

METHYLENE BLUE INHIBITS THE INTERACTION BETWEEN HEPARAN SULFATE AND SARS-COV-2 SPIKE PROTEIN; A REVIEW OF EVIDENCE FOR A HYPOTHESIS

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ABSTRACT

The addition of methylene blue to the standard treatment protocol has been shown to improve respiratory rate and oxygen saturation in COVID-19 patients, reducing morbidity and mortality. Evidence to date suggests that methylene blue inhibits protein-protein interactions between SARS-CoV-2 Spike protein and angiotensin-converting enzyme 2, which in turn inhibits the cell entry of SARS-CoV-2. However, the methylene blue dye-binding characteristics of sulfated glycosaminoglycans suggest additional inhibitory effects of the spike protein-heparan sulfate interaction. We hypothesize that the binding of cationic methylene blue neutralizes polyanionic heparan sulfate molecules on the host cell surface. As a consequence, electrostatic interactions between negatively charged heparan sulfate and the positively charged receptor binding domain of SARS-CoV-2 spike protein will be inhibited. Thus, methylene blue will exhibit a "shielding effect" on the heparan sulfate proteoglycans, inhibiting viral attachment to the cell surface. The proposed mechanism corroborates the possible broad-spectrum antiviral activity of methylene blue against multiple human coronaviruses that exploit the electrostatic interactions with sulfated glycosaminoglycans for virus entry. Methylene blue would exhibit the same anti-adhesive activity at the blood-brain-barrier and olfactory neuroepithelium, corroborating potential benefits in ameliorating post-COVID-19 neurological impairment. However, as cationic dyes can bind to both free glycosaminoglycans in circulation as well as proteoglycans attached to the cell surface, co-administration of intravenous heparin could possibly antagonize the proposed antiviral activity. This critical review focuses on empirical evidence to support the hypothesized heparan sulfate-dependent antiviral activity of MB.

Keywords: Methylene Blue; SARS-Cov-2; COVID-19, Heparan Sulfate; Proteoglycans; Antiviral

INTRODUCTION

Methylene blue (MB) is a medication used for both diagnostic and therapeutic purposes in different areas of the medical field. MB appears to demonstrate multiple biochemical mechanisms to bring about desired effects depending on its use. For instance, intravenous administration of MB for emergency treatment of methemoglobinemia is a Food and Drug Administration (FDA)-approved therapeutic indication where it acts by reducing the oxidized form of Fe³⁺ in methemoglobin to Fe²⁺ (Bistas & Sanghavi, 2020). Even though the remaining therapeutic indications, such as vasoplegic syndrome, septic shock, Plasmodium falciparum malaria, and photodynamic cancer therapy, are not FDA-approved, diverse biochemical mechanisms of the drug are of great interest (Ginimuge & Jyothi, 2010).

Preliminary results of a phase-I clinical trial showed co-administration of MB, vitamin C, and N-acetyl cysteine

decreased inflammatory markers and resulted in overall clinical improvement in COVID-9 patients (Hamidi-Alamdari *et al.*, 2020). Moreover, a randomized, controlled, open-label clinical trial conducted by the same research group showed that the addition of MB to the treatment protocol significantly improved respiratory rate and oxygen saturation in COVID-19 patients, which in turn decreased hospital stay and mortality. Furthermore, a number of therapeutic roles of MB in COVID-19 have been postulated (Hamidi-Alamdari *et al.*, 2021).

