Review Article

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Therapeutic approaches on the interaction between SARS-CoV2 and ACE2: a biochemical perspective

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Abstract: The current conditions in the progression of the SARS-CoV2 pandemic changed the current scientific paradigm, and we now observe a novel rhythm and way of evaluating the collected information. Previous experiences in epidemics with similar viruses (viz., SARS-CoV1, and MERS-CoV) and collected information about the viral transmission and replication can be used to overcome the SARS-CoV2 pandemic. Although SARS-CoV2 emerged very recently, there are plenty of scientific studies about similar viruses to comment on the current situation. Inhibition of SARS-CoV2 spike protein activation, inhibition of virus endocytosis, using a soluble form of ACE2, peptide or non-peptide analogs of ACE2, and sustaining ACE2/Angiotensin-(1–7)/Mas receptor pathway activation can be proposed for use in therapeutic studies. In this review, the biochemical mechanism of SARS-CoV2 and ACE2 binding, virus-cell membrane fusion, and endocytosis of virus to host cells are discussed according to the currently available literature. The significant contribution of this review may be to provide useful information to researchers into the SARS-CoV2 outbreak.

Keywords: ACE2; chloroquine/hydroxychloroquine; SARS-CoV2; diphyllin; targeting tumor necrosis factor- α -converting.

Introduction

Coronaviruses are one of the viruses that may cause severe acute respiratory syndrome (SARS). The first coronavirus associated outbreak caused by SARS-CoV1 was in 2002 in Asia. Later in 2012, the Middle East respiratory syndrome coronavirus (MERS-CoV) was identified in the Middle East and subsequently in several European countries [1]. In December 2019, a new coronavirus SARS-CoV2 was reported in Wuhan, China. This outbreak in China quickly became a threat to all countries [2]. Finally, the World Health Organization (WHO) renamed SARS-CoV2 as Covid-19 and declared the Covid-19 pandemic in March 2020 [3].

SARS-CoV2 appeared as a zoonotic infection, but later human-to-human transmission with close contact through the respiratory tract was confirmed. The first infected individual transmitted the virus in aerosols of his/her cough or sneeze [4, 5].

In early studies, angiotensin-converting enzyme 2 (ACE2) was described as an essential receptor for SARS-CoV1 in 2003 [6]. Although ACE2 expression is prevalent in both small intestine and kidney, SARS-CoV2 is shown in feces but not in urine [7,8]. Detection of SARS-CoV2 in feces (anal swab) was observed in a study after two subsequent respiratory (oropharyngeal) negative swabs five days apart [9]. There can be various human-to-human transmission routes, and detection of SARS-CoV2 simultaneously in all these routes may not be probable. According to the study mentioned above, this raised the consideration that a negative oropharyngeal swab does not rule out a positive fecal swab. Since ACE2 is considered as the host entry receptor of SARS-CoV2, in the development of therapeutic drugs and vaccines targeting SARS-CoV2, consideration of ACE2 receptor expression would be rational [10].

In this review, the biochemical mechanism of SARS-CoV2 and ACE2 receptor binding, virus-cell membrane fusion, and endocytosis of virus to host cells will be discussed according to the current literature. Agents used in previous studies for several purposes but play a

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significant role in biochemical mechanisms in SARS-CoV2 pathogenesis will be discussed as potential therapeutic drugs.

Biochemical and physiological properties of ACE2

It should be noted that ACE2 and Angiotensin-converting enzyme (ACE, EC 3.4.15.1) are not isoenzymes; each catalyzes different enzymatic reactions and have distinct physiological effects. ACE is a membrane-bound carboxypeptidase that can hydrolyze into two amino acids from the C-terminal of Angiotensin I (histidine and leucine) and converts Angiotensin I (a decapeptide) to strong vasoconstrictor Angiotensin II (an octapeptide). ACE2 is a carboxypeptidase that removes an amino acid from the C-terminal of both Angiotensin I and Angiotensin II. ACE2 does not convert Angiotensin I to Angiotensin II, unlike ACE. Then ACE inhibitors do not act as ACE2. ACE2 is a type1 transmembrane protein (N-terminus outside, C-terminus intracellular) (Figure 1) [11].

The physiological effects of these two enzymes, ACE and ACE2, are opposite to each other, and balance in the renin-angiotensin pathway depends on the activity of these enzymes [12].

