soluble guanylyl cyclase, as it forms non-functional heterodimers with the enzyme beta subunits (Oz et al., 2011; Wang et al., 1995; Sobey and Faraci, 1997); *ii*) by direct inhibition of inducible NO synthase (iNOS) enzymatic activity (Lomniczi et al., 2008); and *iii*) by attenuating the expression of iNOS in response to IFN-ɣ, and LPS both in cultured cells and endotoxemic mice, the latter mechanism elicited by the inhibition of the binding affinity of transcription factors (NF-κB and STAT1) on the promoter region of iNOS gene (Huang et al., 2015). Overall, this scenario accounts for the MB-mediated regulation of NO-associated disorders such as vasoplegic syndrome and septic shock (Evora et al., 1997; Evora, 2000; Riedel et al., 2003; Faber et al., 2005; Demirbilek et al., 2006; Kwok and Howes, 2006; McCartney et al., 2018) and endows the molecule with the potential to prevent the noxious effects of NO in SARS-CoV-2 infection.

Sirtuin-1 (SIRT1) and NF-E2-related factor 2 (Nrf2) may mediate the anti-inflammatory effects of MB. It was recently demonstrated that the Nrf2 antioxidant gene expression pathway is inhibited in biopsies acquired from COVID-19 patients, and the agonist of this signaling induced a cellular antiviral program that potently inhibits replication of SARS-CoV2 across cell lines (Olagnier et al., 2020**)**. In sepsis-induced ALI, the increases in SIRT1 activity promotes lung injury and inflammation. MB supplementation activated the expression of prototypical genes known to be activated by the Nrf2-Nrf1/ARE pathway in a murine model of tauopa-

thy (Stack et al., 2014), in mild-age mice (Gureev et al., 2016), in a rat model of colitis (El Sayed and Sayed, 2019**)**, and in human fibroblast (Xiong et al., 2017). Furthermore, in hepatic models, MB treatment up-regulated SIRT1, and thereby decreased PGC-1α acetylation (Shin et al., 2014).

MB inhibits caspase activation

COVID-19 patients suffer from an immune suppression condition named lymphopenia, characterized by sustained reduction of CD4 T and CD8 T lymphocytes, which correlates with infection severity (Liu et al., 2020b). Steady high levels of TNF-α and IL-6, resulting from the uncontrolled cytokines release, might contribute to T-cell apoptosis and/or activation blockade (Channappanavar and Perlman, 2017; Wan et al., 2020) further contributing to lymphopenia. Since SARS-CoV-2-infected T cells may cause cytopathic effects (Azkur et al., 2020), it could be hypothesized an induction of death mechanisms driven by the virus. Concerning this, it has been described upregulation of apoptosis, autophagy, and p53 pathways in peripheral blood mononuclear cells of COVID-19 patients (Xiong et al., 2020). MERS-CoV and SARS-CoV T lymphocytes can undergo apoptosis by the classical intrinsic and extrinsic pathways (Yang et al., 2005; Mubarak et al., 2019). Pyroptosis, the inflammatory form of cell death, has also been suggested as a cause of lymphopenia, based on the high levels of IL1-β in COVID-19 patients (Yang, 2020). This type of non-apoptotic programmed cell death is typically triggered by inflammasome formation, which leads to caspase-1 activation (Brennan and Cookson, 2020; Watson et al., 2000). The lymphopenic state may prolong the viral infection by promoting its host permanence. As COVID-19 is a systemic infection, the related multiple organ and tissues damage beyond the lungs could be mediated by exacerbated death signaling. Therefore, compounds able to inhibit apoptosis or other form of regulated cell death (i.e., pyroptosis, ferroptosis, and necroptosis) could be useful for treating COVID-19. In this regard, MB has proven to inhibit caspase 6 in human colon carcinoma cells (HCT116) and human primary neurons (Pakavathkumar et al., 2015). Mouse liver protein extracts from mice pre-treated with methylene blue and injected with LPS/GALN showed significantly less caspase 3 activity than untreated animals (Pakavathkumar et al., 2015). These authors proposed the oxidation of catalytic cysteine Cys163 as the acting mechanism (Pakavathkumar et al., 2015). The inhibitory effects on caspase-1 were corroborated in BMDMs and human THP-1 cells (Ahn et al., 2017). This molecule also reverses caspase-6-induced cognitive deficits in mice expressing human caspase-6 in hippocampal CA1 neurons by inhibiting caspase-6, and caspase-6-mediated neurodegeneration and neuroinflammation (Zhou et al., 2019). At this point, a cytoprotective effects of MB against the COVID-19-associated toxic inflammation and immune cells death can be envisage due to its ability to inhibit caspases activation.

