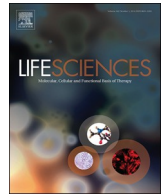




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Review article

Can endolysosomal deacidification and inhibition of autophagy prevent severe COVID-19?

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ABSTRACT

The possibility is examined that immunomodulatory pharmacotherapy may be clinically useful in managing the pandemic coronavirus disease 2019 (COVID-19), known to result from infection by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a positive-sense single-stranded RNA virus. The dominant route of cell entry of the coronavirus is via phagocytosis, with ensconcement in endosomes thereafter proceeding via the endosomal pathway, involving transfer from early (EEs) to late endosomes (LEs) and ultimately into lysosomes via endolysosomal fusion. EE to LE transportation is a rate-limiting step for coronaviruses. Hence inhibition or dysregulation of endosomal trafficking could potentially inhibit SARS-CoV-2 replication. Furthermore, the acidic luminal pH of the endolysosomal system is critical for the activity of numerous pH-sensitive hydrolytic enzymes. Golgi sub-compartments and Golgi-derived secretory vesicles also depend on being mildly acidic for optimal function and structure. Activation of endosomal toll-like receptors by viral RNA can upregulate inflammatory mediators and contribute to a systemic inflammatory cytokine storm, associated with a worsened clinical outcome in COVID-19. Such endosomal toll-like receptors could be inhibited by the use of pharmacological agents which increase endosomal pH, thereby reducing the activity of acid-dependent endosomal proteases required for their activity and/or assembly, leading to suppression of antigen-presenting cell activity, decreased autoantibody secretion, decreased nuclear factor-kappa B activity and decreased pro-inflammatory cytokine production. It is also noteworthy that SARS-CoV-2 inhibits autophagy, predisposing infected cells to apoptosis. It is therefore also suggested that further pharmacological inhibition of autophagy might encourage the apoptotic clearance of SARS-CoV-2-infected cells.

1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a positive-sense single-stranded RNA virus pathogen which is the cause of severe and sometimes fatal respiratory illness, named coronavirus disease 2019 (COVID-19) [1–3].

In general, COVID-19 is a mild illness; symptoms include pyrexia, cough, shortness of breath and, less commonly, diarrhoea. Yet many people infected with the virus remain asymptomatic throughout its

course and unknowingly infect others [4]. However, the weight of evidence suggests that some 20% of patients develop symptoms of a severity that requires hospitalisation. Approximately 40% of these patients develop severe pneumonia and acute respiratory distress syndrome (ARDS) and require prolonged ventilation [5,6]. Once ventilated, the prognosis is poor. Massive alveolar damage leading to a pattern of progressive respiratory failure commonly results in death in over 50% of cases [5,6].

From a histological perspective patients with COVID-19 ARDS

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display evidence of diffuse alveolar damage, increased permeability of epithelial and endothelial cells, fibrin-rich hyaline membranes, significant leakage of fluid into the pulmonary interstitium, gross disruption of gas exchange and signs of hypoxia [5,7–9]. Commonly reported pulmonary immune abnormalities include activated alveolar and bone-derived macrophages carrying markers of pyroptosis [7,10,11] and high levels of neutrophil extracellular trap-producing neutrophils [8,12]. Unsurprisingly, these abnormalities in the pulmonary immune cells are accompanied by severe hypercytokinaemia in the lungs [10,13,14].

The most commonly reported peripheral pro-inflammatory cytokine (PIC) and chemokine profiles of COVID-19 patients includes elevated levels of tumour necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1) and C-X-C motif chemokine 10 (CXCL10) [10,15–19]. Importantly, the levels of immune activation and dysregulation of cytokine and chemokine levels increases with disease severity [10,15–19] and may be 10-fold higher in patients with severe pneumonia and individuals requiring mechanical ventilation than in patients whose symptoms are relatively mild [20].

Systemic inflammation is associated with mortality, with high neutrophil lymphocyte ratios and levels of IL-6 being predictive of a poor outcome [20–23]. Other markers of excessive systemic inflammation such as lymphopaenia, T and NK cell exhaustion and an elevated T helper 17 cell (Th17) to regulatory T cell (Treg) ratio, and the presence of large numbers of TNF- α - and IL-6-secreting macrophages are also all frequently observed in patients with severe COVID-19 [24–27]. There is also evidence of increased NLR family pyrin domain containing 3 (NLRP3) activity and widespread tissue pyroptosis and/or necroptosis in lymph nodes, spleen, liver, heart and the kidney [28,29] reviewed by [26]. This pattern of cellular damage is also observed in epithelial and endothelial cells [30,31]. Such data combined with evidence of a hypercoagulable state [32,33] and the existence of microthrombi in many COVID-19 patients are suggestive of widespread endotheliopathy [34,35].

This pattern of immune and histological abnormalities seen in patients with severe COVID-19 is also seen in sepsis and septic shock [36–40]. Indeed, there is a growing consensus that severe COVID-19 infection may be a distinct form of viral sepsis [1,41,42] which has considerable long-term implications for post-discharge as well as in hospital pathology, as discussed below.

The authors of large observational studies involving over 80,000 patients have concluded that the long-term prognosis for sepsis survivors is poor, with a death rate estimated to be 15% in the first year and 6 to 8% per annum over the next five years [43,44]. In addition, several studies have reported persistent and profound long-term physical disability [45,46] and serious psychiatric sequelae [47,48], reviewed by [49], in survivors of SARS and Middle East Respiratory Syndrome (MERS) infection. Crucially, there is accumulating evidence to suggest that this may be true of survivors of severe COVID-19, with many patients experiencing grossly reduced exercise capacity, extreme fatigue as well as serious neurological sequelae and neuropsychiatric sequelae [50], reviewed by [51].

