

## COVID-19: Drug Targets and Potential Treatments

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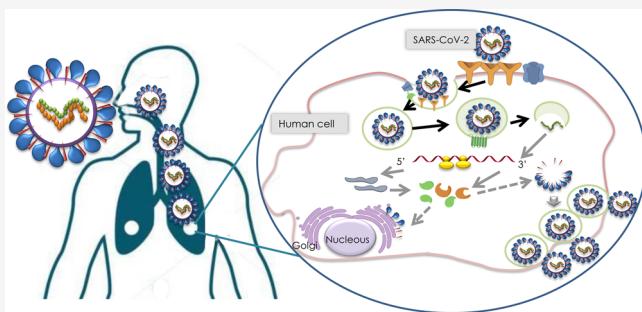


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**ABSTRACT:** Currently, humans are immersed in a pandemic caused by the emerging severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which threatens public health worldwide. To date, no drug or vaccine has been approved to treat the severe disease caused by this coronavirus, COVID-19. In this paper, we will focus on the main virus-based and host-based targets that can guide efforts in medicinal chemistry to discover new drugs for this devastating disease. In principle, all CoV 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.



### 1. INTRODUCTION

The coronavirus pandemic known as 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.<sup>1</sup> This novel CoV belongs to the *Coronaviridae* family, along with 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 HCoV-HKU1), which are responsible for mild respiratory tract infections.<sup>2</sup>

Highly pathogenic CoVs are enveloped, positive polarity, single-stranded RNA betacoronaviruses, and their genomes encode non-structural proteins (nsps), structural proteins, and several accessory proteins.<sup>3,4</sup> The publication of the genome sequence of SARS-CoV-2<sup>5</sup> has allowed researchers to determine that the new virus is closely related to SARS-CoV (82% sequence identity) and, to a lesser extent, to MERS-CoV.<sup>6</sup> 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.<sup>7,8</sup> 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,<sup>1</sup> and cleavage of the S protein by the host transmembrane

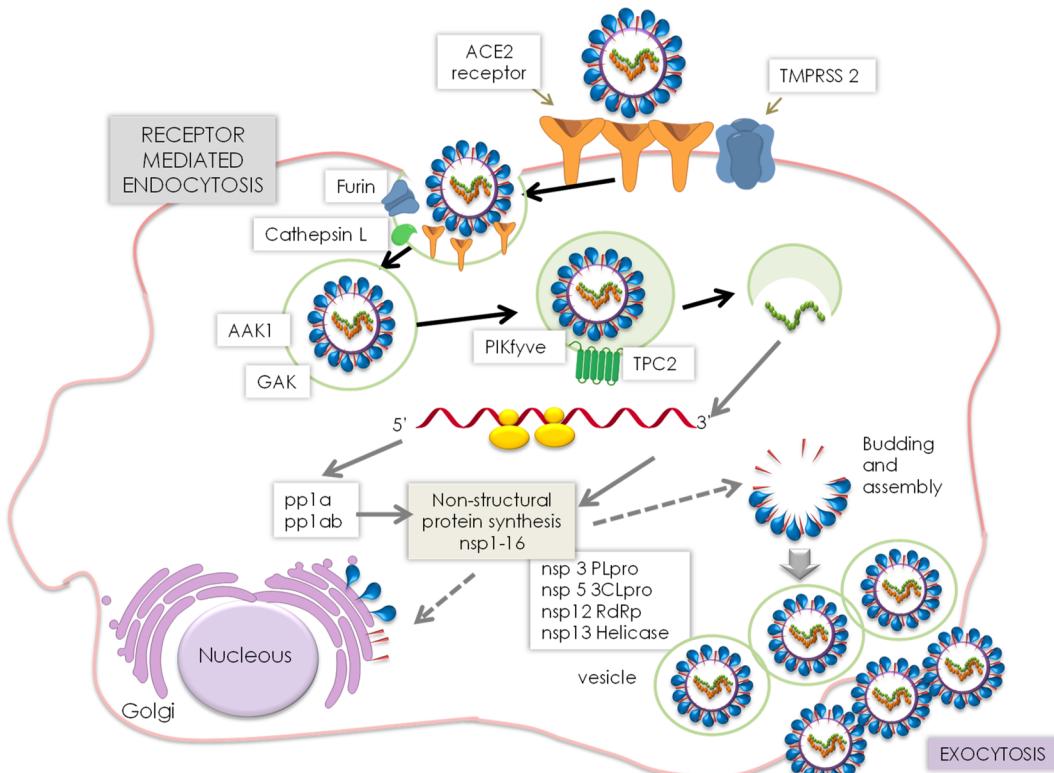
serine protease 2 (TMPRSS2) prior to the fusion to the host cell membrane.<sup>9</sup>

Entry mechanisms of coronavirus were controversial 15 years ago. In early studies, a non-endosomal pathway was initially thought to be 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.<sup>10</sup> The nucleocapsids were then blurred after the virions lost their envelopes, and no endocytic-related events were described. However, recent evidence points to the endosomal pathway as the main entry route for CoVs to infect the cells. Remarkably, Ng et al. had published one year earlier a study with a SARS-CoV isolated from a SARS patient in Singapore.<sup>11</sup> They certainly observed fusion events at the plasma membrane, followed by a movement of spherical viral cores into the cytoplasm within large cellular vacuoles during the first 15 min after infection.

In 2008, Wang and colleagues established the endocytic pathway as an alternative entry pathway apart from direct fusion with the plasma membrane based on their observations of SARS-CoV.<sup>12</sup> They showed that this virus enters the cell via a pH- and receptor-mediated endocytosis-dependent manner. In fact, the spike (S) protein itself or a pseudovirus bearing S

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**Figure 1.** SARS-CoV-2 infection cycle.

protein induced internalization of SARS-CoV receptor ACE2 from the cell surface to cytoplasmic compartments. Furthermore, lysosomotropic drugs blocked the ACE2 receptor in vesicles, impairing their recycling to the plasma membrane. Pseudoviruses were also affected by inhibition of pH acidification, which indicates that SARS-CoV exploits the endocytic pathway to infect the cells, as they found, in a clathrin- and caveolin-independent manner.

Currently, we are immersed in a pandemic caused by the emerging SARS-CoV-2,<sup>13,14</sup> which is severely threatening the public human health care system worldwide. Some years ago, two other coronaviruses also crossed the species barrier, triggering deadly pneumonia in humans: SARS-CoV<sup>15,16</sup> and MERS-CoV.<sup>17</sup> Similarities in the entry pathways of these betacoronaviruses need to be elucidated.

Coronavirus entry relies on the spike (S) protein, and depending on the viral strain and cell type studied, the S protein is cleaved by several different cellular proteases.<sup>18–24</sup> SARS-CoV-2 presents entry requirements similar to those of SARS-CoV. Both viruses are coincident in the cellular receptor ACE2. Similar to SARS-CoV, SARS-CoV-2 infection is profoundly inhibited by lysosomotropic drugs (99% and 98%, respectively) in cells transduced with pseudovirus.<sup>25</sup> These results point out that the endocytic pathway is the preferred route for SARS-CoV-2 entry into the host cell.

Furthermore, SARS-CoV and SARS-CoV-2 pseudovirions' entry is dependent on late endosomal compartments, given their dependence on endosomal acidification. Thus, the inhibition of endosomal maturation would result in the impairment of SARS-CoV-2 infection. In fact, pseudovirions' entry is impaired by treating cells with chemical inhibitors (YM201636 and apilimod) of the PIKfyve enzyme, involved in the phosphoinositides metabolism regulating endosomal

maturity. Other viruses depending on PIKfyve enzyme described have been Ebola virus (EBOV) and African swine fever virus, both dependent on the late endosome for entry.<sup>26–28</sup> SARS-CoV-2 infection is impaired by blocking other proteins characteristic of the late endosomal compartments, like two-pore channel 2 (TPC2) (but not TRMPL1), indicating that TPC2 is important for SARS-CoV-2 pseudovirions' entry.<sup>25</sup>

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

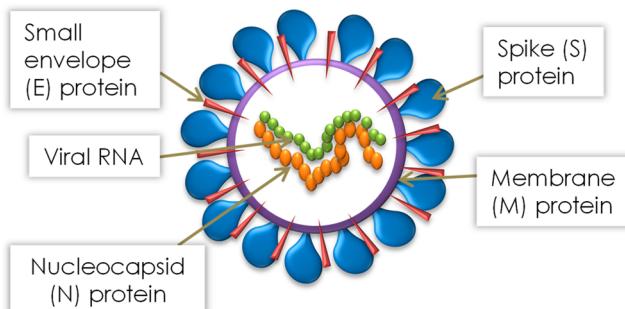
Once the virus enters the host cell, it gets disassembled to release the nucleocapsid and the viral genome. Host ribosomes translate the open reading frame (ORF) 1a/b into two polyproteins (pp1a and pp1ab) that encode 16 nsps, while the remaining ORFs encode structural and accessory proteins. Two proteases 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).<sup>29</sup> 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.<sup>30</sup>

In principle, all CoV 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.

## 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



**Figure 2.** Schematic representation of SARS-CoV-2 and its structural 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 for the viral entry through receptor recognition and membrane fusion.<sup>31</sup> The envelope (E) protein is the smallest of the structural proteins but has a crucial role in assembly, budding, envelope formation, and virulence.<sup>32</sup> The main function of the membrane (M) protein is to promote viral assembly due to its membrane-bending properties.<sup>33</sup> The nucleocapsid (N) protein is a multifunctional protein that packages the viral RNA genome into a ribonucleoprotein complex called nucleocapsid to protect the genome.<sup>34</sup> With regard to the search for new therapeutics based on structural proteins of SARS-CoV-2, some computational studies were carried out to identify the structure and function of E protein<sup>35</sup> and the other structural proteins.<sup>36</sup>

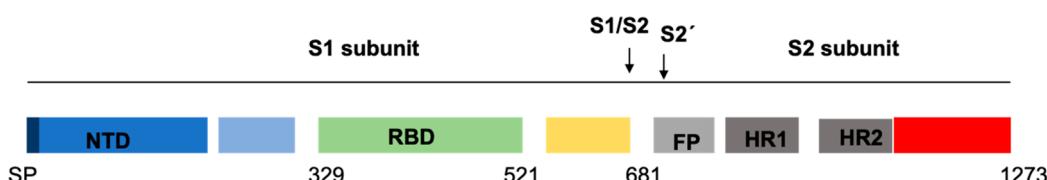
We will focus our attention on the N protein recently crystallized (PDB codes: 6M3M<sup>37</sup> and 6VYO) and on the S protein due to its fundamental role in viral entry and its available structures that will allow structure-based drug design.<sup>38,39</sup>

