A review on the cleavage priming of the spike protein on coronavirus by angiotensin-converting enzyme-2 and furin

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ABSTRACT
The widespread antigenic changes lead to the emergence of a new type of coronavirus (CoV) called as severe acute respiratory syndrome (SARS)-CoV-2 that is immunologically different from the previous circulating species. Angiotensin-converting enzyme-2 (ACE-2) is one of the most important receptors on the cell membrane of the host cells (HCs) which its interaction with spike protein (SP) with a furin-cleavage site results in the SARS-CoV-2 invasion. Hence, in this review, we presented an overview on the interaction of ACE-2 and furin with SP. As several kinds of CoVs, from various genera, have at their S1/S2 binding site a preserved site, we further surveyed the role of furin cleavage site (FCS) on the life cycle of the CoV. Furthermore, we discussed that the small molecular inhibitors can limit the interaction of ACE-2 and furin with SP and can be used as potential therapeutic platforms to combat the spreading CoV epidemic. Finally, some ongoing challenges and future prospects for the development of potential drugs to promote targeting specific activities of the CoV were reviewed. In conclusion, this review may pave the way for providing useful information about different compounds involved in improving the effectiveness of CoV vaccine or drugs with minimum toxicity against human health.

Introduction
At the end of December 2019, Chinese public health officials announced to the World Health Organization (WHO) that a new and unknown virus caused a disease with symptoms similar to pneumonia in Wuhan (Khan & Fahad, 2020). They immediately recognized that the virus was from the coronavirus (CoV) family and was rapidly spreading out of Wuhan. The WHO is seeking to identify, track and restrict a new disease from the CoV family called CoV disease 2019 (COVID-19), which is still affecting many peoples in China and outbreaking to other countries. This type of CoV is also spreading in other countries such as Iran, Italy and South Korea (Memberships & Join, 2020). With the number of CoVs currently increasing, the researchers are trying to figure out what is the main cause of the virus to spread easily and widely. Some genetic and structural analyzes have identified the main characteristic of the virus, which could increase the efficiency and speed of virus transmission between human cells (Belouzard et al., 2012; Jaimes & Whittaker, 2018). Also, some groups are investigating a receptor on cell membranes that introduces the fusion of new CoV into human HCs (Veljkovic et al., 2020; Zheng & Perlman, 2018). Both the cellular and protein receptors of the virus provide potential targets for development of drugs against pathogen (Kaufmann et al., 2018; Papadopoulos et al., 2017). However, some well-developed experiments and promising data are required to ensure such a mechanism. Indeed, exploring the transmission mechanism of the virus is important to prevent its future outbreaks. The COVID-19 spreads much more easily than the SARS virus and infecting easily the people have been already infected with SARS (Jiang et al., 2020; Lai et al., 2020; Liu et al., 2020).

To infect a cell, viruses use spike protein (SP) to bind to the cell membrane, a process activated by specific cellular enzymes such as trypsin, furin, and cathepsin L (Jaimes & Whittaker, 2018; Li et al., 2017; Millet & Whittaker, 2015).

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Genomic analysis of the new CoV has shown that its SP differs from that of other viruses (Du et al., 2017; Li, 2016), indicating that the protein has a site activated by a HC enzyme called furin (Millet & Whittaker, 2015) (Figure 1).