In addition, while severe pneumonia progressing to ARDS is the main cause of mortality [10,33,52–54], serious multi-organ pathology is also commonplace and is a cause of significant levels of morbidity and mortality [5]. For example, acute myocardial injury, often resulting in fatal or non-fatal myocardial infarction, occurs in 20 to 40% of patients [5,54–56] reviewed by [57]. Furthermore, acute renal injury, hepatic dysfunction and various dimensions of ocular damage occur in approximately 30% of patients [6,58,59] reviewed by [57].

Given the numbers of individuals infected and estimates that approximately 20% require hospitalisation, the current pandemic is likely to lead to a considerable burden on health care systems as well as in the medium and longer term. Hence the development of a therapeutic approach which may reduce the numbers of patients progressing to severe disease is of prime importance from an individual and a societal

perspective.

This represents a formidable challenge; however, as the weight of evidence suggests that the main driver of symptoms and severe pathology is the development of a cytokine storm [13,16,60]. In addition, the contribution of the virus and the host immune response to pathology appears to vary over the course of the illness. In particular, the weight of evidence suggests that SARS-CoV-2 and the host immune response are involved in the earliest stages of the illness and thereafter a dysregulated immune response plays the predominant role (2020). Hence an agent or agents with antiviral and immunomodulatory properties would appear to be desirable. Furthermore, the therapeutic window for early intervention appears to be very narrow. The weight of evidence suggests that symptom onset occurs on average four to five days post-infection with peak viral load occurring some five to six days later [61–63]. In addition, patients with severe disease may progress to ARDS in eight or nine days [64]. Hence the therapeutic effects of any approach aimed at preventing the development of severe disease would need to be of relatively rapid onset.

Currently, therapeutic options in the treatment of COVID-19 are extremely limited. Thankfully, there is now evidence that dexamethasone reduces mortality in patients requiring mechanical ventilation [65]. However, the results achieved using remdesivir and tocilizumab have thus far been disappointing [66,67]. In addition, the early hope that hydroxychloroquine might reduce mortality in patients with severe COVID-19 appears to be fading. Despite promising results from small open-label observational studies and randomised controlled trials (RCTs) suggesting that hydroxychloroquine might increase rates of viral clearance and reduce mortality [68–70], a much larger prospective open-label RCT found no evidence of improved mortality [71]. It should be noted that this is consistent with the results from large retrospective studies which also reported no improvement in mortality when administered to hospitalised patients with COVID-19 pneumonia patients requiring oxygen [72,73]. However, other large retrospective analyses suggest that combination therapy involving hydroxychloroquine and azithromycin may reduce mortality in COVID-19 patients if administered early in the course of the illness and may prevent the development of severe disease [74–76].

This is potentially of major therapeutic relevance as there is considerable evidence from observational studies and RCTs that hydroxychloroquine and azithromycin possess immunomodulatory and antiviral activities [77–81]. Moreover, several retrospective analyses and RCTs suggest that fears over potential cardiovascular complications with hydroxychloroquine and/or azithromycin may also be unfounded; large retrospective studies have reported no evidence of increased cardiovascular mortality or life-threatening arrhythmias in COVID-19 patients treated with hydroxychloroquine or azithromycin, whether prescribed singly or in combination, compared with untreated controls [74–76].

In light of the above, the remainder of this paper has three aims. First, to review the evidence relating to the immunomodulatory and antiviral effects of hydroxychloroquine and azithromycin while discussing their respective modes of action. Second, to discuss evidence of safety and the likelihood of serious and minor side effects. Third, to come to a view on the merits of a RCT of a combination of hydroxychloroquine and azithromycin with a particular focus on the dose and duration of therapy as well as patient selection.

2. Hydroxychloroquine

2.1. Immunomodulator in rheumatic diseases

Many research teams have reported significant reductions in mortality in patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) following prolonged ingestion of hydroxychloroquine at doses of 6.5 mg/kg compared with being untreated [82,83] reviewed by [84]. Several authors have also reported large and

significant reductions in organ damage [85–87] and a reduced number of fatal and non-fatal cardiovascular events [88–90]. A recent meta-analysis of 19 RCTs and case-controlled observational studies concluded that hydroxychloroquine administration is associated with a 28% reduction in cardiovascular disease (CVD) in treated SLE patients compared with untreated controls [91]. There is also some evidence to suggest that patients treated with hydroxychloroquine may experience less neurological damage after 20 years of therapy compared with their untreated counterparts [92]. Furthermore, the results from prospective observational studies suggest that use of hydroxychloroquine may lead to a significant reduction in renal damage compared with non-users [93–95]. There may also be less pulmonary damage in SLE patients treated with hydroxychloroquine compared with untreated patients or patients treated with alternative therapies [96].

These are important data, as multiple end-organ damage is relatively common in SLE and is a major determinant of morbidity and mortality in this illness [97] (reviewed in [98]). For example, it is estimated that up to 50% of SLE patients have evidence of cardiac pathology and the incidence of myocarditis may be as high as 15% (reviewed by [99]). In addition, several authors have estimated that as many as 90% of SLE patients display evidence of neurological and neuropsychiatric abnormalities [100,101]. Approximately 50% of SLE patients have underlying renal pathology, and lupus nephritis is a major driver of mortality [102]. Finally, a recent review concluded that there is a six-fold increase in pulmonary disease in SLE patients compared with age- and sex-matched controls [103] and a recent meta-analysis concluded that pulmonary pathology is seen in the majority of patients [104].

There is also considerable observational evidence of reduced levels of mortality in SLE patients treated with hydroxychloroquine compared with age- and sex-matched controls or patients who have discontinued hydroxychloroquine therapy [90,105–107]. The greatest improvement in mortality recorded thus far involved patients enrolled in the LUMINA (Lupus in Minorities; Nature vs. nurture) study. A mortality rate of 5% was reported for hydroxychloroquine adherents and 17% for non-adherents or historically untreated controls [105]. Increased mortality levels in patients who have discontinued hydroxychloroquine have also been highlighted in a recent study. A fourfold increase in mortality was observed in non-adherent patients compared with patients who continued on hydroxychloroquine therapy [108].

