Chloroquine and hydroxychloroquine as antimalarials and antivirals against SARS-CoV-2: The hemin factor

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Abstract

Drugs are urgently needed for COVID-19. In that context, the antimalarial drugs chloroquine and hydroxychloroquine are being used based on their antiviral action against SARS-CoV-2 *in vitro* and results from early clinical studies. The antiviral action of these drugs relies on their accumulation into lysosomes and acidic endosomes that increase pH, making more difficult virus entry and replication. In malaria, these drugs accumulate into the *Plasmodium* acidic vacuole, and interact with hemin released from hemoglobin, inhibiting its crystallization to hemozoin (β -hematin). Hemin is toxic for the parasite (antimalarial effect). Recent results have shown that chloroquine inhibits β -hematin formation, and increases soluble free hemin that is involved in oxidative reactions and inhibition of cysteine proteases, underlining the chloroquine effects mediated by hemin. Therefore, antimalarial and antiviral actions of these drugs differ by the hemin factor. However, could hemin play a role in the antiviral action? Infections by SARS-CoV-2 and acute respiratory distress syndrome (ARDS) induce local lung hemorrhages releasing cell-free hemoglobin which is oxidized and releases free hemin. Besides, SARS-CoV-2 invades epithelial cells of the lung, which express non-erythroid hemoglobin. Chloroquine/hydroxychloroquine could hypothetically interact with that hemin/hemoglobin affecting the host and virus. It could result in oxidative reactions and inhibition of cysteine proteases necessary for viral progression. Remarkably, hemin also exerts antiviral actions against a number of viruses, supporting a possible role in the antiviral effects. Hemin is commonly used for the treatment of acute porphyria attacks, and this might be relevant in possible cases of acute porphyria attacks and COVID-19 treated with antimalarial drugs. Finally, it could be worth of testing the antiviral action of chloroquine/hydroxychloroquine and hemin combined, and that of hemin alone against SARS-CoV-2 (COVID-19).¹

Key words: SARS-CoV-2, COVID-19, *Plasmodium*, malaria, chloroquine, hydroxychloroquine, hemin, hemoglobin, cysteine proteases, oxidative reactions, antiviral, antimalarial, acute porphyria

¹ The information included in this preprint is for scientific use only. The drugs mentioned cannot be taken or used without the legal prescription by a doctor.

1. Background and SARS-CoV-2 pathogenesis

In December 2019, the novel coronavirus SARS-CoV-2 was transmitted from person to person leading to a pneumonia disease called coronavirus disease-19 (COVID-19). The WHO has declared COVID-19 a pandemic. SARS-CoV-2 causes symptoms ranging from mild disease to severe lung injury and multi-organ failure, eventually leading to death. Many people have no symptoms or mild symptoms but some develop severe respiratory illness including acute respiratory distress syndrome (ARDS). The new virus is related to previous Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) that emerged in 2002 and the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) in 2012.

The novel SARS-CoV-2 is an enveloped virus containing one positive-strand RNA genome. It encodes several structural proteins, including the glycosylated spike (S) protein that mediates host cell invasion via binding to a receptor located on the surface of cell membrane. The viral genome encodes non-structural proteins including the RNA-dependent RNA polymerase (RdRp), coronavirus main protease (3CLpro), and papain-like protease (PLpro). Upon entrance to the host cells, the viral genome is released and translated into viral polyproteins using host cell protein translation machinery, which are then cleaved into effector proteins by viral proteases 3CLpro and PLpro. RdRp synthesizes a full-length negative-strand RNA template to be used by RdRp to make more viral genomic RNA (Liu *et al.*, 2020). Proteases and RdRp are critical for viral infection and replication and are key targets for drug discovery. SARS-CoV-2 invades human cells through the angiotensin converting enzyme 2 (ACE2) receptor (Zhang *et al.*, 2020; Zhou *et al.*, 2020) that is expressed in the lung, particularly in type II alveolar cells (ATII), but also in extrapulmonary tissues including heart, kidneys, blood vessels, and intestine (Zhang *et al.*, 2020). This wide distribution may explain the multi-organ dysfunction observed in COVID-19 patients. To enter into the cells, SARS-CoV-2 spike (S) protein binds to ACE2 receptors (Walls *et al.*, 2020; Wan *et al.*, 2020; Zhang *et al.*, 2020), and the process requires priming S protein by transmembrane protease serine 2 (TMPRSS2) of the host cells that cleaves S protein (Hoffmann *et al.* 2020). In addition, other co-receptors or auxiliary proteases, and mechanisms by which viruses enter cells could be also involved (Lukassen *et al.* 2020; Hamming *et al.*, 2004). Coronaviruses can enter into the host cell via endosomal and non-endosomal entry in presence of proteases, but SARS-CoV replication primarily depends on the non-endosomal pathway dependent on proteases (Lukassen *et al.*, 2020). Endosomal cysteine proteases of the host (e.g. cathepsins) could also participate in priming of S protein facilitating virus entry (Hoffmann *et al.*, 2020).