MB protection against hypoxic/ischemic tissues damage

The above-mentioned mechanisms endow MB with the capacity to protect tissues and organs against pathologies or insults involving hypoxia, inflammation and cell death as the pathogenic effectors. This is the case of hypoxic/ischemic tissues damage. Lungs, the first target of SARS-CoV-2 infection are particularly sensitive to MB protection. So, for instance, some researchers claimed that MB protects the isolated rat lungs against ischemia-reperfusion injury by attenuating mitochondrial damage. As expected, it downregulated the mRNA expression levels of TNF-α, IL-1β, IL-6, and IL-18 (Tian et al., 2018). MB also attenuates lung injury induced by hindlimb (Wang et al., 2018) and lungs transplantation (Abreu et al., 2014) ischemia-reperfusion in rats, by inhibiting oxidative stress and inflammation. MB fully protected other organs exposed to ischemic injury namely the pancreas (Weinbroum, 2009), kidneys (Sarac et al., 2015), heart (Cheung et al., 2018), liver (Aksu et al., 2010), and intestine (Morgaz et al., 2020). Protective effects of this compound against other complications induced by xenobiotics or toxics have been examined as well (Chen et al., 2015; Lee et al., 2015). The protection of brain tissue against ischemic damage is the most recurrent effects elic-

ited by MB (Wen et al., 2011; Di et al., 2015; Ahmed et al., 2016; Lu et al., 2016; Yang et al., 2017; Auchter et al., 2020). This is probably because its ability to cross the blood brain barrier (Peter et al., 2000), interrupting simultaneously critical components of ischemic cascade like the overproduction of free radicals, the neuroinflammation, and the initiation of apoptotic signaling (Fisher, 1997; 2011). Thus, MB can protect different organs from insults involving ischemic damage, inflammation, oxidative damage and cell death signaling, which could be beneficial for protection against multi-organ SARS-CoV-2 infection and impairment.

MB could re-adjust cellular metabolism to a mitochondria-centered condition that might deprive the virus from its energetic and structural supplies

Most eukaryotic viruses examined to date induce aerobic glycolysis also known as the Warburg effect. Proteomic data in HIV-1-infected macrophages have unveiled an increase in abundance of enzymes in the glycolytic pathway (pentose phosphate and pyruvate metabolism), together with downregulation of some key mitochondrial enzymes such as glutamate dehydrogenase 2 (GLUD2), adenylate kinase 2 (AK2) and transketolase (TKT) (Barrero et al., 2013). Similar proteomic analysis suggests that hepatitis C virus also induces early perturbations in glycolysis, in pentose phosphate pathway, and the citric acid cycle, which favor host biosynthetic activities supporting viral replication and propagation (Diamond et al., 2010). Influenza virus infection increases glucose uptake, aerobic glycolysis, and he pentose phosphate shunt to help produce more nucleotides. Besides, mitochondrial fatty acid β-oxidation decreases significantly simultaneously with an increase in biosynthesis of fatty acids and membrane lipids (Keshavarz et al., 2020). The alterations of carbon source utilization by infected cells can increase available energy for virus replication and virion production, provide specific cellular substrates for virus particles and create viral replication niches while increasing infected cell survival. As above, SARS-CoV-2 infection might hijack the host metabolic machinery to guarantee enough energetic and structural supplies to facilitate its replication and biogenesis. So, targeting the inhibition of these cellular metabolic pathways by shifting the Warburg effect to a mitochondrial-mediated energetic could stop the infection propagation. MB, as an alternative mitochondrial electron carrier, reverses the Warburg effect, and attenuates anabolism in glioblastoma cells (Poteet et al., 2013). MB in nanomolar range induces PGC1α and SURF1 in normal human lung fibroblast cells (IMR39), which are meaningful signals for mitochondrial and complex IV biogenesis (Atamna et al., 2015). Also, MB increases the ratio NAD^+/NADH, and pAMPK/AMPK (Atamna et al., 2015), which is consistent with the increase in NADH oxidation by mitochondria in MB-treated cells. In this way, this molecule restores mitochondrial oxidative phosphorylation and reduce NADPH, thus limiting the building bricks for virus development.

Recently it was shown that SARS-CoV-2 infection dysregulates the set of genes involved in consumption and biosynthesis of nicotinamide adenine dinucleotide (NAD^+), particularly the non-canonical poly(ADP-ribose) polymerase (PARP) family members genes (Heer et al., 2020). This depressed cellular NAD^+ levels, and provide a plausible explanation as to why aging, where NAD^+ levels decline (Massudi et al., 2012), positively correlate with fatality in COVID-19 patients. At the same time, it also suggests that higher NAD^+ status could protect from infection, which is consistent with the potentially higher NAD^+ status of people who successfully fight off COVID-19 disease (Yang et al., 2020). MB, by restoring cellular NAD^+ levels, may improve PARP function and decreased coronavirus replication. Summarizing, MB could switch the cellular metabolism from a biosynthetic/glycolytic phenotype triggered by SARS-CoV-2 to an energetic, mitochondria-centered hindering specifics energetic and structural virus supplies.