**2.1.1. Nucleocapsid (N) Protein.** As mentioned above, coronavirus N protein is a multifunctional RNA-binding

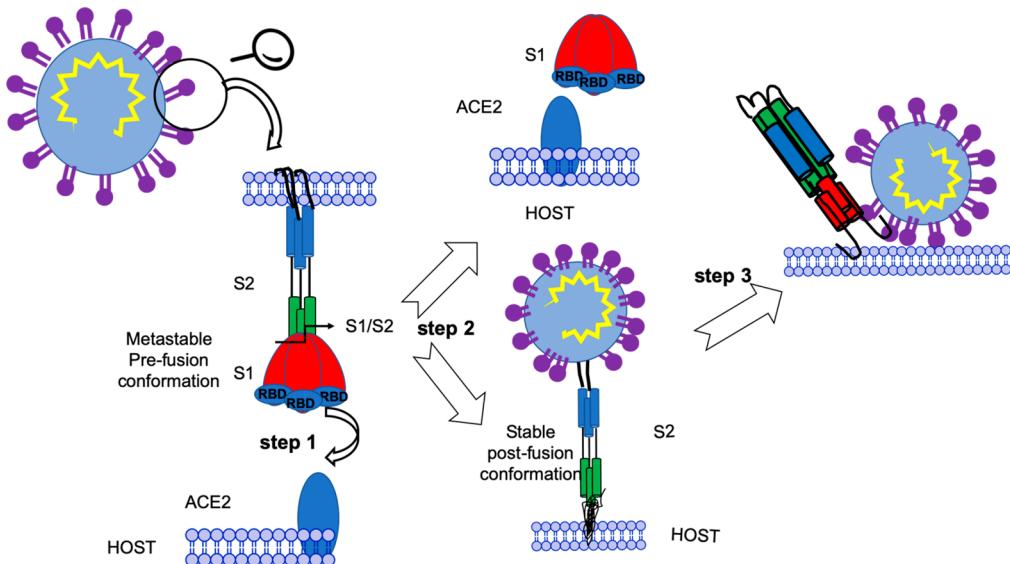
protein considered to be an interesting pharmacological target that merits further attention due to its critical function in viral RNA transcription and replication.<sup>40</sup> This major CoV protein contains two highly conserved domains, an N-terminal RNA-binding domain and a C-terminal dimerization domain, together with a disordered central Ser/Arg-rich linker. Previous studies revealed that the N-terminal domain is responsible for RNA binding, the C-terminal for oligomerization, and the Ser/Arg-rich linker for primary phosphorylation.<sup>41–43</sup> The crystal structure of SARS-CoV-2 nucleocapsid N-terminal domain has been solved (PDB code: 6M3M),<sup>37</sup> showing an overall similarity with the same domain from other CoVs, although the surface electrostatic potential showed a specific distribution. These important structural findings will significantly stimulate the drug discovery of ligands focused on this appealing target to block coronavirus replication and transcription. The success in the development of compounds that interfere with N proteins of other CoVs, such as the recent discovery of stabilizers of the protein–protein interaction of MERS-CoV N protein,<sup>44</sup> reinforces the potential of the N protein as druggable target for SARS-CoV-2 infection. Remarkably, the N protein is highly immunogenic and is being considered as a potential vaccine target and for the development of COVID-19 diagnostic methods.<sup>45,46</sup>

**2.1.2. Spike (S) Glycoprotein.** The S glycoprotein is a structural transmembrane protein of about 1200–1400 amino acid residues per monomer located on the outer envelope of the virion. As has been observed for other viruses of the *Coronaviridae* family,<sup>47</sup> it mediates virus entry by contacting specific host-receptors located on the surface of the cell. Host–guest recognition is virus-specific, and the specificity and selectivity of this process determine both (i) virus tropism and (ii) pathogenesis.<sup>25,39</sup> In its functional form, S protein assembles as a homotrimer. Each of the three monomeric units are formed by two functional groups, the S1 subunit which is responsible for host recognition and the S2 subunit which guides host–guest membrane fusion.<sup>39</sup> The S1 domain is characterized by an N-terminal domain (NTD) and a C-terminal domain (CTD) (Figure 3). The former can recognize carbohydrate moieties and is considered a derivation of ancestral sugar-based recognition domains, while the latter, also known as receptor-binding domain (RBD) or S<sup>B</sup>, mediates host–guest interaction and promotes virus entry by recognizing protein receptors of the infected organism. In particular, the region directly involved in the recognition process is defined as the receptor-binding motif (RBM). A C-terminal, transmembrane domain in the S2 subunit connects the S glycoprotein to the virus membrane.

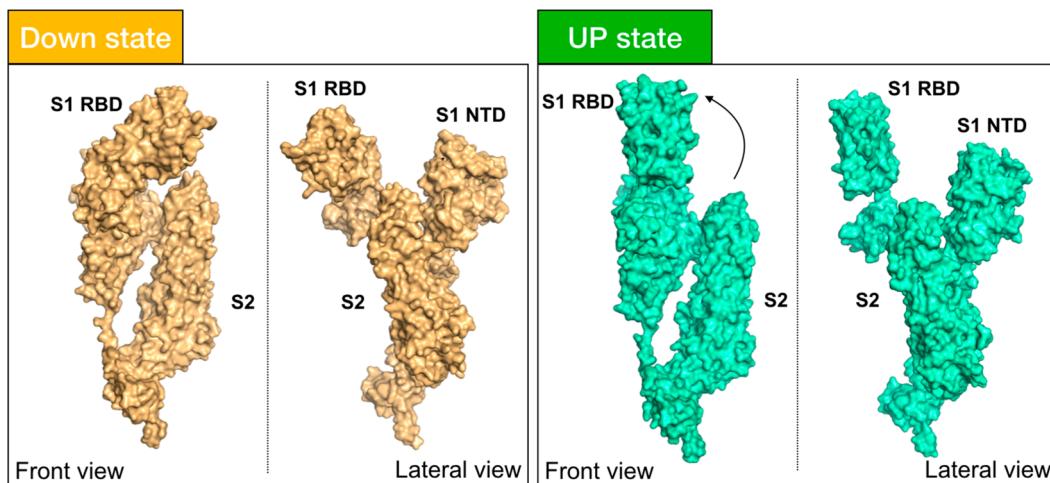
Several conformational states of S exist in a dynamic equilibrium between them: the uncleaved, S0; the cleaved, pre-fusogenic S1/S2; and the post-fusogenic forms. S protein is generally biosynthesized in an uncleaved, fusion-incompetent



**Figure 3.** Structural diagrams of spike glycoproteins of SARS-CoV-2. The spike protein contains an S1 subunit and an S2 subunit, which are divided by the S cleavage sites. Abbreviations: FP, fusion peptide; HR, heptad repeat 1 and heptad repeat 2, RBD, receptor-binding domain, containing the core binding motif in the external subdomain; SP, signal peptide.



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



**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.

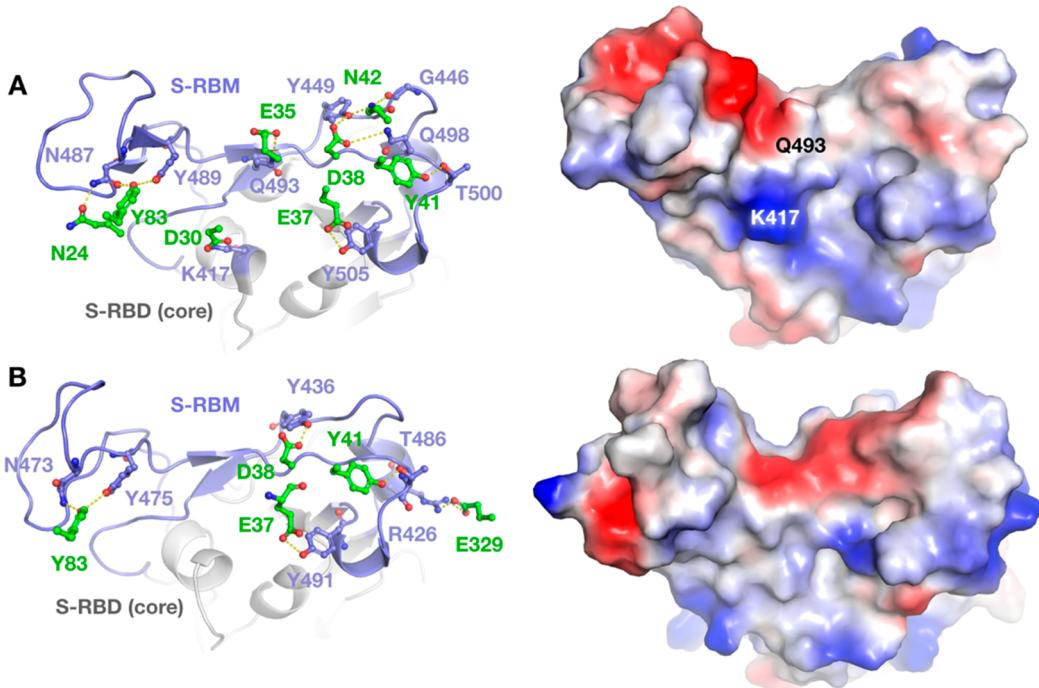
S<sub>0</sub> form, which is then cleaved and activated by proteases. S proteins of less pathogenic CoV are initially exposed uncleaved (S<sub>0</sub>) on the virus membrane, and their activation is later promoted by host proteases. Conversely, S proteins of the highly pathogenic SARS-CoV-2 are cleaved during protein egress and, thus, are exposed on the virion in its cleaved (S<sub>1</sub>/S<sub>2</sub>) form<sup>48</sup> (Figure 4).

In the S<sub>1</sub>/S<sub>2</sub> 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 S<sub>1</sub>. Significant conformational modifications are required for “down-to-up” conversion. A masking and 3D sorting strategy applied on SARS-CoV by Kirchdoerfer et al.<sup>49</sup> suggested that ACE2 does not directly participate in down-to-up conversion of S protein and RBD. It is more likely that ACE2 binds an S-RBD in an already “up” conformation.

So, according to these data, the S protein of SARS-CoV should sample 56% of the triple “down” and 44% of the single

“up” S1 RBD; no double or triple “up” S1 RBD conformations have been observed. Introduction of some stabilizing mutations at positions 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 proved 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.<sup>38</sup>

Priming of the S protein could occur in several ways, depending on the (guest) type of CoV and the pool of available (host) proteases involved in the event.<sup>39</sup> 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



**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.

correspondence of the (R/K)-(2X)n-(R/K) motif of S.<sup>50</sup> Given their undoubted role in virus entry by S priming, all these proteases could represent valuable targets for effective antiviral therapies based on protease inhibition or modulation and are presented in different sections of this Perspective.

Sequence analyses for this region within CoV of the same clade revealed a significant heterogeneity, posing the bases as the explanation for the higher pathogenicity of SARS-CoV-2. Accordingly, a major exposure of the furin-like (S1/S2) cleavage site and, thus, an efficient priming of S protein would derive from insertion of polybasic amino acids at the S1/S2 cleavage site for SARS-CoV-2.<sup>48</sup> Conversely, the S protein of SARS-CoV is inefficiently cleaved by furin at the S1/S2 cleavage site during biosynthesis due to the low exposure of the cleavage site. Unlike SARS-CoV-2, which is exposed in an already cleaved prefusion form, the uncleaved (S0) form seems to be predominant in the case of SARS-CoV, leading to a lower pathogenicity profile.

Conformational changes induced by the first cleavage are not sufficient to promote host–guest membrane fusion, which seems to be finally induced by a second cleavage event at the S2' site (Figure 4).<sup>39,51</sup> This event appears to be subordinated by recognition of attachment host receptors at the level of the S1 domain.

Coronaviruses use different regions of the S1 subdomain to bind the host cell. In SARS-CoVs, recognition is mediated by the RBD of S, which binds an N-terminal  $\alpha$ -helical region (peptidase domain) of the ACE2, which is accommodated in a concave region, named a receptor-binding motif (S-RBM, highlighted in violet in Figure 6), of the S-RBD.<sup>52</sup>

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.<sup>53</sup> In the case of SARS-CoV-2, a total of 18 residues of the RBD contacting 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. The main polar contacts are reported in Figure 6. Interestingly, two additional polar interactions, consisting on one hydrogen-bonding interaction between Q493 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 the S-RBM–ACE2 surface, as a clear consequence of the different electrostatic profiles envisaged for the two CoVs. These additional contacts are facilitated by the deeper insertion of the peptidase domain of ACE2,<sup>54</sup> as a consequence of rearrangement of the receptor-binding ridge (residues 482–485 of S-RBD). The slight reorientation of the peptide domain (PD) of ACE2 also allows a better stabilization of two other hotspots, Q493(S-RBM)–E35(ACE2) and G496(S-RBM)–K353, previously identified as critical in the stabilization of the complex.<sup>55</sup>

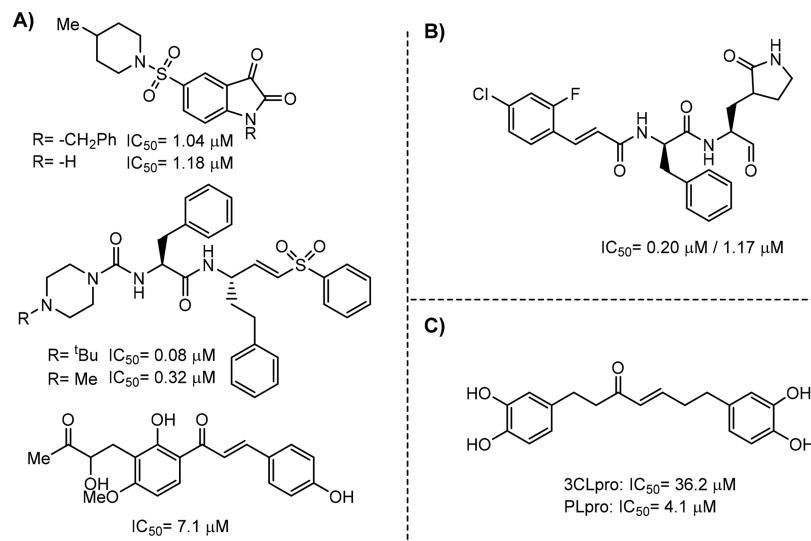
No polar contacts can be observed in the central region of the S-RBM–ACE2 interface for SARS-CoV, where K417 is replaced by a valine residue.

