Title: Artesunate may attenuate the effects of Cytokine Release Syndrome (CRS) in Covid-19 Disease by targeting Interleukin-6 and associated inflammatory response pathways

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Introduction

The coronavirus disease 2019 (COVID-19) pandemic caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an unprecedented global health challenge for which safe, effective and affordable treatments need to be rapidly identified. COVID-19 is currently estimated to have a case fatality rate of 1-10% with different geographical locations globally reporting different mortality estimates¹. The reason for this is unclear at present, although the proportion of the general population tested, genetic factors, demographics and local healthcare capacity may all play a role. Drug repurposing research focusing on antimalarials such as chloroquine and hydroxychloroquine for COVID-19 disease has been of interest globally. However safety concerns, particularly in relation to potential cardiac toxicity in patients with severe COVID-19 have been raised and are currently being investigated. Another class of antimalarial drugs, artemisinins, have a good safety and tolerability profile and warrant further investigation as a possible safe, effective and affordable therapeutic for COVID-19 disease.

COVID-19 and Cytokine Release Syndrome

Similar to the Systemic Acute Respiratory Syndrome (SARS) virus, SARS-CoV-2 is a single-stranded RNA-enveloped virus that targets cells through the viral structural spike (S) protein that binds to the angiotensin-converting enzyme 2 (ACE2) receptor². ACE2 is a membrane-bound receptor richly expressed in the lungs, heart, kidney and gastrointestinal tract. Once bound to the ACE2 receptor, the virus then enters the host cell using host cell receptors and endosomes. TMPRSS2 is a host type 2 transmembrane serine protease that enables cell entry via the S protein. Viral proteins are then synthesized that encode for replicase-transcriptase that enables the virus to synthesize RNA via a RNA-dependent RNA polymerase. Structural proteins are then synthesized that result in viral particle assembly and release³.

In the majority of patients, the natural history of COVID-19 infection is self limiting with a pre- or asymptomatic phase that lasts a few days (Stage 1) followed by symptoms of fever, cough and systemic malaise. After a median period of 5 days, the disease progresses from upper to lower respiratory tract to become a viral pneumonia (Stage 2). Around day 7-10, detectable upper respiratory viral load decreases, antibody responses are generated and patients start to recover. However at 7-14 days, a small proportion of patients develop a cytokine release syndrome (CRS)/cytokine storm (CS), the hallmarks of which appear to be elevated host markers of inflammation (e.g. increased CRP, hyperferritinaemia, D-dimers) and lymphopenia⁴. Patients experiencing CRS often develop progressive respiratory failure (Stage 3) that can then lead to acute respiratory distress syndrome (ARDS) and multi-organ failure (MOF) (Stage 4).

Interleukin-6 (IL-6) is promptly and transiently produced in response to tissue injury and infection, stimulating acute phase responses, hematopoiesis, and immune reactions, which contribute to host defence⁵. However, in chronic inflammation and autoimmune diseases, dysregulated continual synthesis of IL-6 leads to significant pathophysiological effects. Apart from IL-6, other pro-inflammatory cytokines such as IL-1, interferon- γ and TNF are produced during CRS/CS and contribute to pathophysiological processes that result in MOF.

In patients admitted to Intensive Care Units, around 60-70% develop Acute Respiratory Distress Syndrome (ARDS), followed by shock (30%), myocardial dysfunction (20–30%) and acute kidney injury (10–30%)^{6,7,8,9,10,11}. In these critically ill patients, between 42-100% will require mechanical ventilation^{8,9,10,11,12}. Risk factors for developing severe COVID-19 disease include older age, male sex, co-morbidities (including chronic lung disease, cardiovascular disease, diabetes, obesity cancer and organ transplant). Genetic factors and ethnicity may also play a role¹⁴. In various clinical settings, MOF has been shown to correlate with an

increase in nosocomial infections and mortality. The exact mechanisms underlying MOF are complex and not fully elucidated, however systemic inflammation is a common underlying factor in many medical conditions that result in MOF. Several therapeutic agents have been proposed for the treatment of CRS including corticosteroids, intravenous immunoglobulin, selective cytokine blockade and Janus Kinase (JAK) inhibition¹⁵.

