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COVID-19: Drug targets and potential treatments

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Abstract (max 150)

Currently, we are immersed in a pandemic caused by the emerging severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which severely threatens public health worldwide. Until now, no drug or vaccine has been approved to treat the severe disease caused by this coronavirus, COVID-19. We will focus on the main virus-based and host-based targets that can guide medicinal chemistry efforts to discover new drugs for this devastating disease. In principle, all CoVs enzymes and proteins involved in viral replication and the control of host cellular machineries are potentially druggable targets in the search for therapeutic options for SARS-CoV-2. This perspective provides an overview of the main targets from a structural point of view, together with reported therapeutic compounds with activity against SARS-CoV-2 and/or other CoVs. Also, the role of innate immune response to coronavirus infection and the related therapeutic options will be presented.

Key words: SARS-CoV-2, COVID-19, antivirals, drug targets, drug discovery

1 Introduction

The coronavirus pandemic (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This is a highly-pathogenic human coronavirus (CoV) first reported in Wuhan, China, where a pneumonia of unknown cause was detected in December 2019.¹ This novel CoV belongs to the *Coronaviridae* family as well as SARS-CoV and the Middle East respiratory syndrome coronavirus (MERS-CoV). The three of them are zoonotic viruses and have in common their ability to cause severe infection in humans, in contrast to other human CoVs (HCoV-NL63, HCoV-229E, HCoV-OC43 and HCoVHKU1), which are responsible of mild respiratory tract infections.²

Highly pathogenic CoVs are enveloped, positive polarity single-stranded RNA betacoronavirus and their genomes encode non-structural proteins (nsps), structural proteins and several accessory proteins.³⁻⁴ The publication of the genome sequence of SARS-CoV-2,⁵ has allowed to determine that the new virus is closely related to SARS-CoV (82% sequence identity) and in a lesser extent to MERS-CoV.⁶ As a starting point, this sequence identity could pave the way to the identification of druggable targets based on previous studies focused on SARS-CoV and MERS-CoV.⁷⁻⁸ Knowledge of the life cycle of CoVs is essential to achieve this aim (Figure 1).

The SARS-CoV-2 infection process starts with the viral entry mediated by the interaction of the spike (S) glycoprotein with the host angiotensin-converting enzyme 2 (ACE2) receptor,¹ and the S protein cleavage by the host transmembrane serine protease 2 (TMPRSS2) prior to the fusion to the host cell membrane.⁹

Entry mechanisms of coronavirus were controversial 15 years ago. In early studies, a non-endosomal pathway was initially thought as the CoVs mechanism to enter the host cell. In 2004, it was shown that SARS-CoV fused with the cellular surface after attaching the host cell membrane.¹⁰ Then, the nucleocapsids were blurred after the virions lost their envelopes and no endocytic related events were described. However, recent evidences point out to the endosomal pathway as the main entry route for CoVs to infect the cells. Remarkably, Ng. *et al* published one year before, a study with a SARS-CoV

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3 isolated from a SARS patient in Singapore.¹¹ They certainly observed fusion events at
4 the plasma membrane, followed of a movement of spherical viral cores into cytoplasm
5 within large cellular vacuoles during the first 15 minutes after infection.
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8 Then, in 2008, Wang and colleagues established the endocytic pathway as an
9 alternative entry pathway apart from direct fusion with the plasma membrane based in
10 their observations of SARS-CoV.¹² They showed that this virus enters the cell via pH and
11 receptor-mediated endocytosis dependent manner. In fact, the spike (S) protein itself or
12 pseudovirus bearing S protein induced internalization of SARS-CoV receptor ACE2 from
13 the cell surface to cytoplasmic compartments. Furthermore, lysosomotropic drugs
14 blocked the ACE2 receptor in vesicles impairing their recycling to the plasma membrane.
15 Pseudoviruses were also affected by inhibition of pH acidification, which indicates that
16 SARS-CoV exploits the endocytic pathway to infect the cells, as they found, in a clathrin-
17 and caveolin-independent manner.
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28 Currently, we are immersed in a pandemic caused by the emerging SARS-CoV-2¹³⁻¹⁴
29 which is severely threatening public human health care system worldwide. Some years
30 before, two other coronaviruses also crossed the species barrier, triggering deadly
31 pneumonia in humans: SARS-CoV¹⁵⁻¹⁶ and MERS-CoV¹⁷ and similarities in entry
32 pathways of these betacoronaviruses need to be elucidated.
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38 Coronavirus entry relies in the spike (S) protein and, depending on the viral strain and
39 cell type studied, the S protein is cleaved by several different cellular proteases.¹⁸⁻²⁴
40 SARS-CoV-2 presents similar entry requirements to SARS-CoV. Both viruses are
41 coincident in the cellular receptor ACE2. Similar to SARS-CoV, SARS-CoV-2 infection is
42 profoundly inhibited with lysosomotropic drugs (99% and 98% respectively) in cells
43 transduced with pseudovirus.²⁵ These results point out that the endocytic pathway is the
44 preferred route for SARS-CoV-2 entry into the host cell.
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52 Furthermore, SARS-CoV and SARS-CoV-2 pseudovirions entry are dependent on late
53 endosomal compartments given their dependence on endosomal acidification. Also, the
54 inhibition of endosomal maturation would result in the impairment of SARS-CoV-2
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3 infection. In fact, pseudovirions entry is impaired by treating cells with chemical inhibitors
4 (YM201636 and apilimod) of PIKfyve enzyme, involved in the phosphoinositides
5 metabolism regulating endosomal maturation. Other viruses depending on PIKfyve
6 enzyme described have been Ebola virus (EBOV) and African swine fever virus, both
7 dependent on the late endosome for entry.²⁶⁻²⁸ SARS-CoV-2 infection results impaired
8 by blocking other proteins characteristic of the late endosomal compartments, like two
9 pore channel 2 (TPC2), (but not TRMPL1), indicating that TPC2 is important for SARS-
10 CoV-2 pseudovirions entry.²⁵

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18 Protease activation of S glycoprotein is crucial for coronavirus entry. Lysosomal
19 cathepsins are necessary for SARS- and MERS-CoV entry via endocytosis. SARS-CoV-
20 2 also requires cathepsin L for a successful infection since its inhibition decreased 76%
21 the entry of SARS-CoV-2 S pseudovirions, suggesting that cathepsin L should be crucial
22 for SARS-CoV-2 S priming into lysosomes to enter the cells. Conversely, cathepsin B
23 inhibition did not seem to have any effect.²⁵

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Once the virus enters the host cell, it gets disassembled to release the nucleocapsid and
the viral genome. Host ribosomes translate the open reading frame (ORF) 1a/b into two
polyproteins (pp1a and pp1ab) that encode 16 non-structural proteins (nsps), while the
remaining ORFs encode structural and accessory proteins. Two proteases participate in
the cleavage of the polyproteins, the main protease (3CLpro, nsp5) and the papain-like
protease (PLpro, nsp3), to produce nsp2-16 involved in the replication-transcription
complex (RTC).²⁹ Some of those are the RNA-dependent RNA polymerase (RdRp,
nsp12) and helicase (nsp13). In coronavirus, this process is followed by assembly of the
virion components into the endoplasmic reticulum Golgi intermediate compartment
complex and release from the infected cells by exocytosis.³⁰

In principle, all CoVs enzymes and proteins involved in viral replication and the control of
host cellular machineries are potentially druggable targets in the search for therapeutic
options for SARS-CoV-2. In this perspective, we will give an overview of the main virus-
based and host-based targets from a structural point of view, together with reported

therapeutic compounds with activity against SARS-CoV-2 and/or other CoVs. Also, the role of innate immune response to coronavirus infection and the therapeutic options based on this response will be presented.

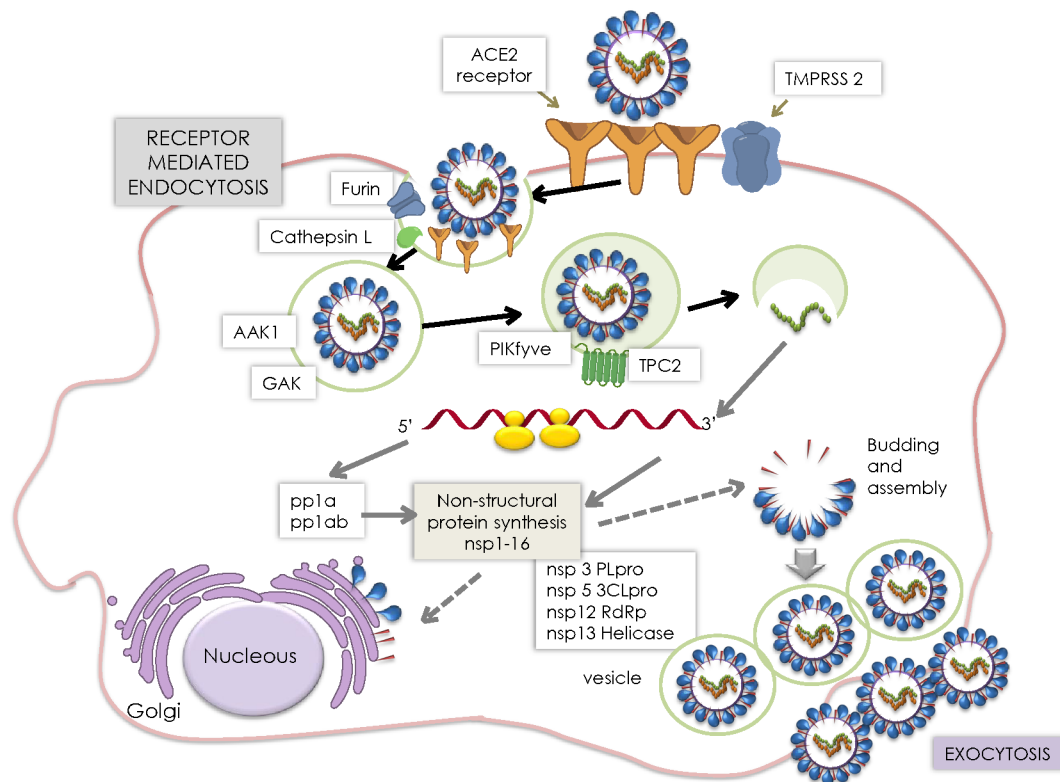


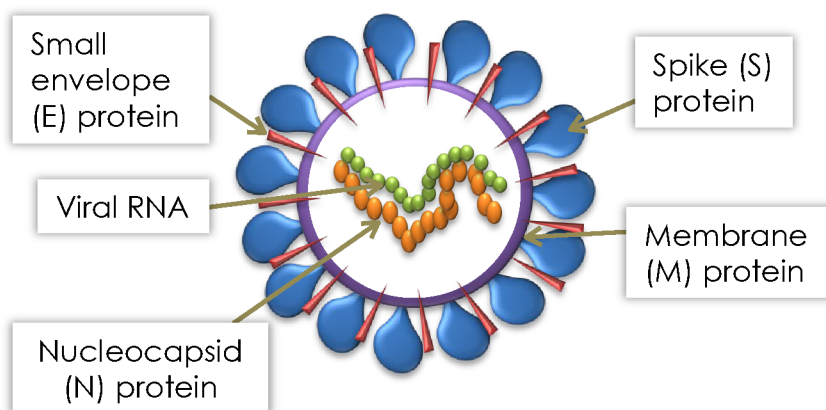
Figure 1. SARS-CoV-2 infection cycle.

2 Virus-based targets

2.1 Structural proteins

The coronavirus structural proteins that form the viral particle are the spike (S) glycoprotein, envelope (E) protein, membrane (M) protein and the nucleocapsid (N) protein (Figure 2). These proteins are less conserved than nsps, playing important functions in the viral life cycle: Spike (S) protein has an important role in virus pathogenesis and organ tropism, being responsible of the viral entry through receptor recognition and membrane fusion.³¹ The envelope (E) protein is the smallest of the structural proteins but has a crucial role in assembly, budding, envelope formation and virulence.³² The main function of the membrane (M) protein is to promote viral

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3 assembly due to its membrane-bending properties.³³ The nucleocapsid (N) protein is a
4 multifunctional protein that packages the viral RNA genome into a ribonucleoprotein
5 complex called nucleocapsid to protect the genome.³⁴ With regard to the search of new
6 therapeutics based on structural proteins of SARS-CoV-2, some computational studies
7 were carried out to identify the structure and function of E protein,³⁵ and the other
8 structural proteins.³⁶
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31 **Figure 2.** Schematic representation of SARS-Cov-2 and its structural proteins
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35 We will focus our attention to the N protein recently crystallized (PDB codes: 6M3M³⁷
36 and 6VYO) and to the S protein due to its fundamental role in viral entry and its
37 available structures that will allow structure-based drug design.³⁸⁻³⁹
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42 43 **2.1.1 Nucleocapsid (N) protein**

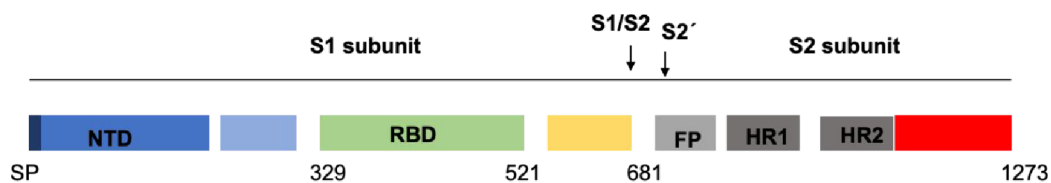
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45 As mentioned above, coronavirus N protein is a multifunctional RNA-binding protein
46 considered as an interesting pharmacological target that merit further attention due to
47 its critical function in viral RNA transcription and replication.⁴⁰ This major CoV protein
48 contain two highly conserved domains, an N-terminal RNA-binding domain and a C-
49 terminal dimerization domain, together with a disordered central Ser/Arg-rich linker.
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51 Previous studies revealed that the N-terminal domain is responsible for RNA binding,
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3 the C-terminal for oligomerization and the Ser/Arg-rich linker for primary
4 phosphorylation.⁴¹⁻⁴³ The crystal structure of SARS-CoV-2 nucleocapsid N-terminal
5 domain has been solved (PDB code: 6M3M),³⁷ showing an overall similarity with the
6 same domain from other CoVs, although the surface electrostatic potential showed a
7 specific distribution. These important structural findings will significantly stimulate the
8 drug discovery of ligands targeting this appealing target to block coronavirus replication
9 and transcription. The success in the development of compounds to interfere with N
10 proteins of other CoVs, such as the recent discovery of stabilizers of the protein-protein
11 interaction of MERS-CoV N protein,⁴⁴ reinforces the potential of the N protein as
12 druggable target for SARS-CoV-2 infection. Remarkably, the N protein is highly
13 immunogenic being considered as a potential vaccine target or for the development of
14 COVID-19 diagnostic methods.⁴⁵⁻⁴⁶
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29 **2.1.2 Spike (S) glycoprotein**

