Diminazene for COVID-19: a missed opportunity?

Angiotensin Converting Enzyme 2 (ACE2) is the receptor the new coronavirus SARS-CoV-2 binds to for cell entry. ACE2 is also an important player in the Renin Angiotensin System (RAS). ACE2 and RAS appears to be central in coronavirus disease (Covid-19) pathophysiology. Covid-19 might be regarded as a virus-induced "RAS disorder". Drugs acting upon RAS components may offer potential therapeutic value in Covid-19. The authors advocate diminazene aceturate (DIZE), a putative ACE2 activator, as a feasible treatment option unduly disregarded *a priori*, likely due to lack of licensing for human use. However, DIZE has been extensively employed in African trypanosomiasis patients with little toxicity reported. With the current pandemic causing health issues on a large scale worldwide, the properties of DIZE remain to be investigated in Covid-19. A drug that is available, inexpensive and with a long track record of safe usage should be considered, particularly while effective alternatives remain scarce.

Introduction

The Renin Angiotensin System (RAS) is an interlinked system considered crucial for adjusting sodium, body fluid volume and maintaining blood pressure, as well as the homoeostasis of several organs. It consists of two opposing axis that counterbalance one another: classical and non-classical or alternative pathway. The classical RAS comprises a cascade of proteolytic enzymes and peptides that leads to the conversion of angiotensin I (AngI) to angiotensin II (AngII), a potent vasoconstrictor, mediated by angiotensin-converting enzyme (ACE). Conversely, the alternative RAS leads to the production of angiotensin (1-7) (Ang(1-7)), a vasodilator, by the action of angiotensin-converting enzyme 2 (ACE2). The effects of AngII mainly result from the stimulation of angiotensin type 1 receptor (AT1R) whereas Ang(1-7) exerts its actions primarily upon stimulation of the Mas oncogene receptor (MasR) (Santos et al., 2018; Bourgonje et al., 2020; Braga et al., 2020; Gheblawi et al., 2020; Verdecchia et al., 2020). Ang-II also combines with AT2R to antagonize Ang-II/AT1R effect, hereby resulting functionally similar to MasR (Wang et al., 2019) (Figure 1). Local RAS is present in numerous tissues and organs including respiratory system (tracheal and bronchial epithelial cells, type 2 pneumocytes, macrophages), heart (endothelium of coronary arteries, myocytes, fibroblasts, epicardial adipocytes), vessels (vascular endothelial and smooth cells), gut (intestinal epithelial cells), kidney (luminal surface of tubular epithelial cells), liver, testes and brain (Hamming et al., 2004; Bourgonje et al., 2020; Nitulescu et al., 2020; Verdecchia et al., 2020). ACE2 is mostly bound to cell membranes as a transmembrane protein and only scarcely present in the circulation in a soluble form (Santos et al., 2018; Gheblawi et al., 2020). RAS dysregulation is involved in lung disease, atherosclerosis and cardiovascular disease, inflammation, renal disfunction and even cancer growth (Qaradakhi et al., 2020). An in-depth description of the RAS escapes the purposes of this paper, for which the authors refer to the excellent reviews available.

It is now well known that, similar to SARS-CoV, SARS- CoV-2 uses the ACE2 receptor for cell invasion (Walls et al., 2020; Yan et al., 2020). It does so through interaction of the virus spike protein (S protein) with the ACE2 receptor, which leads to a conformational change that allows the fusion between the viral and cellular membrane, with subsequent entry of the virus into cell, release of its content, replication, and infection

of other cells. As a result, ACE2 downregulation is produced, thereby causing an imbalance between the ACE2/Ang(1-7)/MasR axis, which results attenuated, and the ACE/AngII/AT1 which remains relatively unopposed (Santos et al., 2018; Bourgonje et al., 2020; Braga et al., 2020; Gheblawi et al., 2020; Verdecchia et al., 2020) . Many of the pathogenetic mechanisms and clinical manifestations of Covid-19 may be explained as the direct or indirect effects of this disbalance, thus making RAS a potential therapeutic target (Bourgonje et al., 2020; Braga et al., 2020; D'Ardes et al., 2020; Gheblawi et al., 2020; Zhang et al., 2020a). The veterinary antiparasitic drug DIZE has been studied as an ACE2 activator in animal models and tissues, offering promising properties for the treatment of acute lung injury (ALI), acute respiratory distress syndrome (ARDS), hyperinflammation, pulmonary hypertension and cardiac dysfunction (Haber et al., 2014; Velkoska et al., 2016a). These properties confer DIZE an excellent profile for potential benefit in Covid-19 which, as far as the authors are aware, at present remains unexplored. Albeit DIZE has never been approved for human use, literature shows that for decades it was extensively used in Africa for human trypanosomiasis with a good overall toxicity profile. The cost of licensing and poor financial prospects, rather than harmful side effects, appears the most likely explanation for the lack of marketing authorization (Pépin and Milord, 1994; Burri et al., 2004; Bacchi, 2009). We therefore would like to propose DIZE as a potential therapeutic alternative for severe Covid-19.

Table 1 and figures 1 and 2 summarize the pharmacological activities of DIZE

DIZE, ACE2 and Acute Lung Injury/ARDS

ACE2 is widely expressed on the pulmonary endothelium, type II alveolar epithelial and smooth muscle cells of the lungs (Santos et al., 2018; Gheblawi et al., 2020; Verdecchia et al., 2020). There is a consensus that ACE2 protects from acute lung injury secondary to different causes (Kuba et al., 2006; Imai et al., 2008; Wösten-van Asperen et al., 2011; Gu et al., 2016; Zhang and Baker, 2017; Wang et al., 2019, 2020) as well as against other pulmonary pathologies (Kuba et al., 2006; Kaparianos and Argyropoulou, 2011) . Its primary function is to metabolize Ang II to Ang(1-7), an heptapeptide that binds to the MasR to exert vasodilatory, antiproliferative, and antifibrotic actions in the pulmonary system (Santos et al., 2018; Gheblawi et al., 2020; Liu et al., 2020a; Verdecchia et al., 2020) . MasR are expressed on the surface of bronchial smooth muscle and alveolar epithelium (Verdecchia et al., 2020). In acute lung injury, ACE, AngII, and the AT1R are related to lung damage (Imai et al., 2005; Kuba et al., 2005; Santos et al., 2018; Bourgonje et al., 2020; Gheblawi et al., 2020). ACE2, on the contrary, exerts antagonist effects and is key for protection from ALI and ARDS. The basic mechanism for lung injury would be as follows: Ang II, via AT1R, produces pulmonary vasoconstriction (for example, against hypoxia) that can result in pulmonary edema. AngII mediated AT1R stimulation progressively leads to endothelial damage, increased vascular permeability, respiratory tree edema, alveolar wall thickening, an inflammatory cascade mediated by several cytokines and infiltrates of inflammatory cells (Imai et al., 2005; Santos et al., 2018; Bourgonje et al., 2020; Gheblawi et al., 2020). The ACE2 downregulation caused by coronaviruses and other etiologies, together with the subsequent decrease in its protective action, would

further increase the unbalance in favor of the ACE/AngII/AT1 arm of the RAS, thereby playing an important role in the pathogenesis and progression of ARDS (Imai et al., 2008; Shenoy et al., 2010) .