The primary sites of gas exchange with the blood in the alveolus are composed of a thin epithelium that covers most of the surface area and contains alveolar type I cells (ATI) and type II cells (ATII). ATII cells produce the pulmonary surfactant, a fluid coating that facilitates the transfer of gases between the blood and alveolar air. ATII cells are capable of cellular division giving rise to ATI and ATII cells when the lung tissue is damaged. Because of the high expression of ACE2 in ATII cells, these cells are more susceptible to infections by SARS-CoV-2 (Lukassen *et al.*, 2020). Within the alveoli, the oxygen travels through a thin wall from air sacs to small blood vessels (capillaries) that deliver oxygen to red blood cells. SARS-CoV-2 damages the wall and the lining cells of

alveoli and capillaries making difficult the transfer of oxygen and producing severe respiratory illness.

Currently, there is no approved treatment for COVID-19. Researchers worldwide are trying to find effective drugs (Guy *et al.* 2020; Liu *et al.*, 2020). Drugs against other virus such as the protease inhibitors, lopinavir and ritonavir (HIV) or the virus replication inhibitor, remdesivir (Ebola), are being used in hospitals (Zhang and Liu, 2020). Clinical trials with these drugs are underway. At the same time, old drugs are being considered and repurposed for COVID-19 (Colson *et al.* 2020; Guo, 2020; Guy *et al.* 2020). Chloroquine and hydroxychloroquine (Figure 1) are two oral antimalarial drugs also used to treat rheumatoid arthritis and autoimmune diseases like lupus. These drugs have reached a remarkable attention in COVID-19 after promising results obtained by Chinese and French researchers that have generated high expectations although not exempt of scepticism for many. As a result, they are actually employed to treat COVID-19 patients in hospitals. A number of clinical trials are underway to determine their efficacy against SARS-CoV-2 as well as their ability to modulate the immune system and mitigate inflammation (the so-called cytokine storm) that ultimately may result in organ failure. These drugs reduce inflammatory cytokines IL-1, IL-6, TNF α produced by various cell types and IFN γ by mononuclear cells (Savarino *et al.*, 2003; Schrezenmeier and Dörner, 2020). They get inside acidic vesicles such as the lysosomal compartment, affect lysosomal activity and autophagy, and inhibit signalling pathways (Schrezenmeier and Dörner, 2020). The inflammatory attack could be somehow reduced by their accumulation in lymphocytes and macrophages. The pharmacology of chloroquine and hydroxychloroquine is similar. They are usually well tolerated and their half-lives are long (40–60 days), explaining the long duration of effects (Schrezenmeier and Dörner, 2020). Following, the antiviral and antimalarial actions of chloroquine and hydroxychloroquine are reviewed and discussed with a focus on the mechanisms of action, and subsequently a possible role for hemin/hemoglobin is considered in SARS-CoV-2 infection (COVID-19).

2. Chloroquine and hydroxychloroquine as antivirals against SARS-CoV-2

Chloroquine (and hydroxychloroquine) has a broad spectrum of antiviral activity against HIV, hepatitis B, Herpes simplex virus type 1, Ebola and also SARS and MERS (Devaux *et al.*, 2020; Savarino *et al.*, 2003). It exerts antiviral effects by inhibiting pH-dependent steps of replication of flaviviruses, retroviruses, and coronaviruses. Savarino *et al.* (2003) already suggested that chloroquine could be used in the future in SARS diseases. Its antiviral action pre- and post-infection against SARS and other coronavirus has been demonstrated in *in vitro* studies (Keyaerts *et al.*, 2004 and 2009; Vincent *et al.*, 2005). Chloroquine affected early stages of SARS coronavirus replication. The effective concentration ($EC_{50}=8.8\text{ }\mu\text{M}$) for the inhibition of SARS-CoV *in vitro* was significantly lower than its cytostatic activity (selectivity index (SI) of 30), and the EC_{50} approximates the plasma concentration of chloroquine during treatment of acute malaria (Keyaerts *et al.*, 2004). Chloroquine showed strong *in vitro* and *in vivo* antiviral activities against human coronavirus HCoV-OC43 (common cold), and treatment with daily doses of chloroquine had a long-lasting protective effect against lethal coronavirus OC43 infection