In several studies, the ACE2/Ang-(1–7)/Mas axis may have negative modulation inflammation over leukocyte migration, cytokine secretion and expression, and fibrinogenic pathways [13]. Thomas et al. [14] have shown that ACE2 deficiency increased vascular cell adhesion molecule (VCAM), tumor necrosis factor- α (TNF- α), monocyte chemoattractant protein-1 (MCP-1), interleukin 6 (IL-6), and matrix metalloproteinases (MMPs) [ed1] in vascular

inflammation and atherosclerosis. But how ACE2 deficiency alters these inflammatory mechanisms is not clarified yet. Then we consider that ACE2 deficiency may have a critical role in the cytokine storm defined in SARS-CoV2 pathogenesis.

The classic renin-angiotensin system is a potent pro-oxidant system in vessels that cause endothelial dysfunction. The ACE2/Ang-(1–7)/Mas receptor axis counteracts these effects. Also, numerous studies showed that Angiotensin (1–7) functions mostly as an anti-thrombotic, anti-proliferative, and antioxidant [15]. The mechanisms of action in circulation have not yet been well established.

Distribution of ACE2 protein in the human organism

In the Human Protein Atlas database, ACE2-mRNA is prevalent in the small intestine, colon, duodenum, kidney, testis, and gallbladder [7]. It postulated that the first transmission of SARS-CoV2 to humans is through the bat feces contaminated fruits ingestion in the Wuhan market because ACE2 is highly expressed on the luminal surface of the intestinal epithelia [16]. The ACE2 receptor expression in the lung is predominantly in alveolar epithelial type II cells (AEC-II). Since AEC-II cells in the lung are responsible for surfactant production, surfactant deficiency may lead to ARDS [17]. Expression of ACE2 in various organs other than the lung may explain multi-organ dysfunction observed in SARS-CoV2 infection.

The entry of the SARS-CoV2 into the host cell

The entrance of SARS-CoV1 to the host cell by direct fusion that is independent of pH was shown with electron microscopy [18] (Figure 2). Consequent studies showed that SARS-CoV might enter the host cell by endocytosis [19]. The ACE2 receptors in the ectodomain site of the host cell bind with SARS-CoV, and ACE2 shedding starts. Then cellular ACE2 levels decrease, but soluble ACE2 increases. Significant SARS-CoV2 related ACE2 shedding occurs in type II alveolar lung cells. Kuba et al. [20] have shown that recombinant Spike-Fc protein reduced human ACE2 (hACE2) expression in A549 human alveolar cells and mouse ACE2 (mACE2) expression in IMCD kidney epithelial cells. In another study, Haga et al. [21] have shown that recombinant SARS-S treatment reduces ACE2 expression level in Vero E6 cells infected with a virus that express S protein

Figure 1: Renin-angiotensin pathway and its physiologic effects.

(S-virus). According to these findings, we may conclude that ACE2 maintenance in type II alveolar lung cells is protective for SARS-CoV2 caused lung injury.

Coronaviruses

Coronaviruses belong to the Coronaviridae family, which are enveloped and have a single-stranded RNA. There are four major structural proteins produced by the viral genome (spike, envelope, membrane, and nucleocapsid). The spike glycoprotein, called 'S', is positioned in the outermost part, has three domains: the receptor-binding domain (RBD), the receptor-binding motif (RBM), and the transmembrane domain (TD) [22]. Detected mutations in the RBD domain of spike glycoprotein, called 'S', are listed as L455Y, F486L, Q493N, and S494D. The N501T mutation is included in this list. An alarming finding states that mutation in N501T is considered to increase the binding affinity between spike protein RBD and human ACE2. Researchers recommend monitoring mutations in N501T [23].

The spike protein has two units; the N-terminal surface unit (termed S1) that has a receptor-binding domain, and a C-terminal transmembrane unit (termed S2) that has three functional elements, a fusion peptide, heptad repeats, and a transmembrane domain [22].

The transmembrane glycoprotein provides viral binding to host cells at proteolytic cleavage sites (S1/S2, putative fusion peptide (S2')) in the S2 domain). Binding to ACE2 receptor with S1 cleaves to S1/S2, with host proteases on the

host cell membrane. The host cell membrane fuses with Spike's S2 site, and this process leads to cell membrane lyses and viral genome entrance to host cell cytoplasm [24, 25].

In an *in vitro* study by Li et al. [6], the ACE2 receptor on the host cell membrane was shown in 2003. In a study with knockout mice in 2005, the ACE2 receptor on the host cell was shown as essential for SARS CoV1 infection [26]. Another study by Glowacka et al. [27] showed that recombinant SARS-CoV1 spike protein decreases ACE2 expression leading to lung injury.