Consistent with the above, in respiratory syncytial virus infected mice, ACE2 deficiency increases the severity of the condition whereas treatment with recombinant ACE2 reduces it(Gu et al., 2016). Something similar occurs with other respiratory viruses (Zou et al., 2014). The protective role of ACE2 against lung injury has been further supported by other studies carried out on ACE2-deficient animals. Knockout mice for ACE2 displayed enhanced vascular permeability, increased lung edema and neutrophil accumulation in experimental models of ARDS, and pulmonary function also improved with recombinant ACE2(Imai et al., 2005). Hyperoxia-induced damaged was inhibited by a MasR agonist in human cell cultures (Abdul-Hafez et al., 2019). Likewise, administration of Ang(1-7) conferred beneficial effects against different models of ALI, effects that were abolished by blocking the Mas receptor(Klein et al., 2013). When intervening upon the ACE/AngII/AT1R arm, blocking the AT1R also attenuated the pulmonary lesions induced by the isolated spike viral protein of the SARS-CoV(Kuba et al., 2005), supporting once more the crucial role of RAS. This last model has the merit of investigating the impact of ACE2 downregulation in the absence of confounding effects of viral invasion and replication (Verdecchia et al., 2020). Hence, it is likely that the decrease in ACE2 activity caused by SARS-CoV-2 unleashes a cascade of deleterious effects that impacts disease progression and clinical worsening(Kuba et al., 2005; Wang et al., 2020)**.**

RAS dysregulation has been found to be involved in lung fibrosis and in pulmonary hypertension. In lung fibrosis, treatment of ACE2 knockout animals with rhACE2 attenuated bleomycin-induced lung fibrosis and improved survival, exercise capacity and lung function in mice (Rey-Parra et al., 2012). Local generation of AngII is required for the pathogenesis of experimental pulmonary fibrosis and both lung ACE-2 mRNA and enzyme activity has been found markedly decreased in lung biopsy specimens from patients with idiopathic pulmonary fibrosis as well as experimental lung fibrosis (Li et al., 2008). Together, these data show that ACE-2 is severely downregulated in both human and experimental lung fibrosis, suggesting that ACE-2 protects against lung fibrogenesis by limiting the local accumulation of the profibrotic Ang II (Li et al., 2008; Meng et al., 2014). Regarding pulmonary hypertension, decreased plasma ACE2 enzymatic activity has been found both in patients with pulmonary hypertension and animal models of pulmonary hypertension (Dai et al., 2013; Hemnes et al., 2018) . In patients with idiopathic pulmonary hypertension, increased lung ACE activity resulted in augmented AngII production by pulmonary endothelial cells together with a marked increase of AT1R within the walls of distal pulmonary arteries due to smooth muscle cell proliferation. In these patients, increased systemic levels of renin, AngI, and AngII were found to be associated with disease progression and mortality (de Man et al., 2012). Conversely, overexpression of ACE2 and Ang(1-7) rendered favorable effects in experimental models (Yamazato et al., 2009; Shenoy et al., 2010).

Interestingly, high plasma levels of AngII are also associated with increased severity and mortality in infections caused by respiratory viruses (Shenoy et al., 2013; Zou et al., 2014; Gu et al., 2016), including SARS- Cov-2 (Liu et al., 2020c). A parallel imbalance

between ACE and ACE2 has also been found in bronchoalveolar lavage fluid of both patients and experimental animals with ARDS (Wösten-van Asperen et al., 2011). In influenza A (H7N9) virus infected patients, plasma Ang II levels measured at different timepoints revealed increasingly higher concentrations until peak values were detected just before fatal outcomes; concurrently, plasma Ang II levels decreased steadily from the early to the late phase of influenza A (H7N9) virus infection in the recovered population (Yang et al., 2015). Similarly, AngII levels in plasma samples from Covid-19 patients have been found to be markedly elevated and linearly associated to viral load and lung injury (Gheblawi et al., 2020; Liu et al., 2020c). Consistent with these observations, several authors have suggested some type of RAS assessment may prove useful as a biomarker for severity and prognosis of related conditions (Huang et al., 2014; Reddy et al., 2019; Oudit and Pfeffer, 2020). This is also the case for Covid-19, where prescreening of ACE2/ACE activity ratios and AngII and Ang(1-7) levels has been proposed for risk stratification (Pang et al., 2020; Ryan and Caplice, 2020) ; as a surrogate and more available alternative, it has also been suggested that drops in plasma potassium concentration might be regarded as markers of RAS activation (Vicenzi et al., 2020).

Evidence shows, therefore, that ACE2 seems to limit the occurrence of ALI and its progression to ARDS, together with the vaso-proliferative, fibrotic, and hypertrophic actions of the classical RAS during lung injury. Consequently, restoring the balance between the vaso-deleterious and the vaso-protective axes of the RAS could be therapeutically beneficial in attenuating Covid-19 pathogenesis and progression to severe forms of the disease (Braga et al., 2020).

DIZE has been reported to exert various organ-protective effects, which are attributed to the activation of ACE2 (Qaradakhi et al., 2020).

In a study aimed to explore the protective role of ACE2 on ALI induced by limb ischemia-reperfusion in mice, DIZE was found to attenuate lung injury. The drug decreased the level of AngII and increased the level of Ang(1-7) in lung tissue; it also increased MasR expression (Li et al., 2018).

In a model of asthma, increased pulmonary expression of ACE and inflammatory markers and decreased expression of ACE2 were observed. DIZE treatment prevented these alterations. Moreover, intra-alveolar interstitial thickening, inflammatory cell infiltration, interstitial fibrosis, oxidative stress and right ventricular hypertrophy in asthma control animals were also reversed by DIZE treatment (Dhawale et al., 2016).

In a different model, hyperoxic lung injury in adult mice was associated to a significantly diminished lung ACE2 expression/activity and elevation of the Ang II/Ang-(1-7) ratio. Lung injury, inflammatory response and oxidative stress were found to be attenuated by DIZE (Fang et al., 2019).

Additionally, levels of ACE2 have been shown altered in failing human heart ventricles from subjects with pulmonary hypertension (Shenoy et al., 2013). Pulmonary overexpression of Ang(1-7) exerted beneficial cardiopulmonary effects in experimental pulmonary fibrosis and pulmonary hypertension (Shenoy et al., 2010). Again, DIZE was found useful in these conditions. DIZE treatment significantly prevented the development of pulmonary hypertension, reduced inflammatory cytokines and

improved cardiac function in rats with pulmonary hypertension in different models: induced by monocrotaline, hypoxia, or bleomycin challenge. These beneficial effects were abolished by C-16, an ACE2 inhibitor. Furthermore, in a curative approach, initiation of DIZE treatment after the induction of pulmonary hypertension arrested disease progression. Enhanced ACE2 enzymatic catalytic activity, which was confirmed *in vitro* using recombinant human ACE2, was considered to be the mechanism underlying the beneficial effect of DIZE in this study (Shenoy et al., 2013). Other works seem to corroborate these findings: monocrotaline induced pulmonary hypertension rodents exhibited a significant increase in right ventricle systolic pressure when compared to control rats, while rats treated with DIZE showed a decrease in this pressure. In this work, treatment with DIZE also improved the autonomic nervous system modulation reversing the imbalance elicited by the pulmonary hypertensive state. It is unclear, though, if these effects were ACE2 mediated (Rigatto et al., 2013). DIZE also proved beneficial in pulmonary fibrosis when combined with exercise in pulmonary lesions induced by bleomycin in mice (Prata et al., 2017).

Besides the above, and very interestingly, DIZE significantly attenuated airway obstruction in a porcine model of cystic fibrosis while being used at a dose below the reportedly required to enhance ACE2, presumably by blocking acid-sensing ion channel (ASIC) (Liao et al., 2020) .

Given the above data, we can conclude that there is reasonable evidence for the therapeutic potential of DIZE in lung disease. In Covid-19 specifically, it has been sufficiently stablished that SARS- CoV-2 binding to ACE2 leads to the downregulation of ACE2 expression (Bourgonje et al., 2020; Gheblawi et al., 2020; Verdecchia et al., 2020) . It is therefore reasonable to anticipate that an hypothetical ACE2 activation by a drug like DIZE might rescue ACE2 activity and counteract the virus deleterious effects, at least partially. Damage to the lungs of patients also occurs by direct viral destruction of alveolar and bronchial epithelial cells and macrophages, and in such mechanism no *a priori* benefits seems to be expected from DIZE.