This enzyme normally cleaves and activates a wide range of substrates in different organisms (Figure 2a) (Braun & Sauter, 2019). This enzyme is found in many human tissues, including the lungs, liver and small intestine, which means that the virus has the potential to invade different organs (Belouzard et al., 2012; Heald-Sargent & Gallagher, 2012; Scamuffa et al., 2006). The furin activation site (FAS) makes the new CoV much different in cell entry than SARS, and probably affects the stability of the virus and, consequently, the transmission process (Li et al., 2015; Millet & Whittaker, 2014; Yamada & Liu, 2009). Several other groups have also identified the site of activation to investigate how the virus spreads among humans (Basak et al., 2007; Kim et al., 2016; Kleine-Weber et al., 2018; Porter et al., 2014). They pointed out that other viruses including influenza family viruses contain these sites that are easily spread among people (Huang et al., 2011; Menachery et al., 2014; Tang et al., 2020; Vlasak et al., 1988). But in these viruses, the site of activation is found on a protein called haemagglutinin, not on the SP (Bernstein et al., 2020; Koopman et al., 2019; Lai et al., 2019). However, some other researchers are more cautious about the significant role of the activation site in helping CoV and facilitating its spread (Amer et al., 2018; Van Doremalen et al., 2013). Other scientists are also skeptical of comparing the influenza viral activation sites with the new CoV transmission channels (Heymann & Shindo, 2020; Yang et al., 2005). The haemagglutinin protein at the surface of influenza viruses is not the same as the SP in CoVs (Yin et al., 2016; Zmora et al., 2018).

In other hand, the influenza virus that has caused the deadliest recorded pandemic lacks even a FAS (Couartard et al., 2020). Therefore, some studies on cellular or animal models are required to assay the function of the activation site. Indeed, CoVs are unpredictable and some experiments should be developed to assess how to modify the activation site. Some other experiments have done to explain why the new CoVs successfully infect human cells (Huang et al., 2020; Letko & Munster, 2020). Their experiments have shown that the SP binds to the human cell receptor (Hulswit et al., 2019). For example, a drug that blocks the receptor may make CoV entry into cells difficult.

**Interaction of furin and angiotensin converting enzyme-2 (ACE-2) with SP**

Furin and furin-like proteases belong to the group of proprotein convertases. Scientists have pointed out that among several targets, the SP as fusogenic envelope glycoprotein of SARS CoV, is a promising site for treatment of infectious diseases (Li et al., 2018; LICITRA et al., 2013). SPs mediate CoV fusion and the entry of the CoV genetic material into the human cell (Senathilake et al., 2020; Struck et al., 2012). Afterwards, a SP-selective strategy, when used at the initial phase of CoV infection is probably considered as a potential approach to mitigate infection inside the body. The furin induces the cleavage of SPs at the RNTR761 EV site (Bergeron et al., 2005) and results in the production of two segments: S1 and S2 (Figure 2b); each segment shows its own biological activity. The S1 segment known as the globular section of SP mediates attachment of CoV to the receptor, ACE-2 with dominance of α-helix structures (Li et al., 2003). A 193-residue fragment of SP has been determined as the protein-binding site (Wong et al., 2004). S2, known as the biomembrane-anchored stalk domain and leads to CoV–human cell fusion (Prabakaran et al., 2004). Some other experiments also exhibited that other biomembrane-anchored proprotein convertases (PrCos), namely PoCo5B and PoCo7 cleave SPs.

In this regard, Basak et al. (2007) employing intramolecularly quenched fluorogenic (IQF) proteins relied on SARS-CoV SP claimed that PoCo5B and PoCo7 beside furin may result in the cleavage of SPs. Kinetic studies showed that the cleavage of peptide occurred potentially by furin as well as recombinant PoCoSB, but with a limited amount by PoCo7. Furthermore, they demonstrated that the cleavage activity could be inhibited by a PoCo-inhibitor, α1-PDX. Circular dichroism intensities revealed that the content of β-sheet conformation in PoCo increases after cleavage by furin (Figure 2c). 1H nuclear magnetic resonance (NMR) spectroscopy displayed that this peptide has a turn motif at its C-terminal part, close to the cleavage site.