However, while the results reported above are encouraging, it should be noted that the majority of the studies discussed are observational or quasi-experimental in nature. In addition, while many such studies are prospective, many are retrospective. These study designs suffer many well-documented limitations owing to lack of randomisation and difficulty in establishing cause and effect associations [109]. Readers interested in detailed treatments regarding the limitations and utility of observational, quasi-experimental and retrospective studies are referred to excellent reviews by [110–112]. However, having emphasised the level of uncertainty in the data discussed above, it should be noted that several meta-analyses of observational studies and RCTs report broadly consistent findings [79,84].

Hydroxychloroquine has also been used in an attempt to alleviate the persistent immune activation experienced by HIV-positive patients without access to antiretroviral (ARV) therapies and in patients well controlled on such regimes [113–117]. However, thus far the evidence in this domain is limited to observational studies and hence these results await confirmation via well designed RCTs.

2.2. Mode of action as an immunomodulator

The weight of evidence suggests that hydroxychloroquine and chloroquine mainly exert their immunomodulatory effects by inhibiting endosomal toll-like receptor (TLR)-3, 7, 8 and 9 activation by increasing endosomal pH, thereby reducing the activity of the acid-dependent endosomal proteases required for their activity and/or

assembly [118–122]. The net effect of such inhibition is the suppression of antigen-presenting cell activity, decreased autoantibody secretion and decreased activity of NF- κ B and levels of PIC production [115,120,121,123,124], (reviewed by [125]). Characteristic changes in immune and inflammatory profiles induced by hydroxychloroquine therapy typically involve reductions in IL-1 β , IL-6, TNF- α , CXCL-10, sCD40L and interferon- α (IFN- α 2) [115–117,123,124,126–128]. Hydroxychloroquine therapy may also reduce B cell activity [129] and Th17 cell differentiation [130,131], while promoting a Th1 to Th2 transition [130] and inhibiting the production of the PICs CCL2 and CXCL10 [132].

2.3. Antiviral action

Several authors have reported inhibition of human coronavirus (HCoV) 229E, HCoV OC43, MERS-CoV, SARS-CoV and SARS-CoV-2 replication following the administration of chloroquine or hydroxychloroquine in vitro in a range of cell types including Vero cells and lung epithelial cells [133–139]. The proposed mechanism underpinning these observations is deacidification of the endolysosomal system and the endoplasmic reticulum (ER) and Golgi apparatus, which is explained below [77].

A hallmark feature of the endolysosome system is its acidic luminal pH [140–142] that is critical for the activity of up to 60 different pH-sensitive hydrolytic enzymes, including proteases, lipases and nucleases [143,144]. The Golgi sub-compartments [145] and Golgi-derived secretory vesicles [140,146] are also mildly acidic and dependent on these conditions for optimal function and structure [145]. Compromised Golgi functions stemming from deacidification include dysregulated processing of proteins and defects in posttranslational modifications, which play a vital role in the function of mature proteins [147,148]. Importantly, raising pH inhibits enzymes involved in glycosylation [148–150]. Other impairments stemming from deacidification include dysregulated or inhibited transfer of proteins from the ER to the Golgi [151] and reverse transport from Golgi to ER [152,153]. Unsurprisingly, deacidification of the Golgi also leads to detrimental effects on protein sorting, transport from the Golgi to secretory vesicles and subsequent transport to the plasma [154].

Chloroquine and hydroxychloroquine are classified as weak diprotic bases [155,156]. In physiological conditions these compounds readily transverse plasma membranes into the cytosol and into the endolysosomal and ER Golgi compartment [157,158]. However, once enclosed within these compartments, they become protonated and thereafter are unable to escape into the cytosol. Over time, the preferential increase in concentration of chloroquine or hydroxychloroquine raises the pH in the lumen of these organelles resulting in a wide range of adverse functional consequences [159].

For example, chloroquine-mediated deacidification compromises endolysosomal trafficking by inducing mis-localisation of endosomes towards the periphery rather than the normal perinuclear region [160]. Other adverse effects include impaired endolysosomal fusion and increased lysosome exocytosis [161,162]. There are also data to suggest that chloroquine administration results in enhanced release of exosomes [163]. Several functional impairments also result from chloroquine administration, including undegraded substrates and atypical cleavages that lead to the generation of toxic intermediates, which are to be expected given the pH-dependent activity of endolysosomal enzymes discussed above [164,165]. Significant enlargement of endosomes and lysosomes also appears to be a characteristic consequence of chloroquine exposure, although the functional consequences of this phenomenon are currently unknown [166,167].

Chloroquine also concentrates in, and raises the pH of, the normally acidic Golgi [145], disrupting cisternal structure [168], impairing protein glycosylation [148] and formation and release of transport vesicles [169,170]. It should also be noted that prolonged chloroquine administration may ultimately result in lysosome membrane

permeabilisation [171,172] resulting in the translocation of lysosomal contents into the cytosol, in turn leading to extreme mitochondrial damage and cell death [164,173]. The therapeutic implications of this phenomenon are discussed below, highlighting the role of the endolysosomal system and the Golgi apparatus in the replication of SARS-CoV-2.

