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Evaluation of mechanisms of action of re-purposed drugs for treatment of COVID-19

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ABSTRACT

Coronavirus disease 2019 (COVID-19) is a global health emergency caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). The rapid worldwide spread of SARS-CoV-2 infection has necessitated a global effort to identify effective therapeutic strategies in the absence of vaccine. Among the re-purposed drugs being tested currently, hydroxychloroquine (HCQ), without or with zinc ion (Zn++), and the antibiotic azithromycin (AZM), has been administered to prevent or treat patients with COVID-19. The outcome of multiple clinical studies on HCQ has been mixed. Zn++ interferes with viral replication by inhibiting replicative enzymes and its entry into cells may be facilitated by HCQ. Another immunomodulatory drug, methotrexate (MTX), is well known for its ability to mitigate overactive immune system by upregulating the anti-inflammatory protein, A20. However, its beneficial effect in treating COVID-19 has not drawn much attention. This review provides an overview of the virology of SARS-CoV-2 and an analysis of the mechanisms by which these anti-inflammatory agents may act in the treatment of COVID-19 patients. We propose a rationale for the combinatorial use of these re-purposed drugs that may help to combat this ongoing pandemic health emergency.

1. Introduction

In the past two decades, the world population witnessed the outbreak of infection by coronaviruses in 2002–2003 by Severe Acute Respiratory Syndrome (SARS) in China and in 2011 by Middle East Respiratory Syndrome (MERS) in Saudi Arabia [1]. After the first epidemic of SARS in China was over, a second interspecies-jumping event occurred in China, resulting in the re-emergence of SARS during the year 2003–2004 [2]. This observation prompted scientists to predict that a major severe respiratory syndrome pandemic caused by a modified coronavirus would infect humans, potentially at any time. This became a reality by the end of 2019 with the emergence of Coronavirus Disease 2019 (COVID-19; SARS-CoV-2) [1]. Each of these outbreaks were caused by viruses that belong to the family Coronaviridae within the order Nidovirales [1]. Thus far, 36 coronaviruses have been identified and are known to cause respiratory or intestinal infections of varying intensities in humans and other animals [2]. In 2003, 8,098 individuals were infected by SARS across 26 nations with a mortality rate of 9% [3]. The present novel coronavirus, SARS-CoV-2, has infected 29.5 million individuals to date (September 15, 2020) across 219 nations with an estimated mortality rate of 4%.

Coronaviruses are a large family of enveloped, positive-sense, single-stranded RNA viruses that infect target cells via angiotensin converting enzyme-2 (ACE-2) receptors (Fig. 1), a host transmembrane glycoprotein [4]. ACE-2 receptors are abundantly present in vascular endothelial cells, heart, lung, kidney and intestine. The viral genome encodes...
several structural proteins and non-structural proteins that are likely targets for the development of future drugs or vaccines and are actively being analyzed. The structural proteins include spike protein (S), nucleocapsid protein, membrane protein, and the envelope protein [4]. The S protein consists of an amino terminal S1 and carboxyl terminal S2 subunits connected by a fusion peptide. The interaction between the S protein and ACE-2 receptor is the primary determinant that enables the coronavirus to infect a host cell [5]. The receptor binding S1 subunit and the membrane-fusing carboxy terminal S2 subunit are critical regions of S protein for binding to the ACE-2 receptor [6]. Upon binding to the ACE-2 receptor, the S protein needs to be “primed” for fusion with host cell membrane to facilitate access to the host cell cytosol. This is generally accomplished by acid-dependent proteolytic cleavage at two specific sites of S protein by a cellular proteases, transmembrane protease serine 2 (TMPRSS2), TMPRSS4, and cysteine proteases cathepsin B and L [5-9]. After proteolytic cleavage, the S protein facilitates viral envelope fusion with the host cell membrane through the endosomal pathway [10,11]. The coronavirus uses the vesicular trafficking system of the secretory pathway of the host cell to release newly synthesized viral particles by exocytosis [11]. In the cytoplasm, positive-sense, single-stranded RNA is translated with the help of host machinery to synthesize polyproteins pp1a and pp1ab [12] (Fig. 1). These protein complexes undergo proteolytic cleavage to produce the viral RNA-dependent RNA polymerase, helicase, and other nonstructural proteins [12]. RNA-dependent RNA polymerase replicates positive-sense, single-stranded RNA into negative-sense, single-stranded RNA. Using negative-sense, single-stranded RNA as template, positive-sense single-stranded RNA is synthesized as well as several mRNA to synthesize viral proteins. Protein assembly encapsulates positive-sense single stranded RNA to become viable coronaviruses [12].