30 The spike (S) glycoprotein is a structural transmembrane protein of about 1200-1400
31 amino acid residues per monomer located on the outer envelope of the virion. As for
32 other viruses of the *Coronaviridae* family,⁴⁷ it mediates virus entry by contacting
33 specific host-receptors located on the surface of the cell. Host-guest recognition is
34 virus-specific, and the specificity and selectivity of this process determine both (i) virus
35 tropism and (ii) pathogenesis.^{25, 39} In its functional form, S protein assembles as
36 homotrimer. Each of the three monomeric units are formed by two functional groups,
37 the S1 subunit which is responsible for host recognition and the S2 subunit which
38 guides host-guest membrane fusion.³⁹ The S1 domain is characterized by an N-
39 terminal (NTD) and a C-terminal (CTD) domains (Figure 3). The former can recognize
40 carbohydrate moieties and is considered a derivation of ancestral sugar-based
41 recognition domains while the latter, also known as receptor binding domain (RBD) or
42 S^B, mediates host-guest interaction and promotes virus entry by recognizing protein
43 receptors of the infected organism. In particular, the region directly involved in the
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3 recognition process is defined receptor binding motif (RBM). A C-terminal,
4 transmembrane domain in the S2 subunit connects the S glycoprotein to the virus
5 membrane.
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18 **Figure 3.** Structural diagrams of spike glycoproteins of SARS-CoV-2. Spike protein
19 contains S1 subunit and S2 subunit, which is divided by the S cleavage sites. FP,
20 fusion peptide; HR, heptad repeat 1 and heptad repeat 2, RBD, receptor binding
21 domain, contains core binding motif in the external subdomain; SP, signal peptide.
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27 Several conformational states of S exist in a dynamic equilibrium between them: the
28 uncleaved, S₀, the cleaved, pre-fusogenic S_{1/S2}, and the post-fusogenic forms. S
29 protein is generally biosynthesized in an uncleaved, fusion-incompetent S₀ form which
30 is then cleaved and activated by proteases. S proteins of less pathogenic CoV are
31 initially exposed uncleaved (S₀) on the virus membrane and their activation is later
32 promoted by host proteases. Conversely, S protein of the highly pathogenic SARS-
33 CoV-2 is cleaved during protein egress and thus, is exposed on the virion in its cleaved
34 (S_{1/S2}) form⁴⁸ (Figure 4).
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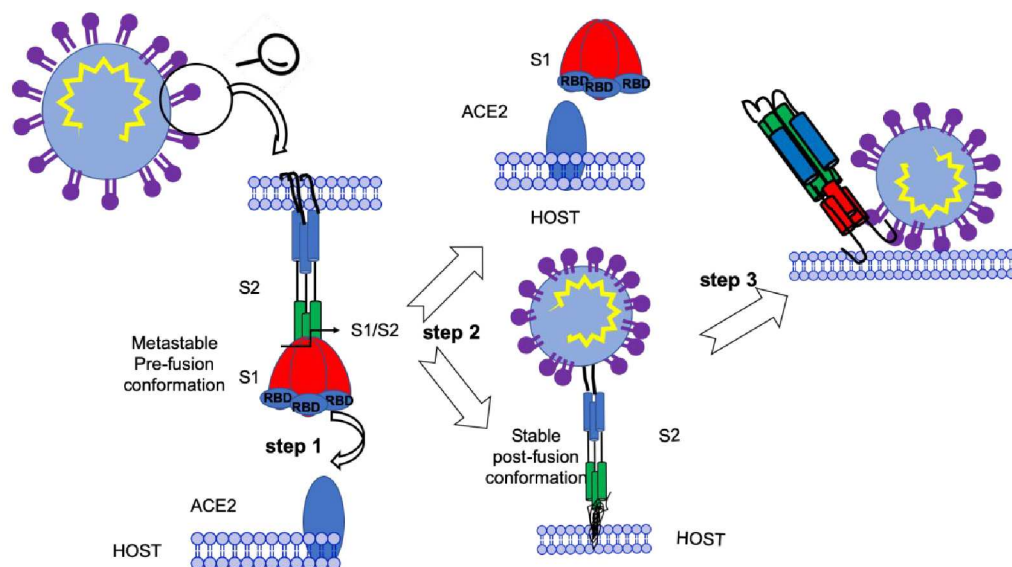


Figure 4. Schematic diagram of SARS-CoV S2-mediated membrane fusion. In receptor binding stage, S protein, which exists as a trimer, binds to the cellular receptor ACE2 via S1-RBD.

In the S1/S2 form, a dynamic equilibrium between open (Up; U) and closed (Down; D) conformations characterizes the three RBD domains of the S protein (Figure 5). The receptor binding site of S is generally occluded when the RBD is in “down” conformation and makes extensive contacts with the NTD of S1. Significant conformation modifications are required for “down-to-up” conversion. Masking and 3D sorting strategy applied on SARS-CoV by Kirchdoerfer *et al*⁴⁹ suggested that ACE2 does not directly participate to down-to-up conversion of S protein and RBD. It is more likely that ACE2 binds an S-RBD in an already “up” conformation.

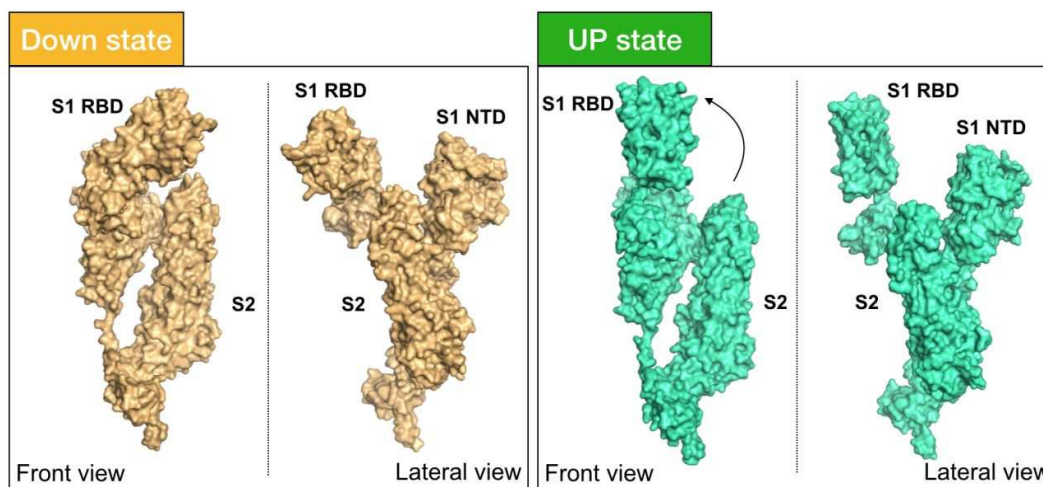


Figure 5. Representation of the open “up” (PDB code: 6VSB) and closed “down” (PDB code: 6VXX) states for the pre-fusion S1/S2 glycoprotein of SARS-CoV-2.

So, according to these data, S protein of SARS-CoV should sample 56% of the triple “down” and 44% of the single “up” S1 RBD and no double or triple “up” S1 RBD conformations have been observed. Introduction of some stabilizing mutations at position 968 and 969 of the S protein seems to move the equilibrium toward the open forms, 58% of the single “up”, 39% / 3% of the double/triple “up” and 0% of the triple “down” S1 RBD. Although intriguing, the exact role and the significance of the dynamic equilibrium of S1 RBD remain unclear. In this regard, it might be interesting to compare with the more pathogenic SARS-CoV-2 but data are not yet available. This strategy has revealed useful to produce pre-fusion stabilized S protein for structure-based drug discovery approaches focused on direct inhibition of the interaction between S-RBD and the host protein ACE2 and has been also applied on SARS-CoV-2.³⁸

Priming of the S protein could occur in several ways, depending on the (guest) type of CoV to the pool of available (host) protease involved in the event.³⁹ As previously introduced, the pre-fusion conformation is originated by the proteolytic cleavage of the S1/S2 site catalyzed by serine-proteases like furin, cathepsin L or TMPRSS2 in correspondence of the (R/K)-(2X)n-(R/K) motif of S.⁵⁰ Given their undoubted role in virus entry by S priming, all these proteases could represent valuable targets for

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3 effective antiviral therapies based on protease inhibition or modulation and are
4 presented in different sections of this perspective.

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6 Sequence analyses for this region within CoV of the same clade revealed a significant
7 heterogeneity, posing the bases for the explanation of the higher pathogenicity of
8 SARS-CoV-2. Accordingly, a mayor exposition of the furin-like (S1/S2) cleavage site
9 and thus, an efficient priming of S protein would derive from insertion of polybasic
10 amino acids at the S1/S2 cleavage site for SARS-CoV-2.⁴⁸ Conversely, S protein of
11 SARS-CoV is inefficiently cleaved by furin at the S1/S2 cleavage site during
12 biosynthesis due to the low exposition of the cleavage site. Unlike SARS-CoV-2, which
13 is exposed in an already cleaved prefusion form, the uncleaved (S₀) form seems to be
14 predominant in case of SARS-CoV, leading to a lower pathogenicity profile.

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16 Conformational changes induced by the first cleavage are not sufficient to promote
17 host-guest membrane fusion which seems to be finally induced by a second cleavage
18 event at the S2' site (Figure 4).^{39, 51} This event seems to be subordinated by recognition
19 of attachment host receptors at the level of the S1 domain.

20
21 Coronaviruses used different regions or the S1 subdomain to bind the host cell. In
22 SARS-CoVs, recognition is mediated by the RBD of S which binds a N-terminal α -
23 helical region (peptidase domain) of the angiotensin-converting enzyme 2 (ACE2),
24 which is accommodated into a concave region, named receptor binding motif (S RBM;
25 highlighted in violet in Figure 6), of the S-RBD.⁵²

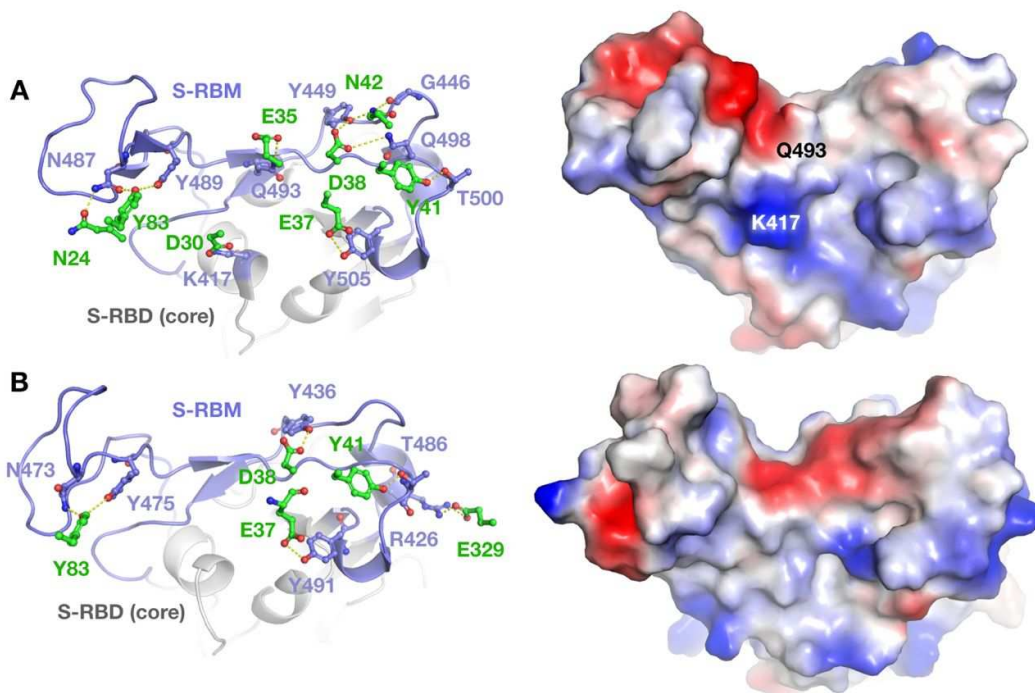


Figure 6. Polar contacts between the S-RBM (in violet) of S-RBD and ACE2 (in green) for SARS-CoV-2 (A) and SARS-CoV (B) and electrostatic potential map for the S-RBD. Some additional polar contacts (Q493-E35 and K417-D30) are visible in the central region of the contact surface for SARS-CoV-2 and ACE2.

A close look at the contact region for SARS-CoV and SARS-CoV-2 with ACE2 revealed a different interaction pattern among the two viruses.⁵³ In case of SARS-CoV-2, a total of 18 residues of the RBD contact 20 residues of the ACE2 can be detected whereas a total of 16 residues of the SARS-CoV RBD contact 20 residues of the ACE2. Main polar contacts are reported in Figure 6. Interestingly, two additional polar interactions, consisting on one hydrogen-bonding interaction between Q439 of S-RBM and E35 of ACE2 and one salt bridge between K417 of S-RBM and D30 of ACE2, can be observed in the central part of S-RBM-ACE2 surface, as a clear consequence of the different electrostatic profile envisaged for the two CoVs. These additional contacts are facilitated by the deeper insertion of the peptidase domain of ACE2,⁵⁴ as a consequence of rearrangement of the receptor-binding ridge (residues 482-485 of S-

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3 RBD). The slight reorientation of the peptide domain (PD) of ACE2 also allows a better
4 stabilization of other two hotspots, Q493(S-RBM)-E35(ACE2) and G496(S-RBM)-K353,
5 previously identified as critical in the stabilization of the complex.⁵⁵
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8 No polar contacts can be observed in the central region of the S-RBM-ACE2 interface
9 for SARS-CoV where K417 is replaced by a valine residue.
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11 Taking together, these findings can contribute to shed some light about the reasons for
12 the pandemic behavior of the new SARS-CoV-2. Anyway, more factors need to be
13 examined in the attempt to properly characterize virus pathogenicity. In this direction, a
14 novel route for virus entry, involving the immunoglobulin-like host protein CD147, well-
15 known to “open the door” to *P. falciparum*, the etiological agent of malaria,⁵⁶ has been
16 proposed by Wang *et al.*⁵⁷ Preliminary *in vitro* antiviral tests indicated that
17 meplazumab, an anti-CD147 humanized antibody, could significantly inhibit virus entry.
18 The formation of the CD147-S-RBD complex has been then confirmed by co-
19 immunoprecipitation and ELISA tests.
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30 Another distinctive trait of obligate parasites as of SARS-CoVs is represented by the
31 high level post-translational modifications. Viral glycans have been proposed to exert a
32 pivotal role contributing to virus functionality, immune selection and thus in its
33 pathobiology.⁵⁸ Viral O- and N-glycosylation, with this last generally occurring on
34 asparagine included in the Asn-X-Ser/Thr sequences, extensively characterizes
35 envelope glycoproteins of HIV-1,⁵⁹ EBOV⁶⁰ and hemagglutinin⁶¹ in Influenza virus.
36 These modifications, originated from selective pressure induced by immune evasion,
37 could contribute to regulate glycoprotein folding, assembly and maturation. The
38 extreme heterogeneity of the number and the distribution of these glycosylated sites
39 makes difficult to discern the exact role on virus life cycle. However, recent
40 advancements on mass spectrometric and chromatographic techniques as well as the
41 availability of recombinant glycosidases have allowed to better study and analyze their
42 impact on folding, structure, sorting, trafficking, and stability of glycoproteins.
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60 Glycosylation can help to stabilize protein folding by enhancing its solubility. Moreover,

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3 they can also participate in intramolecular stabilizing interactions. Another interesting
4 aspect observed for glycoproteins of coronaviruses is that N-glycosylation could help in
5 shielding antigenic sites (as receptor binding sites) to promote immune evasion.⁶² This
6 phenomenon, defined as “glycan shielding”, could represent a critical factor during
7 vaccines design. A direct comparison among S glycoprotein of SARS-CoV and SARS-
8 CoV-2 revealed a different pattern of glycosylation,⁶³ which might contribute to the
9 different profiles in trafficking and interaction with adhesion factors. Some of these
10 differences in glycosylation sites fall in correspondence of antibody-accessible regions
11 of the S protein, thus reinforcing the idea of their effect in hiding the vulnerable region
12 of the protein.⁶⁴

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14 Taken together, these aspects highlight the relevance of a probably overlooked aspect,
15 the glycosylation, during vaccines design.