In the case of SARS-CoV-2, initial entry into cells is enabled by engagement of the viral spike (S) protein with membrane-bound angiotensin-converting enzyme 2 (ACE-2) and subsequent proteolytic cleavage of the latter by a range of host cell enzymes [174,175]. The relative importance of these enzymes in enabling and propagating infection remains a matter of debate. Some research teams have proposed that the presence of the type II transmembrane serine protease TMPRSS2 is the sole determining factor [176] while others have suggested that the presence of TMPRSS2 and furin is required [177]. More recently, another research team has suggested that the presence of furin alone is sufficient [178]. This area is not just of academic importance as evidence suggests that furin activity is pH-dependent, while that does not appear to be the case for activity of TMPRSS2 [176,177,179,180]. Hence, if the presence of TMPRSS2 is the sole requirement for viral entry, then the argument that the use of hydroxychloroquine could prevent SARS-CoV-2 entry would appear to be weak. If, on the other hand, the optimal activity of furin is an indispensable element in viral entry and replication, then the theoretical argument for the prophylactic use of hydroxychloroquine becomes stronger. The matter of SARS-CoV-2 entry into cells may also be more complex than suggested in the evidence discussed above, as it has been proposed that the presence or otherwise of these enzymes determines whether the virus enters via fusion with the plasma membrane or via endocytosis and eventual occupancy within endosomes (reviewed by [181]).

However, the weight of evidence suggests that the dominant route of cell entry is via phagocytosis, with ensconcement in endosomes thereafter proceeding via the endosomal pathway [182–184]. This mode of transport involves transfer between early (EEs) to late endosomes (LEs) and ultimately into lysosomes via endolysosomal fusion [182–184]. This is important as transport from EEs to LEs is a rate-limiting step for coronaviruses [183,185]. Hence inhibition or dysregulation of endosomal trafficking could potentially have a major role in inhibiting the replication of SARS-CoV-2.

The next stage in the replication cycle involves the fusion of the virion with the lysosomal membrane enabled by pH-sensitive lysosomal furins and cathepsins releasing viral RNA into the cytoplasm [178]. Replication in the cytosol is initiated by the translation of the virally encoded replicase protein, leading to the production of a polyprotein subsequently cleaved by a virally encoded protease to produce 16 non-structural proteins (nsps) and the accessory proteins ORF3a, 3b, 6, 7a, 7b, 8, 9b, 9c and 10 [186] [187,188].

In the next stage, the three nsps 3, 4 and 6 stimulate the production of double-membrane vesicles from ER membrane and establish replication transcription complexes (RTCs) [187,188]. Coronavirus RTCs are predominantly an assembly by RNA-dependent RNA polymerase (RdRp) and helicase-containing subunits and are a source of negative-sense RNA, subgenomic and genomic messenger RNAs (mRNAs). The structural viral nucleocapsid (N) protein is translated in the cytoplasm, thereafter binding with genomic mRNAs [189–191]. The other structural viral proteins (M, S and E) are translated in the ER and then migrate to the ER-Golgi intermediate compartment (ERGIC) [192].

All human coronaviruses form virions by budding into the lumen of ERGICs [193,194]. Viral proteins are thereafter glycosylated in the Golgi via pH-sensitive enzymes [195–199]. This is an important point, as the weight of evidence suggests that the glycosylation of proteins plays an indispensable role in enabling the optimal functioning of proteins responsible for suppressing host immune responses, initiating the formation of RCTs and the subversion of the immune response [200–204]. Finally, coronaviruses, including SARS-CoV-2, exit the Golgi in secretory vesicles and are transported to the plasma before

exiting into the intercellular environment via exocytosis [205,206]. Hence, the deacidification of the trans-Golgi network is highly likely to result in dysfunctional virions and impeded exit of such virions into the extracellular compartment. Thus when the data are considered as a whole, the use of hydroxychloroquine or chloroquine would appear to be a rational antiviral therapy so far as SARS-CoV-2 is concerned. In addition, there may be other factors associated with coronavirus replication which may also be targeted via the use of hydroxychloroquine or chloroquine, which are discussed briefly below.

Inducing ER stress and the activation of the unfolded protein response (UPR) is a conserved replication strategy among coronaviruses aimed at inhibiting host proteins involved in metabolism and the immune response to viral invasion [207,208]. Examples of coronavirus proteins which have the capacity to induce ER stress include 3a and ORF-8b of SARS-CoV [195,209]. Broadly, the translation of host proteins is dependent on unphosphorylated eukaryotic initiation factor 2 (eIF2) [207,208] and activation of the UPR leads to phosphorylation of eIF2 by protein kinase R-like ER kinase (PERK or EIF2AK3), thereby inhibiting or terminating host protein synthesis [210]. Importantly, prolonged UPR activation is a common cause of apoptosis in coronavirus-infected cells [208].

Furthermore, there is accumulating evidence that MERS-CoV, SARS-CoV and SARS-CoV-2 inhibit autophagy [211–214] reviewed by [215]. This state of affairs also has deleterious consequences for cell survival by sensitising such cells to apoptosis [216,217]. These data are intriguing given the relative success of hydroxychloroquine and chloroquine as adjuvants to chemotherapy achieved via the inhibition of autophagy and the promotion of ER stress leading to increased rates of apoptosis [218,219]. This offers the tantalising possibility that it might be possible to encourage the apoptotic clearance of SARS-CoV-2-infected cells already predisposed to apoptosis by the administration of hydroxychloroquine at the doses used in the treatment of cancer patients, which appear to be well tolerated and free of serious side effects [218,219].

The replication of viruses such as Ebola, dengue, Zika, HIV and SARS-CoV-2 is heavily dependent on the activity of endosomal and lysosomal proteases, glycosylating enzymes and glycosyltransferases, which in turn is dependent on an acidic pH within these vesicles [118,138,220,221]. The ability to induce endosomal acidification via mechanisms described above has led to research into the use of hydroxychloroquine in the treatment of Ebola, dengue and Zika viral infections, as the replication of these enzymes appears to be inhibited in an environment of decreased endosomal pH [77,222]. These matters are considered in more detail in an excellent review by [223].

There is *in vivo* evidence, albeit from murine models, that hydroxychloroquine administration does indeed lead to the inhibition of SARS-CoV, dengue, Ebola and hand, foot and mouth disease viruses suggesting that the drug at least has the potential to inhibit the replication of these viruses in humans at the correct dosage and duration of therapy [134,222,224,225]. It is also reassuring to note that hydroxychloroquine has demonstrated success in inhibiting the replication of HIV in patients naïve to ARV therapy [226].