The symptoms of SARS-CoV-2 infection appear after an incubation period of approximately 5.2 days [13,14]. The period from the onset of symptoms to death ranges from 6 to 41 days [14]. This time period is dependent on the age of the patient and status of the patient’s immune system. Generally, patients with a good immune system develop immunity against COVID-19 and survive the infection within two to three weeks. The primary clinical symptoms are characterized by a fever, cough, severe headache, loss of taste/smell, rash on skin/discoloration of fingers or toes. About 20 – 30% SARS-CoV-2-infected patients develop respiratory failure. A high mortality rate is strongly associated with the elderly, and particularly those individuals with co-morbidities including asthma, diabetes, obesity, heart disease, immune compromise, etc [15].

2. The role of the cytokine storm induced by SARS-CoV-2 in disease

The main pathogenic manifestations of COVID-19 infection as a respiratory system-targeting virus is severe pneumonia and acute cardiac injury. Several studies have analyzed cytokine profiles from COVID-19 patients and found that the host immune response to the SARS-CoV-2 virus is hyper inflammatory, resulting in the release of a large amount of pro-inflammatory cytokines in an event known as the “cytokine storm”
A cytokine storm refers to an over exuberant inflammatory response leading to the release of massive amounts of cytokines concurrently. The concomitant response to individual cytokines through cytokine-specific receptors, and the autocrine/paracrine action of newly induced mediators, leads to a degree of inflammation that is extremely difficult to counteract therapeutically. While the cytokine storm was first used in the context of sepsis, it has since been used to describe the overproduction of cytokines in response to many infectious diseases. The mortality rate of SARS-CoV-2 infection has been directly linked to the onset of massive inflammation due to the cytokine storm [20]. The cytokine storm was previously associated with avian H5N1 influenza virus infection in 2005 [21]. Similarly, the biological and clinical consequences of cytokine storms by immune system hyperactivity could be a primary reason for lung damage in patients infected with SARS-CoV-2. Individuals infected with SARS-CoV-2 showed increased numbers of immune cells and increased levels pro-inflammatory cytokines in the blood [20]. The cytokine storm causes an overwhelming inflammatory response that drives serious respiratory complications [22]. In the worst case, the cytokine storm initiates viral sepsis and inflammation-induced lung injury with complications such as pneumonitis, acute respiratory distress syndrome (ARDS), shock, organ failure, and potentially death [22]. At the time the individual develops ARDS, ventilators represent the only support system during the patient’s battle. The severe symptoms have been associated with increased fatalities.

ARDS is the primary cause for mortality due to COVID-19 [22]. The increased coronavirus RNA load in lungs and blood is sensed by host antigen-presenting cells through multiple pattern recognition receptors including RIG-I-like Receptors (RLRs) and Toll-like receptors (TLRs) [23] (Fig. 2). TLR7 and TLR8 are SARS-CoV-2 single-stranded RNA-specific TLRs involved in inducing pro-inflammatory cytokines. Sustained activation of TLR7 and TLR8, along with other pattern recognition receptors (PRRs), leads to induction of the cytokine storm (Fig. 2). Cellular damage may lead to the release of host-derived “danger-associated molecular patterns” (DAMPs) that have been shown to trigger both TLR4 and Receptor for Advanced Glycation End products (RAGE) to elicit a potent proinflammatory response to viral infection [24-26]. The pathogenesis of ARDS is associated primarily with injury to the alveolo–capillary membrane that enhances lung permeability and the exudation of pulmonary edema fluid into the airspaces leading “lung leak” and to hypoxia [27].