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17 For viral-based therapeutic options, S protein and its RBD represent a very interesting
18 and intriguing target for antiviral research. The more efficient recognition of the ACE2
19 host protein, together with the expanded host cell tropism given by the existence of a
20 potential new entry route mediated by CD147, highlight S protein as an even more
21 important and central target for therapeutic intervention.

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23 In this direction, common and trusted antiviral strategies directed to S protein consist
24 on inhibition of host recognition, by acting on the S1 RBD and inhibition of the fusion
25 process or by acting at the level of the S2 subunit. Both of these effects could be
26 exerted by small inhibitors, peptides or human monoclonal antibodies, which are able
27 to efficiently recognize these regions of the protein.⁶⁵ A good efficiency has been
28 demonstrated by anti-ACE2 and other S1 human antibodies on SARS-CoV.⁶⁶⁻⁶⁷ The
29 small inhibitor SSA09E2, a piperazine-carboxamide derivative,⁶⁸ was also able to
30 interfere with ACE2 recognition (Figure 7).

31
32 Systemic peptide mapping on SARS-CoV allowed to discover the peptide CP-1, which
33 binds with high affinity the heptad-repeat 2 region of S2 and interferes with the
34 conformational changes leading to the 6-helix bundle formation and thus blocking

virus–cell fusion process (Figure 7).⁶⁹ Recently, a pan-coronavirus fusion inhibitor lipopeptide (EK1C4) has been designed, targeting a structure formed by two specific regions in the S2 subunit.⁷⁰

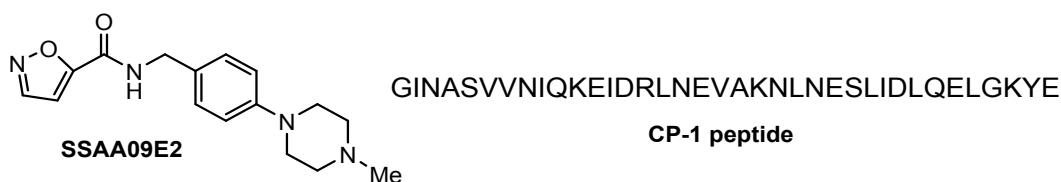


Figure 7. Compounds and peptides directed to S protein.

Collectively, all these strategies could be also applied to the discovery of inhibitors specifically designed to act on the SARS-CoV-2 S protein with a more selective and effective antiviral profile.

2.2 Non-structural proteins (nsps)

SARS-CoV-2, as other coronavirus has 16 nsps that are highly conserved and presented different functions including the formation of the replication-transcription complex (RTC). The specific role of most of them has been reported, although the function of some nsps remains elusive.^{6, 29} Focused on reported studies in SARS-CoV-2, it is interesting to highlight the number of nsp structures of this virus available: the main protease (3CLpro, nsp5) (PDB code: 6Y2E),⁷¹ the papain-like protease (PLpro, nsp3) (PDB code: 6W9C), the RNA-dependent RNA polymerase (RdRp, nsp12) in complex with cofactors nsp7 and nsp8 (PDB code: 6M71),⁷² the methyltransferase-stimulatory factor complex of nsp16 and nsp10 (PDB code: 6W61), the complex nspP10-nsp16 (PDB codes: 6W75 and 6W4H), the nsp9 binding protein (PDB code: 6W4B) and the nsp15 endoribonuclease (PDB code: 6VWW).⁷³

Although any nsp could be exploited as a druggable target,³⁶ the availability of the crystal structure and described ligand together with an essential role in viral infection, significantly increase the chances of success. Based on this premise, we will now

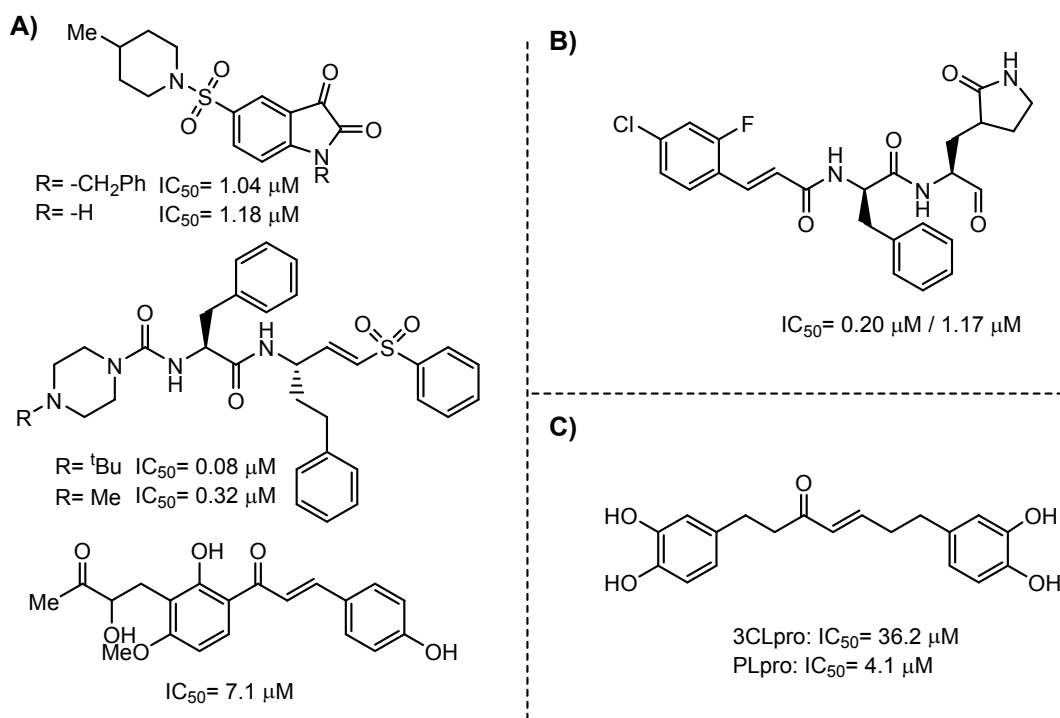
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3 focus in the two proteases (3CLpro and PLpro), the RNA-dependent RNA polymerase
4 (RdRp) and helicase (nsp13).
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8 9 **2.2.1 Proteases 3CLpro and PLpro**

10 SARS CoV-2's genome - as most of the *Coronaviridae* genome - encodes two large
11 polyproteins, pp1a and pp1ab.⁷⁴ These polyproteins are cleaved and transformed in
12 mature nsps by the two proteases 3CLpro (3C-like protease or main protease) and
13 PLpro (papain-like protease) encoded by the ORF 1a/b⁷⁵ as it is mentioned above.
14 Both proteins are crucial for virus replication and controlling the host cell response,
15 therefore they stand as key targets to develop antiviral drugs.
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22 3CLpro forms a dimer and each monomer contains two regions, the N-terminal
23 catalytic region and the C-terminal region.⁷⁶ The sequences of 3CLpro of SARS-CoV
24 and SARS-CoV-2 share 96% of identity, and the minimum differences between both
25 enzymes are in the surface of the proteins. Therefore, inhibitors against SARS-CoV
26 3CLpro are expected to inhibit SARS-CoV-2 3CLpro. Recently, the structure of 3CLpro
27 (PDB code: 6Y2E)⁷¹ has been resolved confirming the high structural similarity
28 between both enzymes.
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36 During the last years, there has been a large development of small-molecules,
37 peptides, and peptidomimetics being able to inhibit SARS-CoV or both SARS-CoV and
38 MERS-CoV 3CLPro, or even both proteases 3CLpro and PLpro.^{7, 77-78} In figure 8, some
39 of the most interesting compounds are classified by the inhibited proteases.
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26 **Figure 8.** Representative CoV protease inhibitors. A) SARS 3CLpro inhibitors, B)
 27 SARS and MERS 3CLpro inhibitors, C) SARS dual 3CLpro and PLpro inhibitor.

31 As 3CLpro is a cysteine protease, therefore covalent inhibitors have been developed
 32 (Figure 8). It is worth to mention a family of vinylsulfone that inhibits SARS-CoV at a
 33 nanomolar range. Indeed, Zhou *et al*⁷⁹ published an *in vivo* study where testing the
 34 combination of the vinylsulfone protease inhibitors and camostast in mice infected with
 35 SARS-CoV. Survival of mice treated with the combination therapy significantly
 36 increased compared to the control group. Very recently Yang's group has determined
 37 the crystal structure of SARS-CoV-2 virus 3CLpro in complex with an irreversible
 38 peptidomimetic inhibitor N3 (PDB code: 6LU7).⁸⁰ In addition, they have performed a
 39 combination of structure-based virtual and high-throughput screening of different
 40 chemical library's including approved drugs, drug candidates in clinical trials, and other
 41 pharmacologically active compounds as inhibitors of 3CLpro. As a result, they have
 42 identified eight compounds with the IC₅₀ in a range from 0.67 to 21.4 μM (Figure 9).
 43 Some of them as disulfiram and carmofur are FDA-approved drugs, whilst ebselen,
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2 shikonin, tideglusib and PX-12 are currently in clinical trials or preclinical studies.
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4 Finally, based in a family of α -ketoamide inhibitors of MERS-CoV, Zhang *et al* have
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6 designed and crystalized a new family of α -ketoamide inhibitors of the SARS-CoV-2
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8 3CLpro, among them compound 13b with an IC_{50} of $0.65\mu M$ ^{71, 81} (Figure 9).
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10 Moreover, there are two SARS-CoV 3CLpro inhibitors approved for the treatment of
11
12 other viral infections, lopinavir and ritonavir, which are being used in patients in the
13
14 currently pandemic.⁸²
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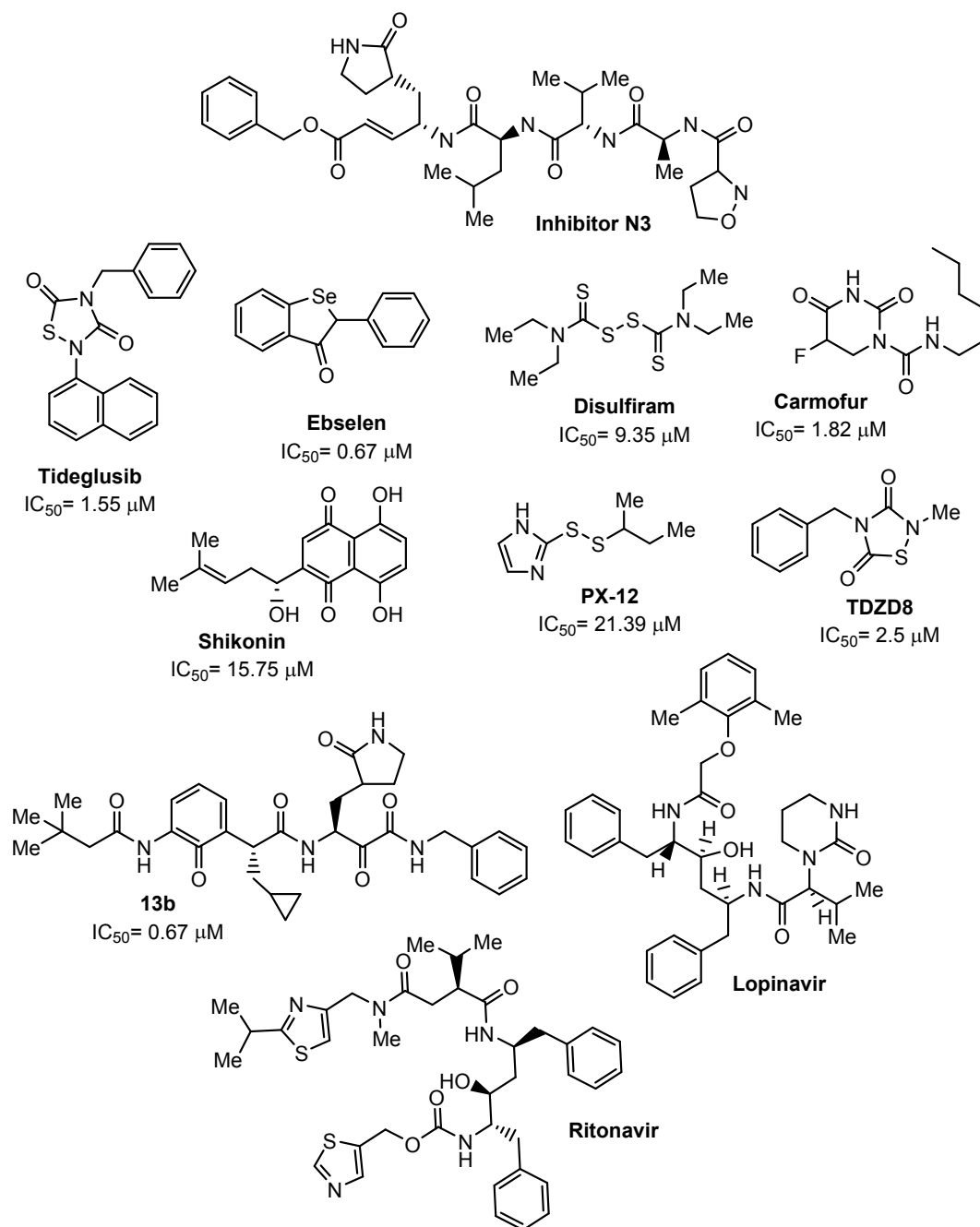


Figure 9. Representative SARS-CoV-2 3CLpro inhibitors.