in newborn mice (Keyaerts *et al.*, 2009). The exact mechanism of the antiviral action of chloroquine and hydroxychloroquine is not completely known. Viruses can enter host cells by endocytosis and target to the lysosomal compartment where the low pH and the action of enzymes disrupt the viral particle liberating nucleic acids and enzymes necessary for viral replication (Figure 2B). Chloroquine and hydroxychloroquine are weak bases that can affect acid vesicles leading to dysfunction of enzymes. They concentrate within acidic organelles such as the endosome, Golgi vesicles and lysosomes, where the pH is relatively low and these molecules are charged. As a result, the pH of these organelles increases and disrupts acid hydrolases inhibiting post-translational modification of the newly synthesized proteins and other enzymes involved in the virus replication pathways (Savarino *et al.*, 2003). Chloroquine impairs replication of viruses by interacting with endosome-mediated viral entry and in the late stages of replication (Savarino *et al.*, 2003). However, SARS-CoV-2's main entryway uses the spike S protein to attach to ACE2 receptor on the surface of human cells instead to endosomes (Kupferschmidt and Cohen, 2020) (Figure 2B). For enveloped viruses such as SARS-CoV-2, post-translational modification of the enveloped glycoproteins involves proteases and glycosyl transferases, some of which require low pH. Chloroquine could inhibit viral replication by affecting proteolytic processing of those proteins. On the other hand, the anti-SARS effect of chloroquine was also due to the inhibition of glycosylation of the SARS coronavirus receptor ACE2 (Vincent *et al.*, 2005).

Chloroquine inhibits the growth of SARS-CoV-2 *in vitro* ($EC_{50} = 1.13 \mu M$; $SI > 88.50$) (Wang *et al.*, 2020). Hydroxychloroquine also inhibits SARS-CoV-2 *in vitro* ($EC_{50} = 0.72 \mu M$), and is more potent than chloroquine ($EC_{50} = 5.47 \mu M$) (Yao *et al.*, 2020). An early clinical study conducted in COVID-19 Chinese patients showed that chloroquine had a significant effect on viral clearance and clinical outcome (reduction in pneumonia severity and time of hospitalization) when compared with control group (Gao *et al.*, 2020). Chinese experts recommended treatment with chloroquine in patients with COVID-19, and started clinical trials (Multicenter collaboration group; Cortegiani *et al.*, 2020). The expectations highly increased with a small clinical study in France suggesting that the treatment of COVID-19 patients with hydroxychloroquine and an antibiotic was useful against the virus (Gautret *et al.*, 2020). Patients received hydroxychloroquine (600 mg) daily and their viral load was tested while untreated patients were included as negative controls. The presence and absence of the virus at day-6 post inclusion was considered the end point. Researchers treated 20 cases and the cases showed a significant reduction of the viral carriage compared with controls. Some patients did not respond to the treatment but it was attributable to metabolic reasons. The antibiotic azithromycin added to hydroxychloroquine significantly improved virus elimination. The researchers concluded that the treatment with hydroxychloroquine was significantly associated with a viral load reduction/disappearance of virus in COVID-19 patients and its effect was reinforced by azithromycin. As a result, they recommended treatment of patients with hydroxychloroquine and azithromycin to cure infection and to limit the transmission of the virus (Gautret *et al.*, 2020). In a recent small clinical trial, the use of hydroxychloroquine (400 mg/day) significantly shortened the time to clinical recovery and promoted the absorption of pneumonia among patients with COVID-19 (Preprint: Chen *et al.* 2020). Despite those initial promising results, there is consensus that further randomized clinical trials and additional studies are needed, and some are underway.

Meanwhile, hydroxychloroquine and chloroquine are currently being administered to hospitalized COVID-19 patients in many countries. Nevertheless, the controversy continues. For example, a recent retrospective non-randomized study with COVID-19 patients showed no benefit of hydroxychloroquine regarding death outcome (Preprint: Magagnoli *et al.*, 2020) whereas in another study increased survival is reported (Preprint: Membrillo *et al.*, 2020). Finally, in a recently reported multinational registry analysis, hydroxychloroquine and chloroquine had no benefit and increased frequency of ventricular arrhythmias (Mehra *et al.* 2020).