Taken together, these findings can shed some light on the reasons for the pandemic behavior of the new SARS-CoV-2. More factors need to be examined in the attempts to properly characterize the virus's pathogenicity. In this direction, a novel route for virus entry, involving the immunoglobulin-like host protein CD147, well known to “open the door” to *Plasmodium falciparum*, the etiological agent of malaria,<sup>56</sup> has been proposed by Wang et al.<sup>57</sup> Preliminary *in vitro* antiviral tests indicated that meplazumab, an anti-CD147 humanized antibody, could significantly inhibit virus entry. Formation of the CD147–S-RBD complex was then confirmed by co-immunoprecipitation and ELISA tests.

Another distinctive trait of obligate parasites such as SARS-CoVs is high-level post-translational modifications. Viral glycans have been proposed to exert a pivotal role contributing to virus functionality and immune selection, and thus to its pathobiology.<sup>58</sup> Viral O- and N-glycosylation, with this last



**Figure 7.** Compounds and peptides directed to S protein.



**Figure 8.** Representative CoV protease inhibitors: (A) SARS 3CLpro inhibitors, (B) SARS and MERS 3CLpro inhibitor, and (C) SARS dual 3CLpro and PLpro inhibitor.

generally occurring on asparagine included in the Asn-X-Ser/Thr sequences, extensively characterizes envelope glycoproteins of human immunodeficiency virus (HIV-1),<sup>59</sup> EBOV,<sup>60</sup> and hemagglutinin<sup>61</sup> in influenza virus (IFV). These modifications, originated from selective pressure induced by immune evasion, could contribute to regulate glycoprotein folding, assembly, and maturation. The extreme heterogeneity of the number and the distribution of these glycosylated sites makes it difficult to discern their exact role in the virus's life cycle. However, recent advancements on mass spectrometric and chromatographic techniques as well as the availability of recombinant glycosidases have allowed researchers to better study and analyze their impact on folding, structure, sorting, trafficking, and stability of glycoproteins. Glycosylation can help to stabilize protein folding by enhancing its solubility. Moreover, glycoproteins can also participate in intramolecular stabilizing interactions. Another interesting aspect observed for glycoproteins of coronaviruses is that N-glycosylation could help in shielding antigenic sites (as receptor-binding sites) to promote immune evasion.<sup>62</sup> This phenomenon, defined as "glycan shielding", could represent a critical factor during the design of vaccines. A direct comparison between S glycoprotein of SARS-CoV and SARS-CoV-2 revealed a different pattern of glycosylation,<sup>63</sup> which might contribute to the different profiles in trafficking and interaction with adhesion factors. Some of these differences in glycosylation sites correspond to antibody-accessible regions of the S protein, thus reinforcing the idea of their effect in hiding the vulnerable region of the protein.<sup>64</sup>

Taken together, these aspects highlight the relevance of a probably overlooked aspect—the glycosylation—during vaccine design.

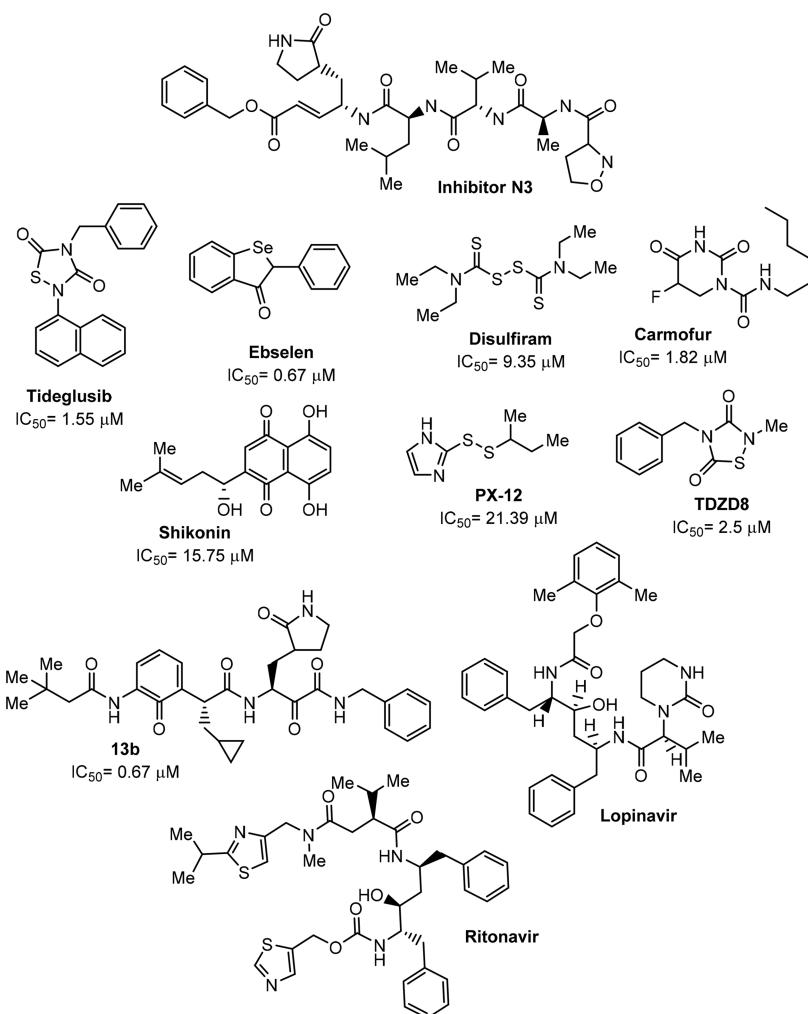
For viral-based therapeutic options, S protein and its RBD represent a very interesting and intriguing target for antiviral research. The more efficient recognition of the ACE2 host protein, together with the expanded host cell tropism given by the existence of a potential new entry route mediated by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

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

Systemic peptide mapping on SARS-CoV led to the discovery of the peptide CP-1, which binds with high affinity the heptad-repeat 2 region of S2 and interferes with the conformational changes leading to the 6-helix bundle formation, thus blocking the virus–cell fusion process (Figure 7).<sup>69</sup> Recently, a pan-coronavirus fusion inhibitor lipopeptide (EK1C4) has been designed, targeting a structure formed by two specific regions in the S2 subunit.<sup>70</sup>

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, like other coronaviruses, has 16 nsps that are highly conserved and present different functions, including the formation of the



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

RTC. The specific roles of most of the nsps have been reported, although the functions of some nsps remain elusive.<sup>6,29</sup> Focused on reported studies on 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),<sup>71</sup> 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),<sup>72</sup> the methyltransferase-stimulatory factor complex of nsp16 and nsp10 (PDB code: 6W61), the complex nsp10-nsp16 (PDB codes: 6W75 and 6W4H), the nsp9-binding protein (PDB code: 6W4B), and the nsp15 endoribonuclease (PDB code: 6VWW).<sup>73</sup>

Although any nsp could be exploited as a druggable target,<sup>36</sup> the availability of the crystal structure and described ligand, together with an essential role in viral infection, significantly increase the chances of success. On this premise, we will now focus on the two proteases (3CLpro and PLpro), the RNA-dependent RNA polymerase (RdRp), and helicase (nsp13).

**2.2.1. Proteases 3CLpro and PLpro.** SARS-CoV-2's genome—like most of the *Coronaviridae* genome—encodes two large polyproteins, pp1a and pp1ab.<sup>74</sup> These polyproteins are cleaved and transformed in mature nsps by the two proteases 3CLpro (3C-like protease or main protease) and PLpro (papain-like protease) encoded by the ORF 1a/b<sup>75</sup> as mentioned above. Both proteins are crucial for virus replication

and controlling the host cell response; therefore, they stand as key targets in the development of antiviral drugs.

3CLpro forms a dimer, and each monomer contains two regions, the N-terminal catalytic region and the C-terminal region.<sup>76</sup> The sequences of 3CLpro in SARS-CoV and SARS-CoV-2 share 96% identity, and the minimal differences between the two enzymes are at the surface of the proteins. Therefore, inhibitors against SARS-CoV 3CLpro are expected to inhibit SARS-CoV-2 3CLpro. Recently, the structure of 3CLpro (PDB code: 6Y2E)<sup>71</sup> has been resolved, confirming the high structural similarity between the two enzymes.

During recent years, there has been a large development of small molecules, peptides, and peptidomimetics that are able to inhibit SARS-CoV or both SARS-CoV and MERS-CoV 3CLPro, or even both proteases 3CLpro and PLpro.<sup>7,77,78</sup> In Figure 8, some of the most interesting compounds are classified by the proteases they inhibit.

As 3CLpro is a cysteine protease, therefore covalent inhibitors have been developed (Figure 8). It is worth mentioning a family of vinylsulfones that inhibit SARS-CoV in the nanomolar range. Indeed, Zhou et al.<sup>79</sup> published an *in vivo* study in which the combination of the vinylsulfone protease inhibitors and camostat was tested in mice infected with SARS-CoV. Survival of mice treated with the combination therapy significantly increased compared to the control group. Very recently, Yang's group has determined the crystal

structure of SARS-CoV-2 virus 3CLpro in complex with an irreversible peptidomimetic inhibitor N3 (PDB code: 6LU7).<sup>80</sup> In addition, they have performed a combination of structure-based virtual and high-throughput screenings of different chemical libraries including approved drugs, drug candidates in clinical trials, and other pharmacologically active compounds as inhibitors of 3CLpro. As a result, they have identified eight compounds with  $IC_{50}$  in a range from 0.67 to 21.4  $\mu\text{M}$  (Figure 9). Some of them, such as disulfiram and carmofur, are FDA-approved drugs, while ebselen, shikonin, tideglusib, and PX-12 are currently in clinical trials or preclinical studies. Finally, based on a family of  $\alpha$ -ketoamide inhibitors of MERS-CoV, Zhang et al. have designed and crystallized a new family of  $\alpha$ -ketoamide inhibitors of the SARS-CoV-2 3CLpro, among them compound 13b with an  $IC_{50}$  of 0.65  $\mu\text{M}$ <sup>71,81</sup> (Figure 9).

Moreover, there are two SARS-CoV 3CLpro inhibitors approved for the treatment of other viral infections, lopinavir and ritonavir, which are being used in patients in the currently pandemic.<sup>82</sup> 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.<sup>7</sup> SARS-CoV-2 PLpro shares 83% sequence identity with SARS-CoV PLpro. That is not as high as it was with 3CLpro; however, the differing residues are located on the surface. It is thus very likely that the SARS-CoV PLpro inhibitors could also be active against PLpro of SARS-CoV-2. Very recently, the structure of PLpro of SARS-CoV-2 has been deciphered (PDB code: 6W9C), but to date, no compound able to inhibit it has been reported.

