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## Review

# Potential therapeutic approaches for the early entry of SARS-CoV-2 by interrupting the interaction between the spike protein on SARS-CoV-2 and angiotensin-converting enzyme 2 (ACE2)

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## ABSTRACT

The COVID-19 pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has quickly spread around the globe. At present, there is no precise and effective treatment for the patients with COVID-19, so rapid development of drugs is urgently needed in order to contain the highly infectious disease. The virus spike protein (S protein) can recognize the angiotensin-converting enzyme 2 (ACE2) receptor on the host cell membrane and undergo a series of conformational changes, protease cleavage and membrane fusion to complete the virus entry, so S protein is an important target for vaccine and drug development. Here we provide a brief overview of molecular mechanisms of virus entry, as well as some potential antiviral agents that act on S/ACE2 protein-protein interaction. Specifically, we focused on experimentally validated and/or computational prediction identified inhibitors that target SARS-CoV-2 S protein, ACE2 and enzymes associated with viral infection. This review offers valuable information for the discovery and development of potential antiviral agents in combating SARS-CoV-2. In addition, with the deepening understanding of the mechanism of SARS-CoV-2 infection, more targeted prevention and treatment drugs will be explored with the aid of the advanced technology in the future.

## 1. Introduction

Coronaviruses (CoVs) are spherical viruses with an envelope on the surface and are approximately 100–160 nm in diameter [1]. This family has the largest viral genome ever discovered and can be further subdivided into four genera according to phylogenetic clustering: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus* [2,3]. The CoVs genomes are single-stranded, positive-sense RNAs with 5'-terminal cap structure and 3'-terminal poly-A tail [4]. About two-thirds of the genome is open reading frame 1a (ORF1a) and ORF1b, which are responsible for encoding two kinds of polyproteins, pp1a and pp1ab [4]. Subsequently, these polyproteins are cleaved into 15 or 16 non-structural proteins (NSPs), such as helicase (NSP13), RNA-dependent

RNA polymerase (RdRp, NSP12), main protease ( $M^{pro}$ , also known as  $3CL^{pro}$ , NSP5) and papain-like protease ( $PL^{pro}$ , NSP3) [2]. These NSPs are involved in the transcription and replication of the virus [2]. ORFs at one-third of the 3'-terminus of the genome are responsible for encoding accessory proteins and four structural proteins involved in virus assembly and infection: spike protein (S protein), membrane protein (M protein), envelope protein (E protein), and nucleocapsid protein (N protein) (Fig. 1). NSPs are more conserved in different CoVs, while structural proteins are more likely to mutate to adapt to new hosts [5]. S protein is a class I fusion protein that induces cell fusion and is responsible for recognizing specific receptor on the host cell surface [6]. M protein and E protein are responsible for assembling the virus and shaping its particle morphology [7,8]. N protein is the only protein in

**Abbreviations:** SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; S protein, spike protein; ACE2, angiotensin-converting enzyme 2; PPI, protein-protein interaction; CoVs, Coronaviruses; ORF1a, open reading frame 1a; ORF1b, open reading frame 1b; NSP, non-structural protein; RdRp, RNA-dependent RNA polymerase;  $PL^{pro}$ , papain-like protease;  $M^{pro}$ , main protease; TMPRSS2, transmembrane protease serine 2; MERS-CoV, Middle East respiratory syndrome coronavirus; NTD, N-terminal domain; RBD, receptor binding domain; HR1, heptad repeat 1; HR2, heptad repeat 2; 6-HB, 6-helix bundle; DPP4, dipeptidyl peptidase 4; SARS-CoV, severe acute respiratory syndrome coronavirus; PD, peptidase domain; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; AAK1, AP2-associated protein kinase 1; AHR, aryl hydrocarbon receptor.