Figure 1. Chemical structures of chloroquine, hydroxychloroquine and hemin.

Figure 2. (A) Chloroquine and hydroxychloroquine as antimalarials. *Plasmodium* breaks down hemoglobin from erythrocytes to amino acids that are used for growth and survival. During this process, heme (Fe³⁺) and H₂O₂ are released. Heme is toxic but it is converted into the non-toxic pigment hemozoin (β-hematin). Chloroquine inhibits this conversion and proportionally increases soluble heme that is involved in peroxidative reactions (oxidation) and inhibition of cysteine proteases (Herraiz *et al.*, 2019). These mechanisms can cause oxidative damage in proteins (enzymes), membranes and DNA, and inhibit hemoglobin degradation, affecting parasite growth and survival. Hydroxychloroquine works similarly. (B) Chloroquine and hydroxychloroquine as antivirals. Chloroquine accumulates into lysosomes and acidic vesicles (endosomes) of host cell and increases pH, affecting enzymes needed for virus entry and infection such as proteases and glycosyl transferases, and enzymes used for replication (Savarino *et al.*, 2003). Hypothetically, in the presence of cell-free hemoglobin, heme and H₂O₂ (ROS) are released. Heme could interact with chloroquine and trigger oxidative reactions and inhibition of cysteine proteases that may affect host cell and virus. Non-erythroid hemoglobin could be degraded into lysosomes, and hypothetically release free heme and ROS that could interact with chloroquine exhibiting oxidative actions and inhibition of cysteine proteases and other enzymes needed for virus replication. Hydroxychloroquine might work similarly. The role of heme/hemoglobin in the graph B and its interaction with chloroquine is hypothetical and needs to be confirmed. This Figure cannot be reproduced without author permission as it contains information that need to be confirmed in scientific reports.

3. Antimalarial actions of chloroquine and hydroxychloroquine

Plasmodium falciparum passes over several stages during its infection cycle in malaria. In its erythrocyte stage, the parasite enters into the red blood cells and digests hemoglobin releasing heme, and peptides and amino acids that are used for growth and survival (Figure 2A). Heme is toxic for the parasite but it has a detoxification system that consists in heme crystallization to afford the insoluble pigment hemozoin (ferriprotoporphyrin IX dimer or β-hematin) (Kumar *et al.*, 2007). The heme crystallization system of *Plasmodium* is a target for antimalarial drugs (Egan, 2002; Kumar *et al.*, 2007; Mossadaque *et al.*, 2018). Chloroquine and other quinolines exert antimalarial actions by

interfering with this system. They accumulate into the acidic digestive vacuole of the parasite (Khun *et al.*, 2007), and prevent heme sequestration resulting in toxicity. The biochemical events underlying the toxic action of heme are poorly understood. In a recent work, we have reported that active quinolines inhibit the formation of β -hematin and proportionally increase soluble free hemin (ferriprotoporphyrin IX) (Herraiz *et al.*, 2019) (Figure 1). Among the quinolines and β -carboline derivatives studied, one of the most active was chloroquine. Chloroquine interacts with hemin by forming soluble complexes (Sullivan *et al.* 1996). We showed that active quinolines increased free hemin that catalyzed peroxidative reactions under conditions occurring in digestive vacuole (Herraiz *et al.*, 2019) (Figure 2A). A significant correlation was found between free hemin increased due to the action of chloroquine and peroxidation (oxidative effects). Then, chloroquine increases oxidative effects in the parasite through increased soluble free hemin. The most active pro-oxidant drugs were chloroquine and quinacrine whereas drugs that did not enhance soluble free hemin such as 8-hydroxyquinoline (a phenolic compound) or β -carboline derivatives did not promote oxidation.