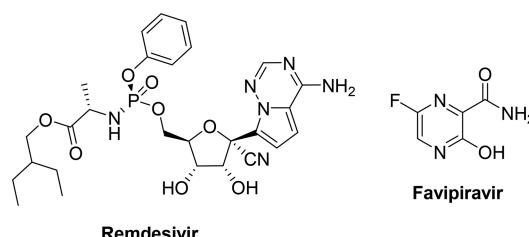
**2.2.2. RNA-Dependent RNA Polymerase (RdRp).** RdRp is a crucial enzyme in the coronavirus life cycle as well as in other RNA viruses. This enzyme is conserved in structure and function among viruses with RNA genomes belonging to different families. RdRp mediates the transcription and replication of the RNA genome during infection. The fact that this enzyme has no human counterpart, together with its essentiality for the virus's life cycle, improves its chances as a drug target for antiviral development,<sup>83</sup> as has been done in other viral infections.<sup>84</sup>

As mentioned above, the replication–transcription complex in coronavirus is formed after cleavage of the polyproteins pp1a and pp1ab into nsps. Moreover, SARS-CoV RdRp requires nsp7 and nsp8 as cofactors to stimulate its polymerase activity. Also, the association with exoribonuclease/N7-guanine cap methyltransferase nsp14 contributes to the formation of a macromolecular assembly for efficient nucleotide polymerization, proofreading, and cap-modifying. Combination of this multifunctional protein assembly with the helicase/RNA triphosphatase nsp13 and the 2'-O-methyltransferase nsp16 could coordinate the replication–transcription machinery of SARS-CoV.<sup>29,85</sup> All these facts are partially elucidated with the structural characterization of some complexes: nsp10/nsp16 complex,<sup>86</sup> nsp14/nsp10 complex,<sup>87</sup> and RdRp with its cofactors nsp7 and nsp8.<sup>88</sup>

The structure of SARS-CoV-2 RdRp in complex with cofactors nsp7 and nsp8 has been recently deposited in the Protein Data Bank (PDB code: 6M71).<sup>72</sup> This structure will significantly accelerate the structure-based drug design toward this essential target. Moreover, a 96% sequence identity between RdRp's from SARS-CoV and SARS-CoV-2<sup>6</sup> contributes to the translation of results with therapeutic agents from one virus to the other. Approved nucleoside analogues acting in other viruses have been successfully used against SARS-CoV

and are now being used in the new coronavirus infection.<sup>89</sup> Molecular modeling studies using the structure of RdRp have identified well-known drugs such as ribavirin, remdesivir, sofosbuvir, galidesvir, and tenofovir as inhibitors of RdRp and potential therapies for COVID-19.<sup>90</sup> Of interest is the reported lack of activity of non-nucleoside analogues in SARS-CoV due to the absence of a hydrophobic pocket in the polymerase to allocate this class of compounds present in other viruses, such as HIV-1 or hepatitis C virus (HCV).<sup>91</sup>

Remdesivir (GS-5734) (Figure 10), an adenosine analogue with broad antiviral spectrum in RNA viruses, has proven to be



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

efficacious against different CoVs *in vitro* and in a mouse model of SARS-CoV infection.<sup>92</sup> During the COVID-19 pandemic, it has been evaluated against a clinical isolate of the new virus,<sup>93</sup> showing a half-maximal effective concentration ( $EC_{50}$ ) of 0.77  $\mu\text{M}$  and a selectivity index of 129.87 (Vero E6 cells). Moreover, the crystal structure of this drug with RdRp has been recently elucidated.<sup>94</sup> Furthermore, several clinical trials around the world have been approved to demonstrate its therapeutic potential in patients.<sup>95</sup> During the preparation of this manuscript, results from the first trial in China were published, and remdesivir was not associated with statistically significant clinical benefits for COVID-19 patients.<sup>96</sup> However, the FDA authorized the use of this intravenous antiviral drug for emergency treatment of COVID-19 patients. Remdesivir can be administered only to hospitalized patients with severe illness, defined as patients with low oxygen in blood or needing breathing assistance.<sup>97</sup>

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

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

**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 triphosphate (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 nidoviruses, it is considered an interesting target for drug development, and several chemical inhibitors have been reported.<sup>103</sup>

Bananins are oligo-oxa-adamantanes that, after conjugation with vitamin 6 (pyridoxal), show antiviral activities (Figure 11). Four derivatives, bananin, iodobananin, vanillinbananin,

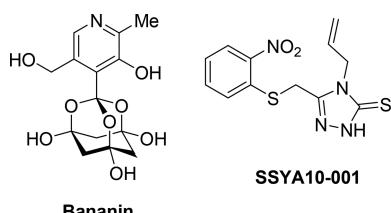


Figure 11. Representative SARS-CoV helicase inhibitors.

and eubananin, inhibited both the ATPase and the helicase activity of the *nsp13* from SARS-CoV. Remarkably, these compounds also inhibited the replication of the virus through a process that occurs after the viral entry.<sup>104</sup> Another family of antivirals acting through the target enzyme are the 5-hydroxychromone derivatives,<sup>105</sup> which were synthesized as bioisosteres of the previous reported aryl diketoacids.<sup>106</sup>

A screen using a FRET-based helicase assay of the Maybridge Hitfinder chemical library allowed the identification

of triazole SSYA10-001 (Figure 11), which specifically blocks the helicase activity of *nsp13* and also shows anti-SARS-CoV activity. Kinetic studies to determine the enzyme inhibition mechanism showed that SSYA10-001 acts as a non-competitive inhibitor of *nsp13* with respect to nucleic acid and ATP substrates.<sup>107</sup> Further studies with this 1,2,4-triazole showed that it is also able to inhibit two other CoVs (MERS-CoV and mouse hepatitis virus). A putative binding pocket was identified corresponding to a conserved pocket in CoVs *nsp13*, where Y277 and K508 are the key residues to maintain the interaction.<sup>108</sup>

At the time of writing of this manuscript, the structure of *nsp13* full-length (PDB code: 6JYT) SARS-CoV is available.<sup>109</sup> So far, no structural information is available on the SARS-CoV-2 helicase, but due to the high sequence similarity (99.8%), the SARS-CoV structure available significantly encourages the search for drugs to treat COVID-19 acting through this target enzyme.

### 3. HOST-BASED DRUGGABLE TARGETS

Coronavirus entry is mainly achieved by the virus binding to the ACE2 host receptor in the cell surface in a receptor-mediated endocytosis pathway. Other molecules such as proteases activate the spike (S) protein and facilitate the fusion with the receptor and cell membrane, allowing entry into the host of viral RNA via an endocytic pathway.

Host-targeted anti-SARS-CoV-2 agents presented here are based on the molecular study of virus entry, identifying key proteins involved in the process. Targeting host proteins may avoid different limitations frequent in antiviral research, probably offering a genetic barrier to viral resistance.

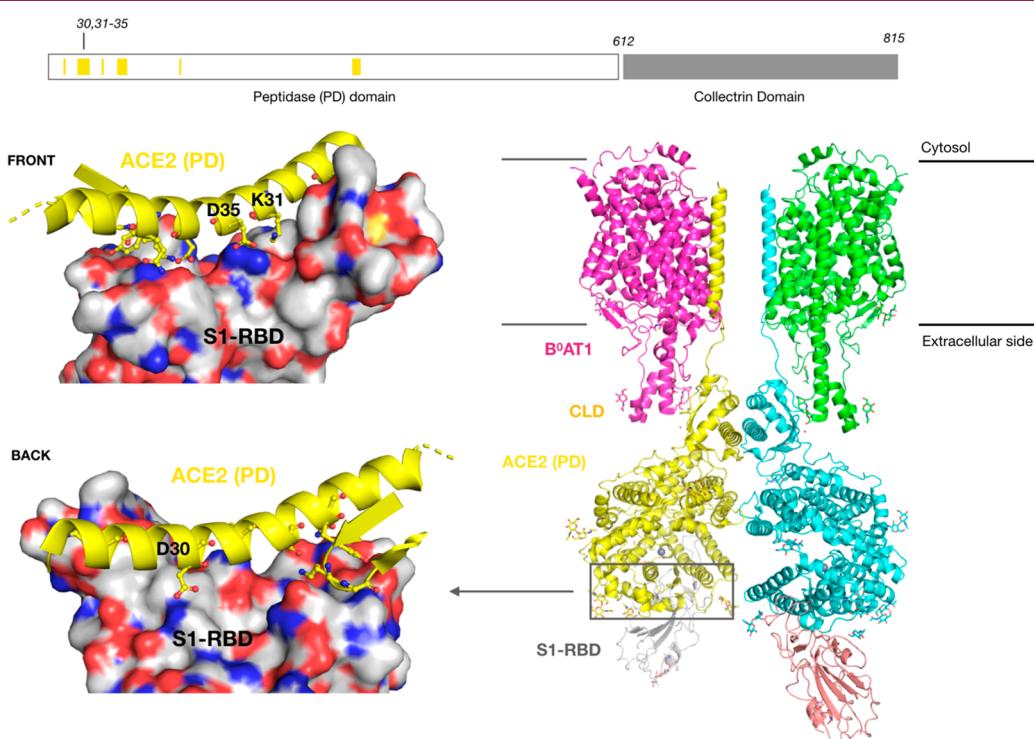


Figure 12. (Right) Cryo-EM structure (PDB code: 6M17) of full-length human ACE2 with the transporter (B<sup>0</sup>AT1) and the receptor-binding domain of the spike glycoprotein (S1-RBD). (Left) S1-RBD–ACE2 interaction interface for SARS-CoV-2 (PDB code: 6M0J), front and back views. Relevant ACE2 residues involved in direct polar interaction with S1-RBD (in particular, D30, K31, and D35) are represented in yellow sticks.

### 3.1. Angiotensin I Converting Enzyme 2 (ACE2)

**Receptor.** Coronavirus entry is mainly produced by the virus binding to different host receptors in the cell surface. In SARS-CoV-2, ACE2 has been recently confirmed as the main virus receptor.<sup>9</sup> Therefore, inhibition or modulation of ACE2 represents one of the proposed host-based strategies for treatment of SARS-CoV-2.<sup>110</sup>

Activity of ACE2 is related to the renin–angiotensin system (RAS), which is involved in the maintenance of blood pressure homeostasis, fluid, and salt balance in mammals.<sup>111</sup> While renin cleaves angiotensinogen to generate angiotensin (Ang) I, the angiotensin-converting enzyme (ACE) catalyzes the formation of Ang II, a critical signaling molecule, through the proteolytic cleavage of Ang I. Although not exclusive,<sup>112</sup> the activity of ACE has been considered to be of pivotal importance in the regulation of Ang II within the RAS.

ACE2, a homologue of ACE, is a multifunctional zinc metalloprotease consisting of 805 amino acids, which can be functionally divided into (i) the amino-terminal catalytic domain and (ii) the carboxy-terminal domain.<sup>110</sup> The enzyme has been observed to negatively regulate RAS through the degradation of Ang II to the heptapeptide Ang 1–7.<sup>112</sup>

ACE2 can be found in epithelial cells of lung, liver, and testis.<sup>110</sup> Unlike ACE, ACE2 populates the apical membrane of respiratory epithelial cells, by which infection can occur. ACE2 receptors have been also observed in nasal and mouth epithelial cells.<sup>113</sup> Interestingly, an enhanced expression of ACE2 receptors in the lungs has been correlated with age, thus partially explaining the higher viral load and severity of symptoms observed in older patients infected by SARS-CoV-2.<sup>114,115</sup>

The cryogenic electron microscopy (cryo-EM) structure of the full-length human ACE2 with the transporter BOAT1, with (PDB code: 6M17) or without (PDB code: 6M18) the RBD of the surface S glycoprotein of SARS-CoV-2, has been recently published.<sup>52</sup> The complex appears as a dimer of heterodimers with the collectrin-like domain (CLD) located among BOAT1 and the peptidase extracellular domain (PD) of ACE2 (Figure 12). CLD consists of a small extra-cellular domain, a long linker, and a single transmembrane (TM) helix; the central region (the neck domain) between the PD and the TM helix constitutes the most relevant part of the dimerization surface. Here, coordination between the two dimers is mediated by extensive polar contacts such as R716'-D713, E639'-R710, N636'-Q653, and N638'-R652. A weaker interaction surface can be also observed between the two N-terminal PDs of ACE2, where Q175' directly interacts with Q139. It was suggested that ACE2 is natively dimeric and thus able to bind two trimeric S proteins.<sup>52</sup>

ACE2 was already known for mediating infection of the less pathogenic SARS-CoV,<sup>116</sup> in particular, by recognizing the receptor-binding domain of the S protein (S1-RBD) with an  $\alpha$ -helical region located in the peptidase domain.