Coutard et al. (2020) revealed that the SP of the SARS-CoV-2 possesses a furin-like cleavage site absent in CoV of the same clade. The genome sequence revealed that SARS-CoV-2 is included in lineage b of β-CoV (Figure 3a). Chen et al. (2020) presented that SARS-CoV-2 as a newly detected CoV shows a high level of similarity to SARS-CoV. The structural analysis of the receptor binding domain (RBD) of SP from the two viruses show 72% similarity in residue sequences (Figure 3b). Molecular modeling studies depicted that SARS-CoV-2 RBD provides a stronger attachment with ACE-2. They found that a distinct Phe residue plays a key role in the binding site due to its interaction with ACE-2.

**Furin cleavage site**

Several kinds of CoV, from various genera, have at their S1/ S2 binding site a furin cleavage site (FCS) (Belouzard et al., 2012). An important phase in the life cycle of the CoV is the human cell entry and fusion derived from proteolytic activity of the relative fusion protein (FP) by respective proteases (Jaimes & Whittaker, 2018). A conventional approach to explore the CoV life cycle is to clone the FP gene into an appropriate vector, transfect the cells, incubate with the related protease, purify the protein and carry out the protein assays (Cong et al., 2019; Li et al., 2019). This approach shows a number of drawbacks: the need of viral genome, expensive synthesizing process, availability of monoclonal antibodies, and time-consuming process. Therefore, Jaimes et al. (2019) reported the fluorogenic peptide cleavage analysis to assay the proteolytic activity of recombinant FP which can be applied in the case of CoV SP in a less labor and time intensive way.
In another study, Xi et al. (2020) isolated one strain of SARS-CoV-2 (ZJ01) in mild COVID-19 patient and reported the presence of more than 30 specific gene mutation. The theoretical analysis of FCS and the case alignment of CoV family determined that FCS may be a crucial site of CoV evolution. ZJ01 showed mutations close to FCS (F1-2), which led to alterations in the conformation and the charge distribution on the surface of the SP. They employed Adaptive Poisson-Boltzmann Solver (APBS) analysis and exhibited that the binding site of furin was covered with a number of negative residues (Figure 4a). The F1 site of SARS-CoV-2, namely ZJ01, Wuhan-Hu-1 and RaTG13 were almost covered with positive moieties while SARS was covered by both mixed negative and positive residues. F1 site of ZJ01 showed more positive charge distribution in its head and more negative residues in its basal site in comparison with Wuhan-Hu-1.

**Figure 1.** Schematic presentation of CoV (i), schematic of a protease cleavage site (ii), ribbon presentation of the structures of three HC proteases (iii), diagram of a CoV life cycle (iv). Abbreviation: transmembrane serine protease (TMPRSS). Reprinted with permission form Ref. (Millet & Whittaker, 2015).

**Figure 2.** (a) Schematic illustration of peptide cleavage induced by furin in different organisms (Braun & Sauter, 2019). (b) Schematic illustration of the residue sequence of SP from human SARS CoV [SP: signal peptide; FP: fusion peptide domain; HR-N: heptad repeat domain NH\(^+\) terminal; HR-C: heptad repeat domain COO- terminal; TMD: transmembrane domain; CT: cytosolic tail (Basak et al., 2007). (c) CD signals of QSARS-4 peptide after incubation with different concentrations of recombinant furin (Basak et al., 2007). Reprinted with permission from Refs. (Basak et al., 2007; Braun & Sauter, 2019).
The F2 site of GZ02 had negative charge distribution while F2 site of Wuhan-Hu-1 and RaTG13 demonstrated a limited level of positive moieties. ZJ01 displayed more positive charge distribution in F2 site than the other species, most likely derived from gene deletion. GZ02 presented a number of negative charge distribution in F3 site while limited negative charge distribution was observed in SARS-CoV-2-associated virus (Figure 4a). Hence, they deduced that, the mutation close to FCS site lead to a significant change in the protein conformation and surface charges, which further affected its interaction with the ligands (Xi et al., 2020).

