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EDITORIAL

Considerations of the effects of commonly investigated drugs for COVID-19 in the cholesterol synthesis pathway

Juan Luis Gomez Marti[a] and Adam M. Brufsky[b]

[a]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA; [b]UPMC Hillman Cancer Center Magee Women's Hospital Suite 4628,Pittsburgh, PA USA

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1. Overview

The management of coronavirus disease 2019 (COVID-19) consists in providing supportive care. Although SARS-CoV-2 and its effect on the host have been extensively characterized, access to effective therapies is scarce. Management of COVID-19 has widely relied on the use of various approved Food and Drug Administration (FDA) drugs since the start of the pandemic [1]. Because the array of established treatments with acceptable efficacy and safety profiles is limited, here we present a rationale for the use of diverse, easily accessible agents that could effectively treat SARS-CoV-2 if used early. These treatments have shown promise in observational studies. Due to increasing evidence linking cholesterol signaling with the pathogenesis of SARS-CoV-2, we consider the effects of SARS-CoV-2 on cholesterol synthesis genes as part of the evaluation of the biological and clinical effects of these drugs.

1.1. Targeting the cell membrane interaction with SARS-CoV-2

Cell membrane receptors that mediate SARS-CoV-2 uptake are common drug targets. Chronic hyperglycemia has been associated with upregulation of ACE2 receptors, leading to hypersensitized viral recognition and severe disease symptomatology [2].

Chloroquine (CQ) analogs such as hydroxychloroquine (HCQ) block the interaction of the S receptor binding domain (RBD) to angiotensin converting enzyme 2 (ACE2) through acidification of the extracellular matrix. This prevents cleavage of the N-terminal S1 by ACE2. The N-terminal of S can also bind to sialic receptors in the mucosa and enter into the cell. By inhibiting sialic acids, CQ/HCQ could prevent SARS-CoV-2 from being internalized [3]. CQ/HCQ analogs also decrease the glycosylation of ACE2 and could help prevent new-onset type 2 diabetes mellitus after COVID-19 disease [4–6].

Basigin (CD147) and Cyclophilin A (CyPA) are membrane receptors used by SARS-CoV. Basigin is a transmembrane glycosylated immunoglobulin and principal receptor of extracellular cyclophilins. Cyclophilins are a family of ubiquitously distributed chemotactic agents that mediate intracellular trafficking and signal conduction. These are released extracellularly during an inflammatory response and commonly exhibited by dying cells. CyPA is the most abundant cyclophilin isoform. The chemotactic effects of CyPA take place primarily after binding to the extracellular domain of basigin. Downstream signaling catalyzes matrix metalloprotease (MMP) release, facilitating leukocyte recruitment [7]. Basigin acts as a receptor for several viruses including measles, HIV-1 and SARS-CoV [8]. Viral binding to basigin induces CyP binding to the N protein of SARS-CoV virions, permitting cellular entry [9]. A preprint of preliminary results in a clinical trial has shown an association between anti-basigin antibodies and decreased hospitalization length and disease severity [10].

Azithromycin (AZT), a macrolide which has been used to treat COVID-19, could interfere with basigin, preventing SARS-CoV-2 uptake and the release of downstream MMPs [11]. Remarkably, on a retrospective observational study, outpatient use of AZT alone or combined with HCQ reduced by half the time to clinical recovery, compared to none or symptomatic treatment (12.9 days, 9.2 and 25.8 days, respectively) [12].

The FDA currently advises against using HCQ/AZT except for critically ill patients or within the context of a clinical trial [13]. The use of HCQ/AZT in the outpatient setting seems more favorable than among inpatients. A small open-label non-randomized trial showed that HCQ use was significantly associated with viral load reduction, which was further enhanced by AZT use [14]. A prospective, outpatient observational non-peer reviewed study found that hospitalization rates dropped from 5.4% to 1.9% upon HCQ use, with hospitalization rates being lower if HCQ was used before day 7 post-symptom onset [15]. A nonsignificant reduction in symptom severity or new onset of illness 14 days was seen in two blinded, placebo-controlled trials that used HCQ within 4 days after exposure [16,17]. Another trial used HCQ within 5-days of symptom duration. This trial found a non-significant 25% reduction in hospitalization rates and a 2-day reduction in time to symptom resolution [18]. A recent case series showed that outpatient low dose use of HCQ combined with AZT and Zn significantly reduced hospitalization rates when compared to untreated controls [19]. A systematic review reported that in studies where HCQ was given early on in the disease course and in the outpatient setting, there was consistent improvement trends in time to symptom resolution and improvement of the hospitalization rates. When HCQ was given on the first