D30, K31, and D35 have been identified as critical residues in the interaction with S1-RBD.<sup>110</sup> Their specific localization with respect to the S-RBD is shown in Figure 12. More details about the S-RBD–ACE2 interaction pattern are given in the Spike (S) Glycoprotein section.

Therapeutic strategies devoted to combat SARS-CoV-2 infection via ACE2 could involve the following:

- Interfering with the dynamics of the virus–host ACE2–S-RBD interface. This can be achieved by using relatively

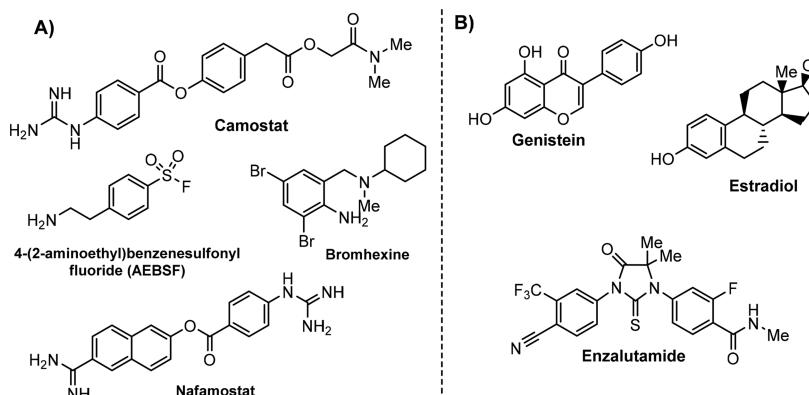
small molecules able to disrupt or negatively affect the efficiency in the dynamic network of protein–protein interaction that guides viral entry. In this regard, 77 molecular candidates have been identified from the FDA database through an *in silico*-guided repurposing study, which combines replica exchange molecular dynamics and ensemble docking, although no experimental decrease of virus infection has been reported.<sup>117</sup> Furthermore, machine learning and ensemble docking simulations have also provided new different scaffolds able to interrupt the spike protein–ACE2 interaction.<sup>118</sup>

- Inhibiting the ACE receptor. Since myocardial injuries caused by SARS-CoV-2 might be related to ACE2, therapies based on the use of ACE inhibitors or angiotensin blockers might be used for organ-protection purposes. In that sense, telmisartan has been proposed as a real option for COVID-19 therapy,<sup>119</sup> and a clinical trial has recently started (NCT04355936). However, the real impact of ACE inhibitors on COVID-19 still remains controversial.<sup>120,121</sup>
- Delivery of soluble ACE2. It has been demonstrated that SARS-CoV-2 downregulates<sup>111</sup> expression of ACE2. Accordingly, it has been proposed that the delivery of soluble ACE2 could exert a beneficial effect by competing with the host ACE2 for binding with S glycoprotein. Soluble recombinant ACE2 or APN01 imitates the human enzyme ACE2, which is used by the virus to enter cells.<sup>110</sup> The virus binds to soluble ACE2/APN01, instead of ACE2 on the cell surface, which means that the virus can no longer infect the cells. At the same time, APN01 reduces harmful inflammatory reactions in the lungs and protects against lung injury due to acute respiratory distress syndrome (ARDS). In this regard, a recombinant human ACE2 (rhACE2; APN01, GSK2586881) has been tested on a small cohort of patients with ARDS, demonstrating encouraging positive results.<sup>122,123</sup> In a very recent paper, researchers tested the potential of direct treatment with soluble human ACE2 to prevent the entrance of the virus to the host cells in engineered human kidney organoids.<sup>124</sup>

In combination with other host-based or virus-based approaches, these strategies offer a vast reservoir of opportunities to combat SARS-CoV-2. Careful examination of their real therapeutic impact and a proper validation are needed to fully address their clinical validity.

### 3.2. Transmembrane Serine Protease 2 (TMPRSS2).

Following receptor interaction, or in addition to it, different host proteases can activate the virus–host cell membrane fusion for subsequent genome delivery. The host cell surface transmembrane serine protease 2 (TMPRSS2) activates S protein present in the highly pathogenic human coronaviruses SARS-CoV and MERS-CoV.<sup>125</sup> Human TMPRSS2 is expressed in the epithelia of the gastrointestinal, urogenital, and respiratory tracts.<sup>126</sup> Cleavage of S protein by TMPRSS2 is preferred for coronavirus infection over other proteases, such as the endosomal cathepsins.<sup>127</sup> Recent research has confirmed that SARS-CoV-2 entry is facilitated by TMPRSS2 and the viral infection is decreased by the use of the protease inhibitor camostat.<sup>9</sup> Moreover, as viral infection is enhanced by TMPRSS2, the Vero E6 cell line overexpressing TMPRSS2 has been described as a useful pharmacological tool for SARS-



**Figure 13.** TMPRSS2 modulators: (A) chemical structure of TMPRSS2 inhibitors and (B) some transcriptional inhibitors of TMPRSS2.

CoV-2 research.<sup>50</sup> Last, but not least, TMPRSS2 is expressed in different cell types of lung tissue, increasing their vulnerability for SARS-CoV-2 infection.<sup>128</sup>

TMPRSS2 has emerged as a useful drug target for antiviral drug discovery,<sup>8</sup> and the lack of influenza and coronavirus infection has been confirmed in TMPRSS2 knock-out mice.<sup>125</sup> COVID-19 may find a potential therapy among different repurposed drugs with inhibitory activity against TMPRSS2. At the moment, only camostat has shown *in vitro* activity against SARS-CoV-2, but other clinical drugs such as nafamostat and 4-(2-aminoethyl)benzenesulfonyl fluoride, all of them protease inhibitors,<sup>129</sup> may offer some therapeutic options for the pandemic (Figure 13A). Repurposing of the mucolytic agent called bromhexine, a TMPRSS2 inhibitor, has been also proposed for COVID-19 therapy.<sup>130</sup> Furthermore, transcriptional inhibition of TMPRSS2 has been proposed as a new therapeutic option. Using computational and experimental methods, estrogen and androgen-related compounds such as genistein, estradiol, and enzalutamide (Figure 13B) have been shown to reduce TMPRSS2 expression in different cell lines.<sup>131</sup> As TMPRSS2 expression in the human lungs 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 target for therapeutic clinical intervention for symptom amelioration in COVID-19 patients.<sup>132</sup>

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

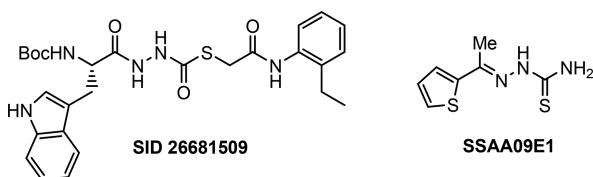
**3.3. Furin.** Recent studies have discovered a “furin-like cleavage site” (FCS) in the S protein of SARS-CoV-2 that may explain the high pathogenicity of the virus.<sup>39,48</sup> Moreover, this highly cleavable site is not found in closely related CoVs.<sup>133</sup> Furin, a type 1 membrane-bound 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 sites in other CoVs, such as the infectious bronchitis virus, increased the pathogenicity, including neural symptoms in infected chickens.<sup>134</sup> As furin is highly expressed in lungs, it is very likely to be involved in SARS-CoV-2 infection, increasing its pathogenicity over other betacoronaviruses, as they lack this cleavage site.<sup>51</sup> Recently, it has been proposed that the FCS may be an important site of coronavirus evolution. In samples isolated from mild COVID-19 patients from Zhejiang Province, China, mutations appeared near FCS (F1–2) that

affected the electrostatic distribution of the S protein surface and its structure, and thus its ability to bind to furin.<sup>135</sup> Experimental results in samples from those patients showed that furin had low protein expression levels in the lungs compared with other tissues, such as colon, glands, liver, and kidney. The FCS may contribute to SARS-CoV-2 infection of these organs. Recent reviews describe the close interaction between ACE2 and furin in the viral infection of SARS-CoV-2,<sup>136</sup> showing a potential relationship between furin activities of different populations and the different clinical scenarios of the SARS-CoV-2 infection.<sup>137</sup>

Inhibition of furin with peptides and, more recently, with small molecules is a strategy pursued to arrest tumor growth,<sup>138</sup> inflammation, and some viral and bacterial infections.<sup>139</sup> However, due to the pleiotropic role of furin-like enzymes in a large number of cellular processes, side effects are a concern.<sup>139</sup> Determination of the crystal structure of furin will aid the design of specific small molecules.<sup>140,141</sup> For example, furin’s unliganded form (PDB code: 5JXG) suggests activation by a substrate-induced mechanism,<sup>142</sup> while the recent complexes with substrate analogue inhibitors (PDB codes: 6EQV, 6EQW, 6EQX)<sup>143</sup> decipher some new pockets to be exploited by a next generation of furin inhibitors that may be a potential therapy for COVID-19.

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

Cathepsin L inhibitors may be therapeutic options for COVID-19 also because they can prevent the progression of pulmonary fibrosis.<sup>144</sup> Furthermore, a synergistic effect may be also achieved by targeting simultaneously cathepsin L and TMPRSS2.<sup>145</sup> 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.<sup>146</sup> Several crystallographic structures of human cathepsin L are available both in the apo form (PDB code:



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

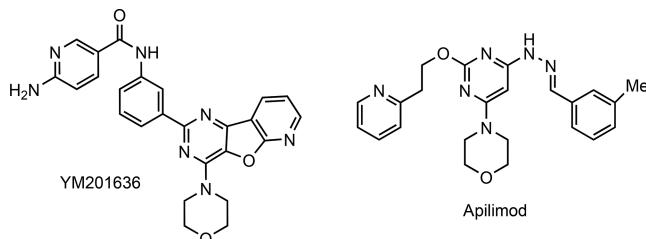
4AXL)<sup>147</sup> and in complex with different nitrile molecules (PDB codes: 2YJC, 2YJB, 2YJ9, 2YJ8, 2YJ2)<sup>148</sup> or (PDB codes: 2XUS, 2XU4, 2XU3, 2XU1).<sup>149</sup>

**3.5. Adaptor-Associated Kinase 1 (AAK1) and Cyclin G-Associated Kinase (GAK).** As already explained, the main entry pathway for SARS-CoVs is receptor-mediated endocytosis. AAK1 and 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.<sup>150,151</sup> AAK1 plays a key role in receptor-mediated endocytosis by specific phosphorylation of adaptor protein 2, which stimulates the binding to cargo proteins. GAK shares some biological functions with AAK1, being a key player in clathrin-mediated trafficking. GAK mediates the binding of clathrin to the plasma membrane and the trans-Golgi network.<sup>152</sup>

Although several molecules have been synthesized to inhibit AAK1, none of these compounds have been optimized and developed as antiviral agents.<sup>153</sup> A rationale for repurposing a combination of the pan-kinase inhibitors sunitinib and erlotinib (Figure 15) based on AAK1 and GAK inhibition has been proposed for treatment of dengue and Ebola patients in future outbreaks. However, these have not been included in current clinical trials until now.<sup>154</sup> Recently, baricitinib, a potent AAK1 and GAK inhibitor, has been proposed as an effective therapy for COVID-19, reducing the viral entry, although no experimental work has been done to prove its mechanism of action.<sup>155</sup> Moreover, as this compound targets also the janus kinase (JNK1/2), it may act also to reduce inflammation in these patients, increasing its therapeutic benefit for COVID-19.<sup>156</sup> However, some data coming from the clinical trial program used for baricitinib registration in Europe showed that the most significant side effect seen was a small increase in upper respiratory tract infections, which may be not well tolerated by COVID-19 patients.<sup>157</sup>