Table 1. Common doses of MB used in the clinical practice.

Disease	Dose	Observation	References
Methemoglobinemia	1-2 mg/kg over 5-10 min		(Clifton 2nd and Leikin, 2003; Ginimuge and Jyothi, 2010)
Sepsis	1-2 mg/kg over 10-30 min	0.25–1 mg/kg/h for 6 h started 2 h after the initial bolus dose	(Kirov et al., 2001)
Vasoplegic syndrome	i.v. single dose 2 mg/kg, 20-minute infusion time	Continuous infusions may be beneficial after the initial bolus for up to 48–72 h	(Leyh et al., 2003)
Bipolar disorder	2-5 mg/kg		(Alda et al., 2017)
Malaria	15 mg/kg orally administered for 3 days		(Dicko et al., 2018; Mendes et al., 2019)

MB pharmacokinetics, usual dosages, safety and contraindications

Table 1 shows the common doses of MB used in the clinical practice. MB intravenously administered displays a multicompartmental pharmacokinetics with plasma half-life of 5–6.5 h (Yang et al., 2017). It is eliminated in bile, feces, and urine as leucomethylene blue (Clifton 2nd and Leikin, 2003; Allegaert et al., 2004). Usually well tolerated, the main side effects are irritation of the gastrointestinal tract when administered orally and burning in the urinary tract. Its continuous peripheral infusion for prolonged duration may lead to local cutaneous necrosis (Dumbarton et al., 2012). Some patients find the urine discoloration worrying. toxic manifestations of MTB (>7 mg/kg) include hemolysis, methemoglobinemia, nausea and vomiting, chest pain, and hypertension (Faber et al., 2005).

According to MB pharmacodynamics, glucose-6-phosphate dehydrogenase deficiency is a relatively common contraindication to its treatment because of the risk of hemolytic anemia (McDonagh et al., 2013). Moreover, it is contraindicated in patients treated with serotonergic agents due to the increased risk of serotonin syndrome (Alda, 2019).

Future perspectives

The epidemiological behavior of COVID-19 is characterized by a continuous increase in morbidity and deaths worldwide, together with the occurrence of new SARS-CoV-2 variants with an unusual large number of mutations that spread more easily and quickly. In this sense, it is urgent to introduce therapies to contain the infection and deaths until the application of vaccines becomes extensive. To date, no therapy has been effective in rescuing patients with severe complications from viral infection. The array of mechanisms involved in COVID-19 pathogenic expressions that lead to the severity of infection and death, prompts to search for multimodal-acting therapeutics agents that target simultaneously several pathological mechanisms. In this context, methylene blue, due to the multiplicity of pharmacological mechanisms potentially related to virus pathogenic pathways, could be an option to be evaluated as part of the protocols applied to critically ill patients.

CONCLUSIONS

The association of an uncontrolled SARS-CoV-2 replication and host-dependent mechanisms in COVID-19 pathogenesis suggests that any therapeutic strategy must combine antiviral drugs and adjuvant therapy to modulate the host's responses. Most of the accepted therapeutics schemes against COVID-19 include, besides antivirals, a combination of anti-inflammatory, immunomodulators, anticoagulants and antioxidants, which could interfere each other at the pharmacokinetic or pharmacodynamics level, thus abrogating an effect or potentiating it to toxic levels. The best adjuvant

therapy would be those that include into one drug most of the mechanisms that can impede the noxious host responses triggered by the virus infection. We are proposing herein such a drug; MB, by a multimodal way of action could simultaneously impact the several mechanisms related to COVID-19 complications, as the severe hypoxia, the hyper-inflammatory reactions, and the increased death signaling leading to an immunosuppressive state (Fig. 3). Likewise, it could re-adjust cellular metabolism to a mitochondria-centered condition that might deprive the virus of energetic and structural supplies. Recently, a preliminary phase I clinical trials based on the use of MB proved to be useful for treating COVID-19 complications by preserving the lives of fourth out of five critically ill patients.

Additionally, recent paper documented a potent *in vitro* virucidal effects of MB at low micromolar concentration. These facts, along with a high safety profile validated by more than 120 years in the clinic, its privileged pharmacokinetics, and low cost, all suggests that MB could help patients to overcome COVID-19 and that its use should be urgently generalized.