2.4. Potential side effects

2.4.1. Cardiovascular complications

Hydroxychloroquine inhibits the action of cardiac potassium channels, which may inhibit myocyte repolarisation and potentially lead to prolongation of the QT interval and arrhythmias such as the life-threatening torsade de pointes (TdP) [227,228]. However, several large retrospective studies investigating the use of hydroxychloroquine in the treatment of COVID-19 found no evidence of increased cardiovascular mortality or life-threatening arrhythmias in patients treated with the drug and reported that increased QT intervals were rarely clinically significant and warranting the discontinuation of therapy [73–76]. Perhaps most importantly, these results are supported by data reported by authors of a recent large RCT who also concluded that the

administration of hydroxychloroquine was not associated with any significant cardiovascular complications [71]. This latter finding is of particular note as the authors of the study used a loading dose of 2500 mg followed by 800 mg/day for a maximum of 10 days [71]. This is considerably higher than the maximum dose of 600 mg/day for 10 days which has been used by authors investigating the use of hydroxychloroquine in early COVID-19 [69,75].

In addition, two narrative reviews have concluded that the occurrence of hydroxychloroquine-associated cardiomyopathy is limited to seven reported cases over many thousands of patient-years of usage in the treatment of SLE and RA [229]. Furthermore, there is evidence to suggest that the phenomenon is influenced by dose and duration of therapy. A slightly higher incidence has been observed in those using hydroxychloroquine over 20 years of therapy (cumulative dose 2419 g) compared with 10 years' duration (cumulative dose 1542 g) [230]. In addition, large meta-analyses, involving almost 50,000 patients, have concluded that there is no evidence of increased instances of arrhythmia following the use of any quinoline derivative in the treatment of malaria [231,232].

2.4.2. Retinopathy

Recent narrative and systematic reviews have concluded that the risk of developing retinopathy with hydroxychloroquine at 5 mg/kg/day is considerably less than 1% irrespective of length of treatment [233,234]. Moreover, the risk of developing this side effect at the more conventional dose of 6 mg/kg/day is related to cumulative dose and duration [235]. In particular, the risk of developing retinopathy at a dose of 6 mg/kg/day over five years is approximately four in 1000, while the risk increases to approximately 1% after five to seven years of continuous therapy, as reviewed in [235]. Hence the risk of developing retinopathy over a relatively short course of treatment would appear to be minimal.

2.5. Consideration of dosage and duration of therapy

The full effect of hydroxychloroquine as an immunomodulator may take between three to six months to develop [236,237]. This is primarily because of the pharmacokinetics of the drug and the time required to saturate lysosomes [157,238], which would be expected given its mode of action and the need to accumulate in deep tissue [239]. The extensive concentration in tissues following hydroxychloroquine administration is reflected in an extremely large volume of distribution (44,257 L) and a very long elimination half-life of between 40 and 96 days [158,240]. Clearly these data suggest that achieving steady-state levels may take several months at a daily dose of 5 to 6.5 mg/kg [240].

In addition, a consideration of other pharmacokinetic parameters suggests that establishing an optimum dose likely to achieve therapeutic levels in most patients enrolled in a clinical trial is at best a complex exercise. For example, while there appears to be a modest correlation between dose and drug plateau levels [241], there is an extremely large interindividual variation in plasma levels following oral ingestion at any trialled dose, which may be as large as 100% [242,243]. This is problematic, as levels of undissociated drug in the plasma determine the distribution of hydroxychloroquine between this compartment and deep tissues which is the dominant factor in the performance of the drug [158]. This unpredictable relationship between dose and therapeutic levels is of major concern in relation to determining an effective dose for a trial as there is evidence to suggest that this feature is associated with the failure of malaria prophylaxis despite apparently adequate dosage and compliance [244].

However, it is encouraging to note that data from animal studies suggest that the relationship between dose and tissue uptake is not linear and that a threefold increase in the dose may lead to a twenty-fold increase in tissue levels during acute and chronic administration [157]. In addition, there is also evidence to suggest that increasing the

dose of hydroxychloroquine to 10 mg/kg could reduce the half-life to two weeks [157]. It is also encouraging to note that the rate of hydroxychloroquine distribution into deep tissues may be increased significantly via the use of a loading dose [245]. Clearly the weight of evidence argues for a high dose, a prolonged course of therapy and the use of a loading dose.

There is evidence to suggest that the efficacy of hydroxychloroquine as an immunomodulator and antiviral agent is dose and duration related. For example, a dose of 800 mg for eight weeks has been shown to reduce viral load in ARV-naïve HIV patients, while a dose of 200 mg appears to produce no effect [226] (reviewed in [246]).

Several research teams have attempted to predict the optimum dose and duration and the results of such studies are elegantly reviewed in [137]. Given the experience with ARV-naïve HIV patients, it is interesting to note that a recommended dose of at least 800 mg per day appears to be the view of the majority of these researchers [139,247]. Indeed, it has been suggested that a dose below that level is unlikely to have any effect in inhibiting the replication of SARS-CoV-2 [248]. In addition, a synthesis of evidence from these *in vivo* studies suggests that the optimal duration may be as long as 10 days [139,249]. It is also noteworthy that one research team concluded that the time needed to achieve steady-state in pulmonary tissue may be considerably longer [139].

However, establishing therapeutic levels *in vivo* is a different challenge from viral inhibition *in vitro*. This would seem to be particularly so in the case of hydroxychloroquine as a treatment of COVID-19 as there is evidence to suggest that uptake of the drug into lysosomes is inhibited in an environment of tissue and cellular inflammation and acidosis [250,251]. This is problematic in the course of a viral infection as both states are present in the intracellular and intercellular environment [252–254]. In addition, it is also worthy of note that the pH of endolysosomes and the ER-Golgi apparatus is maintained within homeostatic norms via the activity of the Na⁺-H⁺ exchanger isoform-1 (NHE-1) membrane pump [255]. This system may take some time to overcome and hence the pH-increasing capacity of hydroxychloroquine may be delayed and/or require a higher dose than predicted from *in vitro* experimentation. These factors further argue against the success of relatively low-dose and/or short courses. In fact, recent evidence from animal studies suggests that short courses of a few days are ineffective with regard to the inhibition of SARS-CoV-2 replication even at doses of 50 mg/kg [256,257]. Given a consideration of the factors discussed above and the presence of these data it would seem reasonable to suggest that any future planned trials using short courses of hydroxychloroquine should not take place.

