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Coronavirus Fusion and Entry are Targets for New Therapies

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Summary

The current treatments for coronavirus infections consist of monoclonal antibodies to prevent spike protein attachment and antiviral agents to block RNA-dependent RNA polymerase interrupting viral replication. Fusion and entry of coronaviruses present new opportunities for therapy and are explained and summarized in this report. Additional effective treatments for current and future coronavirus infections will be of great benefit globally.

Abstract

The current therapies for coronavirus infections are directed at only two targets. Monoclonal antibodies prevent virus attachment to the host cell membrane and antiviral agents attack RNA-dependent RNA polymerase to interrupt replication of the virus. The fusion and entry stage of coronavirus infection can proceed through an endocomal route of a direct plasma membrane route. Molecular virology has provided detailed knowledge of these processes and each step presents an opportunity for therapeutic intervention. Currently available drugs have been repurposed and new compounds have been identified as potential coronavirus treatment options which target fusion and entry of these viruses.

Keywords

Coronavirus, Fusion, Entry, Endosome

Introduction

For many years there was limited study of coronaviruses as they were not associated with severe or deadly human disease. In 2002-2003 the severe acute respiratory syndrome (SARS) emerged affecting about 8,000 people with a death rate approaching 10%. Interest in coronaviruses surged and research accelerated. The SARS epidemic subsided with the implementation of effective public health measures. Then in 2012 the Middle East respiratory syndrome (MERS) appeared with an alarming 35% mortality. Finally, in 2019 the COVID-19 pandemic struck challenging healthcare resources around the world. Researchers have employed all the tools of molecular virology to elucidate the structure and function of these new human pathogens.

Viral infection of host cells may be divided into several steps. The first step is attachment of the virus to a receptor on the host cell membrane. The second step is fusion and entry culminating with the viral genetic material being released into the interior of the cell. The third phase is replication of the virus within the cell. The final phase is release of the mature virions from the cell which can then seek out and infect new cells. Thus far, approved treatment of COVID-19 has been directed at attachment with monoclonal antibodies to the spike protein to prevent binding to host cell receptors and antivirals (remdesivir, molnupiravir, and favipiravir) that inhibit RNA-dependent RNA polymerase to decrease viral replication. However, the fusion and entry step affords numerous potential treatment opportunities to explore.

Infection by Coronaviruses

To successfully infect host cells viruses need to overcome the host cell membrane. Enveloped viruses, like coronaviruses, achieve this by fusion of the viral membrane with the host cell membrane creating an opening through which the viral genetic material can be introduced into the host cell cytoplasm. Coronavirus entry is mediated by the glycoprotein spikes which adorn the capsid resembling a crown or corona [1].

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The coronavirus spike protein is composed of two subunits. The S1 subunit of the outer portion of the spike protein binds to host cell membrane receptor angiotensin-converting enzyme 2 (ACE2). The S2 subunit anchors the spike to the cell membrane after the S1 subunit is split off and completes the fusion process. In some coronaviruses, such as SARS-CoV, cleavage of the spike protein occurs after attachment to the host cell membrane while in others, such as MERS-CoV and SARS-CoV-2, the cleavage occurs during the viral replicative process within the previously infected cell. In the case of SARS-CoV-2 the intracellular cleavage process does not completely separate subunits S1 and S2 resulting in a fragile connection between the two subunits after release from the infected host cell [2]. The mutation of a single amino acid (aspartic acid to glycine) at the 614 position of the spike protein strengthens the connection between the two subunits and thereby increases viral infectivity by lessening extracellular breakup of the subunits rendering the virions incapable of attachment and infection of new cells [3].

Coronavirus genome sequencing has revealed that the SARS-CoV-2 is closely related to at least two bat-derived coronaviruses with 89% of the genes being identical. Fewer genes were shared with SARS-CoV (79%) and MERS-CoV (50%) [4]. As already noted SARS-CoV-2 and MERS-CoV both cleave the spike protein within the infected cell despite their genomic differences.

Routes of Entry

Coronaviruses can enter the host cell by two routes. The first is a direct route which is accomplished by fusion of the viral and host cell membrane. The second is through endocytosis which is a process whereby the host cell engulfs the whole virus after attachment to the ACE2 receptor of the cell membrane introducing it into the cytoplasm contained in a host membrane covered structure called an endosome. Fusion of the viral and endosomal membranes enables the viral RNA to be released into the cytoplasm. Endocytosis is a rapid process and carries the advantage of removing the virus from exposure to the immune system or monoclonal antibodies while the virus is exposed on the surface of the host cell [5]. SARS-CoV-1, MERS-CoV, and SARS-CoV-2 can each use both of these routes of entry. However, SARS-CoV-1 preferentially uses the indirect endocytosis route while SARS-CoV-2 prefers using the direct route through the cell membrane [2].