Tocilizumab is an anti-IL-6 antibody that binds membrane bound and soluble IL-6 receptors, blocking IL-6 from exerting its pro-inflammatory effects. It is currently licensed for the treatment of CRS related to CAR-T cell therapy and rheumatoid arthritis. In a retrospective uncontrolled study of 21 patients from China, tocilizumab was effective in the treatment of severe disease in patients with COVID-19 infection¹⁶.

We hypothesize that artesunate could also be another candidate drug for preventing and attenuating the effects of COVID-19 related CRS that often result in MOF. Artesunate has been consistently shown to suppress proinflammatory cytokines such as IL-6, IL-8, TNF- α , GM-CSF and IL-1 β via inhibition of the mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt and NF- κ B signaling pathways^{17,18,19}. These cytokines are key factors in mediating immune-inflammatory responses and cytokine response syndrome that can contribute to MOF.

Artesunate - an established antimalarial agent with a good tolerability and safety profile

Artesunate is from a family of sesquiterpene trioxane anti-malarial agents known as artemisinins, derived from Sweet wormwood (*Artemisia annua* L). Sweet wormwood (qinghao) has been used in traditional Chinese medicine for two millennia to treat fevers and inflammatory conditions. In the 1970s, as soldiers died in the Vietnam war of malaria, Ho Chi Minh contacted Chairman Mao, requesting China's assistance in researching and developing a new cure for malaria. 'Project 523' was born and Chinese scientist Prof Youyou Tu would go on to lead a team of scientists to identify artemisinins as being the active ingredient of qinghao. In 2015 she was awarded the Nobel Peace Prize in recognition of this discovery.

Artesunate is approved for the treatment of uncomplicated and multidrug-resistant malaria and is on the WHO list of Essential Medicines²⁰. Artesunate has a hemisuccinate group which confers substantial water-solubility and high oral bioavailability and therefore a convenient oral route of administration. Artesunate is extensively hydrolysed by plasma esterases and possibly CYP2A6. Its main metabolite, DHA is metabolised through glucuronidation. The plasma elimination half-life of artesunate is 3-29 minutes whilst its active metabolite DHA has a plasma elimination half-life of 40 to 95 minutes²⁰. Artesunate has an excellent safety and tolerability profile, having been used to treat tens of millions of adults and children globally. For artemisinin-based combination treatments for malaria, artesunate is generally combined with other compounds and notably with quinines (eg chloroquine, amodiaquine, mefloquine) with favourable efficacy and safety profiles. Being off patent it is also affordable at USD1 per dose and would be a feasible treatment particularly for patients in low and middle class income countries (LMICs).

Beyond its antimalarial effects, drug repurposing with artesunate has shown cytotoxic effects against viruses, fungi and a variety of cancers as well as powerful anti-inflammatory effects in animal models of asthma, sepsis, arthritis, pancreatitis, systemic lupus erythematosus and haemorrhagic shock^{21,22,23,24,25,26,27,28,29,30,31}.

Artesunate attenuates Interleukin-6 and associated inflammatory pathway

Several *in vitro* and *in vivo* models have demonstrated protective effects of artesunate against MOF in models of lung, myocardial and renal injury via modulation of key pro-inflammatory pathways. Severe COVID-19 disease results in the generation of pro-inflammatory cytokines

including IL-6 and IL-1b via the toll like receptors (TLR), resulting in the production of active mature IL-1b which is a mediator of fever, lung inflammation and fibrosis⁴. There is a correlation between IL-6 peak levels and severity of pulmonary complications.

Artesunate effects in a model of acute lung injury

In a rat model of liposaccharide (LPS)-induced lung injury, artesunate significantly inhibited the levels of IL-6, IL-1 β and TNF- α . TLR4 expression and NF- κ B activation was also attenuated by artesunate in a dose-dependent fashion. Artesunate upregulated the expression of Nrf2 and HO-1³². In another rat model, acute lung injury (ALI) was stimulated by renal ischemia reperfusion injury (RIR)³³. Artesunate inhibited RIR-stimulated lung inflammation by attenuating serum and pulmonary IL-6, MIP-2, PGE₂, NO and MDA levels and activating the HO-1 pathway.