CONTACT Adam M. Brufsky 300 Halket StPittsburgh, PA 15213 USA brufskyam@upmc.edu

48 h of admission, 67% of studies showed a positive outcome in the HCQ group. When it was used after 48 h, only 40% of studies found HCQ to provide clinical benefit [20].

The use of HCQ in the inpatient setting seems less promising, possibly due to the disease stage at which HCQ is given [21]. In a prospective randomized trial, hospitalized patients with mild-moderate disease were assigned HCQ, HCQ+AZT or standard of care (SOC) usually later than 10 days post-symptom onset; no outcome differences were observed [22]. Similar results were observed in two large trials, RECOVERY [23] and ORCHID [24]. These found no differences in improvement of clinical status at days 28 and 14, respectively. In this context, the RECOVERY trial did not specify the post-symptom onset timeline until HCQ was given, whereas in ORCHID, patients were enrolled within 10 days post-symptom onset. In agreement with what has been previously suggested, the degree of benefit could also be linked to when HCQ is introduced [20,21]. In this regard, a recent large retrospective observational study found a 66% hazard ratio reduction of in-hospital mortality among patients treated with HCQ on day 1 after presentation to the emergency room, further increased to 71% with the addition of AZT, compared to neither treatment [25]. Importantly, reductions in IL-6, troponins and mortality among hospitalized patients with heart disease receiving HCQ has also been reported [26]. It would be of interest to evaluate these effects in non-O ABO groups, who are at increased risk of cardiac injury and mortality compared to other groups [27]. Overall, it appears that if any benefit is derived from HCQ/CQ use, it may come from decreased infection rates based on lower viral shedding [28].

Although mild adverse events are common, severe complications associated with HCQ or AZT seem to be minimal. A controlled retrospective multi-database analysis found that treatment with HCQ, AZT or both has an acceptable safety profile and lack of increased mortality [29].

2. Repressing virion internalization

Coronaviruses undergo early clathrin-mediated endosomal uptake. Early viral fusion takes place at the furin-cleavage site in the S protein. Without this cleavage, endosomes require maturation into lysosomes for viral fusion to occur [30]. Chlorpromazine and CQ/HCQ block this phase of viral infection [1]. These display potent replication inhibition in vitro, and reduced disease severity in a mouse model [1]. Clinically, they do not seem to reduce viral titers or even prevent the development of symptoms [17], but could minimize the clinical course if used post-exposure based on a preprint study [31]. Chlorpromazine constitutes another 'old drug' with a broad spectrum and tolerable safety profile, which is also used for non-psychiatric uses such as nausea and vomiting of pregnancy [32]. A trial record has been posted where chlorpromazine will be added to the standard therapeutic protocol (NCT04366739).

Interactome-based analysis has proposed Sigma-1 receptors to be promoters of SARS-CoV-2 internalization and replication in neurons; which could explain olfactory bulb impairment during COVID-19. In this regard, selective serotonin reuptake inhibitors interact with Sigma-1. Since fluvoxamine has the highest affinity for Sigma-1, its early use could help prevent the complications of COVID-19 [33]. For this reason, fluvoxamine is being evaluated for its ability to prevent dyspnea and hospitalization for shortness of breath are being evaluated in a double-blind clinical trial (NCT04342663).

Endosomal uptake can also be blocked by zinc (Zn)-mediated furin inhibition [34]. In a retrospective observational study, use of zinc sulfate decreased mortality and hospice admission in multivariate analysis [35]. Because CQ/HCQ constitutes a Zn ionophore, it is plausible to support that post-exposure use of both drugs could prevent hospitalization. On a preprint retrospective case series, outpatient use of HCQ +AZT+Zn led to an 84% reduction in hospitalization [36].