Huang et al. recently reported that patients infected with SARS-CoV-2 showed high levels of pro-inflammatory cytokines and chemokines [18]. They demonstrated increased levels of the proinflammatory cytokines IL-1β and IFN-γ, as well as the chemokines CXCL10 and CCL2, and they pointed out that the cytokine storm emerged as a main factor driving a more severe clinical course. Further, they observed that COVID-19 patients requiring ICU admission displayed higher concentrations of G-CSF, CXCL10, CCL2, and TNF-α compared to those in which...
the infection was less severe and did not require an ICU admission.

The uncontrolled release of large amounts of pro-inflammatory cytokines and chemokines by immune effector cells during SARS-CoV-2 infection has been reported, with particular association with IL-6 and IL-1β [15,18,28,29]. Specifically, clinical studies showed that the serum levels of IL-6 are increased in COVID-19 patients and these high circulating serum levels are correlated to disease severity and suggested as predictors for disease severity [30-33]. The hyperimmune systemic-mediated cytokine storm attacks the body and causes ARDS, resulting in multiple organ failure and death in the most severe cases of SARS-CoV-2 infection [22]. For example, TNF-α released by immune effector cells contribute to maintenance of a pro-inflammatory signature, inducing necroptosis in lung epithelial and alveolar cells (leading to further release of DAMPs), and finally, death in severe cases of SARS-CoV-2 infection (Fig. 4).

Additionally, critically ill patients with COVID-19 are prone to thromboembolism/pulmonary embolism/pulmonary intravascular coagulopathy that correlates with disease severity [34-36]. Recent scientific reports on COVID-19 patients suggest that intravascular coagulopathy/fibrin deposition is mainly due to excessive inflammation, hypoxia, and immobilization of patients [36,37]. Clinical reports from the Netherlands and France showed 31% and 20.6% incidence of thrombotic complications in ICU patients with COVID-19 infections, respectively [36,38]. Another study from Wuhan, China reported that patients with n-dimer levels ≥2.0 μg/ml had a higher incidence of mortality compared to the patients with n-dimer levels <2.0 μg/ml [39].

Like SARS-CoV and MERS-CoV, SARS-CoV-2 may use multiple strategies to evade immune responses. One evasion mechanism may be the avoidance of the host immune system by the production of double-membrane vesicles that lack PRRs in which the virions replicate [40]. Studies in mice revealed that Type I IFN (IFN-α and IFN-β) has a protective effect against SARS-CoV and MERS-CoV infection, and more recently, in cases of SARS-CoV-2 [41]. Type I IFNs are key components of the immediate antiviral response and restrict viral replication through IFNAR signaling [42,43]. Unlike patients infected with pathogenic influenza viruses, minimal amounts of IFNs have been detected in the peripheral blood or lungs of patients with severe COVID-19, although SARS-CoV-2 is capable of engaging the IFN-I and IFN-III systems [43,44]. This clearly suggests that host response to SARS-CoV-2 fails to launch type I IFN and –III even though there is robust viral replication. Blanco-Melo et al. analyzed serum samples from COVID-19 patients from two cohorts of individuals and failed to type I IFN. On the other hand, sera from the same COVID-19 patients revealed the significant increase in circulating IL-6, IL1RA, CCL2, CCL8, CXCL2, CXCL8, CXCL9, and CXCL16 levels [43]. Both SARS-1 and MERS inhibit Type I IFN production by preventing nuclear transport of the transcription factor, IFN regulatory factor 3 (IRF3), and thereby inhibit activation of the IFN-β promoter by IRF3 [45], although this has not been established for SARS-CoV-2. A recent report on COVID-19 patients confirmed that patients with undetectable IFN-α levels required invasive ventilation and longer stay in intensive care unit. Further, the viral load inversely correlated with the level of Type I IFN: as expected, the viral load was higher in IFN-negative/low patients with COVID-19 at disease diagnosis [46]. This indirectly suggests that SARS-CoV-2 may also have inhibitory effects on Type I IFN production (Fig. 2). Conversely, several studies have suggested the efficacy of treating COVID-19 patients with type I IFNs [43,47,48]. The fundamental question is whether or not we will be prepared for such global health emergency in the future. In the absence of vaccination and proven antiviral drugs, the clinical management largely depends on supportive care. Clinical management with SARS in the year 2002 led to the identification of certain anti-malarial and anti-viral drugs that are effective against SARS infection and these have been tried in the management of COVID-19. Below, we present data on several potential therapeutics that have been or currently are being used in the treatment of various viral diseases in different parts of the world. The available literature and a basic understanding of their mechanism of action of each drug suggests that a combination of drugs are likely to be potential candidates for the treatment of current COVID-19, and perhaps, in a future outbreak by a novel coronavirus. Using the key words coronavirus, chloroquine (CQ), hydroxychloroquine (HCQ), azithromycin (AZM), Zn2+, and methotrexate (MTX), we retrieved published, as well as preprinted, articles and their cross-references from the literature database.