DIZE, ACE2 and Cardiovascular Disease

Though Covid-19 is primarily a respiratory disease, it seems to move progressively to a vascular disease resulting in more severe clinical manifestations. Patients with preexisting cardiovascular conditions represent large proportions of patients with symptomatic infection, and experience disproportionately worse outcomes. In addition, patients with new Covid-19 infections can also develop cardiovascular complications, such as heart failure, myocarditis, pericarditis, vasculitis, and cardiac arrhythmias. Troponin and brain natriuretic peptides, together with the presence of underlying cardiovascular diseases or cardiovascular risk factors, are highly prognostic of the requirement for intensive care unit admission, ventilation, and death. Between 8% and 28% of patients with COVID-19 infections show evidence of cardiac injury with elevated troponin (Liu et al., 2020b).

Cardiovascular disease was a common comorbidity in patients with COVID-19 predecessors SARS and MERS. A murine model demonstrated a marked presence of

the SARS-CoV virus in the heart of wild-type mice. Among humans, SARS-CoV genome has been detected in autopsied hearts (Oudit et al., 2009). In Covid-19, however, whether the virus can directly proliferate in the heart remains to be dilucidated. Although SARS-CoV-2 RNA has been detected in the myocardium of autopsied hearts (Tavazzi et al., 2020), the results of post-mortem examinations performed on 14 Covid-19 patients raised the question as to whether SARS-CoV-2 can directly cause myocardial injury. Cardiac findings were mostly non-specific and associated with pre-existing comorbidities and the results did not provide direct evidence of myocardial injury by SARS-CoV-2 (Bradley et al., 2020). The exact mechanism of cardiac involvement in COVID-19 therefore remains unclear. It is not known whether the observed cardiac damage is attributable to viral injury or to other causes such as an immunologic response that leads to a “cytokine storm”, myocyte apoptosis due to hypoxia-induced influx of calcium ions or any number of them in combination. One potential mechanism is myocardial involvement mediated by ACE2.

In humans, circulating ACE2, shed from endothelial cells, is a biomarker of hypertension and heart failure. Loss of ACE2 enhances susceptibility to heart failure, and increasing ACE2 levels prevent and reverse the heart failure phenotype (Úri et al., 2014; Liu et al., 2020b). In animal studies, overexpression of Ang II in the heart has been found to induce cardiac hypertrophy and overexpression of the AT1R in cardiomyocytes also induced ventricular hypertrophy and remodeling independently of the systemic blood pressure alterations (Castardeli et al., 2018). ACE2 plays an important role in dampening the hypertrophic response to pressure overload mediated by angiotensin II(Yamamoto et al., 2006). Chronic inhibition of ACE2 causes an accumulation of cardiac Ang II, which in turn exacerbates cardiac hypertrophy and fibrosis without having any further impact on blood pressure or cardiac function (Trask et al., 2010). Cardiac overexpression of ACE2, on the contrary, exerts protective influence on the heart during myocardial infarction (Der Sarkissian et al., 2008).

DIZE treatment has shown to be beneficial in models of cardiovascular diseases such as myocardial infarction or the aforementioned works on pulmonary hypertension. These studies often revealed that the beneficial effects of this drug were via the observed increase in ACE2 activity, since effects were blocked by inhibition of either ACE2 with C16 or MasR with A779 (Qaradakhi et al., 2020).

In a study with experimental animals, in which activation of intrinsic ACE2 was proposed as protective against ischemia-induced cardiac pathophysiology, myocardial infarction significantly increased ACE and ATR1 levels while decreased ACE2 activity by 40%. These changes were reversed by DIZE treatment. DIZE treatment decreased the infarct area and attenuated left ventricular remodeling post myocardial infarction, improving both cardiac hypertrophy and left ventricular function; it also restored normal balance of the cardiac RAS. Additionally, DIZE treatment increased circulating endothelial progenitor cells, increased engraftment of cardiac progenitor cells and decreased inflammatory cells in peri-infarct cardiac regions. All of the beneficial effects associated with DIZE treatment were abolished by the ACE2 inhibitor C-16(Qi et al., 2013). In a different work the role of ACE2 was not so clear, but still DIZE was found beneficial to attenuate pathological remodeling and improve cardiac function after myocardial infarction (Castardeli et al., 2018). Reduction in infarct size associated with

activation of the ACE2/AT1R/MasR signaling pathway and anti-inflammatory effects have been also observed in other studies (Chen et al., 2017; Badae et al., 2019). Besides, DIZE has shown ACE2-mediated myocardium protective effects in a hypertension-induced injury model (Macedo et al., 2016; Wang et al., 2016), pregabalin-induced cardiotoxicity (Awwad et al., 2020) and in hyperglycemia-induced cardiac electrical changes in ventricular repolarization(Coutinho et al., 2014).

Benefits were also found in subtotal nephrectomy in rats, which is associated with elevated blood pressure together with cardiac and renal dysfunction. In this model, increased cardiac ACE, ACE2, AngII and Ang(1-7) levels were observed in nephrectomized rats in vitro compared to control rats. DIZE did not alter circulating RAS components nor improve kidney function. Cardiac ACE2, AngII and Ang(1-7) remained unchanged too. However, DIZE treatment was associated with a reduction in cardiac ACE activity and AngII levels, resulting in a decreased in the ACE/ACE2 activity ratio towards a more favorable and cardioprotective profile, effect that was not found in control rats, which did not show changes in cardiac ACE or ACE2 protein or activity. These changes were accompanied by improvement in cardiac dysfunction and in cardiac fibrosis. This effect on ACE while ACE2 remains seemingly unaffected results somewhat puzzling in view of previous contrary evidence, and requires further clarification. As the authors suggest several factors might be implicated, such as the underlying pathophysiology, whether the study is done *in* vivo or *ex vivo* or the tissue used to assess ACE2 activity (Velkoska et al., 2016b).

Since Covid-19 is a new disease and the pathophysiology underlying cardiac involvement remains under investigation, attempts to anticipate the possible effects of DIZE treatment on cardiac function can only be speculative. It would not be unconceivable, however, to regard the drug profile as initially favorable for cardiac function.

DIZE and vascular endothelium

Among the distinctive features of Covid-19 are the vascular changes associated with the disease. Covid-19 results in both systemic arterial and venous thrombi even in mild infection, as well as a likely localized pulmonary micro-thrombosis. Elevated D-dimer, regarded as a marker of hypercoagulability, have been found in hospitalized patients in a severity-correlated manner. Clinical manifestations suggesting thrombotic microangiopathy, like cutaneous vasculitis signs in patients´ extremities, have been frequently reported (Frazer and Tyrynis Everden, 2020). The presence of microangiopathy and microthrombi in Covid-19 has nonetheless being somehow called into question, as no evidence of widespread microvascular injury was reported in a recent series of Covid-19 *post mortem* examinations (Bradley et al., 2020)**.** Most autopsy studies, however, provide further evidence of micro-thrombosis and even infarction, occasionally despite anticoagulation (Ackermann et al., 2020; Frazer and Tyrynis Everden, 2020). In a sample of lungs obtained during autopsy from patients who died from SARS-CoV-2 infection, the lungs showed distinctive vascular features, consisting of severe endothelial injury associated with the presence of intracellular virus and disrupted cell membranes, widespread thrombosis with microangiopathy, and significant new vessel growth through a mechanism of intussusceptive