Hoffmann et al. (2020) depicted that SARS-CoV-2 cell entry relied on ACE-2 and TMPRSS2 and is limited by a protease inhibitor. Indeed, exploring the cellular factors employed by SARS-CoV-2 for entry might result in providing useful information about viral outbreak and a number of therapeutic approaches. Hoffmann et al. (2020) exhibited that SARS-CoV-2 utilizes the SARS-CoV receptor ACE-2 for specific cellular
internalization and the TMPRSS2 as a serine protease for SP cleavage. A TMPRSS2 inhibitor can be developed in clinical application to block the cellular internalization and might result in the advancement of a therapeutic approach. In general, they demonstrated crucial similarities between SARS-CoV-2 and SARS-CoV infection and reported a promising target for development of anti-viral platforms (Figure 4b) (Hoffmann et al., 2020).

Enzyme inhibitors as therapeutic platforms

The design of enzyme inhibitors as therapeutic platforms against CoV require the control of multiple pharmacologic features beyond the interaction and ACE-2- and furin-targeting drugs (Baron et al., 2020; Kong et al., 2020). A number of these pharmacologic characteristics show their molecular underpinning in chemical pathways within the biological systems. Examples of this include drug transport pathways, blood circulation of drug, drug metabolism, and side effects derived from the interactions of drug with a wide range of enzymes (Zumla et al., 2016). Thus, some thermodynamic and kinetic data in the pharmacological development of drugs during preclinical studies are required.

As we overviewed so far, reducing the levels of ACE-2, might provide a great deal of interest in fighting the CoV. In a point of fact, ACE-2 can induce a protective impact against CoV-stimulated lung damage by enhancing the formation of the vasodilator angiotensin (Imai et al., 2005). Indeed, the attachment of the SP of the CoV to the ACE-2 triggers a reduction in the levels of ACE-2 (Kuba et al., 2005), most likely stimulating lung injury. Xu et al. (2017) reported that vitamin D can be used as a potential candidate to mitigate lipopolysaccharide-induced acute lung damage via control of the renin-angiotensin system. Therefore, it could be suggested that vitamin D can control the outbreak of CoV through inhibition of ACE-2. Gurwitz (2020) claimed that angiotensin receptor blockers can be used as promising SARS-CoV-2 therapeutics. Also, it has been suggested that probable way of fighting the CoV could be the injection of ACE-2 which will result in the preventing the interaction of the CoV to off-infected cells and replenishing ACE-2 in infected cells (Zhang et al., 2020). Some other studies have indicated a close correlation between hypertension and heart disease and CoV infection which may be associated with the prescription of ACE-2 inhibitors (Fang et al., 2020). Indeed, treatment of CoV infection with ACE-2 inhibitors leads to an upregulation of this receptor especially by epithelial cells of the lung followed by facilitation of infection with different kinds of SARS-CoV (Li et al., 2017).

Based on these reports, some attention has been given to the development of furin inhibitors as potential therapeutics platform against SARS-CoV-2 infection. Although, a great deal of research is required to exhibit experimentally this assertion, the inhibition of furin or furin-like enzymes may display a promising anti-viral platform. Actually, it has been recently demonstrated that HCs infected by several kinds of viruses stimulate an interferon-based activity to block the enzymatic activity of furin-like enzymes (Lodermeyer et al., 2013). It was also revealed that virus infection triggers the upregulation of some receptors (Braun & Sauter, 2019; Kim et al., 2015) that inhibit the furin trafficking in post-Golgi compartments.

Also, based on the crystal structure of furin, some potent inhibitors like 2,5-dideoxystreptamine-mediated inhibitor
were developed to be used in clinical trials (Dahms et al., 2017). Because, furin-like enzymes contributed in a several pathways, one crucial point would be to limit the systemic inhibition that may lead to some adverse effects. Consequently, it is most likely that such small molecule or other active agents as promising drugs, probably administered by inhalation and presenting a strong interaction with furin to stimulate a prolonged inhibition, deserve to be quickly analyzed to examine their anti-viral impact against SARS-CoV-2. In general, these details disclosed that inhibitors of furin or furin-like enzymes may play a key role in blocking virus outbreak.

