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Diacerein: A potential multi-target therapeutic drug for COVID-19

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ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes coronavirus disease 19 (COVID-19), was declared pandemic by the World Health Organization in March 2020. SARS-CoV-2 binds its host cell receptor, angiotensin-converting enzyme 2 (ACE2), through the viral spike (S) protein. The mortality related to severe acute respiratory distress syndrome (ARDS) and multi-organ failure in COVID-19 patients has been suggested to be connected with cytokine storm syndrome (CSS), an excessive immune response that severely damages healthy lung tissue. In addition, cardiac symptoms, including fulminant myocarditis, are frequent in patients in a severe state of illness. Diacerein (DAR) is an anthraquinone derivative drug whose active metabolite is rhein. Different studies have shown that this compound inhibits the IL-1, IL-2, IL-6, IL-8, IL-12, IL-18, TNF-α, NF-κB and NALP3 inflammasome pathways. The antiviral activity of rhein has also been documented. This metabolite prevents hepatitis B virus (HBV) replication and influenza A virus (IAV) adsorption and replication through mechanisms involving regulation of oxidative stress and alterations of the TLR4, Akt, MAPK, and NF-κB signalling pathways. Importantly, rhein inhibits the interaction between the SARS-CoV S protein and ACE2 in a dose-dependent manner, suggesting rhein as a potential therapeutic agent for the treatment of SARS-CoV infection. Based on these findings, we hypothesize that DAR is a multi-target drug useful for COVID-19 treatment. This anthraquinone may control hyperinflammatory conditions by multi-faceted cytokine inhibition and by reducing viral infection.

Introduction

The first case of coronavirus disease 19 (COVID-19), a condition caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in the city of Wuhan in Hubei Province, China, on December 2019. Due to its incubation time (1–14 days) and because it is highly contagious over a short time, infection with SARS-CoV-2 has traversed global borders and caused thousands of deaths, mainly in elderly patients. On March 11th, 2020, the World Health Organization declared COVID-19 a pandemic disease. Even though some infected persons are asymptomatic, SARS-Cov-2 infection can cause severe symptoms, including high fever, severe cough and shortness of breath, which often indicates pneumonia. Severe acute respiratory distress syndrome (ARDS) has been identified as the leading cause of COVID-19-associated mortality. In addition, sepsis, acute cardiac injury, and fulminant myocarditis are common critical complications [1,2].

The mortality related to ARDS as well as multi-organ failure in COVID-19-infected patients may be connected with cytokine storm syndrome (CSS), an excessive immune response that severely damages healthy lung tissue [3]. This response may lead to macrophage activation syndrome (MAS) [4,5] or secondary haemophagocytic lymphohistiocytosis (sHLH) with fulminant and fatal hypercytokinaemia [3,6]. In this scenario, a dilemma emerges due to the potential deleterious effects of immunosuppressive agents used to treat hyperinflammation, such as corticosteroids and Janus kinase (JAK) inhibitors [3], on antimicrobial immunity [7].

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Diacerein (DAR), also called diacetylrhein, is an anthraquinone derivative used as a symptomatic slow-acting drug for the management of osteoarthritis (SYSADOA) [8–12] licensed in countries of the European Union, Latin America and Asia for up to 20 years. The drug is administered orally and entirely converted to its active metabolite, rhein, before reaching the systemic circulation [8,12]. The main mechanism of action of DAR is inhibition of the interleukin-1 (IL-1) signalling pathway [8–10,12–20]. In addition, several studies have described the inhibitory effect of this compound on IL-6 [16,20–24] and TNF-α [16,21,25,26]. However, no reported studies have addressed the potential therapeutic use of DAR in patients with COVID-19. Considering the current global health crisis caused by COVID-19, the search for therapeutic alternatives is mandatory. The use of drugs with established mechanisms of action has emerged as a valid tool to identify compounds that can contribute to managing this disease.