Irregardless of the route of entry used the S1-S2 cleavage site is essential for infection of the host cell [6]. The spike protein transitions to a so-called metastable state which is of a lower energy prior to membrane fusion. This spike protein transition is the result of two proteolytic cleavages. The first is localized to the S1-S2 intersection and the second is at the S2' site of the S2 subunit [7]. In SARS-CoV-2 and MERS-CoV the S1-S2 intersect is cleaved by furin in the virus producer cell at the conclusion of viral replication when the virion traverses the Golgi apparatus whereas in SARS-CoV both of the two proteolytic cleavages happen at the time of infection. The S1-S2 cleavage site of SARS-CoV and SARS CoV-2 is between threonine and methionine at positions 696 and 697. SARS-CoV-2 has a second cleavage site which is between arginine and serine at positions 685 and 686 [8]. This second site is shared with MERS-CoV, but not found in SARS-CoV. This second site is the result of a polybasic arginine-arginine-alanine-arginine motif which immediately precedes the site of furin cleavage and is a necessary prerequisite for furin processing. Cleavage at the S2' site must be completed by target cell proteases to finalize the fusion process [2,9]. At the cell surface the enzyme is transmembrane protease serine 2 (TMPRSS2) and within the endosome it is cathepsin L. TMPRSS2 is the important protease for SARS-CoV-2 and MERS-CoV as they prefer to enter the host cell directly through the plasma cell membrane. In the case of SARS-CoV-1, cathepsin L acting in the endosome is the key protease. TMPRSS2 is located in the epithelial cells of the gastrointestinal, respiratory, and urogenital tracts. There are 3 cell types that express both the TMPRSS2 enzyme and ACE2 receptor-type II alveolar cells, ileal absorptive enterocytes, and nasal goblet and/or ciliated cells [2]. Cathepsins are non-specific proteases and are divided into 3 catalytic classes-aspartic, serine, and cysteine. The cysteine protease, cathepsin L, is the most important for coronaviruses, especially SARS-CoV [2].

S1 which mediates binding to the host cell receptor (ACE2) exhibits genetic diversity among coronaviruses, but S2 seems to be conserved. Host cell surface receptors may be of different types and require specific S1 structuring for binding, but the membrane-membrane fusion process remains relatively constant. The unchanging S2 subunit and fusion process have a potential therapeutic advantage over therapies, like monoclonal antibodies, directed at the mutating S1 portion of the spike protein.

Furin Cleavage in the Virus Producing Cell

The initial S1-S2 cleavage by furin within the virus producer cell is the first site of potential therapeutic intervention to be discussed. Precleavage of the spike protein by furin facilitates entry of SARS-CoV-2 through the plasma membrane. This allows the virus to avoid potent endosomal restriction factors which inhibit viral membrane fusion. SARS-CoV-2 virus with deletion of the furin cleavage site was found to be less effective in infecting ferrets and the virus was unable to replicate in high titers in the upper respiratory tract of the ferrets [10]. *In vitro* studies of furin inhibitors using cell culture technique have been reported. Two compounds, decanyl-RVKR-chloromethylketone (CMK) and naphthofluorescein were both found to suppress SARS-CoV-2 production and cytopathic effect in VeroE6 cells. Unfortunately, neither of these compounds which target virus producer cell proteases specifically act on SARS-CoV-2 spike protein and *in vivo* would also affect enzymatic functions normally occurring in cells [11].

The Endocytosis Route

Endocytosis is used by coronaviruses to enter host cells. As previously mentioned, this is the preferred route for SARS-CoV. Endocytosis is an important cellular function used to uptake plasma membrane proteins and is mediated by clathrin. This machinery may be hijacked by bacteria and

viruses to gain access to the cell interior. Clathrin forms a scaffold or cage which stabilizes the plasma membrane and provides a hub for the recruitment of other accessory factors that regulate endocytosis. Using chemical based studies 17,000 small molecules were screened for clathrin inhibition. Two molecules, named Pitstop 1 and Pitstop 2 were identified that selectively inhibited clathrin activity. Using X-ray protein crystallography the two pitstop molecules were found to occupy the same groove in clathrin with a resultant change in the structure of clathrin. *In vitro* studies demonstrated that HIV-1, which requires clathrin-dependent endocytic machinery for cell entry, is inhibited from entering HeLa cells by both pitstop molecules. However, Pitstop 1 exhibits low membrane penetration requiring large doses to show any inhibitory activity which would limit its clinical usefulness [12]. Chloroquine has also been suggested as an inhibitor of clathrin-dependent endocytosis. However, while studies were able to demonstrate reduced nanoparticle uptake in macrophages and Kupffer cells, but not in non-phagocytic cells, suggesting that chloroquine mediated suppression of particle uptake may be phagocytic cell specific [13-15].