3. Drug candidates against SARS-CoV-2 and other viral diseases

Although antibiotics are not effective against viral infections such as COVID-19, they can be used to combat secondary bacterial infections. Several biomedical research laboratories throughout the world are testing a variety of possible treatments. The United States Food and Drug Administration granted permission to use HCQ, an anti-malarial drug, and remdesivir, an anti-viral drug developed for Ebola, to treat severe COVID-19 [49,50]. Along with HCQ, AZM, Zn2+ supplements, and MTX have been proposed as possible treatments to combat severe COVID-19. A list of currently available drugs and their possible mechanisms of action that could be used to treat COVID-19 patients is provided in Table 1.

3.1. HCQ and AZM

It is not unusual that a drug approved for a particular disease ultimately shows beneficial effects in other diseases as well. The best example is the use of anti-malarial drug HCQ in the treatment of rheumatoid arthritis, systemic lupus erythematosus, and currently, as a broad-spectrum anti-viral drug [51]. It has been well established that the HCQ increases the pH of cellular vesicles that are involved in the secretory pathways [51]. Increasing the pH in these acidic vesicles alters the biochemical events such as protein degradation by hydrolases in the lysosome, assembly of macromolecules in the lumen of the endoplasmic reticulum and endosomes, post-translational modification of proteins in the Golgi apparatus, and activation of zymogen formed in the secretory vesicles [51]. Since HCQ alters the pH of various vesicles in a cell, it is theoretically effective against any virus that uses the vesicular pathway for multiplication (Fig. 3). HCQ is well-known to modify cellular autophagy with the pH-dependent steps of endosome-mediated viral entry and late stages of replication of enveloped viruses such as retroviruses, flaviviruses, and coronaviruses [52,53]. CQ or HCQ have been demonstrated to have an anti-HIV-1 effect when used in combination with anti-retroviral drugs in vitro or in patients [54,55]. However, HCQ alone failed to reduce viral replication in HIV-infected patients [55]. Further, scientific reports have validated the anti-viral activity of HCQ that interferes with Japanese encephalitis virus internalization, yellow fever virus replication, and dengue virus maturation [56-58]. Again, HCQ failed to reduce viremia in patients with dengue disease [59]. In addition, CQ modulates biosynthesis of sialic acid by inhibiting quinone reductase-2, a key enzyme involved in the biosynthesis of sialic acid that is essential for receptor recognition and also by binding to the sialic acid-linked transmembrane proteins [60]. Moreover, CQ binds sialic acids with high affinity that may inhibit binding of SARS-CoV-2 [61]. Similarly, HCQ may inhibit the entry of SARS-CoV-2 by modulating biosynthesis of sialic acid and by interacting with sialic acid. Since HCQ has been shown to interfere at the entry level, transportation between vesicles, and at the post-entry level, it has been proposed to be beneficial as prophylactic as well as therapeutic treatment (Fig. 3).