2.1. Inhibition of RNA-dependent RNA polymerase

Inhibition of the RNA-dependent RNA polymerase (RdRp) has been tried with suboptimal results. Reverse transcriptase inhibitors such as remdesivir have modest efficacy in the treatment of hospitalized patients, it does not clearly result in an increased survival rate [37]. The viral replication machinery of SARS-CoV-2 contains exonuclease-based proofreading, which promotes replication and removal of exogenous nucleotide analogs. Deoxyribose-containing RdRp nucleotide analogs have been shown to be more resistant to the exonuclease proofreading complex than those containing ribose rings such as remdesivir. In this regard, sofosbuvir contains a deoxyribose that exhibits more resistance to this proofreading complex, providing more stability than remdesivir [38]. Trial results evaluating sofosbuvir efficacy against COVID-19 are encouraging. A meta-analysis showed that sofosbuvir in combination with daclatasvir improved time to clinical recovery (HR = 2.04), as well as all-cause mortality compared to control arms (risk ratio = 0.31) [39]. Another trial reported a half-reduction in duration of hospital stay compared to ribavirin (5 vs 9 days, respectively), relative risk of death of 0.17, and a number needed-to-treat for benefit of 3.6 [40].

Zn could also be utilized as an RdRp inhibitor. Co-treatment with an ionophore (i.e. chloroquine, ivermectin) could facilitate Zn's membrane transport [34,41]. Its use in combination with sofosbuvir with or without an ionophore should be examined in future trials.

2.1.1. Blockade of virion translocation to the nuclei

Antiparasitic agents have well-defined antiviral properties. During SARS-CoV-2 infection, Orf6 binds to the nuclear membrane importin IMPα/β1, which then translocates to the nuclei, antagonizing the antiviral activity of the transcription factor STAT1. Ivermectin efficiently blocks this transport through the nuclear pore complex; no cellular toxicity was identified in vitro [42]. In a retrospective cohort study (ICON study), use of 200 μg/kg ivermectin on admission was associated with approximately 50% reduction in an all-cause in-hospital mortality rate among patients with severe COVID-19 disease. No signs of toxicity were associated with ivermectin use [43]. A recent trial that evaluated ivermectin+doxycycline vs placebo+standard care, found clinical improvement on day 7 in 60.7% of patients compared to 44.4% in the control. By day 12,

only 23% of ivermectin-users had not shown clinical improvement, compared to 37.2% in the control group. A trial has been registered to further investigate the utility of this drug (NCT04523831) [44].

2.2. Host response. Role of HMGB1 and association with current therapies

HMGB1 is an endogenous damage-associated molecular pattern (DAMP) and DNA-RNA-binder involved in the host response to COVID-19. It is a potent ligand of TLR4, which can be released extracellularly from dying or activated innate cells. Extracellular HMGB1 can access neighboring cells and activate intracellular inflammatory receptors [45]. The receptor for advanced glycation endproducts (RAGE) and TLR4 constitute the main receptors for HMGB1, the former found primarily in alveolar tissue. Development of acute respiratory distress syndrome (ARDS) directly correlates with plasma HMGB1 levels. In this regard, serum levels of HMGB1 are directly involved with specific patterns of cytokine storm in COVID-19, severity of lung injury, d-dimer levels, organ damage, neutrophil/lymphocyte ratio, prediction of ICU stay, and survival [46]. Although specific HMGB1 antagonists are not yet available, lysosome alkalinization with CQ inhibits HMGB1-mediated lysosomal membrane detergent lysis. CQ also decreases the systemic release of HMGB1 during sepsis [47]. Ivermectin blocks LPS-mediated cytokine release, which takes place primarily through TLR4 blockade. This suggests that ivermectin may also block HMGB1-mediated TLR4 activation. Ivermectin also possibly downregulates TLR4 expression [48,49]. On the other hand, ivermectin administration has been associated to HMGB1 release [50].

3. Expert Opinion

Increasing biological and clinical evidence support the rationale that the aforementioned agents act on different parts of the SARS-CoV-2 infection process. Nonetheless, how these medications modulate the response of the host is less discussed.