angiogenesis (nonsprouting). The presence of SARS-CoV-2 virus within the endothelial cells was consistent with other studies (Ackermann et al., 2020). Infection of endothelial cells by SARS-CoV-2 is hypothesized to cause the endothelial damage highlighted by post-mortem studies. Endothelial, smooth muscle cells and perivascular pericytes all have ACE2 receptor (Hamming et al., 2004), which would facilitate viral entry and proliferation. Endothelial cells and activated macrophages can release proinflammatory cytokines likely inducing the acute phase response, with the consequent raised production of adhesion molecules and procoagulant factors. The dysfunctional endothelium would thus become pro-adhesive and procoagulant. With the retreat of ACE2 and activation of AngII, vascular inflammation and prothrombotic state result enhanced (Liu et al., 2020b)**.** In fact, it has been described that AngII promotes vascular leakage and endothelial release of reactive oxygen species (ROS) correlated with endothelial dysfunction. Based on these observations, it has been hypothesized that increased AngII/AT1R axis could contribute to Covid-19 progression to severe forms of the disease through induction of endothelium dysfunction and increased vascular permeability and, consequently, edema (Braga et al., 2020)**.** In any case, the mechanism of endothelial damage remains under debate. Further studies will be required to define the different mechanisms through which SARS-CoV-2 infection causes such marked endothelial cell effects.

Whatever the mechanism specifically involved in Covid-19 thromboembolic complications, it is known that the ACE2/Ang(1-7)/MasR arm exerts anti-thrombotic effects. MasRs are expressed on platelets. Animals knockout for MasR have a shorter bleeding time and increased size of thrombi. In these animals, administration of Ang(1-7) induces a marked antithrombotic effect which is directly related to the plasma levels of Ang(1-7) and is inhibited by A-779, an antagonist of MasR. Chronic Ang(1-7) treatment preserved endothelial function in rat models of myocardial ischemia and in-stent restenosis. Ang(1-7) is therefore regarded as an important player opposing the pro-thrombotic and pro-inflammatory effects of AngII (Verdecchia et al., 2020). These data have led some authors to postulate that activation of intrinsic ACE2 would decrease ROS production and promote beneficial effects on the endothelial function. Indeed, their results showed that the administration of XNT, regarded as an ACE2 activator, improved endothelial function of hypertensive and diabetic rat vessels and also elicited an endothelial-dependent vasorelaxation response mediated by MasR (Langeveld et al., 2005; Fraga-Silva et al., 2013).

Regarding DIZE, several studies have shown interesting properties for vascular repair. CD34+ cells, often designated as endothelial progenitor cells, are bone marrow-derived multipotent stem cells. These cells proliferate and migrate to areas of ischemia and accelerate vascular repair (Sietsema et al., 2019) . Migration to the areas of ischemia is an important property of CD34+ cells since it determines their reparative function (Singh et al., 2015). It has been documented that the ACE2/Ang-(1–7) pathway produces vascular repair-relevant functions of $CD34^+$ cells, whereas Ang II attenuates these functions indirectly by acting on mononuclear cells. While Ang(1-7) induces migration and proliferation in human CD34+, activation of mononuclear cells by AngII has the opposite effect, most likely by stimulating the generation of ROS(Cole-Jeffrey et al., 2018) . The beneficial effects of DIZE on migration of CD34+ cells were shown in a in a study on cells isolated from peripheral blood obtained from healthy volunteers. DIZE was found to stimulate migration and proliferation - functional

signatures of vaso-reparative potential - of human CD34+ cells. AngII, by stimulating the generation of ROS from mononuclear cells, attenuated the functions of CD34+ cells. DIZE enhanced proliferation and migration were blocked by DX-600, an ACE2 inhibitor of CD34+, suggesting that their effects were ACE2 mediated. Interestingly, the presence of Ang II was found necessary for DIZE effects. Overall, the study confirmed that both cardiovascular protective and deleterious arms of RAS modulate the vaso-reparative functions of CD34+ cells. The authors concluded that relative expression of the two axes of local RAS in CD34+ cells could be a good measure of their vaso-reparative functions. An imbalance toward increased expression of ACE/Ang II/AT1R axis, as is seemingly found in Covid-19, would promote dysfunction of $CD34^{+}$ cells and the subsequent vascular damage (Singh et al., 2015). In this regard, DIZE treatment could result of potential benefit for vascular dysfunction.

Endothelial dysfunction also constitutes a hallmark of pulmonary hypertension pathophysiology. In the aforementioned work on pulmonary hypertension models, dysfunctional angiogenic progenitor cells derived from the bone marrow of monocrotaline-challenged rats were repaired by DIZE treatment. Likewise, functional impairment of CD34+ cells isolated from patients with pulmonary hypertension was corrected by DIZE treatment *in vitro*(Shenoy et al., 2013) .

There is evidence that Ang(1-7) protects endothelial function in renal arteries of diabetic patients(Zhang et al., 2015)**.** Furthermore, effective ACE2 signaling has been identified in diabetic patients with vascular complications. ACE2 expression was lower in diabetic CD34+ cells than in non-diabetic controls. A cohort of patients who remained free of microvascular complications despite having a longstanding history of inadequate glycemic controls had higher expression of ACE2/Mas mRNA in CD34+ cells than diabetic patients with microvascular complications matched for age, sex, and glycemic control. Decreased production of Ang(1-7) and reduced migration were also observed in the cohort of patients with complications. It was suggested that the upregulation of ACE2 is supportive of compensatory mechanisms, activating the protective arm of RAS, which could help minimize vascular complications in states of insulin deficiency. Activation of the ACE2/Ang-(1-7)/MasR axis corrected the vaso-reparative dysfunction typically seen in the CD34+ cells isolated from diabetic patients: Ang-(1-7) restored impaired migration and enhanced the survival and proliferation of CD34+ cells from diabetic individuals; DIZE was also beneficial, although less effective in inducing the migration in cells from patients with diabetes compared with non-diabetic controls. Altogether, ACE2/Ang(1-7)\MasR axis activation corrected existing diabetes-induced CD34+ cell dysfunction and was associated with protection from the development of microvascular complications (Jarajapu et al., 2013).

In a different study, DIZE significantly elevated plasma Ang(1-7) levels, attenuated oxidative stress and improved the endothelial function of diabetic rat vessels; the ACE2 inhibitor, DX600, and Ang(1-7) antagonist, A779, reversed some of the effects of DIZE. Furthermore, lack of vaso-protective effect in ACE2 knockout mice confirmed the selectivity of DIZE to activate ACE2. Noticeably, DIZE elevated ACE2 activity in mice aortas and plasma while, by contrast, aortic ACE2 expression was unchanged. It is important to highlight the unchanged ACE2 expression, which would support enhanced ACE2 catalytic activity rather than increased ACE2 expression as a mechanism of action for DIZE. This is of utmost relevance in Covid-19, where an

increased ACE2 expression might be presumed detrimental, since it would theoretically result in facilitating virus cell entry (Zhang et al., 2015).

DIZE and inflammatory response

Upon SARS-CoV-2 virus entrance into the target cell, the viral RNA initiates a cascade of events that results in replication of the virus inside the cell. Consequently, host cells result disabled or destroyed, with the subsequent release of potential danger signals to activate the host's immune responses. Researches have proven that IL-6, IL-17A, and TNF-α are highly expressed in critically ill patients, suggesting that patients with COVID-19 experience a disproportionated immunologic host response (Li et al., 2020). Other findings are lymphopenia - which occurs in above 80% of patients-, marked reductions in circulating levels of CD4+ and CD8+ T lymphocytes, and a relative dominance of mononuclear cells (monocytes and macrophages) in target injury tissues. The degree of lymphopenia is a very important prognostic indicator early in the course of infection. Increasing production of IL-6, on the other hand, can presage an impending cytokine storm (Liu et al., 2020b).