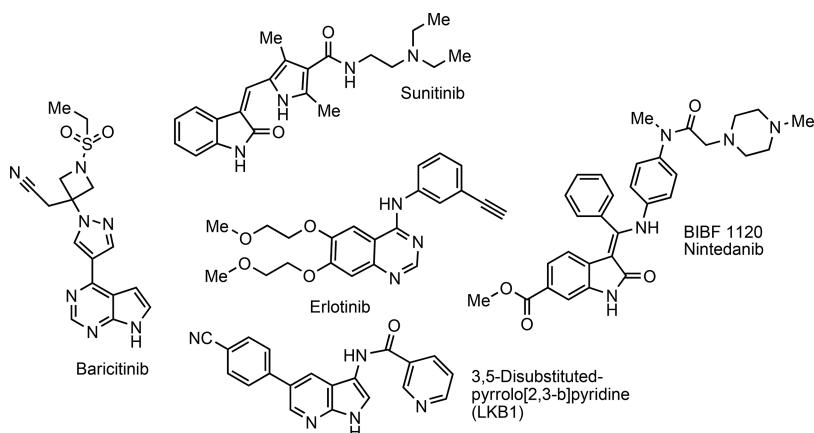
Several crystal structures of AAK1 have been reported in complex with different inhibitors, such as the pan-kinase inhibitor K252a (PDB code: 4WSQ),<sup>158</sup> BIBF 1120 (nintedanib) (PDB code: 5TE0), and LKB1 (PDB code: 5L4Q),<sup>153</sup> offering useful tools for rational drug discovery and/or optimization. The GAK 3D structure presents many different conformations when it is bound to nanobodies (PDB codes: 4C57, 4C58, 4C59)<sup>152</sup> or to gefitinib (PDB codes: 5Y7Z, 5Y80).<sup>159</sup> Recently, it has been discovered that AAK1 and GAK share cysteine residues (C193 and C190, respectively) at equivalent positions that may be targeted by covalent inhibition, offering a good opportunity to develop selective covalent inhibitors.<sup>160</sup> Both structures suggest possibilities for the development of selective AAK1 and GAK inhibitors for viral infections. Nowadays, repurposing marketed drugs or optimizing valuable hits such as 3,5-disubstituted pyrrolo[2,3-*b*]pyridines as potent AAK1 and GAK inhibitors could be a good strategy to prevent SARS-CoV-2 entry.

**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).<sup>161</sup> This phospholipid is synthesized in the late endosome by PIKfyve, an enzyme with lipid and protein kinase activity.<sup>162</sup> PIKfyve plays a key role in several trafficking events associated with the endocytic pathway.<sup>163</sup> 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



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

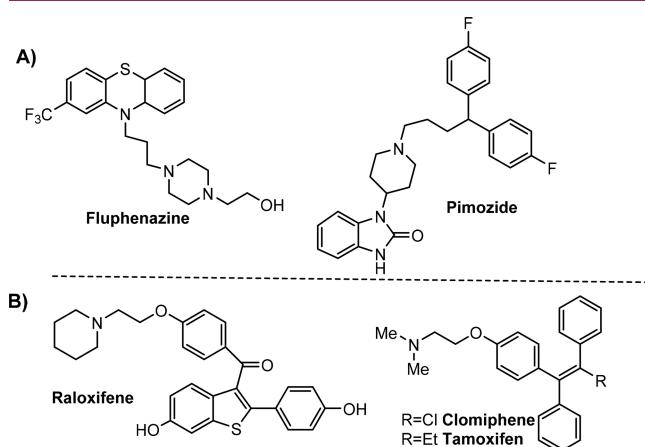
PIKfyve,<sup>25</sup> which was previously identified as an inhibitor of production of interleukins IL-12 and IL-23.<sup>164</sup> The same effect was also observed with YM201636 (Figure 16), another chemically diverse PIKfyve inhibitor.<sup>165</sup> Like apilimod,



**Figure 15.** AAK1 and GAK inhibitors with therapeutic potential for COVID-19.

YM201636 also blocks viral entry and infection by other viruses like African swine fever virus or EBOV.<sup>26,27</sup> These data suggest that PIKfyve is a suitable drug target to modulate infection by viruses that enter through endocytosis, including SARS-CoV-2.<sup>166</sup> At the moment, the 3D structure of this lipid kinase has not been determined yet.

**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.<sup>167</sup> They are voltage-gated channels, and TPC2 is one of the major downstream effectors of PI(3,5)P<sub>2</sub> opening after its binding with such a phosphoinositide.<sup>168</sup> These are involved in the regulation of endolysosomal trafficking and Ebola entry in the host cell.<sup>169</sup> The structure of human TPC2 has been reported using cryo-EM, providing useful structural information about the open, closed, and apo forms for TPC2 (PDB codes: 6NQ0, 6NQ2, and 6NQ1, respectively).<sup>170</sup> By virtual screening, dopamine receptor antagonists and selective estrogen receptor modulators (SERMs) have been recently identified as blockers of TPC2. Specifically, the dopamine antagonists fluphenazine and pimozide, together with the SERMs raloxifene, clomiphene, and tamoxifen, inhibit EBOV infection in experimental models (Figure 17).<sup>171</sup>



**Figure 17.** Clinically used drugs to block TPC2: (A) dopamine antagonists and (B) SERMs.

It has been recently shown that TPC2 plays a relevant role during SARS-CoV-2 infection, and a decrease of SARS-CoV-2 pseudovirus entry was demonstrated after treatment with tetrandrine, a potent calcium blocker used as a pharmacological tool. Altogether, TPC2 emerges as a druggable host-target for SARS-CoV-2 infection, and repurposing of dopamine antagonist such fluphenazine or SERMs as raloxifene merits being tested in clinical trials for COVID-19.

#### 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.<sup>172</sup> For RNA viruses such as coronavirus, viral genomic RNA or replication

intermediates like double-stranded RNA (dsRNA) are recognized either by the endosomal RNA receptors TLR3 and TLR7 or by the cytosolic RNA sensor RIG-I/MDAs, among others. This recognition induces the activation of several signaling 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-κB), and AP-1. Consequently, these factors ultimately promote the production of type I interferons (IFN-I), inflammatory cytokines, and chemokines.<sup>173</sup>

#### 4.1. Viral Proteins Involved in Innate Immune Modulation.

The immune response can be exaggerated, leading to an inordinate inflammatory reaction that causes severe clinical signs of COVID-19. These include pneumonia and lung inflammation, respiratory distress, and a life-threatening cytokine storm and circulatory shock.<sup>174</sup> Other human CoVs have developed strategies to counteract the immune response, which still need to be deeply studied in SARS-CoV-2.<sup>175</sup> SARS-CoV-2 typically reduces IFN-I response and elevates the expression of IL-6 and IL1RA cytokines.<sup>176</sup> SARS-CoV and MERS-CoV infections also suppress IFN synthesis,<sup>177,178</sup> which determines their virulence.<sup>179</sup> In this Perspective, we sum up the main CoVs proteins implicated in innate immune response.

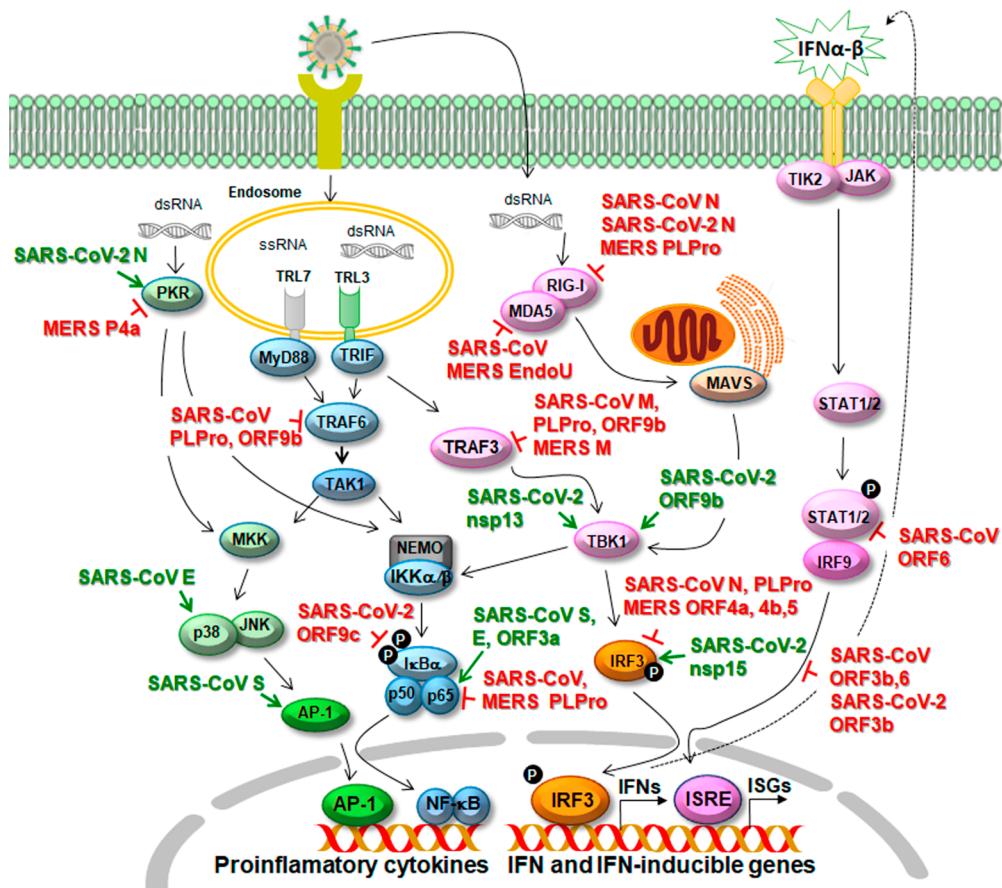
**4.1.1. Structural Proteins' Modulation of Immune Response.** The SARS-CoV and MERS-CoV M protein binds TRAF3 to block its binding and subsequent nuclear translocation of TANK-binding kinase 1 (TBK1), which blocks IRF3-mediated signaling and inhibits type I IFN production.<sup>180,181</sup>

SARS-CoV N protein also interferes with the function of IRF3.<sup>182</sup> The N protein of SARS-CoV targets type I IFN synthesis at early recognition stages of innate immune signaling viral RNA infection.<sup>183</sup> 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.<sup>184</sup>

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

SARS-CoV-2 viral protein interactions and their role in acting against the immune system response are still unknown. However, the first protein–protein interaction studies have elucidated that N protein could target stress granule protein G3BP1, an essential antiviral protein which is known to induce innate immune response.<sup>187</sup> 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 a protein kinase R (PKR)-mediated pathway, which induces pro-inflammatory cytokines<sup>188</sup> (Figure 18).

**4.1.2. Non-structural Proteins' Regulation of the Immune Response.** Nsp and accessory proteins of HCoVs also have important roles in the modulation of innate immunity. SARS-CoV nsp1 affects IFN-dependent signaling,<sup>189</sup> and SARS-CoV PLpro domain (nsp3) protein inhibits IRF3 phosphorylation and nuclear translocation, thereby blocking type I IFN production.<sup>190</sup>



**Figure 18.** Schematic representation of the main pathways of the innate immune response to HCoV. These pathways lead to the activation of nuclear factor kappa B (NF- $\kappa$ B), AP-1, and interferon (IFN), resulting in the secretion of pro-inflammatory cytokines and interferons and the activation of a cellular immune response. Like 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) toward 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.