3. Azithromycin

3.1. Immunomodulator

Several quasi-experimental studies and open-label RCTS have established that the macrolide azithromycin accumulates in macrophages, monocytes and neutrophils and rapidly reaches levels in these immune cells which are up to 2000 times greater than levels seen in plasma [258–261]. Importantly, there is *in vivo* evidence to suggest that azithromycin is an effective NF-κB inhibitor (see Fig. 1) and hence this property of accumulation within macrophages and neutrophils offers the prospect of highly localised immune modulation and minimising the risk of systemic immune suppression [262,263]. From the perspective of treating COVID-19 this is potentially a major therapeutic benefit as there is ample evidence that coronavirus infection results in activation of NF-κB [264–268].

The weight of evidence obtained from observational studies and RCTs suggests that the use of azithromycin therapy in a range of pulmonary and autoimmune illnesses results in increased M2 polarisation of activated alveolar and peripheral macrophages [269–273]. This is accompanied by improved efferocytosis [274] and phagocytosis

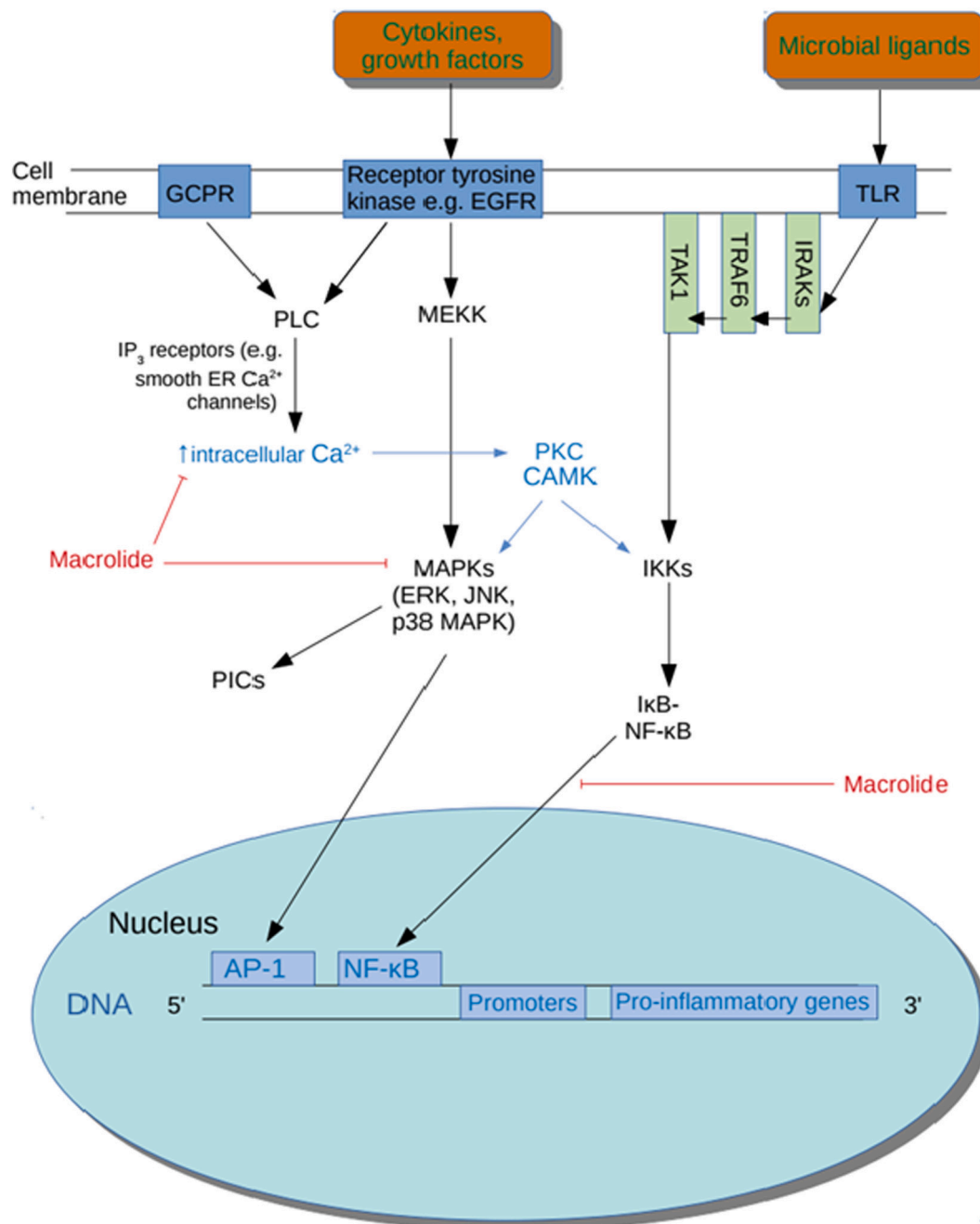


Fig. 1. Actions of macrolides.

[275–277]. These findings are of potential therapeutic relevance assuming that many of the elements driving the pathophysiology of sepsis-induced ARDS apply to COVID-19 as M1 polarisation-compromised phagocytosis and efferocytosis of alveolar macrophages play a pivotal role in the pathophysiology of that condition [36,38,278].