Increased peroxidation produced by chloroquine depended on hemin and it was evidenced in presence of H_2O_2 (Herraiz *et al.*, 2019). Reactive oxygen species (ROS) can participate in the mechanism of action. *Plasmodium* breaks down hemoglobin to peptides and amino acids, and releases heme (Fe^{2+}) which converts into hemin (Fe^{3+}). As a result, it generates superoxide anion ($O_2^{\bullet-}$) that dismutates to H_2O_2 (Egan, 2008) (Figure 2A). H_2O_2 could be involved in peroxidase reactions catalyzed by increased levels of free hemin allowed by the chloroquine action (Herraiz *et al.*, 2019). Results also suggested that proteins (globin and others) potentiate peroxidation catalyzed by free hemin. Although chloroquine contributed to oxidative stress by increasing free hemin, it had no pro-oxidant effects by itself. Peroxidative actions depended on soluble free hemin although hemin could be as hemin-chloroquine complex. Then, the accumulation of chloroquine into the digestive vacuole of *Plasmodium* increases soluble free hemin which can trigger oxidative reactions resulting in oxidative damage to membranes, proteins, and DNA, with the subsequent toxicity for the parasite (Kumar *et al.*, 2005).

Plasmodium uses hemoglobin from red blood cells as a major source of nutrients. Hemoglobin is shuttled to vacuole where it is digested by several proteases, including cysteine proteases such as papain-like falcipains (Goldberg *et al.*, 1990; Rosenthal, 2011). This process results in high levels of globin, peptides, and amino acids that are used for the parasite growth. Cysteine proteases are involved in essential processes for parasite survival and growth such hemoglobin digestion but also erythrocyte rupture and host cell invasion (Lisk *et al.*, 2008; Rosenthal, 2004 and 2011). Therefore, the inhibition of proteolysis is a target for antimalarial drugs (Himangini *et al.*, 2018; Roy, 2017). Recent results indicate that hemin enhanced by the action of chloroquine is able to inhibit different cysteine proteases such as papain, ficin and cathepsin B (Herraiz *et al.*, 2019). Inhibition was due to soluble free hemin (or hemin-chloroquine complexes) allowed by the action of chloroquine but not to chloroquine itself, and occurred in presence of H_2O_2 suggesting a likely involvement of oxidative reactions (Figure 2A). The inhibition of cysteine proteases by hemin increased in presence of proteins such as globin and others. These results suggest that chloroquine could interfere with hemoglobin digestion during the blood stages of malarial life cycle and inhibits lysosomal functions and protein degradation.

Chloroquine and hydroxychloroquine have also antibacterial and antifungal activity (Rolain *et al.*, 2007; Yeo *et al.*, 2020). Hydroxychloroquine is associated with a decreased risk of major fungal infections (*Pneumocystis pneumonia*) in lupus patients (Yeo *et al.*, 2020). Antibacterial activities are mainly due to pH-dependent iron depletion and to increased pH of phagolysosomes (acid vesicles), which inhibit the growth of intracellular organisms.

4. Chloroquine and hydroxychloroquine as antivirals in COVID-19: A role for hemin/hemoglobin

The results discussed above suggest that chloroquine and hydroxychloroquine work differently against virus and *Plasmodium*. The main differential factor is the hemin/hemoglobin. Against virus like SARS-CoV-2, they work by diminishing the efficiency of the virus at entering host cells and decreasing its replication rate. In malaria, they work by poisoning the parasite digestive system (vacuole) via increasing free hemin that is toxic through oxidative stress and enzyme inhibition (cysteine proteases and others). The targeted cells are also different: ACE2-expressing cells such as epithelial ATII cells and other extrapulmonary cells in SARS-CoV-2 infection, and erythrocytes in malaria. In both cases, cell systems where chloroquine or hydroxychloroquine accumulate are involved: lysosomes and related acidic compartments of host cells in SARS-CoV-2, and digestive vacuole in malaria parasite. It is thought that chloroquine and hydroxychloroquine exert their antiviral actions mainly via endosomes. However, the main entryway of SARS-CoV-2 is by the spike S protein instead of endosomes (Figure 2B). This may suggest that additional mechanisms could be involved in their antiviral action.

Most drug candidates investigated in COVID-19 target key proteins of the host cell or the virus. The host's cell proteins are TMPRSS2 protease or cathepsins (used for virus entrance) and enzymes ACE2 (virus receptor). The targeted proteins of the virus are the S protein (a glycoprotein), the RNA-dependent RNA polymerase (RdRp), and the two proteases that are essential for viral replication: main protease (3CLpro), a chymotrypsin-like cysteine protease, and papain-like cysteine protein (PLpro). The 3CLpro utilizes a Cys/His catalytic dyad whereas PLpro has a classical Asp/His/Cys catalytic triad (Preprint: Stoermer, 2020a,b). PLpro possesses a C-terminal zinc-finger like domain bearing four cysteine residues that are critical for catalytic function (Preprint: Stoermer, 2020b). Drugs are being tested as inhibitors of these cysteine proteases in order to block viral replication.