Proposals to inhibit SARS-CoV entrance to host cell membrane

Protease inhibitors targeting SARS-CoV2

This process starts with the ACE2 receptor and SARS-CoV2 spike protein binding. Later, host cell surface transmembrane proteases, protease/serine proteases (TMPRSS2), furin, trypsin, elastase, and plasmin initiates spike protein cleavage (S1/S2). The S2 spike protein then activates the host cell membrane and SARS-CoV fusion. Viral attachment and subsequent fusion are essential processes for SARS-CoV infection [5, 27–29]. Membrane-bound trypsin-like serine; TMPRSS2 on the host cell surface or in the secretory pathway of host cells are type II transmembrane proteins. TMPRSS2's that are prevalent in the respiratory system play a role in

Figure 2: The mechanism of SARS-COV2 entrance host cell.

activation to enter the host cell for several viruses [24, 29]. Recently Hoffmann et al. [28] stated that SARS-CoV2 entrance to host cell is initiated by ACE2 binding, spike cleavage, and activation by TMPRSS2 activity. ACE2 binds with SARS-CoV2 and TMPRSS2 in the lung type II alveolar cell membrane [29]. Serine protease inhibitors, Camostat mesylate, and Nafamostat mesylate are approved to treat chronic pancreatitis, postoperative reflux esophagitis, and anticoagulant. Hoffman et al. [28] showed that Camostat mesylate prevents S protein priming in cell culture, so binding with transmembrane serine protease TMPRSS2 is inhibited. Camostat is used as an alternative drug that can be used orally and substituted for Nafamostat that is used by the intravenous route [30–32]. The half-maximal inhibitory concentration (IC_{50}) of nafamostat was 0.1 μ M, about 10 times lower than that of camostat. Studies on about Camostat mesylate as the therapeutic agent for SARS-CoV2 are very promising.

Among the papain-like protease (PLpro) inhibitors, there are thiopurine inhibitors such as 6-mercaptopurine [33] and natural product inhibitors (Tanshinones, diarylheptanoids, and Geranile flavonoids) [34–36]. The other coronaviral proteases that are essential in virus replication, papain-like protease (PLpro), and 3C-like protease (3CLpro), are the most popular currently. PLpro is related to the ubiquitin pathway [37, 38]. Inhibitors of 3CLpro are *I.* indigotin carrot extract, indigo, sinigrin, aloe-emodin, and hesperetin has significant inhibitory effects for SARS-CoV replication [39]. The inhibition of viral proteases (PLpro, 3CLpro) may be considered in the therapeutic approach since they alter virus replication.

Viral inactivation by tumor necrosis factor- α -converting enzyme (TACE)

A protease ADAM 17, also called TACE activity, cleaves the ectodomains of various transmembrane proteins. It was shown that spike protein and ACE2 binding induces ADAM 17 activity that leads to shedding of ACE2 ectodomain. Although some studies indicate that this shedding is essential for viral replication, TACE inhibitors may help prevent shedding. On the other hand, there are also some other studies indicating that shedding is unnecessary for viral replication [40]. Shedding of ACE2 from the human respiratory epithelium is inhibited by the inhibitor of ADAM 17 [41]. The anti-inflammatory and immunomodulatory properties of “Thalidomide” may inhibit the pro-inflammatory cytokine TNF- α by increasing the degradation of TNF- α mRNA and suppress pro-inflammatory cytokine release in different cell types [42]. Then, one may raise

the consideration of using thalidomide to prevent the cytokine storm of SARS-CoV2.

Proposal to inhibit the entrance of SARS CoV2 into the host cell through endocytosis

Increasing endosomal pH

Endocytosis-regulated SARS-CoV entrance to host cells depends on both pH and ACE2 receptor binding [43]. Endosome maturation has two stages; early endosomes (pH 6.5–6.0) and late endosome (pH 6.0–5.5). The acidification process in the endosome is the regular mechanism for several biochemical and physiological functions, as well as viral replication. This endocytic pathway process ends on lysosomes. A couple of studies showed that SARS-CoV initiates several mechanisms in entering the host cell through endocytosis [18, 44]. Interestingly, it has also been reported that macropinocytosis is an alternative way to enter the host cell [45]. Cathepsin L should activate SARS spike in the host cell endosome for fusion with the endosome membrane and to release its genome to the host cell cytoplasm. SARS-CoV activating proteases; Cathepsin L and TMPRSS2 are found in endosome and host cell membranes, respectively. One may consider that Cathepsin L is very unstable in neutral or low alkaline pH, but increased endosome pH may inhibit Cathepsin L activity [46]. *In vitro* studies it was shown that “MDL28170” inhibits Cathepsin L activity [47]. Agents increasing endosomal pH, NH_4Cl , Chloroquine/hydroxychloroquine, and P9 may all have antiviral activity by increasing endosomal pH.