The role of the cholesterol biosynthesis has increasingly received attention, as it constitutes a primary regulator of innate immunity. Activation of inflammasomes, as well as IL-1b release has been associated to COVID-19 infection [51,52]. In addition, it is known that these become active when the mevalonate pathway is deficient [53,54]. Recently, a study highlighted the importance of the prenylated GTPase Rab7A in mediating viral entry into the host cell. Increased cholesterol biosynthesis, on the other hand, reduced viral infection [55].

Alterations in this signaling pathway could be directly involved with reduced autophagy and the activation of inflammasome-related signals. Prior studies have provided a rationale for arthritis onset as a consequence of blockade of the MVP [56]. A preprint has shown that use of HCQ, on the other hand, significantly upregulates cholesterol signaling genes [57]. Azithromycin has also been found to upregulate cholesterol synthesis genes and reduce proinflammatory cytokine secretion [58] (Figure 1).

Some of the genes involved in the MVP could undergo downregulation following SARS-CoV-2 infection [59]. If hydroxychloroquine deems to be effective when used prophylactically, enhanced mevalonate signaling could constitute one underlying mechanism explaining its preventive effect.

Autophagy provides major protection against developing ARDS by preventing upregulation of inflammasome markers. In a recent in vivo study, deletion of ATG16L1 lead to

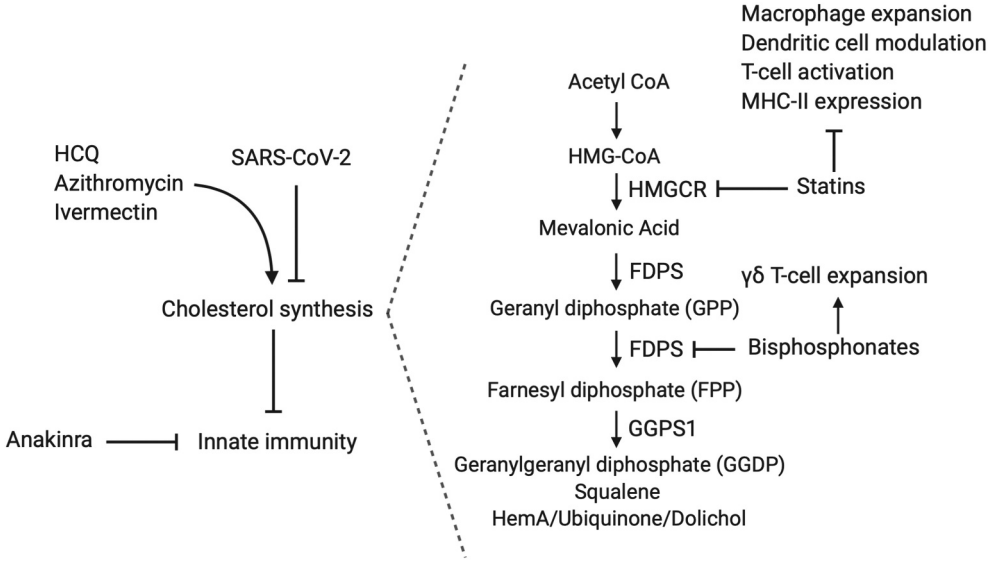

Figure 1. A rationale for the use of drugs that intervene in cholesterol synthesis to control innate response to COVID- 19. (Left) Hydroxychloroquine (HCQ), Azithromycin and ivermectin have been involved in cholesterolsynthesis, which helps preventing activation of proinflammatory cytokines. Anakinra directly involves the blockade of IL-1b and NLRP3 as consequence of downregulation in the mevalonate pathway (MVP). (Right) Statins and bisphosphonates block specific enzymes of the MVP, reducing its downstream activity and modulating immune response. HMGCR: 3-Hydroxy-3-Methylglutaryl-CoA Reductase; FDPS: Farnesyl diphosphate synthase; GGPS1; Geranylgeranyl pyrophosphate synthase. Created with Biorender.com