The hypothesis

SARS-CoV-2 recognizes and binds its host receptor, angiotensin-converting enzyme 2 (ACE2), through the viral spike (S) protein. This binding occurs through the S1 domain of the viral protein, while the S2 domain is responsible for fusion of the viral envelope to the cell membrane [27–29]. Therefore, the level and pattern of human ACE2 expression in different tissues might be critical for differences in susceptibility, symptoms and outcome between different individuals [30]. In addition, it is important to consider that the expression of ACE2, including its related polymorphisms, can differ in the population [30–32]. This enzyme is mainly expressed in endothelial cells of the blood vessels but is also present in other organs, including the heart, lungs and kidneys. This may explain the extra-pulmonary manifestations of the disease as well as some morbidity and mortality factors. At the cellular level, pyroptosis-mediated cell death is strongly involved in the pathophysiology of severe forms of infection with coronaviruses, such as SARS-CoV, MERS-CoV and SARS-CoV-2 (COVID-19), in humans [33]. Oedema and cell membrane rupture are triggered by activation of the NLRP3 inflammasome through the direct action of transmembrane pore-forming viral proteins, such as viroporin 3a. This stimulates the production of gasdermin D (GSDMD) and IL-1β and IL-18 [34]. In addition, cell damage induced by SARS-CoV-2 exposes adjacent cells to damage-associated molecular patterns (DAMPs), activating the NLRP3 inflammasome [35].

The hyperinflammation unleashed during COVID-19 can drive severity [3]. In addition to pulmonary manifestations, CSS-related myocarditis and heart failure should also be considered as important causes of death. The pathophysiology of sHLH and MAS resulting from CSS is associated with elevated levels of pro-inflammatory cytokines, such as IL-1 [4,5,22,36–40], IL-2 [40–42], IL-6 [3–5,36–40], IL-7 [40,41], IL-8 [37,39,40], IL-12 [4,5,37,43], IL-18 [4,5,39], IFNγ [4,5,38,39], and TNF-α [4,5,36,37,40–42]. Interestingly, some suppressor cytokines, such as IL-37 and IL-38, have been suggested as new targets to control inflammation in COVID-19 by inhibiting IL-1β and other pro-inflammatory interleukins [36].

Regarding cardiovascular complications, the dysfunction of endothelial cells induced by some infections results in excessive thrombin generation and fibrinolysis shutdown. This indicates the existence of a hypercoagulable state caused by some infectious diseases [44,45], including COVID-19 [46]. In addition, hypoxia and hypoxia-inducible transcription factor (HIF) activation could increase the expression of coagulant factors and integrins, which promote thrombus formation and stimulate the formation of pro-thrombotic neutrophil extracellular traps [47].

Current efforts for the development of novel preventive and therapeutic strategies for COVID-19 include trials for the production of vaccines [3], licensed antivirals repositioning [3,48], the search for new antivirals and the use of chloroquine and hydroxychloroquine alone or with azithromycin [40,48–58]. Regarding CSS, including that caused by SARS-CoV-2, the therapeutic strategies under study include the use of corticosteroids [3,37,38,59], IL-1 blockade [3,37,38,40], IL-6 blockade [3,37,38], TNF blockade/inhibition [37,38,59] and JAK inhibition [3,37,38,40]. Some of the strategies mentioned, such as the use of vaccines and monoclonal antibodies (MAB), focus on specific targets. On the other hand, other approaches, such as the use of corticoids, present a more nonspecific mechanism of action. It is clear from the data described above that several therapeutic alternatives are under examination. However, at present, no drug for COVID-19 management has been explored from a multi-target perspective.