Artesunate effects in a model of transient myocardial ischaemia

In a rat model of transient myocardial ischemia artesunate appeared to confer myocardial protection via activation of the PI3K/Akt/ERK 1/2 (RISK) pathway, inhibition of glycogen synthase kinase-3 β , inhibition of nuclear factor kappa B, activation of endothelial nitric oxide synthase, and activation of the STAT3 (SAFE) pathway³⁴.

Artesunate effects in a model of nephritis

In a rat model of nephritis, artesunate attenuated IL-6 levels, TNF- α , TGF- β 1, TLR4, and NF- κ B expression³⁵. Artesunate also ameliorated high glucose-induced injury in rat glomerular mesangial cells via suppression of the TLR4/NF- κ B/nod-like receptor protein 3 (NLRP3) inflammasome pathway³⁶. In a mouse lupus nephritis model, artesunate decreased levels of circulating IL-6, TNF- α and IFN- γ and blocked the NF- κ B signaling pathway²⁸.

Artesunate effects in a model of sepsis

In a sepsis model, artesunate inhibited lipopolysaccharide/endotoxin(LPS)-induced cytokine release³⁷. Artesunate inhibited IL-6 and TNF- α release from mouse bone marrow-derived monocytes (BMDMs), peritoneal macrophages (PMs) and RAW264.7 mouse cell line, induced by LPS. LPS is a well recognized pathogenic factor that induces sepsis and has been researched as potential drug target in models of sepsis. The toll-like receptor 4 (TLR4) is utilized by innate immune system monocytes/macrophages to recognize LPS. This then triggers activation of tumor necrosis factor receptor-associated factor 6 (TRAF6) which in turn activates nuclear factor kappa B (NF- κ B). NF- κ B is known to promote the release of downstream pro-inflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- α).

Artesunate effects in a model of haemorrhagic shock induced multi-organ failure

In a rat model of haemorrhagic shock (HS), artesunate attenuated the expression of pro-inflammatory proteins IL6, TNF- α , NF- κ B and nitric oxide synthase. This appeared to be protective against HS induced organ failure³¹. Pathway analysis of RNAseq provided evidence to support an effect of artesunate on the Akt-survival pathway, resulting in IRAK1 down-regulation. Treating HS-rats with artesunate enhanced the phosphorylation (activation) of eNOS and Akt as well as the phosphorylation (inhibition) of glycogen synthase kinase-3 β (GSK- 3 β), confirmed through Western blot analysis. Akt activation has been linked to the prevention of sepsis-induced kidney injury²⁵, sepsis-induced cardiac dysfunction³⁸ and

ventilation induced lung injury³⁹. Akt activation results in phosphorylation and activation of eNOS at Ser117 which then enhances the formation of small amounts of NO which is pivotal for microvascular perfusion preservation, which is preventative against organ injury and multi organ failure. Akt activation has also been associated with increased phosphorylation of GSK-3 β . GSK-3 β is a serine/threonine protein kinase that mediates the addition of phosphate molecules onto serine and threonine amino acid residues and is a kinase for a large number of different proteins involved in a variety of different cellular pathways including cell signaling and cellular transport⁴⁰. GSK-3 β has been shown to regulate immune and migratory processes as well as participating in the innate immune response thorough a number of signaling pathways including pro-inflammatory cytokine and interleukin production^{41,42}.

Discussion

The hallmark of severe COVID-19 infection is a severe inflammatory response caused by a cytokine release syndrome of which pro-inflammatory cytokine IL-6 is a key component. Respiratory distress syndrome, myocarditis and renal failure are CRS/CS sequelae with devastating effects. Artesunate is able to modulate pro-inflammatory cytokine pathways and in doing so prevent multi-organ failure in models of lung, myocardial and renal injury. A safe and affordable treatment that could prevent patients with mild or moderate COVID-19 infection progressing to severe infection as a result of CRS/CS would represent a significant breakthrough globally. Robust clinical trials are indicated to test this hypothesis.

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