PLpro is a key target not only to inhibit viral replication but also to inhibit the dysregulation of signaling cascades in infected cells that may lead to cell death of neighboring, uninfected cells.⁷

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3 SARS-CoV-2 PLpro shares 83% of sequence identity with SARS-CoV PLpro. Hence, it
4 is not as high as it was with 3CLpro, however, the differing residues are located on the
5 surface. Then it is very likely that the SARS-CoV PLpro inhibitors could be also active
6 in PLpro of SARS-CoV-2. Very recently, the structure of PLpro of SARS-CoV-2 has
7 been deciphered (PDB code: 6W9C) but until now, no compound able to inhibit it has
8 been reported.
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17 **2.2.2 RNA-dependent RNA polymerase (RdRp)**

18 RdRp is a crucial enzyme in coronavirus life cycle as well as in other RNA viruses. This
19 enzyme is conserved in structure and function among viruses with RNA genomes
20 belonging to different families. RdRp mediates the transcription and replication of RNA
21 genome during infection. The fact that this enzyme has no human counterpart, together
22 with its essentiality for the virus life cycle, improves its chances as a drug target for
23 antiviral development,⁸³ as occurs in other viral infections.⁸⁴
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30 As mentioned above, the replication-transcription complex in coronavirus is formed
31 after cleavage of the polyproteins pp1a and pp1ab into nsps. Moreover, SARS-CoV
32 RdRp requires nsp7 and nsp8 as cofactors to stimulate its polymerase activity. Also,
33 the association with exoribonuclease/N7-guanine cap methyltransferase nsp14
34 contributes to the formation of a macromolecular assembly for efficient nucleotide
35 polymerization, proofreading and cap-modifying. A combination of this multifunctional
36 protein assembly with the helicase/ RNA triphosphatase nsp13 and the 2'-O-
37 methyltransferase nsp16 could coordinate the replication-transcription machinery of
38 SARS-CoV.^{29, 85} All these facts are partially elucidated with the structural
39 characterization of some complexes: nsp10/nsp16 complex,⁸⁶ nsp14/nsp10 complex⁸⁷
40 and RdRp with its cofactors nsp7 and nsp8.⁸⁸
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52 The structure of SARS-CoV-2 RdRp in complex with cofactors nsp7 and nsp8 has
53 been recently deposited in the PDB (PDB code: 6M71).⁷² This structure will significantly
54 accelerate the structure-based drug design to this essential target. Moreover, a 96%
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3 sequence identity between RdRp from SARS-CoV and SARS-CoV-2⁶ contributes to
4 the translation of results with therapeutic agents from one virus to the other. Approved
5 nucleoside analogues acting in other viruses have been successfully used against
6 SARS-CoV and are now being used in the new coronavirus infection.⁸⁹ Molecular
7 modeling studies using the structure of RdRp have identified well-known drugs such as
8 ribavirin, remdesivir, sofosbuvir, galidesvir and tenofovir as inhibitors of RdRp and
9 potential therapies for COVID-19.⁹⁰ Of interest is the reported lack of activity of non-
10 nucleoside analogues in SARS-CoV due to the absence of a hydrophobic pocket in
11 the polymerase to allocate this class of compounds present in other viruses such as
12 HIV-1 or HCV.⁹¹

22 Remdesivir (GS-5734) (Figure 10), an adenosine analogue with broad antiviral
23 spectrum in RNA viruses has proven to be efficacious against different CoVs *in vitro*
24 and in a mouse model of SARS-CoV infection.⁹² During the COVID-19 pandemic, it has
25 been evaluated against a clinical isolate of the new virus,⁹³ showing a half maximal
26 effective concentration of 0.77 μM and a selectivity index of 129.87 (Vero E6 cells).
27 Moreover, the crystal structure of this drug with RdRp has been recently elucidated.⁹⁴
28 Furthermore, several clinical trials around the world have been approved to
29 demonstrate its therapeutic potential in patients.⁹⁵ During the preparation of this
30 manuscript, results from the first trial in China were published and remdesivir was not
31 associated with statistically significant clinical benefits for COVID-19 patients.⁹⁶
32 However, the FDA authorized the use of this intravenous antiviral drug for emergency
33 treatment of COVID-19 patients. Remdesivir can be administered only to hospitalized
34 patients with severe illness defined as patients with low oxygen in blood or needing
35 breathing assistance.⁹⁷

50 This drug initially showed efficacy for the treatment of Ebola disease, being in fact in
51 clinical development for this devastating infectious disease.⁹⁸ The mechanism of action
52 of this nucleoside prodrug is through the RdRp, by delaying chain termination in EBOV.
53 In CoVs, remdesivir is able, not only to inhibit the RdRp but also to evade the action of
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3 the exoribonuclease (nsp14).⁹⁹ This fact is of utmost importance,¹⁰⁰ because poor
4 activities of some nucleosides such as ribavirin are attributed to their removal by the
5 exoribonuclease.¹⁰¹

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8 Together with remdesivir, other broad-spectrum antivirals targeting RdRp were tested
9 in SARS-CoV-2. The half maximal effective concentration (EC_{50}) and half cytotoxic
10 concentration (CC_{50}) in Vero E6 cells of selected compounds are the following: ribavirin
11 ($EC_{50} = 109.50 \mu\text{M}$, $CC_{50} > 400 \mu\text{M}$), penciclovir ($EC_{50} = 95.96 \mu\text{M}$, $CC_{50} > 400 \mu\text{M}$)
12 and favipiravir ($EC_{50} = 61.88 \mu\text{M}$, $CC_{50} > 400 \mu\text{M}$).⁹³ From this set of compounds, only
13 favipiravir (Figure 10), already approved for the treatment of influenza, was
14 recommended to be further evaluated as a therapeutic option for COVID-19 patients
15 although there are some concerns with regard to the pharmacokinetics properties of
16 this drug.¹⁰²

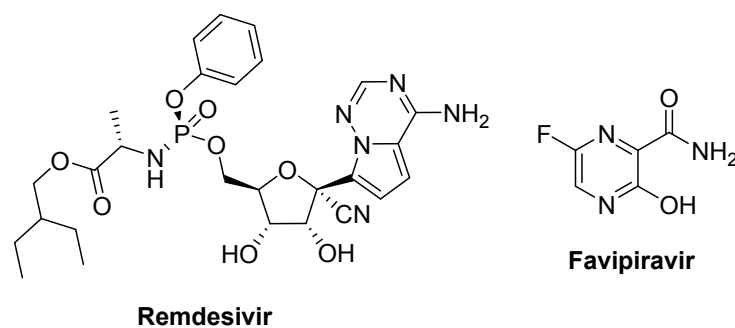


Figure 10. RdRp inhibitors active against SARS-CoV-2 and under clinical trials.

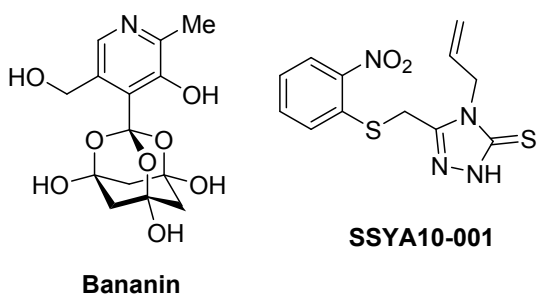
2.2.3 Helicase (nsp13)

NTPase/helicase (nsp13) is a critical protein in the replication-transcription complex of CoVs that catalyzes the separation of duplex oligonucleotides into single strands in a nucleotide tri-phosphate (NTP) hydrolysis dependent manner. Due to the fact that this protein is essential for RNA viral synthesis and one of the most conserved proteins in nidovirus, it is considered an interesting target for drug development and several chemical inhibitors were reported.¹⁰³

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3 Bananins are oligo-oxa-adamantanes that after conjugation with vitamin 6 (pyridoxal)
4 showed antiviral activities (Figure 11). Four derivatives, bananin, iodobananin,
5 vanillinbananin, and eubananin inhibited both the ATPase and the helicase activity of
6 the nsp13 from SARS-CoV. Remarkably, these compounds also inhibit the replication
7 of the virus through a process that occurs after the viral entry.¹⁰⁴ Another family of
8 antivirals acting through the target enzyme are the 5-hydroxychromone derivatives,¹⁰⁵
9 that were synthesized as bioisosters of the previous reported aryldiketoacids.¹⁰⁶

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11 A screen using a FRET-based helicase assay of the Maybridge Hitfinder chemical
12 library, allowed the identification of triazole SSYA10-001 (Figure 11) that specifically
13 blocks the helicase activity of nsp13 showing also activity anti-SARS-CoV. Kinetic
14 studies to determine the enzyme inhibition mechanism showed that SSYA10-001 acts
15 as a non-competitive inhibitor of nsp13 with respect to nucleic acid and ATP
16 substrates.¹⁰⁷ Further studies with this 1,2,4-triazole showed that it is also able to inhibit
17 two other CoVs (MERS-CoV and mouse hepatitis virus). The putative binding pocket
18 was identified corresponding to a conserved pocket in CoVs nsp13 where Y277 and
19 K508 are the key residues to maintain the interaction.¹⁰⁸

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21 At the time of writing, the structure of nsp13 full-length (PDB code: 6JYT) SARS-CoV is
22 available.¹⁰⁹ So far, no structural information is available of the SARS-CoV-2 helicase,
23 but due to the high sequence similarity (99.8%), the SARS-CoV structure available
24 significantly encourages the search of drugs for COVID-19 acting through this target
25 enzyme.
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3 **Figure 11.** Representative SARS-CoV helicase inhibitors.
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8 **3 Host-based druggable targets**

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10 Coronavirus entry is mainly produced by the virus binding to the ACE2 host receptor in
11 the cell surface in a receptor-mediated endocytosis pathway. Moreover, other molecules
12 such as proteases, activate the spike (S) protein and facilitate the fusion with the
13 receptor and cell membrane, allowing the entry into the host of viral RNA via an
14 endocytic pathway.
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20 Host-targeted anti-SARS-CoV-2 agents presented here are based in the molecular study
21 of virus entry, identifying key proteins involved in the process. Targeting host proteins
22 may avoid different limitations frequent in antiviral research, probably offering a genetic
23 barrier to viral resistance.
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32 **3.1 Angiotensin I converting enzyme 2 (ACE2) receptor**

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34 Coronavirus entry is mainly produced by the virus binding to different host receptors in
35 the cell surface. In SARS-CoV-2, the angiotensin I converting enzyme 2 (ACE2) has
36 been recently confirmed as the main virus receptor.⁹ Therefore, inhibition or modulation
37 of ACE2 represents one of the proposed host-based strategies for treatment of SARS-
38 CoV-2.¹¹⁰
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44 Activity of ACE2 is related to the renin-angiotensin system (RAS) which is involved in
45 the maintenance of blood pressure homeostasis, fluid and salt balance in mammals.¹¹¹
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47 While renin cleaves angiotensinogen to generate angiotensin (Ang) I, the angiotensin-
48 converting enzyme (ACE) catalyzes the formation of Ang II, a critical signaling
49 molecule, through the proteolytic cleavage of Ang I. Although not exclusive,¹¹² the
50 activity of ACE has been considered of pivotal importance in the regulation of Ang II
51 within the RAS.
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3 ACE2, an homolog of ACE, is a multifunctional zinc metalloprotease consisting of 805
4 amino acids which can be functionally divided into (i) the amino-terminal catalytic
5 domain and (ii) the carboxy-terminal domain.¹¹⁰ The enzyme has been observed to
6 negatively regulate RAS through the degradation of Ang II to the heptapeptide Ang 1-
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12 ACE2 can be found in epithelial cells of lung, liver and testis.¹¹⁰ Unlike ACE, ACE2
13 populates the apical membrane of the respiratory epithelial cell by which infection can
14 occur. ACE2 receptors have been also observed in nasal and mouth epithelial cells.¹¹³
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16 Interestingly, an enhanced expression of ACE2 receptors in lung has been correlated
17 with age, thus, partially explaining the higher viral load and severity of symptoms
18 observed in older patients infected by SARS-CoV-2.¹¹⁴⁻¹¹⁵

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24 The cryo-EM structure of the full-length human ACE2 with the transporter B0AT1, with
25 (PDB code: 6M17) or without (PDB code: 6M18) the receptor binding domain (RBD) of
26 the surface spike (S) glycoprotein of SARS-CoV-2 has been recently published.⁵² The
27 complex appears as a dimer of heterodimers with the collectrin-like domain (CLD)
28 located among B0AT1 and the peptidase extracellular domain (PD) of ACE2 (Figure
29 13). CLD consists of a small extra-cellular domain, a long linker, and a single
30 transmembrane (TM) helix; the central region (the neck domain) between PD and the
31 TM helix constitutes the most relevant part of the dimerization surface. Here,
32 coordination between the two dimers is mediated by extensive polar contacts as R716'-
33 D713, E639'-R710, N636'-Q653, and N638'-R652. A weaker interaction surface can be
34 also observed between the two N-terminal PDs of ACE2, where Q175' directly interacts
35 with Q139. It was suggested that ACE2 is natively dimeric and thus able to bind two
36 trimeric S proteins.⁵²

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50 ACE2 was already known for mediating infection of the less pathogenic SARS-CoV,¹¹⁶
51 in particular, by recognizing the receptor-binding domain of the S protein (S1 RBD) with
52 an α -helical region located in the peptidase domain.
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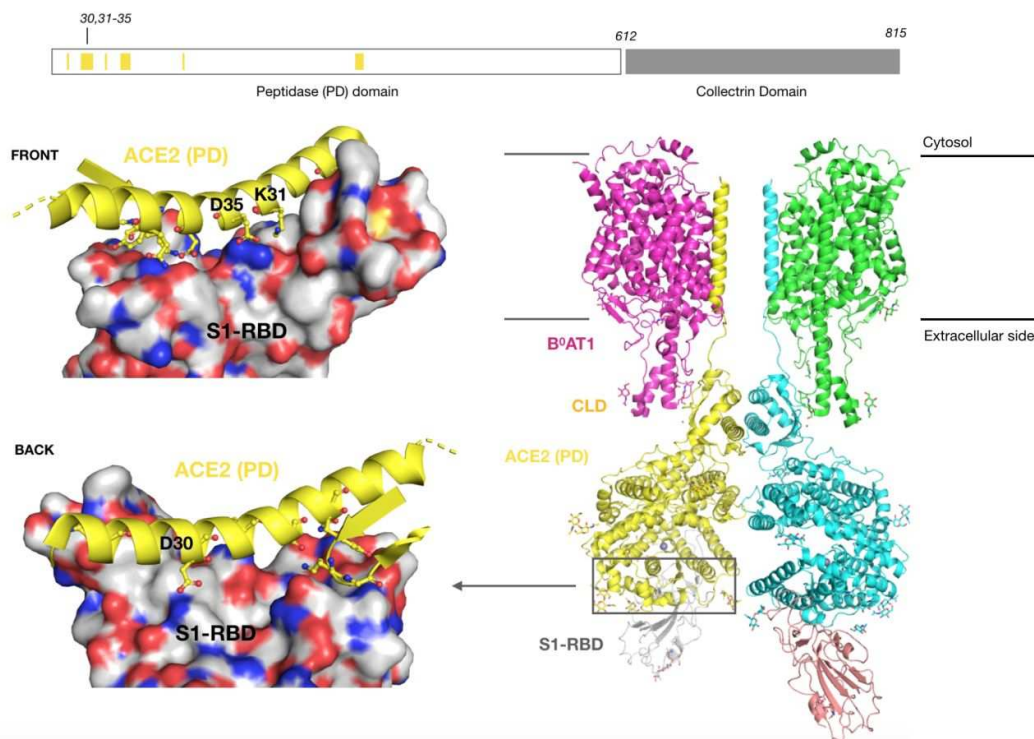


Figure 12. (Right) cryo-EM structure (PDB code: 6M17) of full-length human ACE2 with the transporter, B⁰AT1 and the receptor binding domain of the spike glycoprotein (S1-RBD). (Left) S1-RBD-ACE2 interaction interface for SARS-CoV-2 (PDB code: 6M0J; front and back views). Relevant ACE2 residues involved in direct polar interaction with S1-RBD (in particular, D30, K31, and D35) are represented in yellow sticks.