The active metabolite of DAR, an anthraquinone, is rhein [8,12]. Different studies have demonstrated that the main mechanism of action of DAR is the inhibition of IL-1β production [15,17]. In addition, this drug reduced the number IL-1 receptor (IL-1R) in chondrocytes [15], induced the upregulation of IL-1 receptor agonist (IL-1ra) in cartilage culture [17], and prevented IL-1-induced Nuclear Factor-κB (NF-κB) activation by inhibiting the degradation of its main inhibitor, IκBα [18]. Moreover, rhein was shown to reduce the production of IL-1 converting enzyme (ICE) in cartilage, leading to a reduction in activation of IL-1β from its inactive (pro-IL-1) form [19]. Recently, Chang et al. [60] demonstrated that rhein suppressed caspase-1 protease activity and IL-1β production by interfering with formation of the NLRP3 multi-protein complex. In addition, it may exert its anti-inflammatory action through inhibition of the NF-κB and NALP3 inflammasome pathways [61,62]. Based on these mechanisms of action, rhein could suppress lung inflammatory injury induced by human respiratory syncytial virus in mice [63]. Finally, the inhibitory effect of this molecule on IL-6 [20–24,64,65] and TNF-α [21,25,26,65] has been reported.

The results from several studies conducted using different experimental approaches suggest that rhein also exerts an inhibitory effect on IL-2 [66], IL-8 [20,65,67,68], IL-12 [66,69] and IL-18 [19,63]. Even though we were unable to find studies addressing the effect of rhein on IL-7, one report showed that chrysophanol, another anthraquinone compound, could reduce the expression of IL-7 receptor α [70].

Different studies have shown that anthraquinones may inhibit the replication of different viruses. For instance, Sun et al. [71] studied the inhibition of hepatitis B virus (HBV) replication by *Rheum palmatum* ethanol extract in a stable HBV-producing cell line. Although the focus of the study was on *R. palmatum* extract (RPE), emodin and rhein also showed inhibitory activity against the replication of HBV DNA. Interestingly, at high concentrations, rhein showed higher activity than emodin [71]. Another study showed that rhein significantly inhibited influenza A virus (IAV) adsorption and replication [72]. The authors also observed that rhein decreased IAV-induced oxidative stress and downregulated activation of the TLR4, Akt, p38, JNK MAPK, and NF-κB-mediated signalling pathways and the production of inflammatory cytokines and matrix metalloproteinases *in vitro*.

Studies conducted by different laboratories indicate that anthraquinones may inhibit coronavirus activity by targeting different steps of the infection process. First, a study performed by Ho et al. [73] showed that emodin, an anthraquinone compound derived from plants of the genus *Rheum* and *Polygonum*, significantly blocked the interaction between the S protein of SARS-CoV and ACE2 in a dose-dependent manner. In addition, this compound inhibited the infectivity of S protein-pseudotyped retrovirus in Vero E6 cells. Considering this, the authors suggested emodin as a potential therapeutic agent for the treatment of SARS-CoV infections. In addition, preincubation of rhein with biotinylated S protein slightly inhibited the interaction between the S protein and ACE2 in a dose-dependent manner [73]. Second, a study conducted by Simmons et al. [74] showed that proteolysis of the viral spike S protein by cathepsin L within endosomes is a critical step in SARS-CoV infection [74]. Interestingly, many cathepsin L inhibitors can inhibit cathepsins B and H [75], which can be explained by the high degree of sequence homology between these proteins [76]. In addition, Savarino et al. [77] demonstrated the effect of rhein on the activity of the human liver enzyme cathepsin B *in vitro*. Overall, these observations

prompted us to investigate the potential effect of rhein on cathepsin L and on SARS-CoV-2 cell infection.

Finally, alterations in coagulation parameters are associated with a worse prognosis in patients with COVID-19-associated pneumonia [78–80]. Interestingly, rhein was able to protect myocardial H9c2 cells against hypoxia/reoxygenation-induced injury via the AKT/GSK3β/p38 pathway [81]. In addition, many anthraquinones inhibit platelet aggregation [82]. For example, chrysophanol-8-O-glucoside, an anthraquinone derivative in rhubarb, exerts anti-platelet and anti-coagulant activities by inhibiting platelet phosphatidylserine exposure and prolonging the activated partial thromboplastin time. This molecule did not alter prothrombin time or directly inhibit intrinsic factors [83]. Moreover, another study performed with obtusifolin and aurantio-obtusin suggested that these molecules are strong thrombin inhibitors [84]. The data described raise the possibility that rhein, as an anthraquinone, may exhibit similar anti-coagulant effects. This hypothesis warrants further research.