Chloroquine is not a good inhibitor of cysteine proteases. However, it interacts with hemin and increases oxidative reactions (oxidative stress) and inhibition of cysteine proteases (Herraiz *et al.*, 2019). These events might hypothetically occur in the host and virus during infection. Cell cultures might contain residual hemoglobin (methemoglobin) and hemin eventually influencing the antiviral action of chloroquine or hydroxychloroquine. *In vivo*, hemoglobin is confined into red blood cells, which in contrast to *Plasmodium*, are not presumably targets of SARS-CoV-2. Recent results suggest that the virus might use CD147 receptor as an additional gate into host cells and the same receptor is used by *Plasmodium* to enter into red blood cells (Preprint: Wang *et*

al., 2020; Zhang *et al.*, 2018). However, noticeable hemolysis has not been reported as a common feature in COVID-19 patients although ferritin appears to increase. Nevertheless, there are possible sources of extravascular local cell-free hemoglobin and heme that should be considered. Hemoglobin could be locally released from red blood cells breakdown in capillaries close to alveolar cells damaged during infection. Thus, SARS-CoV infections caused hemorrhages in the alveolar space of the lungs (Gralinski and Baric, 2015), and lung hemorrhages in animal models infected with coronaviruses are significantly reduced with antivirals (Sheahan *et al.*, 2020). SARS-CoV-2 attacks blood vessels and some patients show coagulation dysfunction and develop blood clots while others develop pulmonary embolism. The lung pathology seen in patients with COVID-19 shows marked microvascular thrombosis and hemorrhage linked to extensive alveolar and interstitial inflammation (Preprint: McGonagle *et al.*, 2020). Clinical pathological studies of lung biopsies and autopsies of COVID-19 patients indicate alveolar hemorrhage along with small vessel thrombus formation in the lung periphery (Fox *et al.*, 2020; Preprint: Luo *et al.*, 2020). Alveolar hemorrhage is also a common feature of the acute respiratory distress syndrome (ARDS), a condition associated with COVID-19. ARDS patients have elevated levels of cell-free hemoglobin within the alveolar space from red blood cell breakdown (Janz and Ware, 2015; Shaver *et al.*, 2016), and it may contribute to the pathogenesis of acute lung injury. Cell-free hemoglobin resulted in increased inflammation, loss of alveolar capillary barrier integrity, and epithelial cell injury (Shaver *et al.*, 2016). It converts to methemoglobin and releases free heme (Schaer *et al.* 2013a). Heme increased alveolar-capillary permeability (barrier dysfunction) but did not cause alveolar inflammation or epithelial cell injury in the lungs (Shaver *et al.*, 2016). Finally, the drugs chloroquine and hydroxychloroquine might also induce erythrocyte breakdown in some patients (Chou and Fitch, 1980; Juurlink, 2020). Extracellular heme/hemoglobin released from red blood cell breakdown might be a target for the action of chloroquine and hydroxychloroquine (Figure 2B).

On the other hand, hemoglobin is expressed in non-erythroid cells such as epithelial cells and activated macrophages. Expression of this protein has been observed in several human tissues including the lungs, particularly in alveolar epithelial cells type II (ATII) that produce the pulmonary surfactant essential for lung function (Bhaskaran *et al.*, 2005; Ghosh *et al.*, 2018; Grek *et al.*, 2011; Ishikawa *et al.*, 2010; Newton *et al.*, 2006; Saha *et al.*, 2014). Hypoxia greatly increased hemoglobin expression in ATII cells, and decreased surfactant proteins (Grek *et al.*, 2011). Hemoglobin in these cells might be involved in sensing oxygen concentrations, and facilitation of oxygen transport across air-blood barrier (Grek *et al.*, 2011), and it might have a role in ARDS. Remarkably, epithelial ATII cells that are main targets of SARS-CoV-2 infection also express hemoglobin, and this expression highly increases during hypoxia, a phenomenon occurring during viral infection. Hemoglobin/heme expressed in ATII cells might be a target for the action of chloroquine and hydroxychloroquine (Figure 2B).