D30, K31 and D35 have been identified as critical residues in the interaction with S1-RBD.¹¹⁰ Their specific localization with respect to the S-RBD is showed in Figure 12. More details about the S-RBD-ACE2 interactions pattern are given in the S glycoprotein section.

Therapeutic strategies devoted to combat SARS-CoV-2 infection via ACE2 could consist in:

i) Interfering with the dynamics of virus-host ACE2-S-RBD interface. This can be achieved by using relatively small molecules able to disrupt or negatively affect the

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3 efficiency in the dynamic network of protein-protein interaction that guides viral entry. In
4 this regard, 77 molecular candidates have been identified from FDA database through
5 an *in silico* guided repurposing study which combines replica exchange molecular
6 dynamics and ensemble docking, although no experimental decrease of virus infection
7 has been reported.¹¹⁷ Furthermore, machine learning and ensemble docking
8 simulations have also provided new different scaffolds able to interrupt spike protein-
9 ACE2 interaction.¹¹⁸

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16 ii) Inhibition of ACE receptor. Since myocardial injuries caused by SARS-CoV-2 might be
17 related to ACE2, therapies based on the use of ACE inhibitors or angiotensin blockers,
18 might be used for organ-protection purposes. In that sense, telmisartan has been
19 proposed as a real option for COVID-19 therapy¹¹⁹ and a clinical trial has recently started
20 (NCT04355936). Anyway, the real impact of ACE inhibitors on COVID-19 still remains
21 controversial.¹²⁰⁻¹²¹

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28 iii) Delivery of soluble ACE2. It has been demonstrated that SARS-CoV-2
29 downregulates¹¹¹ expression of ACE2. According to this, it has been proposed that the
30 delivery of soluble ACE2 could exert a beneficial effect by competing with the host
31 ACE2 for binding with spike glycoprotein. Soluble recombinant ACE2 or APN01
32 imitates the human enzyme ACE2, which is used by the virus to enter cells.¹¹⁰ The
33 virus binds to soluble ACE2/APN01, instead of ACE2 on the cell surface, which means
34 that the virus can no longer infect the cells. At the same time, APN01 reduces the
35 harmful inflammatory reactions in the lungs and protects against acute lung injury
36 respiratory distress syndrome (ARDS). In this regard, a recombinant human ACE2,
37 (rhACE2; APN01, GSK2586881) has been tested on a small cohort of patients with
38 ARDS, demonstrating encouraging positive results.¹²²⁻¹²³ In a very recent paper,
39 researchers tested the potential of direct treatment with soluble human ACE2 to avoid
40 the entrance of the virus to the host cells in engineered human kidney organoids.¹²⁴

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In combination with other host-based or virus-based approaches, these strategies offer
a vast reservoir of opportunities to combat SARS-CoV-2. Anyway, careful examination

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3 of their real therapeutic impact and a proper validation are needed to properly address
4 their clinical validity.
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8 9 **3.2 Transmembrane serine protease 2 (TMPRSS2)**

10 Following receptor interaction or in addition to it, different host proteases can activate the
11 virus-host cell membrane fusion for subsequent genome delivery. The host cell surface
12 transmembrane serine protease 2 (TMPRSS2) activates S protein present in the highly
13 pathogenic human coronaviruses SARS-CoV and MERS-CoV.¹²⁵ Human TMPRSS2 is
14 expressed in the epithelia of the gastrointestinal, urogenital, and respiratory tracts.¹²⁶
15 Cleavage of S protein by TMPRSS2 is preferred for coronavirus infection over other
16 proteases, such as the endosomal cathepsins.¹²⁷ Recent research has confirmed that
17 SARS-CoV-2 entry is facilitated by TMPRSS2 and the viral infection is decreased by the
18 use of the protease inhibitor camostat.⁹ Moreover, as virus infection is enhanced by
19 TMPRSS2, the Vero E6 cell line overexpressing TMPRSS2 has been described as a
20 useful pharmacological tool for SARS-CoV-2 research.⁵⁰ Last, but not least, TMPRSS2
21 is expressed in different cell types of lung tissue increasing their vulnerability for SARS-
22 CoV-2 infection.¹²⁸
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36 TMPRSS2 has emerged as a useful drug target for antiviral drug discovery⁸ and the lack
37 of influenza and coronavirus infection has been confirmed in TMPRSS2 knock-out
38 mice.¹²⁵ COVID-19 may find a potential therapy among different repurposed drugs with
39 inhibitory activity on TMPRSS2. By the moment, only camostat has shown *in vitro*
40 activity against SARS-CoV-2 but other clinical drugs such as nafamostat and 4-(2-
41 aminoethyl)benzenesulphonyl fluoride, all of them protease inhibitors,¹²⁹ may offer some
42 therapeutic options for the pandemic (Figure 13A). Repurposing of the mucolytic agent
43 called bromhexine, a TMPRSS2 inhibitor, has been also proposed for COVID-19
44 therapy.¹³⁰ Furthermore, transcriptional inhibition of TMPRSS2 has been proposed as a
45 new therapeutic option. Using computational and experimental methods, estrogen and
46 androgen-related compounds, such as genistein, estradiol and enzatumamide (Figure
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13B) have shown to reduce TMPRSS2 expression in different cell lines.¹³¹ As TMPRSS2 expression in the human lung seems to be modulated by estrogens and androgens, data suggest that the activation of estrogen pathways or inhibition of androgen pathways may be a new therapeutic clinical intervention for symptom amelioration in COVID-19 patients.¹³²

Currently, the crystal structure of TMPRSS2 is not available and target-based drug discovery and design should be done using different homology models based in other well-known serine protease structures.

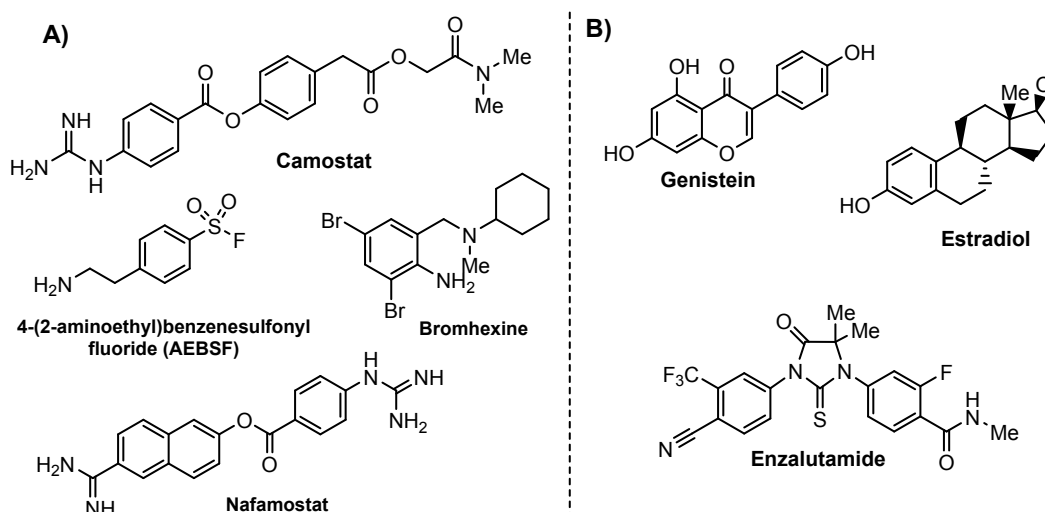


Figure 13. TMPRSS2 modulators. A) Chemical structure of TMPRSS2 inhibitors. B) Some transcriptional inhibitors of TMPRSS2.

3.3 Furin

Recent studies have discovered a “furin-like cleavage site” (FCS) in the S protein of SARS-CoV-2 that may explain the high pathogenicity of the virus.^{39, 48} Moreover, this highly cleavable site is not found in closely related CoVs.¹³³ Furin, a type 1 membrane-bound protease expressed in multiple tissues, belongs to the subtilisin-like proprotein convertase family. This family includes proteases with specific roles in the secretory pathway. The insertion of such cleavage site in other CoVs such as the infectious bronchitis virus increased the pathogenicity, including neural symptoms in infected

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3 chickens.¹³⁴ As furin is highly expressed in lungs, it is very likely to be involved in SARS-
4 CoV-2 infection increasing its pathogenicity over other betacoronaviruses as they lack
5 this cleavage site.⁵¹ Recently, it has been proposed that the FCS may be an important
6 site of coronavirus evolution. In samples isolated from mild COVID-19 patients of
7 Zhejiang province, mutations near FCS (F1-2), that affect the electrostatic distribution of
8 the S protein surface and its structure, thus its binding ability to furin, appeared.¹³⁵
9 Experimental results in samples of those patients showed that furin had low protein
10 expression levels in lungs compared with other tissues, such as colon, glands, liver, and
11 kidney. The FCS may contribute to SARS-CoV-2 infection of these organs. Recent
12 reviews describe the narrow interaction between ACE2 and furin in the viral infection of
13 SARS-CoV-2,¹³⁶ showing a potential relationship between furin activities of different
14 populations and the different clinical scenarios of the SARS-CoV-2 infection.¹³⁷
15 Inhibition of furin with peptides and more recently with small molecules, is an strategy
16 pursued to arrest tumor growth, inflammation and some viral and bacterial infections.¹³⁸
17 However, due to the pleiotropic role of furin-like enzymes in a large number of cellular
18 processes, side-effects should be avoided.¹³⁹ Determination of the crystal structure of
19 furin will aid the design of specific small molecules.¹⁴⁰⁻¹⁴¹ For example, furin unliganded
20 form (PDB code: 5JXG) suggests activation by a substrate-induced mechanism,¹⁴² while
21 the recent complexes with substrate analogue inhibitors (PDB codes: 6EQV, 6EQW,
22 6EQX)¹⁴³ decipher some new pockets to be exploited by a next-generation of furin
23 inhibitors that may be a potential therapy for COVID-19.
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46 **3.4 Cathepsin L**

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48 Activation of SARS-CoV-2 spike protein by proteases cleavage is a key step in virus
49 infection. Different lysosomal cathepsins were relevant in human coronavirus entry
50 through endocytosis.⁷⁹ In a recent study, it has been shown that only cathepsin L and not
51 cathepsin B, or calpain are involved in SARS-CoV-2 endocytosis entry.²⁵ Treatment of
52 HEK 293/hACE2 cells with the cathepsin L selective inhibitor SID26681509, reduced
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more than 76% the entry of SARS-CoV-2 pseudovirus, underlying the potential role of cathepsin L for priming of SARS-CoV-2 S protein in the lysosome.⁹ Previously, the cathepsin L inhibitor named as SSAA09E1 was revealed as a novel antiviral agent for SARS-CoV infection (Figure 14).⁶⁸

Cathepsin L inhibitors may be a therapeutic option for COVID-19 also because they can prevent the progression of pulmonary fibrosis.¹⁴⁴ Furthermore, synergistic effect may be also achieved targeting simultaneously cathepsin L and TMPRSS2.¹⁴⁵ The main challenge for the design of specific cathepsin inhibitors is to obtain selectivity. In this sense, several computational methods have been developed to solve this problem using information from the known 3D structures.¹⁴⁶ Several crystallographic structures of human cathepsin L are available both in the apo form (PDB code: 4AXL)¹⁴⁷ or in complex with different nitrile molecules (PDB codes: 2YJC, 2YJB, 2YJ9, 2YJ8, 2YJ2)¹⁴⁸ or (PDB codes: 2XU5, 2XU4, 2XU3, 2XU1).¹⁴⁹

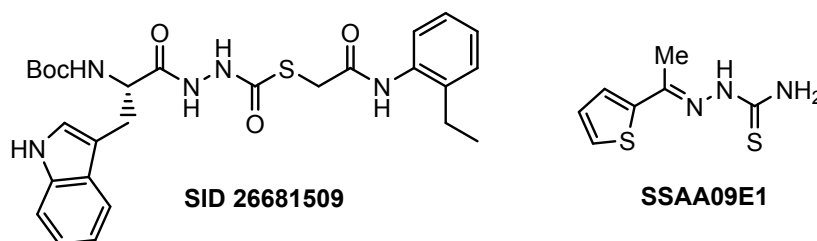


Figure 14. Chemical probes targeting cathepsin L with inhibitory activity against SARS-CoV-2 and SARS-CoV infection.