Based on these findings, we hypothesize that DAR is a multi-target drug useful for COVID-19 treatment. This drug may have an impact on viral infection and disease onset, progression and outcome by controlling hyperinflammatory conditions through multi-faceted cytokine inhibition and potentially acting on viral cell infection.

Evaluation of the hypothesis

Effect on pro-inflammatory and anti-inflammatory cytokines

Monolayer cultures of lung, cardiac, endothelial and epidermal cells will be maintained under standard cell culture conditions. The different cell lines will be seeded in 24-well plates and infected with SARS-CoV-2 at different multiplicity of infection (MOI) values. After 48 h, cell cultures will be treated with rhein at different concentrations (1 μM to 400 μM) for 3, 6, 12, 24, 48 and 72 h. Production of the pro-inflammatory and anti-inflammatory cytokines IL-1b, IL-2, IL-6, IL-7, IL-8, IL-10, IL-12, IL-18, IL-37, IL-38 and TNF-α in cell extracts and the supernatants and whole-cell extracts of the cell cultures described will be determined by Western blotting, flow cytometry, Luminex assay and enzyme-linked immunosorbent assay (ELISA). Additionally, release of the damage-associated molecules APT and HMGB-1 in the cell culture supernatants will be measured by ELISA. The expression and function of the NACHT, LRR and PYD domains-containing protein (NALP3) inflammasome in the cell lines treated with rhein will be analysed by Western blotting and RT-polymerase chain reaction (PCR). All the experiments will be performed in triplicate. Moreover, the levels of activated caspase-1 and HMGB1 will be tested.

Effect of rhein on SARS-CoV-2 infection

The effect of rhein-mediated inhibition of the interaction between the S protein and ACE2 on SARS-CoV-2 will be evaluated as described by Ho et al. [73]. The method will consist of purification and biotinylation of recombinant SARS-CoV-2 S protein, biotinylated ELISA, immunofluorescence assay (IFA), and the infection of Vero cells and lung-, cardiac-, endothelial- and epidermal-derived cell lines with S protein-pseudotyped retrovirus. Cell viability will be evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The study will be performed in the presence of rhein at varying concentrations (1 μM-400 μM).

In vivo effect of rhein treatment on virus-induced respiratory syndrome in mice

C57 Black/6 (7 to 9 weeks old) mice will be infected with the mouse-adapted SARS-CoV-2 MA15 strain [85]. For infection, mice will be intranasally inoculated with 1×10^4 PFU mouse-adapted SARS-CoV-2 in 50 μl of PBS. Treatment of 3 experimental groups and equivalent control and placebo-treated groups with rhein (1 μM to 400 μM) will start at 2, 6 or 12 h after viral inoculation. Body weight and pulmonary function (using a single-chamber, whole-body plethysmograph (Buxco Eletronics Inc., NC, USA)) will be assessed daily until the 5th day post-inoculation. At day 5, the blood, lungs, heart, kidneys and peripheral lymph nodes will be harvested after euthanasia. The lungs will be scored for haemorrhage, and the inferior right lobe will be used for viral load determination by Vero E6 cell infection according to the protocol described by Sheahan et al. [86]. For lung damage measurements, the lungs will be fixed and embedded in paraffin. Scores will be given according to the American Thoracic Society scoring tool, which takes into consideration neutrophil infiltration in the alveolar space, neutrophils in the interstitial spaces, hyaline membranes, proteinaceous debris in air spaces, and alveolar septal thickening. Moreover, necrosis, haemorrhagic areas and cellular sloughing will be examined. The hearts and kidneys will also be fixed and embedded for histology to search for necrosis and haemorrhagic areas. The lymph nodes will be mechanically dissociated into single-cell suspensions. Cells will be labelled with Proliferation Cell Dye (BD Biosciences, Carlsbad, CA) and stimulated with 10 mg/ml TPA and 1 μg/ml ionomycin for 4 days. After that period, cell culture supernatants will be harvested for cytokine secretion determination (Th1/Th2/Th17 CBA kit, BD Biosciences, Carlsbad, CA), and cells will be harvested and labelled with anti-CD4, anti-CD8, anti-CD19 and anti-CD25 to determine the proliferation of specific populations. Serum isolated from the blood will be used to determine the cytokine concentration by Luminex and the lactate concentration to evaluate acidosis using a colorimetric assay (Merck/Sigma).