Local extracellular hemoglobin or hemoglobin expressed in ATII cells could be oxidized to methemoglobin (Fe^{3+}). This oxidation produces reactive oxygen species (ROS) (H_2O_2), and may occur in presence of inflammatory-cell-derived oxidants (Balla *et al.*, 1993). Methemoglobin is toxic, generates ROS, and releases free heme (Bunn and Jandl, 1968) which can be incorporated into endothelial cells (Balla *et al.*, 1993, Vercellotti *et al.*, 1994), and perhaps other cells as well (Schaer *et al.* 2013a). Endothelium

defends itself from heme/hemin by induction of the heme degrading enzyme heme oxygenase, and produces large amounts of the iron binding protein ferritin (Vercellotti *et al.*, 1994). Chloroquine and hydroxychloroquine could modulate those actions. For example, their interaction with free hemin could trigger oxidative reactions and inhibition of cysteine proteases of the virus and host, which could result in antiviral actions. Chloroquine could also limit degradation of extracellular hemoglobin by alveolar macrophages. During disposition of extracellular hemoglobin in hemoglobin scavenger receptor CD163-positive HEK cells, chloroquine action resulted in intracellular hemoglobin trapping compromising clearance and abolished expression of heme oxygenase-1 (HO-1) (Schaer *et al.*, 2013b). Chloroquine blocked the elimination of hemoglobin (or methemoglobin) by interfering with lysosomal hemoglobin degradation, and caused intracellular accumulation of heme and globin (Schaer *et al.*, 2013b). These results are in line with chloroquine effects by increasing free hemin (and hemoglobin) to potentiate oxidative reactions (oxidative stress) and inhibition of cysteine proteases (Herraiz *et al.*, 2019) that might affect the host and virus (Figure 2B). However, in order to be effective, this mechanism would need the presence of sufficient local free hemin/hemoglobin to interact with chloroquine. It is currently unknown whether this really occurs during infection in COVID-19. In a recent preprint by Liu and Li (2020), it is reported that non-structural proteins of SARS-CoV-2 could attack hemoglobin and heme to release porphyrins used for the virus. This might be interpreted that the virus degraded hemoglobin. However, the report is a bioinformatics study and not an experimental work, and does not prove the breakdown of hemoglobin or heme to porphyrins, that would need a kind of heme oxygenase.

As described here, chloroquine and hydroxychloroquine exert diverse biological effects as shown also by their anti-inflammatory actions in autoimmune (lupus) and rheumatoid arthritis diseases (Ponticelli *et al.*, 2017). They are also used in porphyria such as porphyria *cutanea tarda* (Singal *et al.*, 2012). As discussed here, an interaction of these drugs with heme is expected. In this regard, intravenous hemin (hydroxyhemin or hemin arginate) is used for the treatment of acute porphyria attacks. It helps to overcome the relative deficiency of heme in the liver and reduces the supply of porphyrins and precursors needed for heme production. During the current COVID-19 pandemic, porphyria patients could be infected with SARS-CoV-2 and still be treated with hemin (<https://porphyriafoundation.org/for-patients/COVID-19-and-porphyria/>). Harmful drugs are avoided during treatment with hemin although hydroxychloroquine or chloroquine might be probably safe based on evidence that is either inconsistent or insufficient to be conclusive (<https://porphyriafoundation.org/drugdatabase/drug-safety-database-search/>). So far, it is unknown whether possible simultaneous cases of acute porphyria attack and COVID-19 have been treated with hemin and chloroquine or hydroxychloroquine but it would be of interest to know the outcome because an interaction of hemin with these drugs is expected.

Remarkably, hemin exhibits antiviral actions against several viruses such as HIV, Zika, influenza, or Ebola. This action could be related with the induction of heme oxygenase enzyme. Therefore, the antiviral action of hemin supports a possible mechanism for antiviral action of chloroquine and hydroxychloroquine with involvement of hemin, as discussed above. Hemin inhibited HIV replication *in vitro* and reduced viral load in mice (Devadas and Dhawan, 2006). It highly inhibited replication of Zika virus *in vitro* (Huang *et al.*, 2016). Hemin showed anti-influenza virus activity *in vitro* and *in vivo*