Evidence suggests that the RAS is also important in regulating inflammatory responses through at least two mechanisms: on one hand, ACE2 can directly interact with macrophages in inflammatory processes; on the other, ACE2 reduces the levels of AngII, which is directly proinflammatory and pro-oxidant. AngII contributes to the inflammatory process by increasing the release of pro-inflammatory cytokines, chemokines, cell adhesion molecules, growth factors, and ROS through the AT1R. Overexpression of ACE2 reverses the imbalance of ACE/ACE2, reducing Ang II and increasing Ang(1-7) levels. Ang(1-7), in turn, has been shown to exerts inhibitory effects on inflammation acting via MasR and also reduce key signaling pathways and molecules thought to be relevant for fibrogenesis (Simões e Silva et al., 2013; Santos et al., 2018; Gheblawi et al., 2020).

Several studies have shown that DIZE has certain effect on the host immune system.

In human retinal pigment epithelia cell cultures, DIZE increased ACE2 expression and exerted anti-inflammatory actions, theoretically through activation of the protective axis of RAS, ACE2/Ang(1-7)/MasR. Findings that supported this hypothesis were: DIZE significantly reduced the AngII levels, reduced the expression of AT1R and resulted in a dramatical increase of Ang-(1-7); furthermore, a small interfering RNA of ACE2 and an Ang-(1-7) antagonist A779 abrogated the anti-inflammatory effect of DIZE (Tao et al., 2016).

In another interesting work, DIZE was used in two breeds of mice infected with *Trypanosoma congolense*. The infection caused systemic inflammatory response syndrome and cytokine storm. One type of mice was relatively resistant and survived more than 100 days; the other, more susceptible, usually succumbed at 8-10 days due to hyperimmune activation - particularly of macrophages and T cells- and subsequent shock. In both cases, DIZE modulated the host immune response to the parasite in a manner that dampened excessive immune activation and production of pathology-promoting pro-inflammatory cytokines (IL6, TNF-a, IL-12, and IFN-c). In the case of susceptible mice, it also prolonged their survival to an indefinite date. The dampening

effect on pro-inflammatory cytokine production was mediated by the effect of DIZE on CD11b+ cells, including splenic and liver macrophages. In view of the fact that similar effects were observed in DIZE-treated uninfected mice, the authors considered it unlikely that the drop in parasite load resulting from the trypanolytic effect of DIZE was solely responsible for such an striking reduction in pro-inflammatory cytokines and alteration in cellular immune responses (Kuriakose et al., 2012). The molecular mechanisms through which DIZE alters pro-inflammatory cytokine production by macrophages were further investigated by the same authors. Globally, their studies showed that treatment with DIZE reduces cytokine production in different models of microbial molecule-induced proinflammatory cytokine induction in macrophages both *in vivo* and *in vitro*. Their findings suggested that the compound inhibitory effects on cytokine production takes place through a wide range of targets. DIZE significantly downregulated phosphorylation of MAPKs (mitogen-activated protein kinase), STATs (signal transducer and activator of transcription) and NF-kB (nuclear factor-kB), which are critical signaling molecules involved in the production of proinflammatory cytokines in immune cells. DIZE also was found to upregulate SOCS1 (suppressor of cytokine signaling 1) and SOCS3 expression, proteins that act as negative regulators of cytokine signaling and have been shown to inhibit intracellular signaling pathways mediated by MAPKs and STATs (Kuriakose and Uzonna, 2014; Kuriakose et al., 2014). Inhibition of the MAPK and NF-kB pathways by DIZE were also found in the aforementioned work in human retinal pigment epithelia cell cultures (Tao et al., 2016). Interestingly, the fact that DIZE downregulated cytokine production in different models strongly suggests that the effect of the drug is non pathogen- specific.

In chronic liver injury, fibrosis is driven by a range of proinflammatory cytokines such as TNF-α, MCP-1 and IL-6 released from activated Kupffer cells and hepatic stellate cells. In a work conducted in two different biliary fibrosis models in mice and also in cell lines (human hepatic stellate and mouse Kupffer), DIZE markedly inhibited the activation of fibroblastic stellate cells, both *in vivo* and *in vitro*. This was associated with a reduced activation of Kupffer cells, with diminished cytokine secretion. The changes produced by the drug led to a reduction in biliary fibrosis. The inhibitory effect of DIZE on cytokine secretion might have been mediated by reduced NF-kB activity, as the levels of phosphorylated (activated) NF-kB were significantly downregulated by DIZE treatment in Kupffer cells. In contrast to previous studies, though, it was found that DIZE treatment did not affect ACE2 gene expression or activity, nor any other RAS components, strongly suggesting that the effects of DIZE in the liver were ACE2 independent (Rajapaksha et al., 2018).

Additionally, DIZE has been shown to block histamine induced responses in mammalian tissues and exert some anti-histaminic and anti-inflammatory effects in vivo(Kuriakose and Uzonna, 2014). DIZE induced stimulation of ACE2 also ameliorated the renal deleterious consequences induced by γ-radiation via activation of the protective ACE2/Ang(1–7)/MasR axis in rats(Hasan et al., 2020). In a mouse model of lethal inflammatory liver disorder, treatment with DIZE inhibited expression of inflammatory markers, alleviated histopathological damage and improved survival rates (Ge et al., 2018).

In severe Covid-19 patients in which warning signals such as lymphopenia or rising inflammatory markers are observed, diverse strategies have been considered in an effort to restore immune balance: anti- L1 (anakinra) or anti-L6 (tocilizumab) approaches, immunoglobulin and recovered serum, etc. The suppression of proinflammatory cytokines production in macrophages, both *in vivo* and *in vitro,* suggests that DIZE has strong and potent anti-inflammatory properties, whether ACE2 mediated o ACE2 independent. We suggest, therefore, that those properties add extra therapeutic potential for DIZE in Covid-19 treatment which, to date, remains regrettably uninvestigated.

Other conditions

Besides the beneficial effects in experimental models of lung disease, cardiovascular disease or inflammation, other studies have focused on the actions of DIZE.

DIZE has demonstrated reno-protective effects in several studies. The drug improved renal function after renal ischemia/reperfusion injury (Malek and Nematbakhsh, 2014). In a rat model of type 1 diabetes, DIZE increased glomerular ACE2 and prevented nephropathy (Goru et al., 2017). When administered orally to hypertensive rats, DIZE improved renal alterations and inflammation induced by renovascular hypertension (Kangussu et al., 2019). As mentioned previously, DIZE ameliorated the biochemical and histopathological damage induced by radiation (Hasan et al., 2020).

Regarding metabolic profile, evidence is being gathered pointing out that ACE2/Ang(1-7)/MasR axis activation is able to enhance glucose tolerance and insulin sensitivity and improve metabolic parameters(Macedo et al., 2015; Bruce et al., 2018). Oral DIZE lowered fat deposition, body weight, serum cholesterol and serum triglycerides in mice (Macedo et al., 2015). It also resulted in a significant reduction of food intake and body weight in both young and old obese animals (Bruce et al., 2018). In addition, DIZE administration was unexpectedly found to reduce serum cholesterol concentrations, specifically very low-density lipoprotein-cholesterol, in wild-type but not in ACE2-deficient mice, suggesting ACE2 mediated regulation. In the same work, DIZE reduced the size, severity, and incidence of AngII-induced abdominal aortic aneurysms, again in wild-type, but not in ACE2-deficient mice, supporting an ACE2-dependent mechanism of the compound (Thatcher et al., 2014).