Chloroquine/hydroxychloroquine

According to the “Henderson Hasselbalch” rule, protonated lysosomotropic weak bases Chloroquine and Ammonium chloride (NH_4Cl) are trapped in an endosome. In conclusion, these weak bases increase endosome pH leading to Cathepsin L inactivation that also prevents spike protein activation in an endosome. Several *in-vitro* or *in-vivo* studies state that antimalarial drugs Chloroquine/hydroxychloroquine decreases viral reproduction and viral load for several viruses. Prevention of SARS-CoV replication with Chloroquine IC_{50} dose at $8.8 \pm 1.2 \mu$ M was shown [48–50].

One of the two other mechanisms about chloroquine/hydroxychloroquine for SARS CoV inactivation is the inhibition of post-translational modification of viral

envelope glycoprotein with proteases and glycosyltransferases; and the other mechanism is immune modulation. In a couple of studies, it was shown that chloroquine/hydroxychloroquine prevents SARS-CoV activity in cell culture by interfering with terminal glycosylation of cellular receptor ACE2 and sialic acids [49]. In this way, SARS-CoV binding to ACE2 can be prevented. The use of hydroxychloroquine in rheumatic diseases is primarily for its immune modulator effect and decrease in phospholipase A1 activity that leads to an anti-inflammatory effect. Other than antiviral activity, the mechanism of action of chloroquine and hydroxychloroquine is quite comprehensive and complex. The immune modulator, anti-inflammatory, pH increasing effects of Chloroquine/hydroxychloroquine needs further research for SARS-CoV-2 management [51].

P9

A 30-aminoacid long basic protein P9 that is produced from “ β -defensin-4 (mBD4)” by mice inhibits both SARS-CoV and MERS-CoV both *in vitro* and *in vivo*. This basic protein prevents viral attachment to the host cell by binding to viral spike proteins and prevents endosome acidification from employing its own basic structure. This basic protein “ β -defensin-4 (mBD4)” is produced by mucosal membranes to prevent enveloped viruses entrance to the respiratory, digestive, urinary, and reproductive system. It was shown that the IC_{50} of P9 against SARS-CoV was approximately five $\mu\text{g/mL}$. In particular, it has been suggested that P9 at concentrations higher than 25 $\mu\text{g/mL}$ can prevent more than 95% of SARS-CoV and MERS-CoV infections [52, 53]. This information, may indicate that broad-spectrum antiviral activity of P9 should be investigated in further studies particularly for SARS-CoV2.

Blocking Vacuolar-ATPase (V-ATPase)

Primarily protons are pumped to organelle with V-ATPase from the cytoplasm. V-ATPase has two subunits: a transmembrane V_0 complex; and a V_1 cytosolic complex. These subunits transfer protons through membranes, and hydrolyze ATP to convert chemical to mechanical energy, thereby force proton displacement, respectively [53]. A macrolide “Bafilomycin A1” isolated from the fermentation of *Streptomyces* spp prevents ATP dependent proton translocation from cytoplasm to endosome by V-ATPases. It was shown that Bafilomycin A1 induced pH increases in endosome might inactivate SARS-CoV [54, 55]. The inhibitor of V-ATPases “Concanamycin A” acts by binding

to V_0 subunit c [56]. Diphyllin, used to treat Zika, Dengue, Yellow fever, tick-borne encephalitis, Japanese encephalitis, West Nile and Ebola virus infections, may also be used to treat SARS-CoV by preventing endosomal acidification. Diphyllin is a lignan compound used for its antitumor activity in Chinese medicine and has broad-spectrum antiviral activity as a potent vacuolar ATPase (V-ATPase) inhibitor. It was reported that glycosylated diphyllin (patentiflorin A) was isolated from *Justicia gendarussa* and had *in vivo* and *in vitro* inhibitor effect on the Zika virus. Diphyllin/glycosylated diphyllin blocks not only the Zika virus but also other flaviviruses [57–61]. Thus, inhibitors of V-ATPases may be another option in SARS-CoV2 therapy.