In the context of SARS-CoV-2, the cytokine storm is highly detrimental and it may be initiated via single stranded RNA-sensing endosomal TLRs (e.g., TLR7 and TLR8) and RLRs (e.g., RIG-I), in monocye-derived macrophages and dendritic cells [62,63]. Activation of endosomal TLRs occurs within acidified endolysosomal compartments and leads to production of pro-inflammatory cytokines [64]. HCQ has been reported to abrogate endosomal TLRs (TLR3, TLR7, TLR8, and TLR9).
Currently available possible drug candidates to treat COVID-19 patients.

| Drugs                          | Target          | Pre-clinical/clinical trial | Results                                                                 |
|-------------------------------|-----------------|-----------------------------|-------------------------------------------------------------------------|
| Chloroquine (CQ)              | Viral replication, TLR-7 and TLR-8 signaling | Clinical trial was conducted for 100 COVID-19 patients in China | Increased viral clearance compared to control. Effective in inhibiting the exacerbation of pneumonia compared to control [70]. Lethality. High dose: 39%; Low dose: 15% [110]. |
| Hydroxychloroquine (HCQ)      | Viral replication, TLR-7 and TLR-8 signaling | Randomized clinical trial: 400 mg/kg for 5 days | Effective against pneumonia. HCQ: 80.6%, Control: 54.8% [72]. Significant reduction of viral load. HCQ: 57.1%, HCQ + AZM: 100% [111]. No significant difference in mortality rate compared to control [112]. |
| Lopinavir and ritonavir        | 3-chymotrypsin-like-protease | Randomized clinical trial: Lopinavir: 400 mg and ritonavir: 100 mg twice a day for 14 days | No significant difference in viral clearance, clinical improvement and mortality rate compared to control [113]. |
| Famotidine                    | Papain-like protease | Treatment options for non-hospitalized patients: 80 mg three times daily for a median of 11 days. Retrospective cohort study: 10 mg, 20 mg and 40 mg for a median of 5.8 days. | Famotidine is well tolerated and associated with improved self-reported outcomes in non-hospitalized patients with COVID-19 [114]. Significantly reduced risk for the composite outcome of death or intubation [115]. |
| Umifenovir                    | Viral membrane lipids | Chinese clinical trial: 0.4 g/day for a median of 9 days Retrospective study: 0.2 g three times a day | Improved the discharging rate and decreased the mortality rate [116]. No significant improvement of viral clearance [117]. |
| Ivermectin                    | Cell-transport protein | Preclinical study (in vitro): IC50 2.8μM | Reduction of virus (~5000 folds) in cell culture [118]. Reduction of viral load after ivermectin treatment in 1-5 days [119]. |
| Corticosteroid                | Cytokines       | CoDEx randomized clinical trial: Deamethasone 20 mg/day for 5 days, then 10 mg/d for 5 days + standard care | Significant increase in the number of ventilator-free days compared to control [120]. Reduces disease progression, while having a negligible impact on the viral clearance [121]. |
| Remdesivir                    | Viral RNA-dependent RNA polymerase | Randomized clinical trial: 200 mg loading dose on day 1, followed by 100 mg daily for up to 9 additional days | Median recovery time of 10 days as compared with 15 days placebo received group [122]. |

Table 1 (continued)

Responses by preventing acidification [64]. Thus, in addition to its anti-viral actions, HCQ may also act as an anti-inflammatory molecule responsible for reduced pro-inflammatory cytokines by inactivating RNA-sensing TLRs in the endosomes [65] (Fig. 2). Furthermore, HCQ has been shown to prevent the release of the host protein and TLR4 agonist, High Mobility Group Box 1 (HMG1), upon influenza infection [66]. Interestingly, both TLR4 and HMG1 antagonists protect therapeutically against influenza-induced death in mice [67-69].