Another pharmacological property still poorly researched is the inhibition of acid-sensitive ion channels (ASICs) by DIZE (Schmidt et al., 2017; Krauson et al., 2018)**.** ASICs are related to the development of various diseases such as rheumatoid arthritis (Neidhart et al., 2014) or neurological disorders. Local acidosis is associated with neuro-inflammation and can have significant effects in multiple sclerosis, brain ischemia, spinal cord injury and epilepsy (Wemmie et al., 2013) . Therefore, pharmacological modulation of ASICs is regarded as a new promising strategy for future therapies aimed at neuro-protection. DIZE ameliorated demyelination and axonal damage in experimental autoimmune encephalomyelitis affected mice (which is an animal model used to study multiple sclerosis), probably by modulation of ASICs (de Ceglia et al., 2015). DIZE also induced peripheral anti-hyperalgesia in a rat model of chronic inflammatory pain, likewise probably by ASIC inhibition (Lee et al., 2018) **.** DIZE

neuroprotective properties have also been observed in models of cerebral ischemia (Mecca et al., 2011) and glaucoma (Foureaux et al., 2013)**,** in both of which RAS regulation is seemingly implicated, likely alongside other mechanisms. In addition, DIZE has also been found beneficial in a model of Alzheimer disease, in which the involvement of RAS peptides was not clear (Evans et al., 2020)**.**

Apparently unrelated actions of DIZE have additionally been found in the gastrointestinal tract, where it could promote re-epithelialization and regeneration of gastric tissue in different injury models (Souza et al., 2016).

Pharmacological activities and safety issues on DIZE

DIZE is an aromatic diamidine, with antiparasitic effect. Currently, it is used in veterinary medicine against trypanosomiasis and babesiosis and not approved for human use. Nevertheless, DIZE was used for decades to treat African trypanosomiasis in humans (both caused by *Trypanosoma brucei gambiense* and caused by *T. brucei rhodesiense*).

DIZE selectively blocks kinetoplast DNA (kDNA) synthesis, through interference with mitochondrial, although not nuclear, topoisomerase II of *Trypanosoma* spp.(Shapiro and Englund, 1990; Shapiro, 1993; Burri et al., 2004). It presents great affinity for sequences of adenine-thymine base pairs in kDNA(Kuriakose et al., 2012). It may also indirectly affect the host's immune system by decreasing pro-inflammatory cytokine (IL-6, IL-12 and TNF) production in macrophages *in vivo* and *in vitro* following stimulation with *Trypanosoma* spp and lipopolysaccharide. The proposed mechanism is through the downregulated phosphorylation of MAPKs, STATs and NFκB, key signaling molecules involved in the production of proinflammatory cytokines(Kuriakose and Uzonna, 2014; Kuriakose et al., 2014).

DIZE is an analogue of pentamidine. Unlike diminazene, pentamidine is approved for human use. Pentamidine has been used against African trypanosomiasis, but there is more data available on its toxicity from its administration to patients with *Peumocystis jiroveci* infection. The adverse effects of pentamidine are: reversible nephrotoxicity, syncope, nausea, vomiting, abdominal pain, cardiotoxicity, headache, peripheral neuropathy, hypoglycemia, pancreatitis, dermatitis, and hypocalcemia (Van Nieuwenhove, 1999).

DIZE has been used orally and intramuscularly, at a dose of 5-7 milligrams per kilogram of weight every 48 hours for six days, or 2 milligrams per kilogram of weight daily for seven days (Pepin). Between 1962 and 1984, several series of patients with early-stage African trypanosomiasis, treated with diminazene, were published, mostly in Uganda (Table 2). There are more than 200 reported cases, the majority treated intramuscularly, with infrequent relapses (3-15%) and reversible adverse effects (Hutchinson and Watson, 1962; Bailey, 1968; Onyango et al., 1970; Temu, 1975; Abaru et al., 1984).

There is a case of a patient with babesiosis who did not respond to chloroquine and was treated with DIZE (first IM then orally) after obtaining an informed consent. The patient showed an excellent response, especially regarding fever and parasitemia, but

developed Guillain-Barré syndrome after several days. A probable causal relationship was established, due to both DIZE and pentamidine have been associated to peripheral neuropathy (Cassaday et al., 1979). Reactive encephalopathy was also found after the administration of DIZE prior to melarsoprol in a patient with stage II trypanosomiasis (De Raadt et al., 1965). However, in Bailey's series (Bailey, 1968), the administration of an ampoule of DIZE before treatment with melarsoprol in 28 patienst with meningoencephalitis caused by Trypanosimiasis spp reduced the incidence of encephalopathy associated to malarsoprol, with no other toxic effects added.

Nieuwenhove(Van Nieuwenhove, 1999) proposes that the adverse effects of DIZE are reversible and similar to those of pentamidine, but DIZE presents several advantages over pentamidine, such as shorter treatment schemes and a much cheaper cost. For Burri et al(Burri et al., 2004), it would be hard to approve suramine or pentamidine for commercialization at the present time but fortunately, they were approved years ago and have saved many lives since. They refer to the use of DIZE intramuscularly (5 mg/Kg every 48 hours, three doses, o 2-5 mg/Kg/daily during 7-10 days) in 400 patients before the existence of other drugs for the treatment of African trypanosomiasis.

The medium life of DIZE is 12 hours in sheep and >60 hours in cows. The most frequent adverse effects include nausea, vomiting and albuminuria. Rare adverse effects are paralysis and reversible coma. The fact that brain hemorrhages have been described in other animals, probably due to a species-specific effect, is a cause for concern regarding its use in humans, even before clinical trials are carried out.

A cycle of three injections of DIZE used to have a cost of 2 dollars. It is highly likely that DIZE has also been used more recently in Africa, despite it not being approved for human use. Pepin et al (Pépin and Milord, 1994) report that in endemic countries in Africa, physicians have used this drug profusely in humans for its efficacy and good tolerance, even though the data has not been published due to the unjustifiable ethical dilemma surrounding its use.

The aforementioned toxic effects in certain animals are the reason DIZE production was discontinued for human use. Since then, the pharmaceutical industry has never shown any interest in its commercialization for human trypanosomiasis (a good example of a neglected infectious disease), probably due to the costs involved in the development of toxicological studies in exchange for a very limited market.

Currently, facing the SARS-CoV-2 emergency and considering the potentially beneficial effects of diminazene-aceturate on the ACE2 receptor, we are provided with an opportunity to use it in a MEURI (*Monitored Emergency Use of unRegistered and experimental Interventions*) framework (Weltgesundheitsorganisation, 2016).

Discussion

Despite the predominance of respiratory symptoms, Covid-19 may present as a systemic disease leading to cardiovascular, kidney, eye, skin, gut and liver abnormalities. This is consistent with the ubiquitous distribution of the ACE2 receptor. ACE2 is a cell surface bound enzyme, with its catalytic site exposed to the extracellular