Effect of rhein on SARS-CoV-2 replication

The effect of rhein on SARS-CoV-2 replication will be evaluated as described by Sun et al. [71] with modifications. Briefly, the different cell lines described above will be infected with SARS-CoV-2 virions and cultured in the presence of rhein at different concentrations. After 72 h, the effect of rhein on viral replication will be determined by measuring viral load by quantitative real-time PCR. The expression levels of SARS-CoV-2 surface antigens S and E will be determined by ELISA and a virus neutralization test (VNT) after rhein treatment. Rhein will be tested under the conditions described above. The half-maximal inhibitory concentration (IC50) will be calculated.

Effect of rhein on redox state and major cell signalling pathways in SARS-CoV-2-infected cells

Total extracts from monolayer cell cultures infected with SARS-CoV-2 and treated with rhein under the conditions described above will be analysed using commercially available protein arrays to determine the levels and activation state of proteins involved in the TLR-, Akt-, MAPK-, and NF-κB-regulated signalling pathways. Data will be analysed using ImageJ software. The levels of reactive oxygen species (ROS)/reactive nitrogen species (RNS) will be determined in the total cell extracts and supernatants from monolayer cell cultures infected with SARS-CoV-2 and treated with rhein under the conditions described above.

Anti-platelet aggregation and anti-coagulation activity

In vitro and in vivo platelet aggregation studies will be performed following a previously described method [83]. Platelet-rich plasma (PRP) will be preincubated with rhein at different concentrations for 5 min in the cuvette of an aggregometer before stimulation with aggregating agents (collagen, 2 μg/mL; thrombin, 0.4 U/mL; AA, 100 μM; or ADP, 10 μM). Platelet aggregation will be recorded for 5 min after agonist addition. For in vivo evaluation, the effects of DAR at different concentrations (3, 10, 25, 50 mg/kg) will be compared with those of

aspirin (50 mg/kg). Two hours after oral administration of the drugs, rat blood samples were collected, and platelet aggregation will be monitored as described for the *in vitro* test. The anti-coagulation activity of rhein will be evaluated by measuring plasma clotting times as previously described [87]. Prothrombin time (PT) and activated partial thromboplastin time (aPTT) will be measured using an Automated Coagulation Laboratory 100 instrument (Instrumentation Laboratory Co.) with platelet-poor plasma (PPP).

Epidemiologic profiles of DAR users

DAR is a symptomatic slow-acting drug for the management of osteoarthritis (SYSADOA), and products based on DAR have been available in the European Union, Latin American and Asian countries for up to 20 years. The optimal dosage is 100 mg/day, administered orally. Considering that age is a risk factor for both diseases, it is very probable that some people infected with SARS-CoV-2 have osteoarthritis and, because of this, eventually use DAR for osteoarthritis management. Thus, prospective and retrospective cohort studies, including other non-interventional studies, constitute a very important tool to test our hypothesis in humans and, based on their results, accelerate the translational development of this potential therapeutic alternative for COVID-19.

Discussion and conclusion

Considering the data presented above, we hypothesize that DAR is a multi-target drug useful for COVID-19 treatment. The mechanisms of action involved include the control of hyperinflammatory conditions by multi-faceted cytokine inhibition of IL-1, IL-2, IL-6, IL-8, IL-12, IL-18 and TNF-α; anti-platelet aggregation activity; and potential effects on viral infection and replication.

Conflict of interest statement

Pedro Gonçalves de Oliveira is responsible for R&D activities at TRB Pharma Indústria Química e Farmacêutica Ltda. TRB Pharma is the owner of the product ARTRODAR®, a diacerein-based product for osteoarthritis treatment.

The other authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mehy.2020.109920.

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