There are multiple reports supporting or refuting the use of HCQ in conjunction with AZM in COVID-19 patients. Early on, a Chinese group showed that chloroquine phosphate is effective in treating COVID-19-associated pneumonia in patients [70]. In this clinical study with more than 100 patients, the authors demonstrated that chloroquine phosphate treatment inhibited the exacerbation of pneumonia, improving lung pathology and shortening the disease course. In support of this, a study from France reported the efficacy of HCQ in clearing viral nasopharyngeal carriage of SARS-CoV-2 in COVID-19 patients within three to six days of infusion. They found a significant difference between HCQ-treated patients and controls [71]. According to this study, HCQ treatment cured viremia in 70% of patients compared to 12.5% in the control group [71]. Recently, another report in support of HCQ treatment of COVID-19 patients for its anti-viral activity concluded that HCQ treatment significantly reduced the recovery time for body temperature and cough remission [72]. Interestingly, the comparative analysis of the chest CT of patients showed significant improvement in patients treated with HCQ [72]. Very recently, Catteau et al. from Belgium have shown the beneficial action of HCQ alone and HCQ with AZM in a large clinical trial. The authors have reported that fatality rate was lower in the HCQ group than in the group without HCQ. The significant decrease in mortality rate was observed in the patients group administered with HCQ monotherapy at a dosage of 2400 mg over 5 days compared with patients treated without HCQ [73].

The synergistic effect of HCQ and AZM has also been reported. AZM is known to prevent severe respiratory tract infections when administered to patients suffering viral infection, although the mechanism is
not well understood [74]. Very recently, the combinatorial effect of HCQ and AZM with good clinical outcome and decreased viral burden in a large population (1,061) of patients (91.7%) was reported [75]. While it has been surmised that the AZM acts by preventing the enhanced secondary bacterial infection after virus infection, it is possible that it also acts by inducing anti-inflammatory “alternatively activated” (M2) macrophages. Previously, it was shown that M2 macrophages were necessary for resolving the lung pathology associated with respiratory syncytial virus (RSV) infection [69,76]. Administration of M2-inducing agents therapeutically, including AZM, resulted in resolution of RSV-induced pathology. This clearly suggests that AZM may not only prevent secondary bacterial infection, but also, blunts viral-induced pathology by creating anti-inflammatory environment.

In contrast to studies supporting the use of HCQ, other clinical studies failed to show significant difference between HCQ-treated and control responses. A study by Mallat et al. observed that the duration of hospital stay was longer in HCQ-treated with COVID-19 patients [77]. Another recent report with a larger patient population suggested that HCQ treatment did not provide beneficial support to use in patients with COVID-19 who require oxygen [78]. They found that additive HCQ treatment to standard care did not reduce patient admissions to the intensive care unit. Also, the rate of survival without ARDS did not increase in HCQ-treated patients compared to standard care alone. Very recently, Boulware and colleagues tested the efficacy of HCQ as COVID-19 post-exposure prophylaxis in a randomized clinical trial with asymptomatic individuals [79]. They found that HCQ failed to prevent illness with COVID-19 when used as post-exposure prophylaxis within 4 days after a high-risk or moderate-risk exposure [79]. In another clinical study with 807 veterans from the United States, HCQ treatment with or without co-administration of AZM did not improve mortality or reduce the need for mechanical ventilation [80]. In this report, they analyzed multiple parameters that are critical to assess the severity of COVID-19 patients including SpO2, respiratory rate, heart rate, temperature, blood pressure, liver enzymes, d-dimer, CRP, troponin I so on. Although some parameters were significantly different between HCQ alone or HCQ + AZM compared to the control group, no improvement in mortality compared to control group was observed [80]. Thus, while HCQ alone, or HCQ with AZM treatment of COVID-19 patients has been used in these desperate times, the efficacy of such treatment has not yet been confirmed in a placebo-controlled randomized clinical trial. Hopefully, such data will be forthcoming soon and will reveal if the mixed reports of efficacy are due to dosing, timing, or other environmental parameters not carefully studied. The paradoxical effect of HCQ monotherapy or combined with AZM could be due to the co-morbidities, such as cardiovascular complications of the COVID-19 patients. HCQ along with AZM might be beneficial to combat COVID-19 in patients without cardiovascular complications. For patients, especially in the elderly with a history of cardiovascular disease, HCQ and AZM may be detrimental that prolong QT interval [81]. No instances of arrhythmogenic death were reported [82]. Additionally, HCQ treatment induces rash and headache in some patients. Also, the timing, severity of illness, and dose of HCQ will be very important to consider before treating COVID-19 patients. Finally, it is very unfortunate that the use of HCQ has become highly politicized. However, careful retrospective analysis of all studies reported may reveal specific populations or specific times during which this drug is most efficacious (https://www.tabletmag.com/sections/science/articles/hydroxychloroquine-morality-tale).