Proposals for activation of ACE2/Ang-(1–7)/Mas receptor pathway

Several studies have reported that ACE2 protects against acute lung damage [15, 62]. Thus, the balance of ACE/AngII/AT1R and ACE2/Ang-(1–7)/Mas receptor axis appears to play a critical role in the pathogenesis of SARS-CoV2 (Figure 1). SARS-CoV2 uses ACE2 to enter the host cell, so decreased cellular ACE2 expression may be responsible for the cytokine storm. So, a novel approach may be to find new ways to increase ACE2 activity. Limited ACE2/Ang-(1–7)/Mas pathway in the lung may be the cause of aggravated cytokine storm and ARDS, so increasing ACE2 activity in the lung may be useful rather than harmful viral entrance to the host cell. Increased viral load in the respiratory system may have a harmful effect since ACE2/Ang-(1–7)/Mas pathway is slowed down in the absence of ACE2 also. The following options may be used to sustain the ACE2/Ang-(1–7)/Mas pathway.

Using recombinant human ACE2 (rhACE2) to activate ACE2/Ang-(1–7)/Mas pathway

One of the possibilities of activating the ACE2/Ang-(1–7)/Mas axis is the use of rhACE2. ACE2 has two functional units. These are membrane-bound ACE2 and soluble ACE2 (sACE2) [63]. The extracellular domain of ACE2 is hydrolyzed by an enzyme known as sheddase, and sACE2 is formed [64]. If recombinant ACE2 replacement is sustained, the binding of coronavirus to ACE2 on the cell membrane can be reduced, thereby reducing the entry and replication of the virus into the host cell.

Circulating ACE2

There is sACE2 in circulation; also, pathologies related to SARS-CoV2 in other organs may be alleviated. As far as we know, we do not have sufficient information yet to use hrACE2 in humans. Future studies about antibodies hrACE2 or anti-ACE2 are needed [9].

Using peptide or non-peptide analogs

The most frequently studied Ang (1–7) analog is non-peptide “AVE 0991” that is orally active and a physiologically well-tolerated imidazole derivative. However, short half-life and rapid degradation of AVE 0991 in gastrointestinal system may be a limitation. Beneficial effects of “AVE 0991” are reported as reno-protection, cardio-protection, and anti-atherogenesis. Even its use in diabetic foot is being investigated (phase II trial). Angiotensin (1–7) exerts its action through stimulation of the specific G-protein coupled Mas receptor. Mas receptor stimulation increases phosphorylation of endothelial nitric oxide synthase and nitric oxide release [65–68].

Increasing ACE2 receptor activation

A Decrease in ACE2 activity occurs in the elderly in certain chronic disorders (hypertension, cardiovascular diseases, and diabetes, etc.). Since SARS-CoV-2 infection decreases ACE2 activity and increases ACE2 receptor consumption imbalance between Ang II and ACE2 may increase the severity of pathophysiological mechanisms during SARS-CoV2 infection [69].

Cheng et al. [70] found that ACE2 expression in rat lung is dependent on age and gender since ACE2 expression is high in young rats.

Although there is no registered drug yet, other alternatives to increase ACE2 activity, thereby increasing Ang-(1–7) synthesis, are to use agents modulating ACE2 gene expression. In 2008, two ACE2 activators were discovered; xanthenon (XNT) and resorcinolnaphthalein [71].

Diminazen aceturate (DIZE) is another ACE2 activator used as a veterinary drug. DIZE is approved by the FDA, is an antitrypanosomal drug, and increases *in vivo* ACE2 activity. The protective effects of DIZE are associated with the activation of the overprotective axis of the lung renin-angiotensin system, decreased inflammatory cytokines, improved pulmonary vasoreactivity, and enhanced cardiac function [72, 73]. The data presented shows great promise

in animal models, but, unfortunately, lack necessary human data as yet.

Conclusion

The contribution of this review may provide useful information to researchers who are studying or plan to study the SARS-CoV2 outbreak. Therapeutic approaches listed in biochemical perspective to overcome SARS CoV-2 are shown below.

- (1) Inhibition of TMPRSS2 proteolysis for SARS-CoV2 Spike Protein
- (2) Inhibition of SARS-CoV2 proteases
- (3) Inhibition of vesicle-mediated endocytosis of SARS-CoV2
 - Direct inhibition of cathepsin L
 - Increasing the endosome pH
 - V-ATPase inhibition
- (4) Using a soluble form of ACE2
- (5) Peptide or non-peptide analogs of Angiotensin-(1–7) for ACE2 activation

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