In the light of evidence coming from initial clinical trials, MB has been a point of discussion in several hypotheses and literature reviews as a multifunctional agent. Among the pathogenic mechanisms of COVID-19 that can be targeted by MB are inhibition of SARS-CoV-2-host cell interaction, phagosome maturation, translation of viral RNA, viral replication, hypoxic

damage, hyper-inflammatory reaction, etc (Pardo Andreu, 2021). Moreover, MB has other potential applications in the COVID-19 pandemic, including decontamination of personal protective equipment (Lendvay *et al.*, 2021), and pathogen reduction of COVID-19 convalescent plasma (Kostin *et al.*, 2021). Additionally, some studies suggest the prospective use of photodynamic therapy as an effective method for the prevention and treatment of COVID-19 (Svyatchenko *et al.*, 2021). However, MB has also demonstrated *in vitro* antiviral activity in non-photodynamic assays (Gendrot *et al.*, 2020). This critical review focuses on empirical evidence to support the heparan sulfate-dependent antiviral activity of MB.

METHODOLOGY

The present hypothesis proposes that aggregation of methylene blue on heparan sulfate proteoglycans on the host cell surface will inhibit the binding of SARS-CoV-2 spike proteins. A systematic approach was applied to substantiate the hypothesis which was conceptualized based on three complementary empirical findings.

1. Methylene Blue inhibits cellular entry of SARS-CoV-2.
2. Methylene blue binds to and forms complexes with polyanionic glycosaminoglycans (GAGs).
3. Adhesion of SARS-CoV-2 through spike protein-heparan sulfate interactions facilitates the cellular entry of SARS-CoV-2.

Online electronic bibliographic databases such as PubMed, Scopus, and Google Scholar were explored using specific search strings limiting the search to the title, abstract, and keywords. The search terms used were "methylene blue", "antiviral", "heparan", "glycosaminoglycan", "SARS-CoV-2", "coronavirus", and "COVID-19". Search results were pooled and duplicates were removed. Irrelevant papers are eliminated by examining abstracts while the relevant scholarly literature is perused and woven into the hypothesis. Additional sources were also retrieved from the reference lists of eligible papers and review articles.

The subsequent sections of the present paper are stratified in terms of the three complementary empirical findings that led to the hypothesis.

RESULTS

Methylene Blue Inhibits Cellular Entry of SARS-CoV-2

MB has been found to exhibit potent antiviral activity against SARS-CoV-2 in the absence of UV- activation *in vitro* (Cagno *et al.*, 2021). It has been demonstrated that the drug inhibits the entry of a SARS-CoV-2 spike bearing pseudovirus into angiotensin-converting enzyme 2 (ACE2)-expressing cells (Bojadzic *et al.*, 2020). Subsequently, a study conducted by the same research group suggested that MB inhibits protein-protein interactions between SARS-CoV-2 Spike protein and ACE2 (Bojadzic *et al.*, 2021). On the other hand, another study showed that the addition of MB to the cell cultures inhibits the replication of SARS-CoV-2 at both "entry" and "post-entry" stages supporting the multimodal nature of its action (Gendrot *et al.*, 2020; Gendrot *et al.*, 2021). Therefore, the drug may exhibit multiple mechanisms of action at both entry and post-entry stages of the infection, in addition to inhibition of SARS-CoV-2 spike protein-ACE2 interaction. The inhibition of SARS-CoV-2-heparan sulfate interaction has not been proposed previously as a potential mechanism of action.

Interactions between heparan sulfate and SARS-CoV-2 spike protein

SARS-CoV-2 uses ACE2 as the primary entry receptor (Hassanzadeh *et al.*, 2020). Nevertheless, directed expression or selective cleavage of ACE2 had no measurable effect on viral adhesion (Milewska *et al.*, 2014). Conversely, ablation of genes involved in heparan sulfate biosynthesis or incubating cells with a heparan sulfate mimetic inhibited Spike-mediated viral entry (Zhang *et al.*, 2020). Therefore, these findings suggest that SARS-CoV-2 initially binds to cell surface heparan sulfate increasing virus density at the cell surface and facilitating the interaction between SARS-CoV-2 and ACE2 for virus entry (Reis *et al.*, 2021), as illustrated in Fig. 1A. Moreover, microarray binding experiments have suggested that ACE2 and heparan sulfate can simultaneously engage with the receptor-binding domain (RBD) of SARS-CoV-2 spike protein, and probably no dissociation between heparan sulfate and RBD is required for binding to ACE2 (Liu *et al.*, 2021). Furthermore, studies have revealed significant colocalization between SARS-CoV-2 and syndecan (a proteoglycan), suggesting a jointly shared internalization pathway. Among syndecans, the lung abundant syndecan-4 was found to be the most efficient in mediating SARS-CoV-2 uptake (Hudák *et al.*, 2021). In line with the aforementioned findings, heparan sulfate has been recognized as an important attachment factor for SARS-CoV-2 infection in human lung