3.5 Adaptor-associated kinase 1 (AAK1) and cyclin G-associated kinase (GAK)

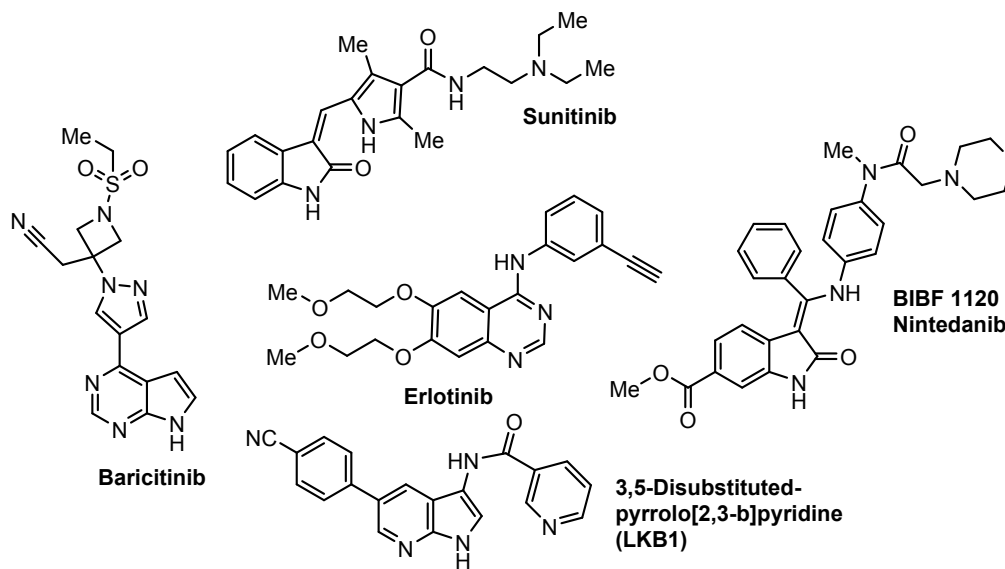
As already explained, the main entry pathway for SARS-CoVs is the receptor-mediated endocytosis. The adaptor-associated kinase 1 (AAK1) and cyclin G-associated kinase (GAK) are host serine–threonine protein kinases that regulate intracellular viral trafficking during entry, assembly and release of multiple unrelated RNA viruses such as rabies, Ebola, dengue or hepatitis C virus.¹⁵⁰⁻¹⁵¹ AAK1 plays a key role in receptor-

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3 mediated endocytosis by specific phosphorylation of adaptor protein 2, which stimulates
4 the binding to cargo proteins. GAK shares some biological functions with AAK1, being a
5 key player in clathrin-mediated trafficking. GAK mediates the binding of clathrin to the
6 plasma membrane and the trans-Golgi network.¹⁵²
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10 Although several molecules have been synthesized to inhibit AAK1 none of these
11 compounds have been optimized and developed as antiviral agents.¹⁵³ A rationale for
12 repurposing a combination of the pan-kinase inhibitors sunitinib and erlotinib (Figure 15)
13 based in AAK1 and GAK inhibition have been proposed for dengue and Ebola patients
14 treatment in future outbreaks. However, these have not been included in current clinical
15 trials until now.¹⁵⁴ Recently, baricitinib, a potent AAK1 and GAK inhibitor has been
16 proposed as an effective therapy for COVID-19 reducing the viral entry, although no
17 experimental work has been done to prove its mechanism of action.¹⁵⁵ Moreover, as this
18 compound targets also the janus kinase (JNK1/2), it may act reducing also the
19 inflammation in these patients, increasing its therapeutic benefit for COVID-19.¹⁵⁶
20 However, some data coming from the clinical trial program used for baricitinib
21 registration in Europe showed that the most significant side-effect seen was a small
22 increase in upper respiratory tract infections, which may be not well tolerated by COVID-
23 19 patients.¹⁵⁷
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38 Several crystal structures of AAK1 have been reported in complex with different
39 inhibitors such as the pan-kinase inhibitor K252a (PDB code: 4WSQ),¹⁵⁸ BIBF 1120
40 (nintedanib) (PDB code: 5TE0) and LKB1 (PDB code: 5L4Q),¹⁵³ offering useful tools for
41 rational drug discovery and/or optimization. GAK 3D-structure was released presenting
42 many different conformations when it is bound to nanobodies (PDB codes: 4C57, 4C58,
43 4C59)¹⁵² or to gefitinib (PDB codes: 5Y7Z, 5Y80).¹⁵⁹ Recently, it has been discovered
44 that AAK1 and GAK share cysteine residues (C193 and C190, respectively) at an
45 equivalent position that may be targeted by covalent inhibition, offering a good
46 opportunity to develop selective covalent inhibitors.¹⁶⁰ Both structures suggest
47 possibilities for the development of selective AAK1 and GAK inhibitors for viral
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3 infections. Nowadays, repurposing marketed drugs or optimizing valuable hits such as
4 3,5-disubstituted-pyrrolo[2,3-*b*]pyridines as potent AAK1 and GAK inhibitors could be a
5 good strategy to avoid SARS-CoV-2 entry.
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31 **Figure 15.** AAK1 and GAK inhibitors with therapeutic potential for COVID-19.
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3.6 Phosphatidylinositol 3-phosphate 5-kinase (PIKfyve)

In endocytosis, one of the molecules that regulates the dynamic process of endosome maturation is phosphatidylinositol-3,5-bisphosphate (PI(3,5)P₂).¹⁶¹ This phospholipid is synthesized in the late endosome by phosphatidylinositol 3-phosphate 5-kinase (PIKfyve), an enzyme with lipid and protein kinase activity.¹⁶² PIKfyve plays a key role in several trafficking events associated with the endocytic pathway.¹⁶³ Very recently, a significantly reduced entry of SARS-CoV-2 pseudovirus on 293/hACE2 cells was found after the treatment with apilimod (Figure 16), a potent inhibitor of PIKfyve,²⁵ which was previously identified as an inhibitor of production of interleukins IL-12 and IL-23.¹⁶⁴ The same effect was also observed with YM201636 (Figure 16), another chemically diverse PIKfyve inhibitor.¹⁶⁵ As apilimod, YM201636 also blocks viral entry and infection of other viruses like African swine fever virus or Ebola.²⁶⁻²⁷ These data suggest that PIKfyve is a suitable drug target to modulate the infection of viruses that enter through endocytosis, including SARS-CoV-2.¹⁶⁶ At the moment, the 3D-structure of this lipid kinase has not been determined yet.

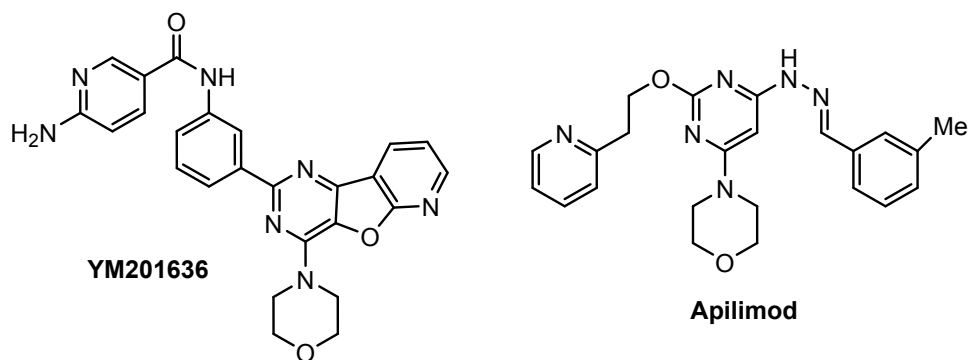
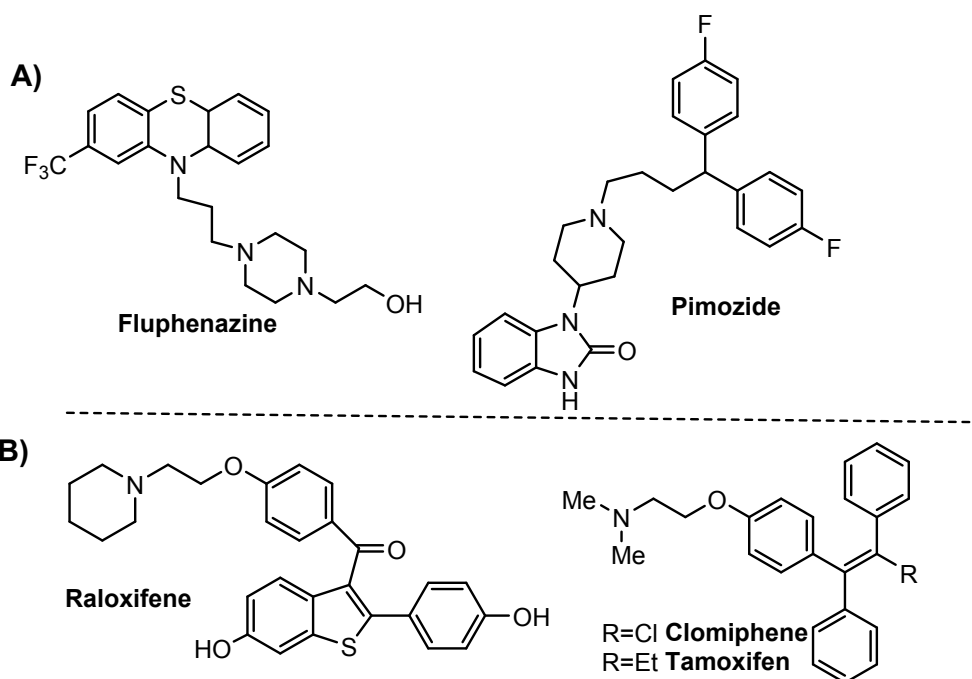


Figure 16. PIKfyve inhibitors active on SARS-CoV-2 infection in cell culture.

3.7 Two pore channel (TPC2)

Among different channels in the endolysosomal system, the two-pore channels (TPC1-3) regulate the conductance of sodium and calcium ions across cellular membranes.¹⁶⁷ They are voltage-gated channels, and TPC2 is one of the major downstream effectors of

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3 PI(3,5)P2 opening after its binding with such phosphoinositide.¹⁶⁸ These are involved in
4 the regulation of endolysosomal trafficking and Ebola entry in the host cell.¹⁶⁹ The
5 structure of human TPC2 has been reported using cryo-electromicroscopy (Cryo-EM)
6 providing useful structural information about the open, closed and apo forms for TPC2
7 (PDB codes: 6NQ0, 6NQ2 and 6NQ1 respectively).¹⁷⁰ By virtual screening, dopamine
8 receptor antagonists and selective estrogen receptor modulators (SERMs) have been
9 recently identified as blockers of TPC2. Specifically, the dopamine antagonists
10 fluphenazine and pimozide together with the SERMs raloxifene, clomiphene and
11 tamoxifen inhibit Ebola virus infection in experimental models (Figure 17).¹⁷¹
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13 During SARS-CoV-2 infection, it has been recently shown that TPC2 plays a relevant
14 role, and a decrease of SARS-CoV-2 pseudovirus entry was demonstrated after the
15 treatment with tetrandrine, a potent calcium blocker used as pharmacological tool.
16 Altogether, TPC2 emerges as a druggable host-target for SARS-CoV-2 infection, and
17 repurposing of dopamine antagonist such fluphenazine or SERMs as raloxifene merit to
18 be tested in clinical trials for COVID-19.
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55 **Figure 17.** Clinical used drugs blockers of TPC2. A) Dopamine antagonists, B) SERMs.

4 Host immune response

Innate immune cells display an effective antiviral response to coronavirus infection. This response is based on detection of viral infection by using pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). PRRs include C-type lectin-like receptors, Toll-like receptor (TLR), NOD-like receptor (NLR), RIG-I-like receptor (RLR) and free-molecule receptors in the cytoplasm, such as cGAS-STING, IFI16 and so on.¹⁷² For RNA virus such as coronavirus, viral genomic RNA or replication intermediates like double-stranded RNA (dsRNA), are recognized by, either the endosomal RNA receptors TLR3 and TLR7, or by the cytosolic RNA sensor RIG-I/ MDA5, among others. This recognition induces the activation of several signalling pathways resulting in inflammation and initiation of cellular immune response. These pathways activate crucial transcription factors including interferon regulatory factor 3 (IRF3), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), and AP-1. Consequently, these factors ultimately promote the production of type I interferons (IFN-I), inflammatory cytokines and chemokines.¹⁷³

4.1 Viral proteins involved in innate immune modulation

The immune response can be exaggerated leading to an inordinate inflammatory reaction that cause severe clinical signs of COVID-19. This is characterized by severe pneumonia and lung inflammation, respiratory distress and a life-threatening cytokine storm and circulatory shock.¹⁷⁴ Other human CoVs have developed strategies to counteract the immune response, which await to be deeply studied in SARS-CoV-2.¹⁷⁵ SARS-CoV-2 typically reduces IFN-I response and elevates the expression of IL-6 and IL1RA cytokines.¹⁷⁶ SARS-CoV and MERS-CoV infection also produces the suppression of IFN synthesis,¹⁷⁷⁻¹⁷⁸ which determines their virulence.¹⁷⁹ In this perspective, we sum up the main CoVs proteins implicated in innate immune response.

4.1.1 Structural proteins modulation of immune response

The SARS-CoV and MERS-CoV M protein binds TRAF3 to block its binding and subsequent nuclear translocation of TANK-binding kinase 1 (TBK1), which blocks IRF3- mediated signaling and inhibits type I IFN production.¹⁸⁰⁻¹⁸¹

SARS-CoV N protein also interfere with the function of IRF3.¹⁸² The N protein of SARS-CoV targets type I IFN synthesis at early recognition stages of innate immune signaling viral RNA infection.¹⁸³ Potentially, it may also act on other viral RNA recognition strategies of the host. The N protein of SARS-CoV also binds to the SPRY domain of TRIM25 and inhibits TRIM25-dependent RIG-I activation, thereby suppressing its ubiquitination and type I IFN production.¹⁸⁴

It was also described that purified SARS-CoV spike (S) protein induces an inflammatory response, possibly through TLR2 activation.¹⁸⁵ Finally, the SARS-CoV E protein, is a viroporin that aids NLRP3 (NLR family) inflammasome, thereby secreting IL-1 β .^{32, 186}

SARS-CoV-2 viral protein interactions and its role to act against the immune system response are still unknown. However first protein-protein interactions studies have elucidated that N protein could target stress granule protein G3BP1, an essential antiviral protein which is known to induce innate immune response.¹⁸⁷ Indeed this interactome identifies that N protein binds stress granule related factors G3BP1/2, the mTOR repressors LARP1, and the kinases CK2. Stress granule induction depends on dsRNA recognition by protein kinase R (PKR)-mediated pathway, which induces proinflammatory cytokines¹⁸⁸ (Figure 18).

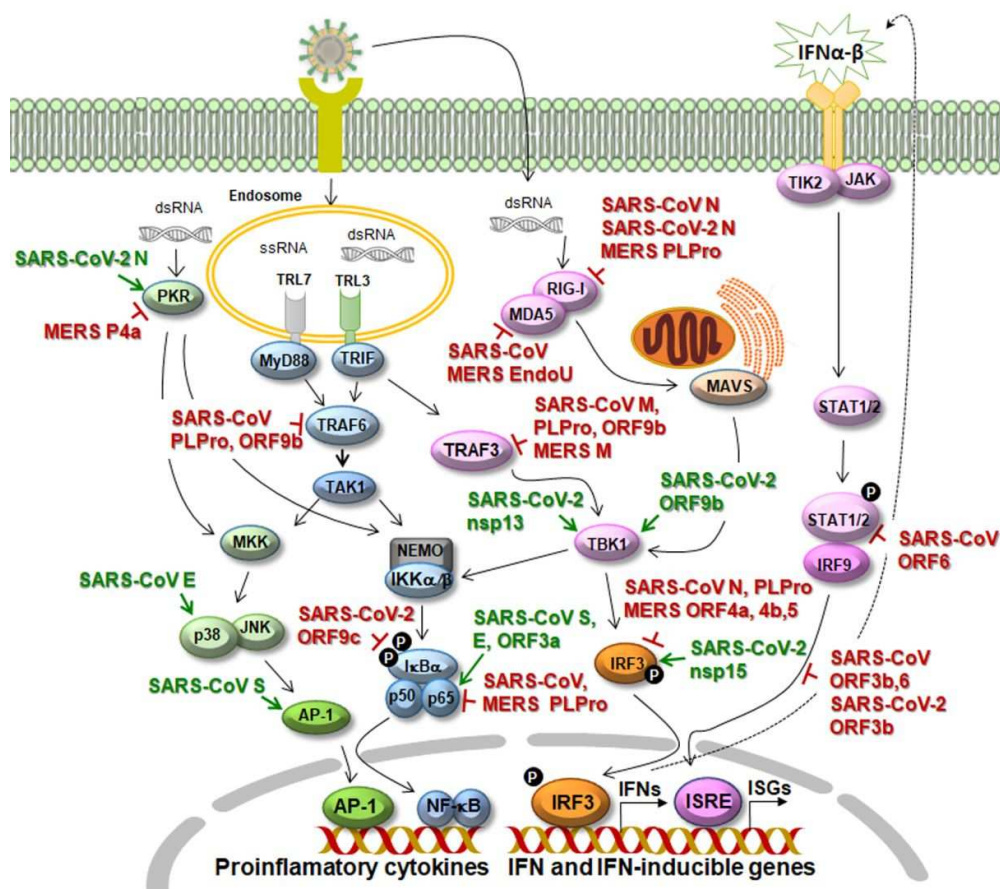


Figure 18. Schematic representation of the main pathways of the innate immune response HCoV. These pathways lead to the activation of nuclear factor kappa B (NF- κ B), AP-1 and interferon (IFN) resulting in the secretion of proinflammatory cytokines and interferons and the activation of a cellular immune response. As most viruses, HCoVs have evolved mechanisms to evade the innate immune response. Most of their proteins have been found to be inhibitory (depicted in red) of several arms of the innate immune response. Remarkably, SARS-CoV-2 ORF9b and nsp15 activate (in green) the IFN route, while other HCoVs proteins have the opposite effect leading to destruction of both the cell and the virus. Also, SARS-CoV-2 activates PKR, while MERS - and several other viruses- typically inhibit this enzyme for recovery from ER stress and viral protein synthesis shut-off.