**Ongoing challenges and future perspective**

ACE-2 exhibited the same primary structures patterns in vertebrates’ lineages (Imai et al., 2005). Structural investigations indicated that ACE-2 from these systems can efficiently interact with RBD of SARS-CoV-2, inducing them all to serve as promising hosts for the virus infection (Mathewson et al., 2008; Poon & Peiris, 2020). Furthermore, it can be deduced that small molecular and ligands inhibitors that can limit the interaction of ACE-2 with RBD should be developed to combat the spreading CoV epidemic (Chen et al., 2020; Senathilake et al., 2020). Isolating and cultivating CoV *in vitro* may not usually be practical or demand particular facilities that are not accessible in every bioresearch laboratory. Therefore, there is a necessity for developing some strategies to assay the human health response to spread of emerging CoV that can be performed in normal laboratory systems. Apart from viruses targeting humans, several animal viruses also have identical FPs, hence, exhibiting the comparable features than their human sites. Purifying these kinds of viruses can display some inevitable biological challenges, making the application of pioneering devices to examine them unavoidable.

Current drugs have limited efficacy in treating CoV in different populations and species. Given the high incidence of CoV resistance, especially in immunocompromised patients, the design of new drugs that target specific activities of the virus and stop one or more stages of its infection cycle is essential (Prajapat et al., 2020; Yang et al., 2020; Zhou & Zhao, 2020). In recent years, most research has focused on blocking virus transmission to the HC, RNA polymerase activity of the virus, and HC-virus interactions (Schaack & Mehlle, 2019). Genetic changes, reapparance and emergence of antigenic variants and transmission of CoV to humans require extensive measures to control globalization. Vaccination, drug follow-up, and immediate protection are important tools for dealing with viral infections (Ahmed et al., 2020).

Due to the possible genetic modification of CoV, producing a suitable vaccine against this disease is difficult (Kim et al., 2016). Any changes in the antigenic sites of surface proteins, especially SP, which is the most important surface antigen of the virus, give rise to appearance of new strains (Du et al., 2017; Kleine-Weber et al., 2018). Changes in these regions affect the antibodies produced against the former strains, and therefore have no role in the immunity against this disease (Stebbing et al., 2020).

The emergence of resistant strains under drug selective pressure and their limited availability in high-risk cases further exacerbates the need for new therapeutic strategies (Zu et al., 2020). In recent years, compounds affecting different stages of the virus’s life cycle have been introduced and a wide range of anti-viral strategies have been proposed, including inhibiting the entry and stopping of viral replication or targeting intracellular signal transduction pathways (Peeri et al., 2020). In recent decades, targeting viral proteins inducing humoral and cellular immune responses have received a great deal of attention in development of anti-viral compounds (Zumla et al., 2016). The ability of biomolecular systems such as cytokines, interleukins, and bacterial derivatives to improve immunogenicity and xenografts is being evaluated as a novel strategy, although immune system regulatory proteins have received more attention.

**Conclusion**

CoVs are RNA viruses replicating in the cytoplasm of HCs. To transfer their genetic materials into the human HCs, they are dependent on the interaction of their envelope with the human HC biomembrane. The SP mediates CoV entry and conducts the interaction of CoV with receptor (ACE-2) on the HCs as well as mediating the fusion of HC biomembrane and viral envelope. Also, SARS-CoV-2 furin substrate site can facilitate the cleavage of the SP. This review discussed an overview on the role of ACE-2 and furin in the binding of CoV with HC biomembrane mediated by SP. Also, we surveyed the contribution of FCS on the CoV outbreak. Moreover, we considered the ability of small molecular inhibitors on the limiting the interaction of ACE-2 and furin with SP to be used as promising antiviral drugs or vaccines. This paper may provide promising information about the development of useful strategies to combat the spreading CoV epidemic.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

All authors read and approved the final manuscript.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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