surface. A soluble form of ACE2 can be released from the membrane through proteolytic cleavage mediated by ADAM17 (ADAM metallopeptidase domain 17) resulting in loss of ACE2 protection against tissue classical RAS (Xu et al., 2017; Liu et al., 2020b; Verdecchia et al., 2020; Wang et al., 2020). Viral cellular entry induces ADAM-17 activation and further ACE2 ectodomain shedding leading to a detrimental positive feedback cycle. Furthermore, loss of membrane ACE2 promotes Ang II accumulation, which also activates ADAM-17 activity, thus perpetuating membrane shedding of ACE2 and subsequent classical RAS overactivation (Gheblawi et al., 2020; Oudit and Pfeffer, 2020; Wang et al., 2020). The imbalance of the tissue RAS from SARS–CoV-2 infections results in a shift away from the protective ACE2/Ang(1–7)/MasR axis towards the disease state, giving way to a number of pathophysiological effects in the tissues or organs affected, such as edema, inflammation, fibrosis or thrombi formation. This, in turn, translates into the wide range of clinical presentations observed in Covid-19 patients, from the more frequent respiratory manifestations to other features such as cardiovascular disorders, thromboembolic complications, kidney or liver dysfunction, gut abnormalities, etc. Regardless of other possible additional mechanisms, such as secondary infection, the ubiquitous presence of ACE2 represents the underlying factor linking the multiple organ systems affected by the SARS-CoV-2 virus (Clerkin et al., 2020; Gheblawi et al., 2020; Liu et al., 2020b; Verdecchia et al., 2020; Wang et al., 2020). Furthermore, ACE2 receptor involvement may also help to explain laboratory findings in Covid-19(Liu et al., 2020b). Elevated circulating levels of soluble ACE2 resulting from ACE2 shedding have been postulated as markers of increased activity of the classical RAS and worse prognosis (Xu et al., 2017; Verdecchia et al., 2020). Similarly, plasma Ang II levels have also been proposed as biomarkers, since they have been found elevated in a severity-correlated manner in Covid-19(Gheblawi et al., 2020; Liu et al., 2020c). It is also noteworthy that preexisting conditions associated with a higher risk of Covid-19 infection share a variable degree of RAS imbalance towards an overactive classical RAS (Gheblawi et al., 2020; Wang et al., 2020). Loss-of-function experiments using ACE2 knockout mice and RAS acting compounds have revealed that dysregulated RAS occurs in these comorbidities, including hypertension, microvascular complications, inflammation, fibrosis, diastolic and systolic dysfunction, oxidative stress(Wang et al., 2020), diabetes mellitus(Luther and Brown, 2011), obesity(Rüster and Wolf, 2013) and lung disease(Kuba et al., 2006; Shenoy et al., 2010; Kaparianos and Argyropoulou, 2011; Wösten-van Asperen et al., 2011). In humans, elevated circulating ACE2 has been reported peripherally in heart failure, hypertension, severe acute respiratory syndrome and Type 1 diabetes (Úri et al., 2014; Xu et al., 2017; Sama et al., 2020; Verdecchia et al., 2020), among others. This way, RAS imbalance provides a plausible justification for both Covid-19 features and epidemiology.

Although there is a consensus about the crucial role of the ACE2 receptor in Covid-19 pathogenesis, the underlying mechanisms remain incompletely understood, thus conditioning the feasibility of therapeutic approaches. A variety of therapeutic targets have been aimed at, including inflammatory response and viral invasion (Bourgonje et al., 2020; Clerkin et al., 2020). Little has been proposed, however, directed towards the virus-induced RAS dysregulation observed in Covid-19 patients. As far as the authors are aware, recombinant human ACE2 (rhACE2) has emerged as the one postulated treatment directly addressing the RAS imbalance resulting from SARS-CoV-2 infection.

Although additional studies are needed, the potentially protective action of rhACE2 against viral infection provides new hope in the fight against the pandemic. The effects of rhACE2 may be principally mediated by its competing effects with ACE in limiting the generation of Ang II, while potentiating Ang(1–7); simultaneously, *rhACE2* functionally sequesters circulating viral particles to prevent S-protein interactions with endogenous ACE2 (Gheblawi et al., 2020; Pang et al., 2020; Zhang et al., 2020a). DIZE, by contrast, would not be expected to exert these dual actions, since viral particles sequestration is not to be expected. However, ACE2 enhancement by DIZE might also hypothetically achieve a parallel Ang(1–7) potentiation and subsequent beneficial results. It has been previously shown that DIZE rescues in vivo ACE2 functions within the RAS involving lung function, fibrosis, inflammation, cardiovascular system and endothelial function. Because of these properties, DIZE provides a potential weapon for the more severe clinical pictures found in Covid-19 patients. In addition, DIZE features other advantages worth considering. DIZE is a small compound that exerts its effects at the target-tissue. This is not attainable through rhACE2 treatment, since soluble ACE2 lacks the transmembrane domain and thus cannot stabilize its cell-surface localization (Hashimoto et al., 2012)**.** Additionally, because of its large molecular size, rhACE2 penetrance at tissue level can be presumed limited (Gheblawi et al., 2020), whereas DIZE may pass through multiple barriers (Qiu et al., 2014)**.** The cost of manufacturing the recombinant protein, its stability, short half-life and repetitive intravenous dosing present other challenges for rhACE2 therapy (Shenoy et al., 2014; Guignabert et al., 2018). DIZE, on the other hand, has been effectively administered in humans as a single daily dose by intramuscular(Hutchinson and Watson, 1962; Onyango et al., 1970; Burri et al., 2004) or in occasions oral routes (Bailey, 1968; Van Nieuwenhove, 1999; Burri et al., 2004); 48-hour dosing intervals have also been used (Pépin and Milord, 1994; Van Nieuwenhove, 1999)**.** At this point it may also be worth noticing the subsequent restriction of undesirable exposure of healthcare workers through repeated drug administration. Easy dosage regimens together with minimal cost of treatment render DIZE a potential universally available treatment for the present pandemic.

One major objection to DIZE treatment may come from the hypothetical increased susceptibility to infection linked to DIZE-induced ACE2 upregulation (Pang et al., 2020). Since the virus is primarily respiratory, special attention must be given to the airway and lungs as the main portal of entry. ACE2 is widely expressed on the pulmonary endothelium, type II alveolar epithelial and smooth muscle cells of the lungs (Hamming et al., 2004; Bourgonje et al., 2020). This, together with the extensive surface of alveolar epithelial cells, has been suggested as a likely explanation for the vulnerability of this organ to SARS-Cov-2 invasion (Verdecchia et al., 2020). Studies have shown that ACE2 may be upregulated in the lungs of patients with diseases reported to be comorbidities associated with severe COVID-19 (Pinto et al., 2020). Therefore, the possibility of viral entry facilitation by a drug that presumably might increase ACE2 expression demands caution. Nevertheless, there has been a great deal of speculation regarding the role of ACE-2 expression in virus invasion. This has been clearly illustrated throughout the well-known controversy as to whether ACE inhibitors (ACE-Is) and angiotensin receptor blockers (ARBs) elevated morbimortality through potential ACE2 upregulation. As it turned out, the use of ACE-Is/ARBs has been associated with

lower mortality compared with ACE-Is/ARBs non-use(Zhang et al., 2020c) and it has even been suggested that it may be protective or, at the minimum, not necessarily harmful(Bean et al., 2020; Guo et al., 2020; Gurwitz, 2020; Murthy et al., 2020; Vaduganathan et al., 2020; Zhang et al., 2020b). The balance between ACE2 facilitated viral entry into pneumocytes and the beneficial effects of increasing the expression and the activities of ACE2 remains a conundrum to be solved.