epithelial cells (Chu *et al.*, 2021). Importantly, SARS-CoV-2 spike glycoprotein has shown a significantly higher affinity to human lung heparan sulfate than bat lung heparan sulfate (Yan *et al.*, 2021). The higher infectivity of the SARS-CoV-2 can be partly attributed to the high affinity of the spike glycoprotein to heparan sulfate (Yue *et al.*, 2021).

Molecular docking studies suggest that SARS-CoV-2 spike protein interacts with heparan sulfate and ACE2 through multiple binding sites on the RBD (Clausen *et al.*, 2020; Schuur *et al.*, 2021). Furthermore, the SARS-CoV-2 spike protein is slightly more positively charged than that of SARS-CoV since it contains four more positively charged residues and five less negatively charged residues. Therefore, it has been suggested that SARS-CoV-2 spike protein exhibits an increased affinity to bind with negatively charged adhesion molecules (Hassanzadeh *et al.*, 2020). Heparan sulfate, which belongs to the group of sulfated GAGs, are polyanions with high negative charge density (Soares da Costa *et al.*, 2017). Hence, heparan sulfate can form strong electrostatic interactions with RBD of SARS-CoV-2 spike protein. Therefore, drugs targeting the interaction between heparan sulfate and SARS-CoV-2-related proteins have been proposed as an important strategy for developing novel therapeutics for the ongoing COVID-19 pandemic (Yu *et al.*, 2021; Zhang *et al.*, 2020). Highly sulfated GAGs such as heparin and tri-sulfated heparan sulfate have higher charge density and binds SARS-CoV-2 spike protein with a higher affinity. The position of sulfation within heparin was also found to be important for its successful binding to SARS-CoV-2 spike protein (Hao *et al.*, 2021; Kim *et al.*, 2020). Therefore, non-anticoagulant derivatives of heparin, heparan sulfate mimetics, and other sulfated and sulfonated molecules have been suggested to have a therapeutic potential against SARS-CoV-2 infection as competitive inhibitors (Gasbarri *et al.*, 2020; Jin *et al.*, 2020; Kwon *et al.*, 2020; Mycroft-West *et al.*, 2020; Tandon *et al.*, 2021; Wells *et al.*, 2021). Importantly, inhibition of viral cellular entry is one of the potential non-anticoagulant roles in heparin therapy of COVID-19 patients (Buijssers *et al.*, 2020).

Intriguingly, attachment of the virus to heparan sulfate proteoglycans on host cells is a common step in the lifecycle of multiple viruses, including herpesviruses, human immunodeficiency virus, dengue virus, human papillomavirus, as well as coronaviruses (Koganti *et al.*, 2021). Therefore, discovering an effective drug targeting heparan sulfate

will open up a new paradigm in antiviral therapeutics. In line with this prospect, a study revealed that bovine lactoferrin binds to heparan sulfate proteoglycans, thereby blocking viral attachment to the host cell. The same study showed that lactoferrin has broad-spectrum antiviral activity against multiple coronaviruses including SARS-CoV-2 (Hu *et al.*, 2021). Interestingly, lactoferrin did not have an inhibitory effect on the spike protein-ACE2 interaction (Lang *et al.*, 2011). Therefore, it could be hypothesized that heparan sulfate-binding drugs that inhibit spike protein-heparan sulfate interaction will show a broad-spectrum antiviral activity. Several drugs and heparin/heparan sulfate binding peptides that inhibit viral entry by targeting heparan sulfate have been proposed and studied (Suryawanshi *et al.*, 2021; Tavassoly *et al.*, 2020; Zhang *et al.*, 2020). Human monoclonal antibodies targeting the SARS-CoV-2 spike protein have already entered clinical trials while other viral attachment sites such as heparan sulfate proteoglycans have been suggested as potential targets (Sun and Ho, 2020). Despite this interest, to the best of our knowledge, no research or hypothesis has focused on the potential heparan sulfate-dependent anti-adhesive activity of MB against SARS-CoV-2.