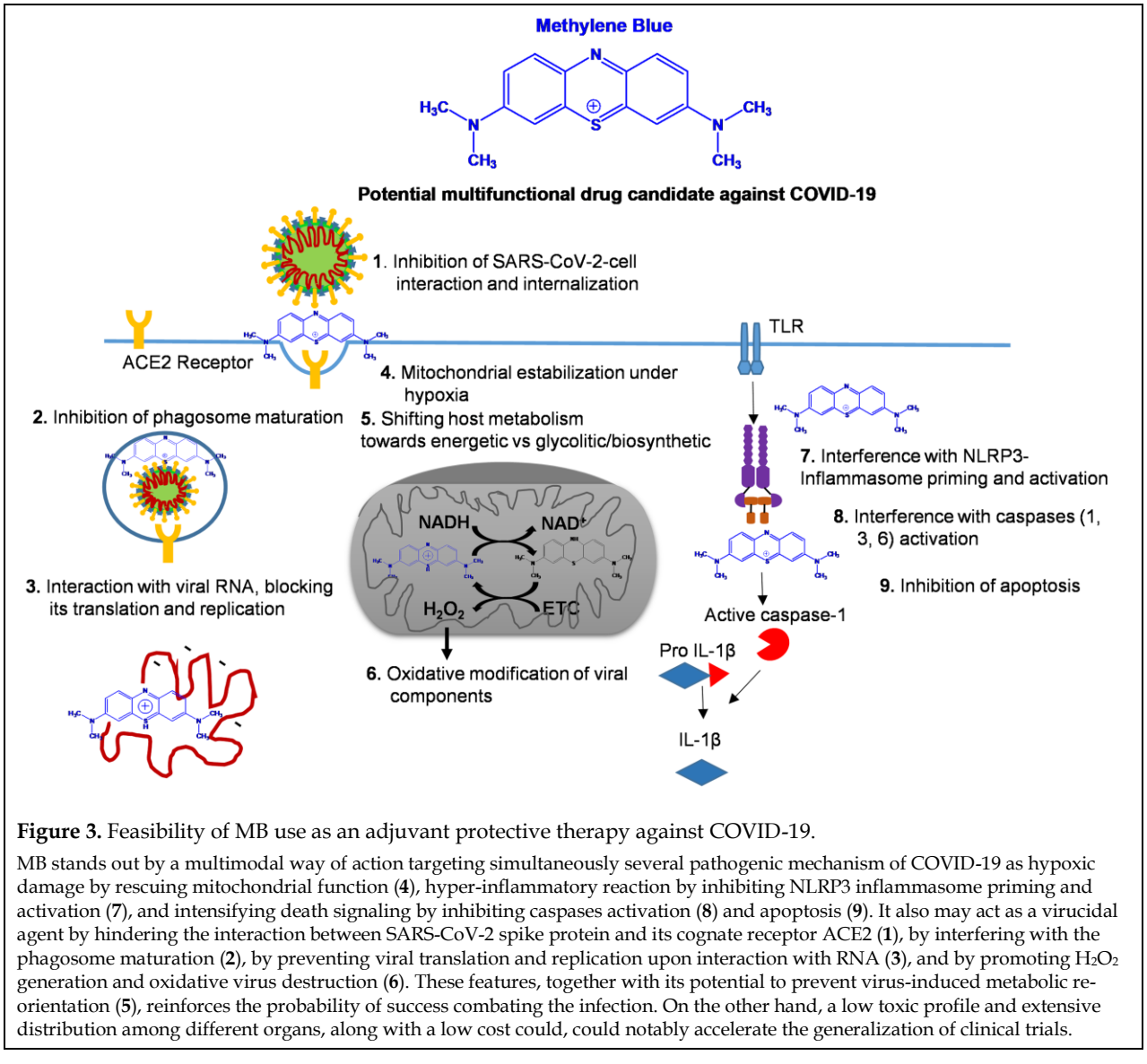

Figure 3. Feasibility of MB use as an adjuvant protective therapy against COVID-19.

MB stands out by a multimodal way of action targeting simultaneously several pathogenic mechanism of COVID-19 as hypoxic damage by rescuing mitochondrial function (**4**), hyper-inflammatory reaction by inhibiting NLRP3 inflammasome priming and activation (**7**), and intensifying death signaling by inhibiting caspases activation (**8**) and apoptosis (**9**). It also may act as a virucidal agent by hindering the interaction between SARS-CoV-2 spike protein and its cognate receptor ACE2 (**1**), by interfering with the phagosome maturation (**2**), by preventing viral translation and replication upon interaction with RNA (**3**), and by promoting H_2O_2 generation and oxidative virus destruction (**6**). These features, together with its potential to prevent virus-induced metabolic re-orientation (**5**), reinforces the probability of success combating the infection. On the other hand, a low toxic profile and extensive distribution among different organs, along with a low cost could, could notably accelerate the generalization of clinical trials.

CONFLICT OF INTEREST

The author declares no conflicts of interests.

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