(Wang *et al.*, 2017). It ameliorated influenza pneumonia and attenuated virus-induced lung injury, lymphocytopenia and local inflammation in a mouse model. Hemin protected mice from death and body weight loss caused by influenza A virus infection (Wang *et al.*, 2017). Hemin had an important effect in the reduction of Ebola virus replication. Treatment of different human cells with hemin reduced infection by >90% and showed minimal toxicity to infected cells (Huang *et al.*, 2016). This inhibition could be partly due to heme oxygenase (HO-1) activity and expression. The reverse transcriptase activity of a murine leukemia virus was inhibited by hemin and inhibition increased 1000-times when hemin solution was aged (Tsutsui *et al.*, 1987). Hemin also potentiated the activity of artemisinin against Hepatitis C virus showing synergistic effects (Paeshuyse *et al.*, 2006). Taken together, the antiviral effects of hemin are noticeable and could be useful in the current context of absence of effective treatments in COVID-19. In this regard, the action of hemin, and the combined action of hemin and chloroquine or hydroxychloroquine may be worth of testing in SARS-CoV-2 (COVID-19) studies evaluating antiviral action and effects on the disease. Nevertheless, possible safety and toxic effects should be also considered as hemin may induce toxic actions (Van Avondt *et al.*, 2019), and chloroquine and hydroxychloroquine are not exempt of undesirable effects such as heart problems and retinopathy (Chorin *et al.*, 2020; Juurlink, 2020).

5. Overview and future directions

In COVID-19, SARS-CoV-2 infects lung epithelial ATII cells and damages the alveolar wall making difficult oxygen exchange. To enter into the cells, SARS-CoV-2 uses spike (S) protein that binds to ACE2 receptors in host cells, and is primed by membrane protease TMPRSS2 and other proteases (cathepsins) of the host. The virus expresses two key proteases, the main protease (3CLpro) and the papain-like protease (PLpro), and a polymerase (RpRp), which are needed for replication. Drugs against SARS-CoV-2 are urgently needed and a number of molecules are being tested and evaluated in clinical trials. Chloroquine and hydroxychloroquine have shown broad antiviral effects *in vitro* against different viruses including SARS-CoV-2. Early clinical results suggest that these drugs could be useful in COVID-19 patients and on this basis, they are currently being used in hospitals. However, these drugs are not yet approved for COVID-19 and clinical trials are underway. Their antiviral action is mainly attributed to an increase of pH in lysosomes and acidic vesicles of the host cell that makes difficult the entrance of the virus, and also affects enzymes needed for virus progression. Chloroquine and hydroxychloroquine are approved as antimalarials and in autoimmune diseases. They exert anti-inflammatory effects that could be useful against inflammatory developments occurring in COVID-19. As antimalarials, they get inside the parasite vacuole in infected red blood cells, and inhibit hemin crystallization to hemozoin (β -hematin). Soluble hemin is toxic for the parasite. Recent findings have shown that chloroquine inhibits the formation of β -hematin and increases soluble free hemin that is involved in peroxidative reactions (oxidative stress) and inhibition of cysteine proteases. These mechanisms can underline the toxic effects of chloroquine against the parasite.

The mechanisms of action of chloroquine and hydroxychloroquine as antivirals and antimalarials differ. The main differential factor is the heme/hemoglobin. However, local hemorrhages occur in COVID-19 and ARDS patients with a subsequent release of cell-free hemoglobin (extracellular) which is oxidized to methemoglobin and releases heme. On the other hand, lung epithelial ATII cells invaded by SARS-CoV-2 express non-erythrocytic hemoglobin, which is highly increased during hypoxia. In the presence of sufficient hemoglobin/heme, chloroquine and hydroxychloroquine could have antiviral effects mediated by heme. These drugs could interact with heme and increase oxidative actions and inhibition of cysteine proteases of the host and virus. These processes could affect the virus and host with potential effects on the disease. In the short future, the efficacy and safety of chloroquine and hydroxychloroquine should be conveniently substantiated in clinical trials of COVID-19. Moreover, studies could investigate whether their mechanism of action against SARS-CoV-2 involves oxidative reactions (ROS) and inhibition of cysteine proteases of the virus and host, and the role of heme/hemoglobin. Remarkably, heme exerts antiviral actions against a number of viruses both *in vitro* and *in vivo*. This fact supports a role of heme in the antiviral action of chloroquine and hydroxychloroquine. Heme is used for the treatment of acute porphyria attacks. In this regard, it would be of interest to know whether possible simultaneous cases of porphyria attacks and COVID-19 have been treated with heme and chloroquine/hydroxychloroquine, and to know the outcome because an interaction among these drugs is expected. Under current need of drugs, it could be worth of testing the antiviral action and safety of chloroquine/hydroxychloroquine combined with heme and that of heme alone against SARS-CoV-2 (COVID-19).¹

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Disclosure of Potential Conflicts of Interest

There are no conflicts of interest.

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