Fig. 3. Possible sites of inhibition of viral replication by combination of HCQ, Zn++ and MTX. The combination of HCQ, Zn++ and MTX interferes with viral replication at several stages. HCQ interferes directly by inhibiting fusion of virus to host membrane receptors and indirectly acts as ionophore that transports Zn++ that inhibits viral RNA polymerase. HCQ directly interacts with sialic acid and interferes in the attachment of virus to membrane glycoproteins. MTX interferes at viral replication stage by depleting the nucleotide pool.
3.2. Zinc

Zinc deficiency is common in young children and elderly persons in the developing world and these individuals have suppressed immune responses and are more susceptible to a diverse range of infectious diseases [83,84]. The common cold caused by rhinovirus is one of the most widespread illnesses that affects people of all ages and is more severe in zinc-deficient populations [85]. Administration of zinc for the treatment of the common cold is very well-established. Clinical studies have found that zinc reduces the duration of colds and decreases the upper respiratory infection in children [86]. In addition, zinc is an immunomodulator and helps the host fight infection and heal wounds [83]. Several studies revealed that the therapeutic effect of zinc is due to its interference with viral replication [87-89]. In cell culture studies, increased levels of intracellular zinc, along with the ionophores that facilitate zinc cellular import, were found to inhibit RNA replication of different RNA viruses, including influenza virus, RSV, polio virus, arteri virus, coronavirus, and several picornaviruses [87]. For some viruses, this effect has been attributed to the inhibition of certain proteolytic cleavages in the processing of the viral polyproteins. With arteri virus and coronaviruses, zinc interferes in viral replication by inhibition the RNA-dependent RNA polymerase enzyme [87]. Intracellular mobilization of zinc is crucial for its anti-viral activity and is facilitated by compounds like hinokitiol, pyrrolidine dithiocarbamate, and pyrithione that stimulate cellular import of zinc. Apart from affecting vesicular pH, HCQ is an excellent ionophore of zinc and increases the intracellular concentration of zinc within short time [90]. The combination of HCQ with zinc is more effective in regulating the viral multiplication by interfering at various steps of its life cycle (Fig. 3).

3.3. Methotrexate (MTX)

MTX, a disease modifying anti-rheumatic drug (DMARD), is used clinically for rheumatoid or psoriatic arthritis and systemic lupus erythematosus with good therapeutic results [91-93]. It is a folic acid analogue that is either used as a monotherapy or in combination with other synthetic DMARDs. MTX is polyglutamylated inside the cell and MTX-polyglutamates (MTX-Pgl) constitute the active form of the drug. MTX-Pgl acts by inhibiting various enzymes such as dihydrofolate reductase (DHFR), thymidylate synthase (TS), and aminoimidazole carboxamide ribonucleotide formyl transferase enzyme (AICART) [94] that are involved in the synthesis of precursor nucleotides required for RNA and DNA synthesis (Fig. 3). AICART inhibition reduces inosine monophosphate and increases aminoimidazole carboxamide ribonucleotide (AICAR) which has anti-inflammatory effect. Impaired nucleic acid synthesis suppresses rapidly dividing cells such as immune cells, thereby disrupting the S phase of the cell cycle. Thus, in rheumatological diseases, MTX is an immunosuppressive drug that reduces inflammation and joint destruction [95]. Apart from inhibiting immune cell division, MTX also inhibits the synthesis of purine and pyrimidine pools in host cells. This decreased nucleotide pool impairs viral replication (Fig. 3).