Interaction between heparan sulfate and cationic dyes

Metachromatic dyes such as MB, dimethylmethylene blue (DMMB), and toluidine blue are positively charged and react with negatively charged polyanions in aqueous solutions. The binding of the dye with the polyanions neutralizes the positively charged dye. Consequently, the non-polar aromatic ring of the dye binds with the adjacent dye by Van der Waals forces releasing the water bound to the dye molecules and resulting in dye-to-dye aggregation. The overall binding of the dye becomes stronger due to the Van der Waals forces and the absorbance spectrum of the dye shifts to a shorter wavelength (Dey, 2018; Jakubowska *et al.*, 2014; Jiao and Liu, 1999). This principle is used to detect GAGs in biological samples (Stone *et al.*, 1994; Templeton, 1988; Zhang *et al.*, 2004). MB can bind to both free and protein-bound forms of heparan sulfate, given that the size is over four disaccharide units (Németh-Csóka *et al.*, 1975). Therefore heparan sulfate proteoglycans on the host cell surface will possibly exhibit the aforementioned dye-binding characteristics during MB therapy.

DISCUSSION

In the light of empirical evidence, it can be conceivably hypothesized that cationic dyes such as MB can form

aggregates on heparan sulfate molecules covalently linked to core proteins (proteoglycans) and interfere with electrostatic interactions between negatively charged heparan sulfate and positively charged patch in RBD. Thus, cationic dye molecules will exhibit a “shielding effect” on the heparan sulfate proteoglycans as illustrated in Fig. 1B. This will inhibit the heparan sulfate-mediated viral attachment and decrease the local concentration of pathogens on the cell surface. Thus, it renders the interaction of SARS-CoV-2 with the entry receptors less efficient. Therefore, cationic dyes such as MB may exhibit anti-adhesion properties in addition to direct inhibition of spike protein-ACE2 interaction.

Considering the non-specific nature of the proposed mechanism of action, it can be further hypothesized that cationic dyes could exert broad-spectrum antiviral activity against multiple human coronaviruses that exploit the electrostatic interactions with sulfated GAGs for virus entry. However, cationic dyes can bind to both free GAGs in circulation as well as proteoglycans attached to the cell surface. On the other hand, the charge density of heparin is higher than that of heparan sulfate. Therefore, co-administration of intravenous heparin can possibly antagonize the proposed antiviral activity of MB.

Transneuronal spread through the olfactory nerves and hematogenous route after breaching the blood-brain barrier have been proposed as possible routes for SARS-CoV-2 brain entry (Kumar *et al.*, 2020). Expression of the viral binding receptors in non-neural cells of the olfactory epithelium and myelin-forming cells are implicated in facilitating the invasion of olfactory nerve. Interestingly, the olfactory neuroepithelium has been shown to express heparan sulfate important and strong binding sites for herpes virus entry (Milho *et al.*, 2012). Moreover, brain microvascular endothelial cells express high levels of heparan sulfate and chondroitin sulfates which act as binding sites for brain-tropic viruses via electrostatic interactions between basic residues in viral glycoproteins and negatively charged sulfated GAGs (Bobardt *et al.*, 2004). This evidence suggests that SARS-CoV-2 may exploit its electrostatic interactions with sulfated GAGs to enter and migrate through the blood-brain barrier and olfactory epithelium to invade the brain. Therefore, cationic dyes would possibly exhibit the same anti-adhesive activity at the blood-brain-barrier and olfactory neuroepithelium corroborating potential benefits in ameliorating post-COVID-19 neurological impairment. Therefore, this

may add an incremental value to MB therapy as a neuroprotective agent in COVID-19.