Apart from its protease activity, SARS-CoV and MERS-CoV PLpro also has de-ubiquitinating activity.<sup>191,192</sup> PLpro of SARS-CoV inhibits TLR7 signaling by removing K63-linked ubiquitin chains from TRAF3 and TRAF6.<sup>193</sup> A subsequent analysis showed that PLpro of both SARS-CoV and MERS-CoV also recognized another ubiquitin-like modifier, interferon-stimulated gene 15 (ISG15). In those cases, PLpro acts as a de-ISGylating enzyme. This domain downregulates mRNA levels of pro-inflammatory cytokines such as CCL5, IFN- $\beta$ , and CXCL10.<sup>194</sup> Furthermore, it has been identified that the ADP-ribose-1-monophosphatase macrodomain encoded within nsp3 in HCoV-229E and SARS-CoV is responsible for suppressing IFN induction.<sup>195</sup>

The SARS-unique domain encoded in nsp3 of SARS-CoV can also enhance a cellular E3 ubiquitin ligase called ring-finger and RCHY1, which leads to proteasomal degradation of p53.<sup>196</sup>

Endonuclease U (EndoU) domain is encoded in nsp15, and it is an important component of CoVs RTC. EndoU activity prevents dsDNA recognition by PPRs such as MAD5, thereby evading early innate immune response.<sup>197</sup> CoVs also encode ribonucleases that counteract dsRNA antiviral response. This is the case of the exoribonuclease domain of nsp14, which modulates dsRNA levels and innate immune sensing.<sup>198</sup>

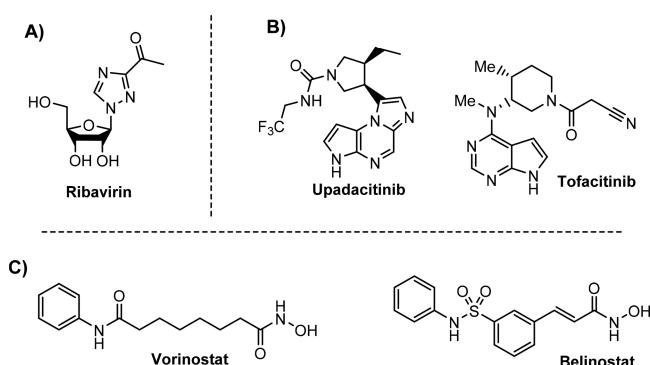
Despite being dispensable in viral replication, HCoV accessory proteins get involved in processes such as cell proliferation, apoptosis, and interferon signaling.<sup>199</sup> SARS-CoV, ORF3b, and ORF6 are shown to interfere with IFN- $\beta$  synthesis and IFN signaling by preventing IFN- $\beta$ -induced activation of interferon-stimulated response element (ISRE), found in the promoter region of ISG.<sup>182</sup> SARS-CoV ORF3a induces TRAF3-mediated ubiquitination of apoptosis-associated speck-like protein containing a caspase recruitment domain, which activates NLRP3 inflammasome and NF- $\kappa$ B pathway.<sup>200</sup> The SARS-CoV ORF6 protein was shown to interact with nuclear pores and blocks p-STAT1 import into the nucleus, reducing innate immune responses and increasing pathogenesis.<sup>201</sup> The accessory proteins of MERS-CoV, ORF4a, ORF4b, and ORF5, could similarly suppress IRF3 nuclear translocation,<sup>181</sup> while P4a suppresses PKR-dependent stress response, an additional antiviral response.<sup>202</sup> Moreover, SARS-CoV ORF9b might also hijack a ubiquitin E3 ligase called AIP4 to trigger the degradation of MAVS, TRAF3, and TRAF6, thereby significantly suppressing IFN responses.<sup>203</sup> Indeed, SARS-CoV accessory protein P6 interacts with the IFN-signaling pathway-mediating protein Nmi and promotes its ubiquitin-dependent proteasomal degradation, thereby

potentially modulating the virus-induced innate immune response.<sup>204</sup>

SARS-CoV-2 protein–protein interaction maps have shown so far that nsp5 (3CLpro) could interact with the epigenetic regulator histone deacetylase 2 (HDAC2).<sup>188</sup> A previous study demonstrated that HDAC2 mediates inflammation and interferon response.<sup>205</sup> The same study showed that nsp13 interacts with two elements of the IFN signaling pathway, TBK1 and TBK1-binding protein 1 (TBKBP1), modulating the NF- $\kappa$ B inflammatory response. E3 ubiquitin ligase RNF41/Nrdp1 is targeted by nsp15 protein, which would increase type I interferon production.<sup>206</sup> Two other E3 ubiquitin ligases, TRIM59 and MIB1, regulate antiviral innate immune signaling and are hijacked by ORF3a and nsp9.<sup>207</sup> ORF9c protein was found to modulate I $\kappa$ B kinase and NF- $\kappa$ B signaling pathway, and ORF9b interacts with a mitochondrial import receptor, Tom70, a linker between MAVS and TBK1/IRF3, inducing IRF-3 activation<sup>188</sup> (Figure 18). Nsp3, ORF3b, and ORF6 of SARS-CoV-2—with less homology with SARS-CoV proteins—could induce IFN-I sensitivity.<sup>208</sup> However, it has been described that truncated ORF3b of SARS-CoV-2 acts as an IFN antagonist more efficiently than does that of SARS-CoV.<sup>209</sup> These studies at the cellular level await further clinical or animal model investigations before the role of the immune response in this disease can be fully understood.

**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.<sup>210</sup> Clinical findings showed exuberant inflammatory responses during SARS-CoV-2 infection, further resulting in uncontrolled pulmonary inflammation, likely leading to fatality. The repurposing of host-based therapeutics to control the immune response may counterattack COVID-19.<sup>211</sup>

Some of these treatments include the use of recombinant IFN- $\alpha$  and IFN- $\beta$  as antiviral cytokines that inhibit viral replication in targeted cells. Some studies have reported that IFN- $\beta$  alone has more effect against SARS-CoV-2 than IFN- $\alpha$  *in vitro*. In fact, combinations of IFN- $\alpha$  and IFN- $\beta$  with other antivirals such as ribavirin (Figure 19) and/or lopinavir/ritonavir (HIV treatment) (Figure 9) have a synergistic effect *in vitro*<sup>212,213</sup> and in animal models.<sup>214,215</sup> Recent results from an open-label, randomized, phase 2 trial (NCT04276688) conducted in China proved that the triple therapy (IFN/ $\beta$ -lopinavir/ritonavir) is safe and more effective than lopinavir/



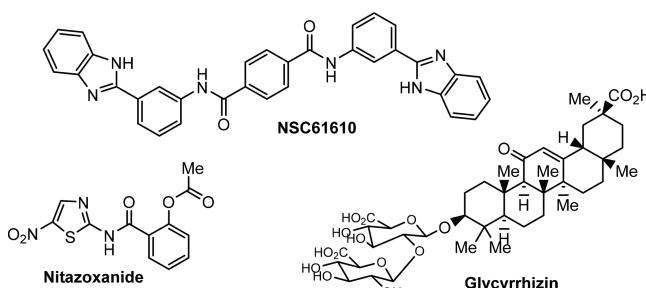
**Figure 19.** Potential immunosuppression treatments: (A) antiviral used in combination with IFN $\alpha$  and IFN $\beta$ , (B) JAK inhibitors, and (C) HDAC inhibitors.

ritonavir alone in alleviating symptoms and shortening the duration of viral shedding in mild to moderate COVID-19 cases.<sup>216</sup> *In vitro* and *in vivo* studies show the protective effect of Type III IFN (IFN- $\gamma$ ) treatment against SARS-CoV infection<sup>217,218</sup> 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.<sup>219,220</sup> Another immunosuppressor, tocilizumab, a humanized monoclonal antibody against the interleukin IL-6 receptor, reduces pro-inflammatory response in COVID-19 patients.<sup>221</sup> 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),<sup>222</sup> blinatumomab,<sup>223</sup> and HDAC inhibitors, such as vorinostat or belinostat (Figure 19B,C).<sup>224</sup> Baricitinib (Figure 15), as mentioned before, is also a potent inhibitor of AAK1 and may also lead to a decrease in viral infectivity, making it a good candidate for clinical trials of COVID-19.<sup>155</sup>

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

Nitazoxanide is another potent type I IFN inducer that has been used in humans as an antiparasitic agent (Figure 20). It is



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

a synthetic nitrothiazolyl–salicylamide derivative that exhibits broad-spectrum antiviral activities against both RNA and DNA viruses.<sup>226</sup> It is effective in the treatment of HCV and IFV-A and -B infection, currently in Phase II and Phase III clinical studies,<sup>227,228</sup> and could be also tested in SARS-CoV-2 infection. This drug could inhibit expression of the viral N protein and pro-inflammatory cytokines (IL-6) in MERS-CoV-infected mice.<sup>229</sup>

Other IFV antivirals could be useful in SARS-CoV-2 therapeutic treatments. Glycyrrhizin (Figure 20) is an antioxidant, anti-inflammatory, immunomodulatory, and anti-viral agent<sup>230,231</sup> that inhibits IFV-A infection through inhibiting pro-inflammatory gene expression.<sup>232</sup> Glycyrrhizin can suppress SARS-CoV infection at the early virus entry and the late replication stages in Vero E6 cells.<sup>233</sup> 14-Deoxy-11,12-dehydroandrographolide suppresses pro-inflammatory cytokines and chemokines as well as IFV-A replication.<sup>234</sup> NSC61610 (Figure 20) induces lanthionine synthetase C-like 2 (LANCL2), a therapeutic target for treating infectious diseases such as IFV-A infection.<sup>235</sup> The *Ligustrum purpureascens* extract phenylethanoid glycoside (LPG) induces IFN- $\gamma$ ,

which can inhibit IFV-A replication *in vitro* and *in vivo*.<sup>236</sup> Some of these agents could be considered in clinical trials for treating COVID-19, although adverse effects need to be deeply studied.<sup>237</sup>

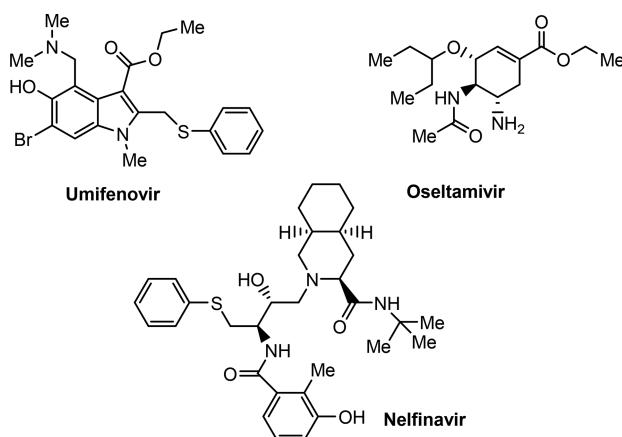
Other strategies attempting to control host immune response in HCoV include cyclophilin-targeting drugs such as cyclosporin A and alisporivir. SARS-CoV-2 uses not only ACE2 receptor for cell entry but also CD147, as described for SARS-CoV.<sup>238,239</sup> CD147 receptor regulates cytokine secretion 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 treatment, e.g., imatinib mesylate, dasatinib, trametinib, saracatinib, etc.<sup>240,241</sup> or the immunosuppressor mycophenolic acid.<sup>242</sup>

## 5. THERAPEUTIC OPTIONS IN CLINICAL TRIALS FOR COVID-19

Antiviral lopinavir/ritonavir (Figure 9) has been recommended for clinical treatment for COVID-19. Recent results from clinical trials do not confirm any benefit in hospitalized adult patients with severe COVID-19,<sup>243</sup> but the combination of these two antivirals with interferon, as previously mentioned, is more promising.<sup>216</sup>

Remdesivir (Figure 10) is an adenosine analogue RdRp inhibitor with antiviral protection against SARS-CoV-2<sup>93</sup> and other viruses.<sup>99</sup> In addition, intravenous administration has been found to be efficacious in an American patient with COVID-19.<sup>244</sup> 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.<sup>97</sup>

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



**Figure 21.** Antivirals used in clinics as potential COVID-19 treatments.

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

- Antivirals against viral components: EBOV viral polymerase inhibitor remdesivir (Figure 10),<sup>93</sup> developed for EBOV, IFV antiviral favipiravir (Figure 10),<sup>90</sup> and others developed for HIV or HCV, such as lopinavir/ritonavir (Figure 9), capable of inhibiting 3CLpro.
- Antivirals developed against host targets: viral endocytosis inhibitors such as chloroquine and hydroxychloroquine (Figure 22), which are drugs for malaria that decrease endosomal acidity, i.e., kinase inhibitors.
- Anti-inflammatory drugs, corticosteroids, or immunosuppressors.