Dynamin is a protein that is critical for the pinching and release of a completed endosome from the plasma membrane. Chlorpromazine has been shown to inhibit clathrin-mediated endocytosis by inhibiting dynamin [16]. Chlorpromazine has also been found to have activity against SARS-CoV-2 in monkey VeroE6 cells and human alveolar basal epithelial A549-ACE2 cells at concentrations that are achievable in lungs and saliva with usual clinical doses [17]. Using a colorimetric assay researchers screened approximately 16,000 small molecules for their ability to inhibit the GTPase activity of dynamin and found 34 with activity. Based on other desirable characteristics one molecule was selected for further study. This molecule was named dynasore and was found to be a potent inhibitor of the endocytic pathway [18].

Once the endolysosome has been formed the spike protein must be cleaved to begin the fusion process with the endosomal membrane. In the case of SARS-CoV this entails cleavage at the S1-S2 site as well as S2'. For MERS-CoV and SARS-CoV-2 which have already been cleaved at the S1-S2 site by furin only the S2' site requires further cleavage. Cathepsins, especially cathepsin L, are capable of these cleavages. Cathepsin L inhibitors have been extensively reviewed and some promising drugs have been identified [19,20]. Investigators screened a 1,000 compound library using a chemical assay for inhibition of cathepsin L and found one (MDL28170) to be a potent inhibitor and these results were confirmed using a cellular assay [21]. Other researchers screened 466 compounds for transcriptional expression levels of cathepsin L using a cell based assay. Amantadine was among the top 5 most active in down regulating cathepsin L [8]. Further studies by another group of investigators using cell cultures as well as humanized mice showed that amantadine has suppressive effects on SARS-CoV-2 infection [22]. The glycopeptide antibacterial agent, teichoplanin, inhibits cathepsin L in a dose-dependent manner in cell culture [23]. The potential for teichoplanin for treatment of SARS-CoV-2 has already been examined in a small retrospective controlled study of seriously ill intensive care unit patients in Italy (Tei-COVID study). There was a slight improvement in both mortality and clearance of virus, but neither was statistically significant [24].

Cathepsins require an acid environment (pH between 4.5 and 5.0) for optimal activity [20]. Chloroquine is a weak base that increases the pH of vesicles and therefore warranted investigation in coronavirus infection. In 2005 chloroquine was reported to be effective in preventing the spread of SARS-CoV in cell culture when the cells were treated prior to or after SARS-CoV infection [25]. These studies were repeated using Vero E6 cells infected with SARS-CoV-2 with similar results [26]. These promising *in vitro* studies were followed by disappointing results in COVID-19 clinical trials. Later studies revealed that hydoxychloroquine efficiently blocks the endosomal viral entry medicated by cathepsin L, but not the plasma membrane viral entry mediated by TMPRSS2 which is preferred by SARS-CoV-2 [27].

Chloroquine inhibits the spread of SARS-CoV-2 in the African green monkey kidney derived cell line Vero E6, but engineered expression of TMPRSS2 renders these cells insensitive to chloroquine. Additionally, chloroquine does not block infection with SARS-CoV-2 in the TMPRSS2 expressing human lung cell line Calu-3 [28]. These studies fully explained why chloroquine and hydroxychloroquine were ineffective in treating COVID-19 despite early encouraging results with *in vitro* studies (Table 1).

After attachment to the ACE2 receptor of the host cell membrane coronaviruses may fuse with the plasma membrane enabling their RNA to enter the cytoplasm. As MERS-CoV and SARS-CoV-2 have been already been cleaved

Table 1: Coronavirus inhibitors of fusion and entry.

Precleavage in virus producing cell	
Furin inhibitors	Decanyl-RVKR-chloromethylketone (CMK)
	Naphthofluorescein
Entry by endocytosis:	
Clathrin inhibitors	Pitstop 1
	Pitstop 2
	Chloroquine
Dynamin inhibitors	Chlorpromazine
	Dynasore
Cathepsin L inhibitors	MDL 28170
	Amantadine
	Teichoplanin
	Chloroquine/ hydroxychloroquine
Entry through the plasma membrane	
TMPRSS2 inhibitors	Nafamostat
	Camostat
	Gabexate
	MM3122

at the S1-S2 boundary by furin in the virus producing cell all that remains to activate the fusion process is cleave of the S2 subunit at the S2' site. In 2016, studies demonstrated that the serine protease inhibitor nafamostat was effective against MERS-CoV membrane fusion using the Calu3 human lung cell assay [29]. A biochemical assay has been developed to evaluate the ability of potential therapeutic agents to inhibit TMPRSS2. Three serine proteases have been found to be effective. These proteases in rank order of inhibitory ability are nafamostat, camostat and gabexate [30]. Nafamostat has been shown to block SARS-CoV-2 infection of human lung cells with higher efficacy than camostat [31]. Using a sophisticated structure based design employing X-ray crystallography a novel TMPRSS2 inhibitor (MM3122) has been identified. This small molecule ketobenzothiazole has greater inhibitory activity than camostat or nafamostat when assessed using SARS-CoV-2 infection of Calu3 cells [32].

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Conflicts of Interest

None.

Authorship

The author is solely responsible to the content of this manuscript.

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