The profuse data available paradoxically shed little light on the issue. It has been pointed out, for instance, that if we consider that the susceptibility to SARS-Cov-2 infection should increase with increasing ACE2 levels, women should be more prone to the infection than men, in contrast to the epidemiological data available up to the present (D'Ardes et al., 2020) . Also, conflicting affirmations have been hypothesized regarding plasma and membrane-bound ACE2 correlation: while on one side it has been postulated that high soluble ACE2 could indicate high membrane-bound ACE2(Sama et al., 2020; Swärd et al., 2020), it has also been suggested that increased ACE2 shedding should expectedly lead to lower membrane-bound ACE2 and correspondingly higher soluble ACE2 and vice versa(Larouche-Lebel et al., 2019; Leow, 2020). Indeed, ACE2 expression seems variable and complex, as well as subject to numerous influencing factors including sex, age, genetics, drug treatment or disease (Bourgonje et al., 2020; Ghafouri-Fard et al., 2020). Variability has also been observed in different tissues and situations. This way, for example, a dissociation between tubular and glomerular ACE2 expression has been reported, particularly in diabetic kidney disease, where ACE2 expression is increased at the tubular level but decreased at the glomerular level (Soler et al., 2013). Noticeably, on the other side, ACE2-expressing organs do not equally participate in COVID-19 pathophysiology, implying that other mechanisms are involved. Likely, ADAM17 and transmembrane serine proteases also play an important role in promoting virus uptake and activation (Heurich et al., 2014; Meng et al., 2020). Moreover, the ubiquity of ACE2 and high binding affinity of the virus suggest that the SARS-CoV-2, once present in the circulation, can spread easily through the body. For this reason, it has been considered unlikely that a mild or moderate ACE2 deficiency may protect from viral invasion (Verdecchia et al., 2020). In addition, animal data support elevated ACE2 expression as conferring potential protective pulmonary and cardiovascular effects (Imai et al., 2005; Kuba et al., 2005; Oudit et al., 2009; Shenoy et al., 2010, 2013; Zou et al., 2014; Yang et al., 2015). Taken together, therefore, there are no robust data to clearly suggest an independent link between increased ACE2 expression and susceptibility to Covid-19 infection. In consequence, whether the level of *a priori* ACE2 expression is high or low may not be a key factor affecting the prognosis of patients with COVID-19. Alternatively, a key factor may rather be the global RAS status, where ACE2 activity together with ACE2 expression as well as other RAS peptides are likely involved. A possible overall disbalance towards the classical RAS, rather than ACE2 expression, might be an underlying explanation for Covid-19 pathophysiology and epidemiology, where conditions involving the RAS (obesity, diabetes, lung and cardiovascular disease) have certainly been established as risk factors (Bourgonje et al., 2020; Ebinger et al., 2020; Ghafouri-Fard et al., 2020; Gheblawi et al., 2020; Gil-Rodrigo et al., 2020; Jordan et al., 2020; Verdecchia et al., 2020). The downregulation of ACE2 induced by viral invasion could be especially detrimental in individuals with a baseline RAS

dysregulation (due to, for example, diabetes or hypertension), since it would place them in an *a priori* disadvantageous position to face further attack to an already precarious balance. In this setting, biomarkers such as plasma AngII or other type of RAS assessment may shed some light and prove useful for risk assessment.

Given the above premises, even if the use of ACE2-stimulating drugs were to facilitate the entry of SARS-CoV-2 into pneumocytes, questions arise regarding situations where the viral load has already overcome physiologic compensating mechanisms. In such cases, hindering viral invasion may not take on as much priority as re-establishing RAS homeostasis. Hence, the benefits of ACE2 activation may outweigh the theorized detrimental effects due to ACE2-mediated increase in viral entry. In fact, as we already mentioned, data on pneumonia suggest the potential utility of ACE-Is and ARBs to reduce lung inflammation and mortality (Caldeira et al., 2012; Klein et al., 2013; Vaduganathan et al., 2020; Verdecchia et al., 2020).

At the same time, special notice should be given to other postulated DIZE mechanisms of action. While increased ACE2 expression by DIZE has been reported (Foureaux et al., 2013; Goru et al., 2017; Hasan et al., 2020), a number of other actions have also been observed, occasionally in an ACE2-independent manner (Haber et al., 2014; Neidhart et al., 2014; Krauson et al., 2018; Rajapaksha et al., 2018; Liao et al., 2020).

DIZE has been shown to produce a variety of effects, including boosting of MasR (Bruce et al., 2018), increasing ACE2 activity (Kulemina and Ostrov, 2011; Qi et al., 2013; Shenoy et al., 2013; Qiu et al., 2014; Zhang et al., 2015; Bruce et al., 2018), increasing MasR/AT1R and ACE/ACE2 ratios (Qi et al., 2013; Shenoy et al., 2013; Velkoska et al., 2016b), increasing AT2 receptor (Goru et al., 2017), decreasing ACE expression (Bruce et al., 2018), AT1R suppression (Tao et al., 2016; Kamel et al., 2018) and non-ACE2 mediated anti-inflammatory effects (Rajapaksha et al., 2018) and airway protection (Liao et al., 2020), all of which present potential benefit. Above that, in silico docking studies identified DIZE as an ACE2 activator able to bind to a pocket in the ACE2 hinge-bending region and stabilize the enzyme in an open conformation. This open conformation is important because it accelerates peptidase activity, thereby boosting generation of Ang(1-7) form AngII. When studied *in vitro*, DIZE enhanced cleavage of AngII in a biphasic dose-response curve: at low concentrations of DIZE the enzyme is activated whereas at high concentrations it is partially inhibited (Gjymishka et al., 2010; Kulemina and Ostrov, 2011). Such findings have been both challenged (Haber et al., 2014) and confirmed (Shenoy et al., 2013) by further studies with no clear explanation found for these discrepancies. This results a crucial issue: indeed, DIZE as an enzymatic activator could lead to an ideal scenario where ACE2 enhancement would be achieved without the theoretical risk of facilitated viral entry, this way potentially rescuing ACE2 activity and helping to re-establish RAS homeostasis.

Another important challenge is posed by lack of human pharmacological studies and licensing, which represents an obvious major obstacle. Nonetheless, DIZE is far from being a new compound, and experience using it in human beings is not entirely lacking. Indeed, hundreds - if not thousands- of patients did benefit from it decades ago, although intended for a totally different purpose (Pépin and Milord, 1994; Van

Nieuwenhove, 1999; Burri et al., 2004; Delespaux and Dekoning, 2007; Bacchi, 2009). Besides indirect evidence, a documented long track record of safe usage is available and should not be disregarded. Published reports detailing numbers and characteristics of patients (Hutchinson and Watson, 1962; Bailey, 1968; Onyango et al., 1970; Temu, 1975; Cassaday et al., 1979; Abaru et al., 1984), in despite of the legal and ethical issues involved, suggest a widespread familiarity with the drug that was likely an open secret. Very few side effects were noted and all were reversible. Among the reported experiences with the use of DIZE there is even a study on primary school children, albeit topically applied (Lynen and Van Damme, 1992).

Bearing in mind that the development of a new drug normally takes a decade of expensive research, previous experience with DIZE presents an advantage, particularly when therapeutic alternatives are scarce. Undoubtedly, further investigation is required to better establish the precise molecular mechanisms by which DIZE exerts its effects. Research into the action of DIZE on cell cultures infected by SARS- CoV-2 and experimental models may prove useful. Pharmacological studies and clinical evidence are also needed to determine the relative benefits and risks in Covid-19 patients. Whilst international ethical and scientific research guidelines should not be undermined, providing a timely response results equally essential. This includes the appropriate clinical trials as well as exceptional interventions following the WHO recommendations for MEURI *("Monitored Emergency Use of unRegistered and experimental Interventions")* (Weltgesundheitsorganisation, 2016). The authors believe available data from laboratory findings and animal studies together with previous use of DIZE in humans could provide legitimate support for DIZE treatment in selected patients, in which the therapeutic arsenal has been exhausted and the prognosis is grim. Depriving patients of a feasible alternative to improve their condition, even if representing a last option, carries a risk of overriding the principle of beneficence that has guided the pandemic-induced unavoidable off-label interventions to date.

This paper is intended as a call for consideration to be given to an old drug that is affordable, easy to administer, well tolerated and potentially useful in a context of overstretched healthcare systems worldwide and a pressing need to reduce morbimortality, together with the social and economic consequences caused by the Covid-19 pandemic.

DISCLOSURES

None

Declaration of competing interest

The authors declare they have no conflict of interest.

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