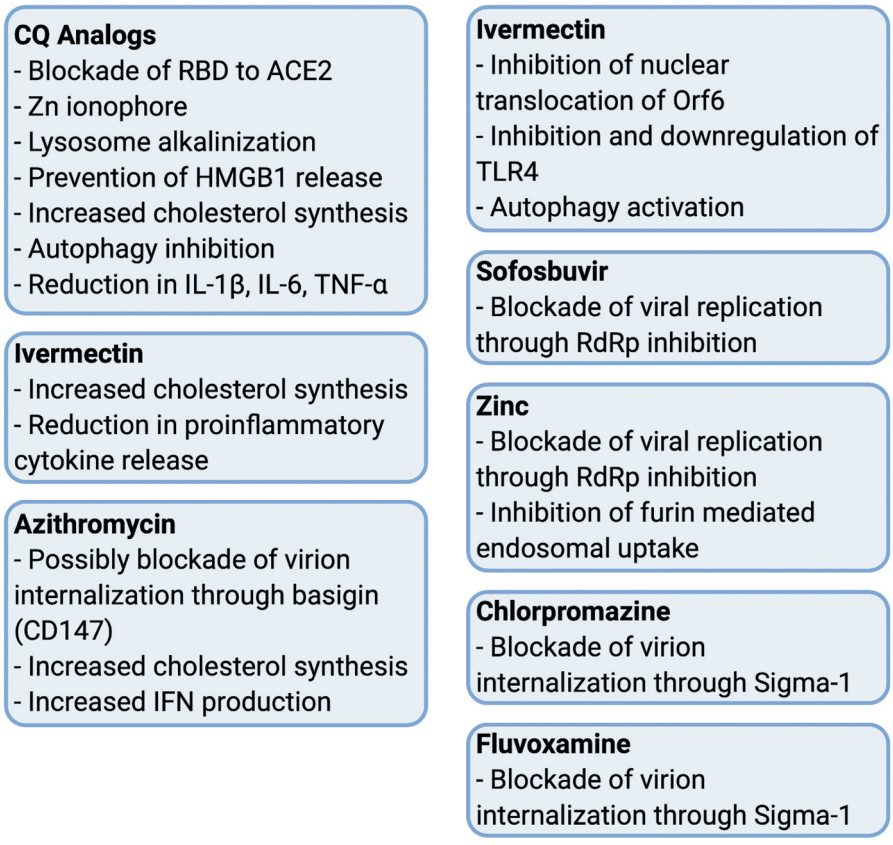

Figure 2. Schematic of the biological evidence of pharmacological agents against COVID-19 mentioned in this manuscript. RBD: Receptor binding domain; ACE2: Angiotensin converting enzyme 2; HMGB1: High mobility group box 1; TLR4: Toll like receptor 4. Created with Biorender.com

increased hypoxemia and severe lung injury with high IL-1b. Induction of autophagy improved recovery and decreased production of IL-1b [60]. Importantly, the IL-1b and NLRP3 inflammasome inhibitor anakinra has already been proposed and found to be safe and effective in reducing mortality among patients with severe COVID-19 [61] (Figure 1). Interestingly, ivermectin is also an autophagy inductor [50].

HCQ is a well-known autophagy inhibitor, but it also has nevertheless shown to reduce IL-1b, IL-6 and TNF-α secretion, among other cytokines [62]. These seemingly contradictory results could be explained on the one hand by direct inhibition of autophagosome-lysosome fusion; and on the other hand, by increasing the expression of genes related to cholesterol synthesis, which would prevent inflammasome activation after SARS-CoV-2 infection. In this regard, preserving these pathways by using HCQ early on in the disease progression may be a clinically promising approach. Mechanisms underlying this double-edge effect deserve further investigation.

Drugs that interfere in the MVP are showing promise. In an observational retrospective study, statins were associated with reduction in-hospital all-cause mortality among patients with COVID-19 (HR = 0.58) [63]. Although statins block the mevalonate pathway, they are also known to modulate dendritic cell phenotypes, decrease antigen endocytosis, macrophage expansion, T-cell activation, MHCII expression, and leukocyte migration, all of which are critical in the development of COVID-19 progression [64]. Similarly, bisphosphonates could also promote γδ-T cell expansion preventing the γδ-T cell depletion landscape of COVID-19 [65] (Figure 1).

In summary, considering the effects that SARS-CoV-2 might have on the metabolic reprogramming of cholesterol synthesis may facilitate the understanding of how some commonly used drugs have not always provided consistent results, and why starting therapy earlier to 'build up' the innate immune system should be considered. A schematic of the drugs discussed in this article is shown in Figure 2.

Declaration of Interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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