In addition to interfering with the synthesis of precursor nucleotides, MTX has been shown to exert an immunomodulatory role [91]. Recently, Municio et al. [92] showed that long-term low-dose MTX treatment increased the expression of A20 in macrophages stimulated with TLR ligands or TNF-α (Figs. 2 & 4). A20 is a TNF-responsive cytoplasmic protein that inhibits TNF-induced NF-κB activity [96]. It has been reported that A20 inhibits ubiquitin-dependent NF-κB signaling by exhibiting deubiquitinating capacity, ubiquitin binding, and E3 ligase activities [96]. Moreover, MTX reduced LPS/TNF-α-dependent pro-inflammatory cytokine production, MAPK activation, iκBα degrada
tion, and NF-κB activity via A20 [92]. Therefore, the involvement of A20 in MTX response validates the anti-inflammatory role of MTX, widely used drug in inflammatory diseases with an excellent safety record. Moreover, TNFAIP3 gene single nucleotide polymorphisms (SNPs) are strongly associated with psoriasis, a well-studied inflammatory disease [97]. Interestingly, the reduction in A20 expression caused notably increased levels of IL-1β, IL-6, TNF-α, and IL-12 p40 in splenocytes, supporting the anti-inflammatory role of A20 in inflammatory diseases [97]. Overall, the immunomodulatory action of MTX might be exploited to treat COVID-19 patients since the cytokine storm is thought to be a major cause of morbidity and mortality (Fig. 4). Apart from these, MTX administration can provoke the release of an anti-inflammatory molecule, adenosine, that exerts its effects on immune cells [98]. Upon release, adenosine activates intracellular signaling cascade through specific receptors that results in the reduced production and release of pro-inflammatory cytokines [99]. It is well known that MTX modulates of reactive oxygen species (ROS) and reduces production of IL-6 which is associated with COVID-19 patient severity. In addition to reduced IL-6, MTX potently suppresses the JAK/STAT signaling pathway which is activated by multiple pro-inflammatory cytokines [100]. Further, High Mobility Group Box 1 (HMGB1), a host-derived “danger-associated molecular pattern,” has been associated with respiratory virus-mediated lung pathology by inducing inflammation through its receptors, receptor for advanced glycation end products (RAGE) and TLR4. Interestingly, MTX can inhibit the action of HMGB1 by directly binding to RAGE [99]. The range of MTX side effects is characterized by nausea, vomiting, diarrhea, myelosuppression, pancytopenia, liver dysfunction, acute renal failure, pulmonary symptoms, mucositis, stomatitis, ulceration of the gastrointestinal system and cutaneous ulcerations [101,102].

3.4. Dexamethasone (DEX)

DEX is a synthetic glucocorticoid with 20 to 30 times the binding affinity for glucocorticoid receptors of endogenous cortisol. DEX is being used in a wide range of conditions for its anti-inflammatory and immunosuppressant effect [103]. Corticosteroids are widely used to treat autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, psoriasis and eczema. They are being used as immunosuppressant following organ transplant and also in treating certain cancers [104]. Very recently, clinical trial results from the United Kingdom claimed that a low dose of dexamethasone, a corticosteroid, reduces mortality in patients with COVID-19 on ventilators and requiring oxygen [105,106]. In support of this, an earlier study by a team of physicians from China recommended short courses of corticosteroids at low to moderate doses for critically ill patients with COVID-19-induced pneumonia [107]. In contrast, a clinical study by Russell and colleagues provided the evidence that corticosteroid treatment did not support treatment for 2019-nCOV-induced lung injury [108]. With all these, dexamethasone is well known for its side effects [109]. Thus, we propose that low dose of MTX, alone or in combination with dexamethasone, could be an effective immunotherapy to protect health care workers and to treat COVID-19 patients.

4. Concluding remarks

SARS-CoV-2 has affected the human population worldwide by causing disease associated with ARDS. Although some clinical studies have supported the use of HCQ to treat patients with COVID-19, others have failed to support its effectiveness in treating SARS-CoV-2. HCQ might not be effective in critically ill COVID-19 patients, but it may help patients recover from SARS-CoV-2 infection if administered during very early stages along with AZM and Zn2+. MTX is known to suppress TLR/ TNF-α-induced NF-κB activation, and could be beneficial in combating lung pathology caused by SARS-CoV-2. In conclusion, we suggest the combinatorial use of drug candidates, such as HCQ with AZM/Zn2+ or MTX will require additional randomized, placebo-controlled clinical trials to unambiguously determine their efficacy as therapeutic approaches to combat COVID-19.
Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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