4.1.2 Non-structural proteins regulation of the immune response

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3 Nsps and accessory proteins of HCoV have also an important role in the modulation
4 of innate immunity. SARS-CoV nsp1 affects IFN-dependent signalling¹⁸⁹ and SARS-
5 CoV PLpro domain (nsp3) protein inhibits IRF3 phosphorylation and nuclear
6 translocation, thereby blocking type I IFN production.¹⁹⁰
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10 Apart from its protease activity, SARS-CoV and MERS-CoV PLpro also has
11 deubiquitinating activity.¹⁹¹⁻¹⁹² PLpro of SARS-CoV inhibits TLR7 signaling removing
12 K63-linked ubiquitin chains from TRAF3 and TRAF6.¹⁹³ A subsequent analysis showed
13 that both PLpro of SARS-CoV and MERS-CoV also recognized another ubiquitin-like
14 modifier interferon-stimulated gene 15 (ISG15). In those cases, PLpro acts as a
15 delISGylating enzyme. This domain downregulates mRNA levels of pro-inflammatory
16 cytokines such as CCL5, IFN- β , and CXCL10.¹⁹⁴ Furthermore, it has been identified
17 that the ADP-ribose-1-monophosphatase macrodomain encoded within nsp3 in HCoV-
18 229E and SARS-CoV is responsible for suppressing IFN induction.¹⁹⁵
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21 SARS-unique domain encoded in nsp3 of SARS-CoV can also enhance a cellular E3
22 ubiquitin ligase called ring-finger and RCHY1, which leads to proteasomal degradation
23 of p53.¹⁹⁶
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26 Endonuclease U (EndoU) domain is encoded in nsp15 and it is an important
27 component of CoVs replicase-transcriptase complex (RTC). EndoU activity prevents
28 dsDNA recognition by PPRs such as MAD5, thereby evading early innate immune
29 response.¹⁹⁷ CoVs also encode ribonucleases that counteract dsRNA antiviral
30 response. This is the case of exoribonuclease domain of nsp14, which modulates
31 dsRNA levels and innate immune sensing.¹⁹⁸
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34 Despite being dispensable in viral replication, HCoV accessory proteins get involved in
35 process such as cell proliferation, apoptosis and interferon signalling.¹⁹⁹ SARS-CoV,
36 ORF3b and -6 are shown to interfere with IFN- β synthesis and IFN signaling by
37 preventing IFN- β -induced activation of interferon-stimulated response element (ISRE),
38 found in the promoter region of ISG.¹⁸² SARS-CoV ORF3a induces TRAF3- mediated
39 ubiquitination of apoptosis-associated speck-like protein containing a caspase
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3 recruitment domain, which activates NLRP3 inflammasome and NF- κ B pathway.²⁰⁰ The
4 SARS-CoV ORF6 protein was shown to interact with nuclear pore and blocks p-STAT1
5 import into the nucleus, reducing innate immune responses and increasing
6 pathogenesis.²⁰¹ The accessory proteins of MERS-CoV, ORF4a, -4b and -5, could
7 similarly suppress IRF3 nuclear translocation,¹⁸¹ while P4a suppresses PKR-
8 dependent stress response, an additional antiviral response.²⁰² Moreover, SARS-CoV
9 ORF9b might also hijack an ubiquitin E3 ligase called AIP4 to trigger the degradation of
10 MAVS, TRAF3 and TRAF6, thereby significantly suppressing IFN responses.²⁰³
11 Indeed, SARS-CoV accessory protein P6 interacts with the IFN-signaling pathway-
12 mediating protein Nmi and promotes its ubiquitin-dependent proteasomal degradation,
13 thereby potentially modulating the virus-induced innate immune response.²⁰⁴
14 SARS-CoV-2 protein-protein interaction maps showed so far, that nsp5 (3CLpro) could
15 interact with the epigenetic regulator histone deacetylase 2 (HDAC2).¹⁸⁸ Previous
16 studies demonstrate that HDAC2 mediates inflammation and interferon response.²⁰⁵
17 The same study shows that nsp13 interacts with two elements of IFN signaling
18 pathway including TBK1 and TBK1-binding protein 1 (TBKBP1), modulating the NF- κ B
19 inflammatory response. E3 ubiquitin ligase RNF41/Nrdp1 is targeted by nsp15 protein,
20 which would increase type I interferon production.²⁰⁶ Two other E3 ubiquitin ligases,
21 TRIM59 and MIB1, regulate antiviral innate immune signaling and are hijacked by
22 ORF3a and nsp9.²⁰⁷ ORF9c protein was found to modulate I κ B kinase and NF- κ B
23 signaling pathway and ORF9b interacts with a mitochondrial import receptor, Tom70, a
24 linker between MAVS and TBK1/IRF3, inducing IRF-3 activation¹⁸⁸ (Figure 18). Nsp3,
25 ORF3b and ORF6 of SARS-CoV-2 -with less homology with SARS-CoV proteins could
26 induce IFN-I sensitivity.²⁰⁸ However, it has been described that truncated ORF3b of
27 SARS-CoV-2 acts as an IFN antagonist more efficiently than that of SARS-CoV.²⁰⁹
28 These studies at the cellular level await further clinical or animal model investigations
29 to fully understand the role of the immune response in this disease.
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4.2 Therapeutic targeting of the innate immune response

It has been known that a cytokine storm results from an overreaction of the immune system in SARS and MERS patients.²¹⁰ Clinical findings showed exuberant inflammatory responses during SARS-CoV-2 infection, further resulting in uncontrolled pulmonary inflammation, likely leading case fatality. The repurposing of host-based therapeutics to control immune response may counterattack COVID-19.²¹¹

Some of these treatments include the use of recombinant IFN- α and IFN- β as antiviral cytokines that inhibit viral replication in targeted cells. Some studies have reported that IFN- β alone has more effect against SARS-CoV-2 than IFN- α *in vitro*. In fact, combinations of IFN- α and IFN- β with other antivirals such as ribavirin (Figure 19) and/or lopinavir/ritonavir (HIV treatment) (Figure 9) have a synergistically effect *in vitro*²¹²⁻²¹³ and in animal models.²¹⁴⁻²¹⁵ Recent results from an open-label, randomised, phase 2 trial (NCT04276688) conducted in China has proved that the triple therapy (IFN- β -lopinavir-ritonavir), is safe and more effective than lopinavir-ritonavir alone in alleviating symptoms and shortening the duration of viral shedding in mild to moderate COVID-19 patients.²¹⁶ *In vitro* and *in vivo* studies show the protective effect of Type III IFN (IFN- γ) treatment against SARS-CoV infection,²¹⁷⁻²¹⁸ that possibly in combination with IFN-I- could be an effective treatment for SARS-CoV-2.

A recent study showed the potential benefits from low-dose corticosteroids treatment in SARS-CoV-2 critically ill patients.²¹⁹⁻²²⁰ Another immunosuppressor, tocilizumab, an humanized monoclonal antibody against the interleukin IL-6 receptor reduces pro-inflammatory response in COVID-19 patients.²²¹ Immunosuppressor treatments successfully used against other viruses, could be also used for COVID-19. These would include JAK inhibitors such as tofacitinib, baricitinib, and the recently approved upadacitinib, previously used in rheumatoid arthritis,²²² blinatumomab²²³ and HDAC inhibitors, such as vorinostat or belinostat (Figure 19B and 19C).²²⁴ Baricitinib (Figure 15), as mentioned before, is also a potent inhibitor of AAK1 and may also influence in decreasing viral infectivity, being a good candidate for clinical trials of COVID-19.¹⁵⁵

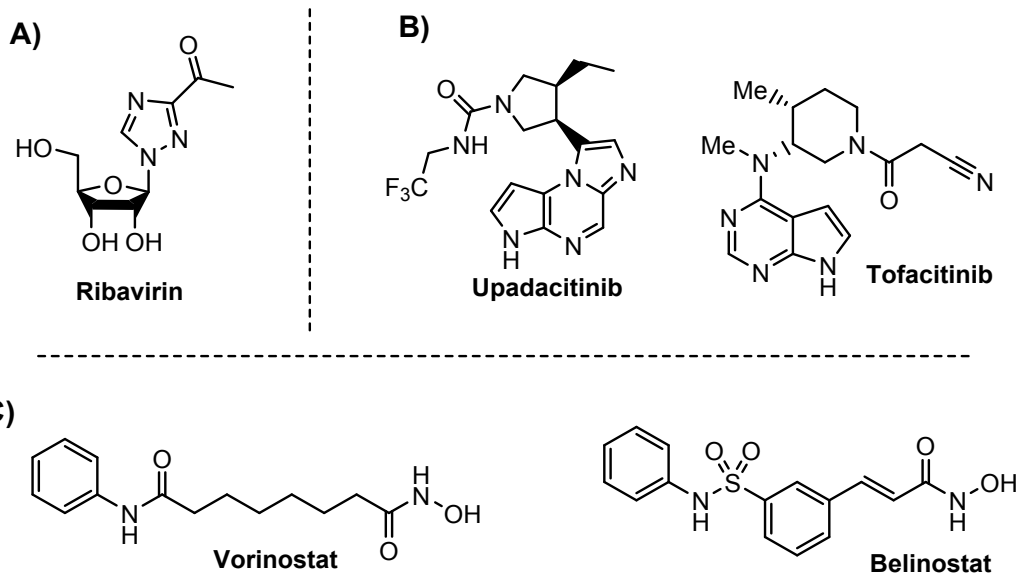


Figure 19. Immunosuppression potential treatments. A) antiviral used in combination with IFN α and IFN β , B) JAK inhibitors, C) HDAC inhibitors.

Another therapy that could control SARS-CoV-2 would be polyinosinic:polycytidylic acid (poly(I:C)), which is a synthetic analogue of dsRNA that induces type I IFN and decreases MERS-CoV load in BALB/c.²²⁵

Nitazoxanide is another potent type I IFN inducer that has been used in humans as antiparasitic agent (Figure 20). It is a synthetic nitrothiazolyl-salicylamide derivative that exhibits broad-spectrum antiviral activities against both RNA and DNA viruses.²²⁶ It is effective in the treatment of HCV and influenza (IFV)-A and -B infection, currently at Phase II and Phase III clinical studies²²⁷⁻²²⁸ and could be also tested in SARS-CoV-2 infection. This drug can inhibit expression of the viral N protein and proinflammatory cytokines (IL-6) in MERS-CoV infected mice.²²⁹

Other IFV antivirals could be useful in SARS-CoV-2 therapeutic treatments. Glycyrrhizin (Figure 20) is an antioxidant, anti-inflammatory, immunomodulatory and antiviral agent²³⁰⁻²³¹ that inhibits IFV-A infection through inhibiting proinflammatory gene expression.²³² Glycyrrhizin can suppress SARS-CoV infection at the early virus entry and the late replication stages in Vero E6 cells.²³³ 14-deoxy-11,12-

dehydroandrographolide suppresses proinflammatory cytokines and chemokines as well as IFV-A replication.²³⁴ NSC61610 (Figure 20) induces lanthionine synthetase C like 2 (LANCL2), a therapeutic target for treating infectious diseases such as IFV-A infection.²³⁵ The *Ligustrum purpurascens* extract phenylethanoid glycoside (LPG) induces IFN- γ , which can inhibit IFV-A replication *in vitro* and *in vivo*.²³⁶ Some of these agents could be considered in clinical trials for treating COVID-19, although adverse effects need to be deeply studied.²³⁷

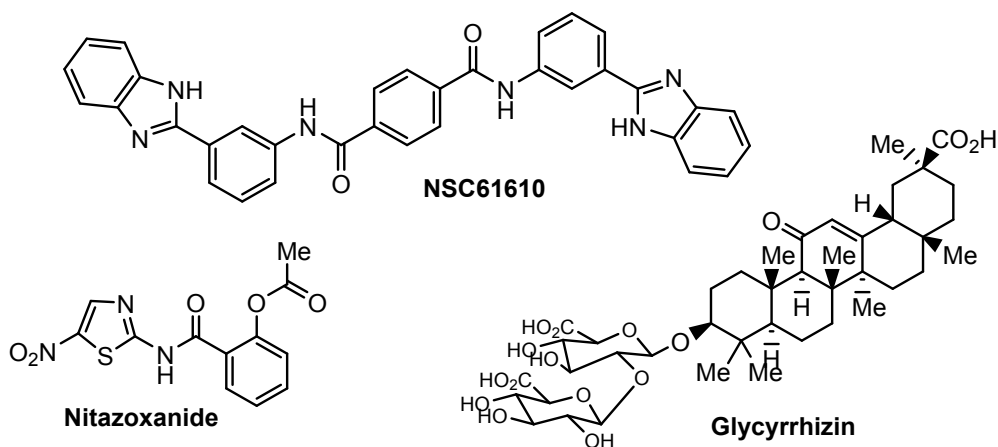


Figure 20. Anti-IFV-A agents with potential therapeutic effects on SARS-CoV-2.