MB is a safe medication when used within its therapeutic range <2 mg/kg. It is water-soluble and can be administered by oral, intranasal, inhalational as well as intravenous routes. The drug is inexpensive and widely available across the world. Moreover, compared to protein therapeutics such as antibodies, MB therapy has a relatively lower propensity for unwanted immunogenicity and hypersensitivity. The other advantages over conventional antivirals include inhibition of excessive production of reactive species and cytokines, and amelioration of post-COVID-19 blues (Magoon *et al.*, 2021; Scigliano & Scigliano, 2021). Further, it has the possible advantage of having antibacterial effects against secondary bacterial infections commonly encountered among COVID-19 patients. Nevertheless, MB is contraindicated in pregnancy and glucose-6-phosphate dehydrogenase deficiency (Bistas & Sanghavi, 2020). MB can also give rise to falsely lower estimates of oxygen saturation in COVID-19 patients due to its interference with the pulse oximeter's light emission (Ginimuge & Jyothi, 2010).

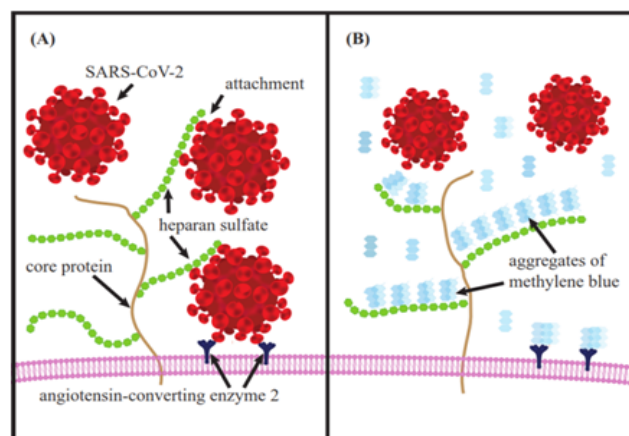


Figure 1; (A) SARS-CoV-2 initially binds to cell surface heparan sulfate and facilitates the interaction between SARS-CoV-2 and angiotensin-converting enzyme 2. (B) Methylene blue forms aggregates on heparan sulfate molecules and blocks viral attachment.

CONCLUSIONS

A plethora of evidence corroborates the proposed hypothesis in which MB binds to heparan sulfate and inhibits SARS-CoV-2-heparan sulfate interactions. We believe that the present hypothesis will form a base for future studies focusing on the effect of cationic

molecules on heparan sulfate-mediated viral adhesion/attachment. We suggest that the hypothesized effect of MB can be tested by conducting flow cytometry-based and confocal microscopy-based virus adhesion experiments on cells with heparan sulfate proteoglycan expression. Due to the prevailing COVID-19 pandemic, the attempts to repurpose the existing drugs for the treatment of COVID-19 are pertinent. MB appears to be a good candidate for drug repurposing with both preventive and curative prospects, considering the evidence supporting antiviral and other beneficial actions. More broadly, future research should also examine the approaches for novel drug discovery targeting heparan sulfate-mediated virus adhesion.

Authors' Contributions

NLR. Indika conceived the hypothesis, created the graphical illustrations, reviewed the literature and drafted the manuscript. NG. Fonseka participated in developing concept outline, interpretation of literature and revising the manuscript critically for important intellectual content. All the authors have read and approved the final manuscript.

Conflict of interest

The authors declare that they have no competing interests in writing this article.

ACKNOWLEDGEMENT

The authors would like to thank Dr. Udara D. Senarathne who moderated this paper and in that line improved the manuscript significantly.

Funding

This work did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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