A therapeutic combination (including a and c) has been selected for a randomized large clinical trial launched by WHO, called Solidarity, to collect clinical data from several thousand patients from dozens of countries.<sup>95</sup> Other antivirals used against viral RNA polymerase include ribavirin (Figure 19)<sup>214,247</sup> and galidesivir (BCX4430) (Figure 22).<sup>248</sup> Others are selected on the basis of their protease inhibition activity, such as disulfiram (a PLpro inhibitor)<sup>249</sup> or camostat mesylate (a TMPRSS inhibitor) (Figure 13),<sup>116</sup> or their spike protein inhibition, such as griffithsin.<sup>250</sup> Some other antivirals used are clathrin-mediated endocytosis inhibitors. This is the case of chlorpromazine (Figure 22) and fluphenazine (Figure 17), dopamine inhibitors which are FDA-approved drugs,<sup>171</sup> and miscellaneous compounds (Figure 22) such as resveratrol,<sup>251</sup> gemcitabine, mefloquine,<sup>240</sup> and loperamide.<sup>252</sup> Losartan and, more recently, telmisartan have been used as ACE2 receptor inhibitors. Furthermore, ivermectin has recently shown SARS-CoV-2 inhibition *in vitro*.<sup>253</sup>

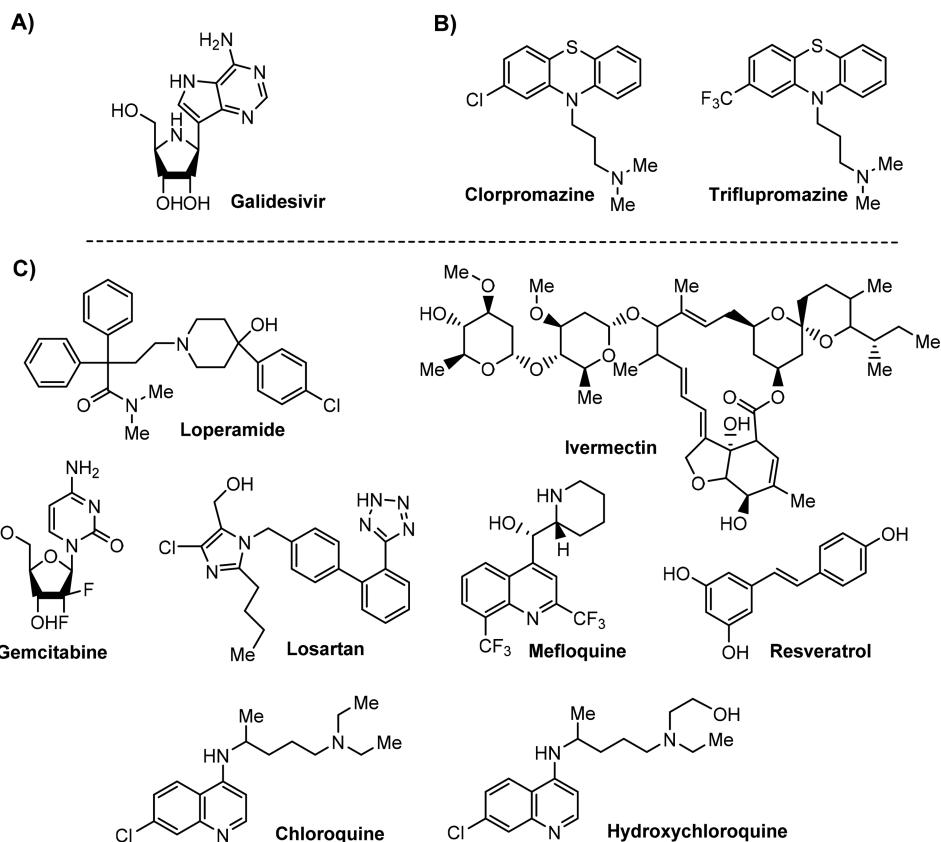
## 6. CONCLUSIONS

As mentioned in the Introduction, a deep knowledge of 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 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 nsps. Of 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

**Table 1.** Drugs Currently Used in Clinical Trials<sup>a</sup>

target	antiviral treatment	use
RNA polymerase	remdesivir* favipiravir, ribavirin, oseltamivir, galidesivir, sofosbuvir, umifenovir	Ebola virus (EBOV) influenza virus (IFV), hepatitis C virus (HCV), human immunodeficiency virus (HIV)
3CL protease	lopinavir/ritonavir*, ivermectin	HIV
PL protease	disulfiram	
protein S	griffithsin	HIV transmission
<i>Inhibition of Virus Entry</i>		
inhibition of TMPRSS2	camostat mesylate, nafamostat, bromhexine, enzatulamide	
inhibition of endocytosis	chlorpromazine, fluphenacine	other uses
acidification of endosome	chloroquine/hydroxychloroquine	malaria
inhibition of entry	convalescent plasma	antibodies
kinase inhibitors	imatinib, baricitinib	antitumor agents
<i>Immune Response Control</i>		
anti-inflammatory drugs	corticosteroids interferons* nitazoxanide, mycophenolic acid tocilizumab cyclosporin/alisporvir	inflammatory response HCV interferon induction or synergy IL6 antagonist immune suppressors
Miscellaneous	resveratrol, loperamide, losartan, telmisartan, etc.	control organ damage

<sup>a</sup>The WHO clinical trial Solidarity compares treatment strategies with several compounds, marked with asterisks.



**Figure 22.** Approved drugs in clinical trials for COVID-19: (A) inhibitors of viral RNA polymerase, (B) antipsychotics effective in clathrin-mediated endocytosis, and (C) miscellaneous drugs.

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 escape 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.<sup>254</sup> In fact, drugs currently under clinical trials were initially approved for other indications. However, the past and present coronavirus outbreaks require our preparedness not only for the current situation but also for a future potential re-emergence of novel coronaviruses. In this sense, it is of utmost importance to design drugs acting as pan-coronavirus antivirals or through a multi-target approach to avoid a lack of effectiveness by viral mutation escape.

**Addendum (May 29, 2020).** Since the submission of this manuscript, the number of tridimensional structures determined for different SARS-CoV-2 proteins available in the PDB has been increasing dramatically. On average, in February 2020, a total of 4 structures were found deposited in the PDB, increasing by 99 in March, by 45 in April, and by 65 in May. As of late May 2020, a total of 214 structures were available in the PDB, although some of them have not yet been published (Table S1). Different experimental techniques in addition to X-ray diffraction have been used, including electron microscopy and NMR, and the structure of the SARS-CoV-2 main protease has been the most studied, with more than 136 different crystals deposited. This huge data explosion confirms the relevance of proteomic data in relation with the pandemic.

As the number of PDB structures, as well as the compounds with potential antiviral action against SARS-CoV-2 being studied, will increase sharply in the next months, essential Web resources have been made available where the COVID-19 information that may be of utmost importance for medicinal chemists is continuously updated. These Web sites are summarized in Table S2.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c00606>.

3D structures available (PDB) of SARS-CoV-2 proteins (Table S1); Web resources for COVID-19 drug discovery (Table S2) ([PDF](#))

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<https://pubs.acs.org/10.1021/acs.jmedchem.0c00606>

### Notes

The authors declare no competing financial interest.

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**Tiziana Ginex** received her degree in Pharmaceutical Chemistry (CTF) in 2012 and her European Ph.D. in Food Sciences from the University of Parma (Italy) in 2016. From 2016 to 2019, she was a postdoctoral researcher in the Computational Biology and Drug Design group at the University of Barcelona (Spain). Financed by an HPC-Europa3 grant, she was a visiting researcher in the CCBioSim group of the University of Bristol (UK). In 2019, she was awarded a PRACE Preparatory Access (2010PA5217) related to the study of Influenza A. She is currently a postdoctoral researcher in the Translational Medicinal and Biological Chemistry group at CIB-CSIC (Spain). Her area of expertise covers computational methods applied to the study of mechanistic events related to neurodegenerative and infectious diseases.

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**Vanesa Nozal** received her M.Sc. in Organic Chemistry in 2016 from the Complutense University of Madrid (Spain) and her degree in Chemistry from the University of Valladolid (Spain) in 2015. Currently, she is working on her Ph.D. thesis under the supervision of Prof. Ana Martínez at CIB-CSIC, funded by a FPU fellowship. Her main research interest is the design and synthesis of new drugs for the treatment of unmet diseases.

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**Jesús Urquiza** received his Biology degree from the Alcalá de Henares University of Madrid (Spain). He spent 6 months in an internship in Klinikum Rech der Isar, München (Germany), and obtained a Virology Master degree from the Complutense University of Madrid (Spain). Currently, he is a Ph.D. Microbiology student at Autónoma University of Madrid (Spain), under a predoctoral grant at the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) in Madrid. His fields of interest cover virus–host interactions and therapeutic developments thereby.

**David Ramírez** received his Ph.D. in Applied Sciences in early 2017 from the Center for Bioinformatics, Simulation and Modelling (CBSM) at the University of Talca (Chile) in the laboratory of Prof. Wendy González. He spent one year of postdoctoral fellowship in the same group. Then, he joined the Laboratory of Prof. José Argüello for one year as a postdoctoral fellow at the Worcester Polytechnic Institute (USA). In early 2019 he was appointed as Assistant Professor at the Biomedical Sciences Institute, Autónoma University (Chile). His research is focused on membrane proteins using both experimental and theoretical approaches, pharmacology, and modulation of ion channels as well as transport mechanisms in plant and bacteria transporters.

**Covadonga Alonso** received her medical degree with honors from the Complutense University of Madrid (Spain) and obtained her Ph.D. from the Autónoma University of Madrid (Spain). After postdoctoral work in the Microbiology and Molecular Biology Department of the Tufts University School of Medicine in Boston (USA), she became a Staff Researcher at the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) in Madrid. She has been Director of the Biotechnology Department at INIA and recently became Professor of Research at the same institution. She has developed target-based antiviral peptides after the characterization of the interaction between viral proteins and microtubular motor proteins and is cofounder of the biotech company Algenex.

**Nuria E. Campillo** received her Ph.D. from Autónoma University of Madrid in 1997. After a postdoctoral position at Cambridge

University (UK), she became an Associate Researcher (2001–2003) at Instituto de Química Médica-CSIC (Madrid, Spain) and Staff Scientist (2003–2014) later. At present, Dr. Campillo works at CIB-CSIC. Her research interests are focused on the design, synthesis, and pharmacological evaluation of new compounds as potential drugs for neurodegenerative disorders and neglected diseases. She is also interested in the use of machine learning and deep learning for the prediction of biological properties (activity, ADMETox).

**Ana Martínez** received her organic chemistry degree and Ph.D. in medicinal chemist from Complutense University (Spain). Since 1990, she is a tenured research staff member of CSIC. She has great experience in technological transfer, being R&D Director of NeuroPharma from 2002 to 2008 and founder of Ankar Pharma in 2014. Currently, she is a research professor at CSIC and head of the Medicinal and Biological Chemistry group at CIB. Her interests are focused on drug discovery and development for severe unmet diseases, having some compounds in clinical trials.

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## ABBREVIATIONS USED

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

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