Other strategies attempting to control host immune response in HCoV include cyclophilin targeting drugs as cyclosporin A and alispovir. SARS-CoV-2, not only uses ACE2 receptor for cell entry, but also CD147, as described for SARS-CoV.²³⁸⁻²³⁹ CD147 receptor regulates cytokine secretion and chemotaxis of inflammatory cells through cyclophilin A and cyclophilin B. Another group of drugs with potential applications are other kinase inhibitors used in cancer, imatinib mesylate, dasatinib, trametinib, saracatinib etc.²⁴⁰⁻²⁴¹ or the immunosuppressor mycophenolic acid.²⁴²

5 Therapeutic options in clinical trials for COVID-19

Antivirals lopinavir/ritonavir (Figure 9) has been recommended for clinical treatment for COVID-19. However, recent results from clinical trials do not confirm any benefit in

hospitalized adult patients with severe COVID-19²⁴³, but the combination of these two antivirals with interferon, as previously mentioned, is more promising for patients.²¹⁶

Remdesivir (Figure 10) is an adenosine analogue RdRp inhibitor with antiviral protection against SARS-CoV-2⁹³ and other viruses.⁹⁹ In addition, intravenous administration has been found to be efficacious in an American patient with COVID-19.²⁴⁴ Based on these results, Gilead Company provided the compound to China to perform the first clinical trials in SARS-CoV-2-infected individuals (Clinical trials NCT04257654/6). As it has been mentioned before, the FDA has approved the emergency use of this drug for COVID-19 patients with severe symptoms.⁹⁷

Arbidol™ (umifenovir) is able to block viral fusion against influenza viruses. The antiviral activity of umifenovir against SARS-CoV-2 has been confirmed *in vitro*.²⁴⁵ First clinical data from patients with laboratory-confirmed COVID-19 points to a superior efficacy of umifenovir monotherapy to lopinavir/ritonavir treatment.²⁴⁶ Also, other drugs used for influenza as Avigan™ (favipiravir) (Figure 10) and Tamiflu™ (oseltamivir) have been used in patients (Figure 21).

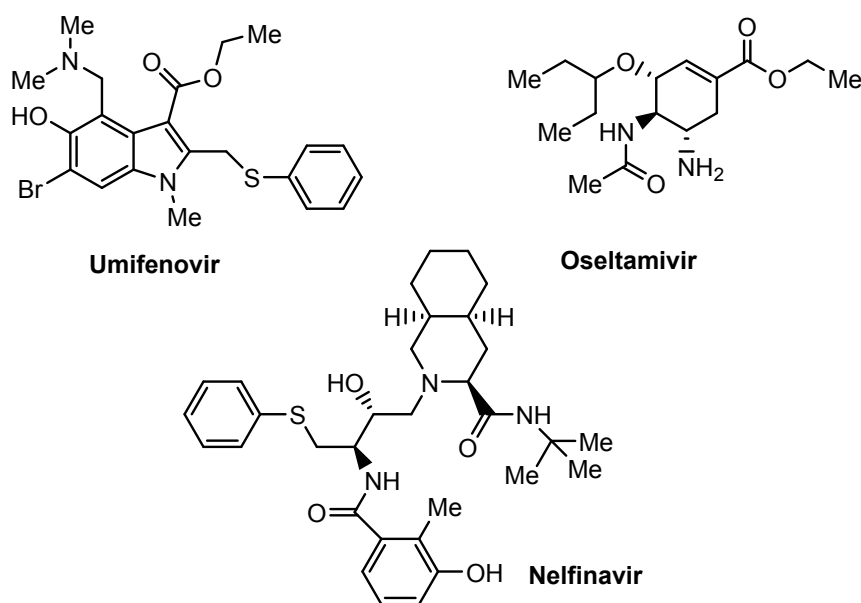


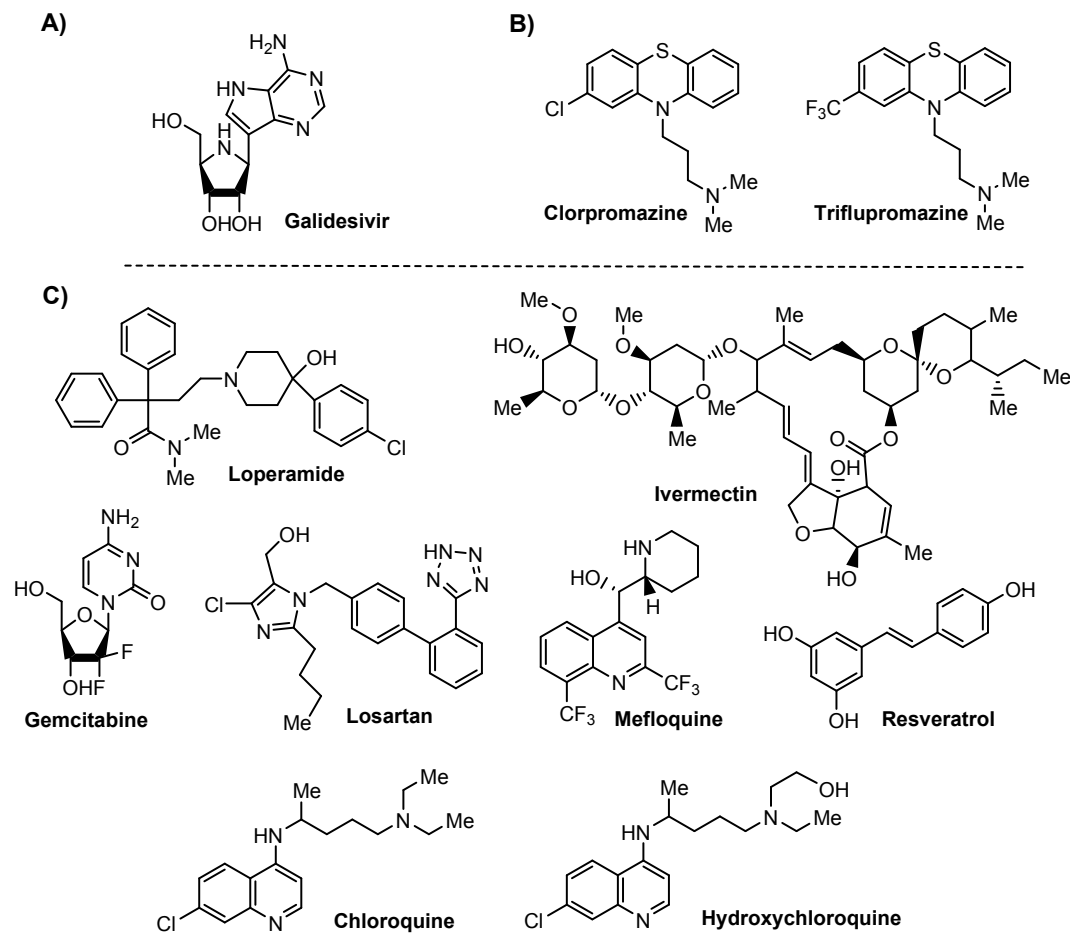
Figure 21. Antivirals used in clinic as potential COVID-19 treatment

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3 Importantly, in this pandemic, clinical trials have been started from the beginning
4 (Table 1). The clinical management of the critically-ill COVID-19 patient includes not
5 only compounds targeting the viral replication, but also therapeutic compounds seeking
6 to modulate the immune dysregulation and the inflammatory storm that causes the
7 severe disease and frequent fatalities. Interestingly, some compounds have been
8 shown to control hyper-activated immune response and simultaneously, they are able
9 to inhibit viral replication at some extent. Antiviral therapeutic options under clinical
10 trials could be classified as:

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18 a) antivirals against viral components: EBOV viral polymerase inhibitor remdesivir
19 (Figure 10),⁹³ developed for EBOV, IFV antiviral favipiravir (Figure 10)⁹⁰ and
20 others developed for HIV or HCV: lopinavir-ritonavir (Figure 9) capable to inhibit
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26 b) antivirals developed against host targets: viral endocytosis inhibitors such as
27 chloroquine and hydroxychloroquine (Figure 22), which are drugs for malaria
28 that decrease endosomal acidity, kinase inhibitors and, finally,
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32 c) anti-inflammatory drugs, corticosteroids or immunosuppressors.
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34 A therapeutic combination (including a and c) has been selected for a randomized
35 large clinical trial launched by WHO, called Solidarity, to collect clinical data from
36 several thousand patients from dozens of countries.⁹⁵ Other antivirals used against
37 viral RNA polymerase include ribavirin (Figure 19),^{214, 247} and galidesivir (BCX4430)
38 (Figure 22).²⁴⁸ Others are selected based in their protease inhibition activity such as
39 disulfiram (a PLpro inhibitor),²⁴⁹ or camostat mesylate (a TMPRSS inhibitor) (Figure
40 13)¹¹⁶ or the spike protein inhibition, such as griffithsin.²⁵⁰ Some others antivirals used
41 are clathrin-mediated endocytosis inhibitors. This is the case of chlorpromazine (Figure
42 22) and fluphenazine (Figure 17), dopamine inhibitors which are FDA-approved
43 drugs,¹⁷¹ and miscellaneous compounds (Figure 22) such as resveratrol,²⁵¹
44 gemcitabine, mefloquine²⁴⁰ and loperamide²⁵². Losartan, and more recently, telmisartan
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3 have been used as ACE2 receptor inhibitor. Furthermore, ivermectin has recently
4 shown SARS-CoV-2 inhibition *in vitro*.²⁵³
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37 **Figure 22.** Approved drugs in clinical trials for COVID-19. A) Inhibitors of viral RNA
38 polymerase, B) antipsychotics effective in clathrin-mediated endocytosis, C)
39 miscellaneous drugs
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Table 1. Drugs currently used in clinical trials (WHO clinical trial Solidarity compare treatment strategies with several compounds, marked with asterisks).

Target	Antivirals	
RNA polimerase	Remdesivir*	Ebola Virus
	Favipiravir, ribavirin, oseltamivir, galidesivir, sofosbuvir, umifenovir	Influenza virus, HCV, HIV
3CL protease	Lopinavir/ritonavir*, ivermectin	HIV
PL protease	Disulfiram	
Protein S	Griffithsin	HIV transmission
Inhibition virus entry		
Inhibition TMPRSS2	Camostat mesylate nafamostat, bromhexine enzatulamide	
Inhibition endocytosis	Chlorpromazine, fluphenacine	Other uses
Acidification endosome	Chloroquine/hidroxi-Chloroquine	Malaria
Inhibition entry	Convalescent Plasma	Antibodies
Kinase inhibitors	Imatinib, baricitinib	Antitumor agents
Immune response Control		
Anti-inflammatory drugs	Corticosteroids	Inflammatory response
	Interferons*	HCV
	Nitazoxanide Micofenolic acid	Interferon induction or synergy
	Tocilizumab	IL6 antagonist
	Cyclosporin/alisповir	Immune suppressors
Miscelaneous	Resveratrol, loperamide, losartan, telmisartan, etc.	Control organ damage

Conclusions

As mentioned in the introduction, a deep knowledge the life cycle of SARS-CoV-2 is essential to identify druggable targets that will allow the development of effective therapeutics against this coronavirus. Through the journey across the life cycle of the virus presented in this perspective, we have considered several virus-based and host-based targets as objectives for pharmacological intervention as well as the host immune response. Among the virus-based targets, the importance of the structural spike (S) protein is remarkable due to its key role in the SARS-CoV-2 entry through the interaction with the host receptor ACE2. We have also reviewed structural druggable sites and determinants of antibodies efficacy against S protein. This coronavirus has 16 non-structural proteins. Special relevance for virus replication, and thus, relevant as drug targets are the two proteases (3CLpro and PLpro), the RNA-dependent RNA polymerase (RdRp) and the helicase. Those are highly conserved proteins and represent suitable targets for the development of pan-coronavirus antivirals. Study of the molecular basis of virus entry pointed to key cellular proteins involved in this process. This is the case of the already mentioned host receptor ACE2, host proteases like TMPRSS2, furin or cathepsin L, or kinases that are implicated in the regulation of intracellular viral trafficking during endocytic entry such AAK1, GAK or PIKfyve. TPC2 is also an important host channel involved in the regulation of endolysosomal trafficking. Host immune modulation has proven to be a useful alternative for the clinical management of viral diseases lacking specific treatment. Moreover, targeting human proteins is an excellent alternative to avoid viral scape by mutation. Another promising alternative could be the combination of antiviral drugs acting through different targets in a multi-target strategy that has proven to increase efficacy and overall prevent viral resistance.

Given the urgency of the COVID-19 pandemic, the repurposing of approved drugs is the only alternative to find a timely effective treatment.²⁵⁴ In fact, drugs currently under clinical trials were initially approved for other indications. However, the past and

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3 present coronavirus outbreaks require our preparedness not only for the current
4 situation but also for a future potential reemergence of novel coronaviruses. In this
5 sense, is of utmost importance to design drugs acting as pan-coronavirus antivirals or
6 through a multi-target approach to avoid lack of effectiveness by viral mutation escape.
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10 11 12 **Addendum (May 29, 2020)**

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14 Since the submission of this review, the number of tridimensional structures
15 determined for different SARS-CoV-2 proteins available in the PDB has been
16 increasing dramatically. On average, in February 2020, a total of 4 structures were
17 found deposited in the PDB, increasing by 99 in March, by 45 in April, and by 65 in
18 May. Today (late May 2020), a total of 214 structures are available in the PDB,
19 although some of them have not yet been published (Table S1). Different experimental
20 techniques in addition to X-ray diffraction have been used, including electron
21 microscopy and NMR, and the structure of SARS-CoV-2 main protease has been the
22 most studied, with more than 136 different crystals deposited. This huge data explosion
23 confirms the relevance of proteomic data in relation with the pandemic.
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27 As the number of PDB structures, as well as the compounds with potential antiviral
28 action against SARS-CoV-2 being studied will increase sharply in the next months,
29 essential web resources have been made available where the COVID-19 information
30 that may be of utmost importance for medicinal chemists, is continuously updated.
31 These websites are summarized in table S2.
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34 35 36 **Supporting information**

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38 The Supporting Information is available free of charge on the ACS Publications
39 website.
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42 3D structures available (PDB) of SARS-CoV-2 proteins (Table S1); Web resources for
43 COVID-19 drug discovery (Table S2).
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32 **Ana Martinez** received her organic chemistry degree and PhD in medicinal chemist
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10 **Abbreviations used**

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12 AAK1, Adaptor-associated kinase 1; ACE2, angiotensin-converting enzyme 2; Ang,
13 angiotensin; ARDS, acute respiratory distress syndrome; 3CLpro, 3C-like protease or
14 main protease; COVID-19, coronavirus infection disease (2019 pandemia); CoV,
15 coronavirus; E protein, envelope protein; EBOV, Ebola virus; FCS, furin-like cleavage
16 site; GAK, cyclin G-associated kinase; IFN, interferon; IRF3, interferon regulatory factor
17 3; ISRE, interferon-stimulated response element; M protein, membrane protein; MERS-
18 CoV, Middle East respiratory syndrome coronavirus, N protein, nucleocapsid protein;
19 Nsp, non-structural proteins; NTD, N-terminal domain; ORF, open reading frame;
20 PIKfyve, phosphatidylinositol 3-phosphate 5-kinase; PI(3,5)P2, phosphatidylinositol-
21 3,5-bisphosphate; PLpro, papain-like protease; RAS, renin-angiotensin system; RBD,
22 receptor binding domain; RBM, receptor binding motif; RdRp, RNA-dependent RNA
23 polymerase; S protein, spike protein; SARS-CoV-2, severe acute respiratory syndrome
24 coronavirus 2; SERMs, selective estrogen receptor modulators; TLR, Toll-like receptor;
25 TMPRSS2, transmembrane serine protease 2; TPC2, two pore channel 2.
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