Prolonged azithromycin treatment also exerts a range of positive effects on neutrophils leading to improved function and decreased survival resulting in large decreases in the synthesis and release of PICs [279–282]. One mechanism involved in decreased neutrophil survival following azithromycin therapy involves the inhibition of granulocyte-macrophage-colony stimulating factor (GM-CSF) levels [283,284]. This may be a very important therapeutic benefit as elevated levels of GM-CSF are a major driver of extreme lung damage and increased mortality in many illnesses including ARDS and severe pneumonia [285,286]. In addition, several authors have reported reduced neutrophil infiltration

into the lungs and the amelioration of pre-existing neutrophilia in study participants following prolonged azithromycin therapy [287–290]. When viewed as a whole, the effects of azithromycin on neutrophils may be of major importance as increased neutrophil recruitment and impaired neutrophil apoptosis also play a major role in the pathogenesis of ARDS [278,291,292]. Finally, there is evidence to suggest that azithromycin inhibits the activity of the NLRP3 inflammasome and the subsequent release of IL-1 β and IL-18 [259,293]. This may also be a major advantage in a therapy aimed at the treatment of COVID-19 as the activation of this complex and cellular pyroptosis appears to play a major role in the early and later stages of COVID-19 and in the development of ARDS [38,294]. The main elements believed to be involved in the pathophysiology of ARDS are represented in Fig. 2.

There is also some evidence, albeit from retrospective studies, to suggest that azithromycin may reduce intensive care unit stay and 60-

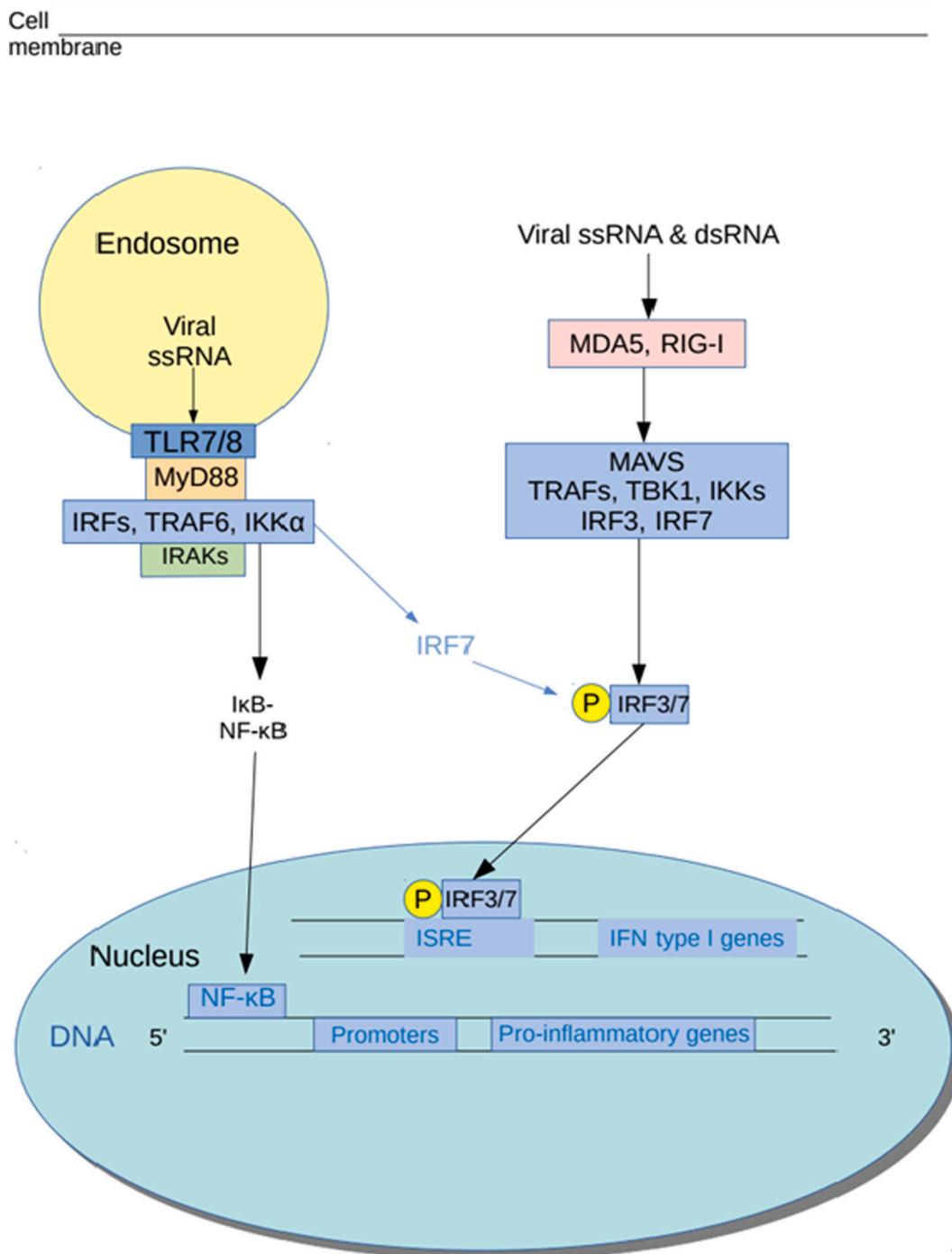


Fig. 2. Elements involved in the pathophysiology of ARDS.

day mortality in patients suffering from sepsis [295–297]. These findings are broadly supported by prospective studies examining the effects of azithromycin on survival using animal models of sepsis [298–300]. Moreover, the results from the latter studies strongly suggest that the improvements in mortality following azithromycin administration are related to immunomodulation rather than antibacterial effects [298,300].

3.2. Antiviral action

There is copious, albeit in vitro, evidence of direct antiviral effects of azithromycin (reviewed in [301]). Importantly, these effects have

been observed in upper airway cells [78,302,303] and appear to be achieved via the upregulation of type I and type III INF responses [78,304]. These are significant data as recent evidence suggests that interferon production is grossly suppressed in patients with severe COVID-19 [305]. In addition, the mechanisms underpinning these effects seem to involve increased transcription and activity of melanoma differentiation-associated protein 5 (MDA5) and retinoic acid-inducible gene I (RIG-1) [78,304]. This is of particular importance in suggesting an antiviral treatment for early COVID-19 as, in line with other pathogenic human coronaviruses, the truncated SARS-CoV-2 endonuclease inhibits MDA5 activity; MDA5 is the main pattern recognition receptor for coronavirus and thus this action delays $\text{INF}\gamma$

signalling [306–308]. There is also evidence of significant inhibition of autophagy in bronchial epithelial cells, suggesting another potential vehicle for synergy between azithromycin and hydroxychloroquine as antiviral agents [309–311]. Finally, it is encouraging to note that *in vitro* data suggest that a combination of azithromycin and hydroxychloroquine displays higher antiviral activity than either agent alone [312].

3.3. Safety considerations in the treatment of COVID-19

A large observational study by Ray and fellow workers reported that the use of azithromycin was associated with an approximately threefold increase in the risk of a fatal myocardial infarction compared with amoxicillin or placebo in patients with a high risk of developing CVD [313]. However, a meta-analysis of 12 RCTs involving the long-term use of azithromycin in a range of chronic conditions involving 15,558 patients concluded that the use of azithromycin was not associated with increased cardiovascular events [314]. These conclusions have been supported by a more recent meta-analysis of 33 observational studies and RCTs and data on 22,601,032 patients [315].

As previously discussed, large retrospective studies involving the combination of azithromycin and hydroxychloroquine for the treatment of COVID-19 reported no increase in cardiovascular complications in treated patients compared with untreated controls [74–76]. These findings are consistent with smaller observational studies examining the potential increased cardiovascular risk in patients prescribed a combination of azithromycin and hydroxychloroquine for the treatment of COVID-19 [316,317]. However, thus far there is no information regarding the safety of hydroxychloroquine and azithromycin in the cardiovascular arena so far as RCTs are concerned and hence there is a clear need for caution. In this regard, it should be noted that there are several published guidelines detailing protocols regarding matters such as baseline electrocardiography and cardiac monitoring for selecting appropriate patients and their subsequent management, from the perspective of mitigating against any potential cardiac pathology and maximising patient safety; these have been developed in anticipation of cardiovascular complications resulting from the use of hydroxychloroquine. Excellent examples of this approach have been produced by [227,318].

3.4. Consideration of dosage and duration of therapy

Azithromycin is unique within the macrolide class of antibiotics in being a weak diprotic base and a potent lysosomotropic compound [319,320]. The “trapping” of the drug within acidic lysosomes results in a massive increase in concentration in intracellular compartments in a similar manner to hydroxychloroquine and chloroquine [321,322], largely explaining the rapid accumulation into macrophages and neutrophils discussed above [323,324]. This property also leads to sustained drug levels in tissues [325], a comparatively large volume of distribution of 31 L/kg [326,327] and an elimination half-life of approximately five days [326]. In addition, high tissue levels persist for days following cessation of therapy [328,329].

The absorption and distribution of azithromycin into tissues are far more rapid than is the case for hydroxychloroquine and concentrations in tissues may be some 300-fold higher than in plasma [261]. Doubling the dose from 500 mg to 1000 mg shortens the half-life and produces a dramatic increase in the concentration of the drug in the lung [330–332].

The weight of evidence gleaned from animal and human studies strongly suggests that the immunomodulatory effects of azithromycin are related to dose and duration of therapy and would seem to be maximal at a dose of 100 mg/kg, although 20 mg/kg also results in immunomodulatory activity [298–300]. A synthesis of the data provided by these authors suggests that intravenous administration results in more rapid effects than oral administration [298,300].

Importantly, the results of an *in vivo* quasi-experimental prospective study investigating the effects of azithromycin on MDA5 activity suggest that the effective dose is 50 mg/kg, which in humans of average body mass would correspond to approximately 4 g daily [78]. More generally, there is *in vivo* evidence reporting that an inhibitory effect of azithromycin on influenza is achieved at a dose of 20 mg/kg [333]. In addition, the results of human RCTs examining the antiviral effects of azithromycin suggest that early administration is vital [334] and the macrolide may take two weeks to display antiviral, or indeed immunomodulatory, activity [335].

Finally, it should be emphasised that the potential therapeutic window for the delivery of an effective therapy aimed at inhibiting the replication of SARS-CoV-2 would appear to be extremely narrow. For example, thus far no research team has detected viable virus in any patient after nine days post-infection [336]. In addition, there is evidence to suggest that peak viral levels may occur within five days of infection [336,337] and potentially while many individuals are yet to display symptoms [338,339]. Moreover, a recent study reported viral levels in hospitalised COVID-19 patients were almost two orders of magnitude lower than those in COVID-19 patients in the community, further emphasising the need for early treatment [340].

4. Conclusion

It is concluded that the safety profiles of hydroxychloroquine and azithromycin are acceptable, either alone or in combination, as therapeutic agents aimed at preventing the development of severe COVID-19, although routine cardiac monitoring is clearly indicated. It is further concluded that hydroxychloroquine is a highly effective immune modulator in chronic usage but its slow onset of action may limit its usefulness as an immunomodulator in the treatment of COVID-19. Azithromycin, on the other hand, displays potential as an immunomodulator targeting many elements involved in the pathogenesis of COVID-19 if administered at a high dose early in the course of the illness. The potential synergy of these molecules as antiviral agents is intriguing and offers a rational therapeutic intervention. However, issues remain regarding optimal dosage and duration of treatment, but short courses and/or low doses are very unlikely to succeed. Finally, the narrow window of opportunity for therapeutic intervention must be emphasised and the greatest chance of success is probably confined to patients in early disease; future trials should be focused on patients in a community setting.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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