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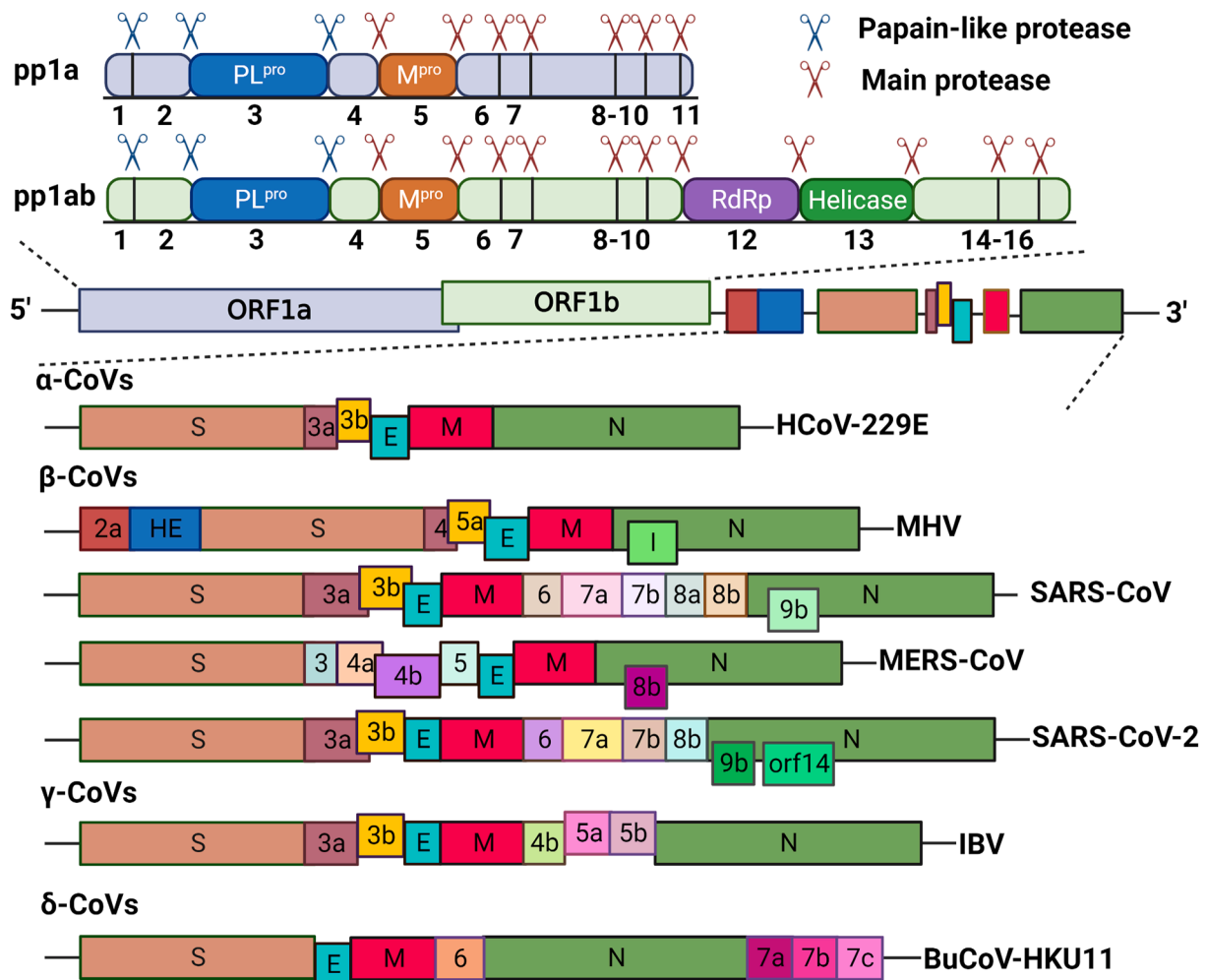
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the nucleocapsid that binds to the viral RNA genome and forms a ribonucleoprotein core to protect the viral genetic material [3,9]. However, the S protein is the major immunogenic antigen and is crucial for the interaction between the virus and the host cell receptor [10]. Therefore, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) S protein serves as an attractive target for the development of anti-SARS-CoV-2 therapeutics and vaccines. This review attempts to describe the mechanism of SARS-CoV-2 infection, summarize the agents that block the interaction between S protein and angiotensin-converting enzyme 2 (ACE2), and highlight the necessity of developing broad-spectrum antiviral drugs based on new targets and new mechanisms.

## 2. ACE2 mediating the entry of SARS-CoV-2 to the host cell by binding to the receptor binding domain of the S protein

Each CoV S protein contains an average of 1300 amino acids and is a very large transmembrane trimer protein, which constitutes the unique spike on the surface of the CoV [2,11]. The ectodomain of CoV S protein can be cleaved into the S1 and S2 subunits by a variety of host proteases, such as transmembrane protease serine 2 (TMPRSS2), factor Xa, and cathepsin L [12–15]. In the structure, the S1 subunit contains two subdomains, N-terminal domain (NTD) and C-terminal domain (CTD), and

either NTD or CTD can act as the receptor binding domain (RBD) to recognize host cells depending on the virus; the sequence structure of the S2 subunit includes a putative fusion peptide and two heptad repeat sequences, heptad repeat 1 (HR1) and heptad repeat 2 (HR2) [2]. The RBD in S1 subunit binds to the cellular receptor and initiates the receptor-mediated conformational change of S2 subunit from a metastable pre-fusion form to a more stable post-fusion form [16]. As a result, the fusion peptide is inserted into the host cell membrane and combined with HR1 and HR2 to form a 6-helix bundle (6-HB) fusion core structure to participate in the fusion process [2]. Notably, CoVs use different domains of the S1 subunit to recognize various types of host cell receptors [17]. For example, human coronavirus 229E (HCoV-229E) uses human aminopeptidase N (APN) as a receptor to invade host cells [18], Middle East respiratory syndrome coronavirus (MERS-CoV) invades cells through dipeptidyl peptidase 4 (DPP4/CD26) [19], both human coronaviruses OC43 (HCoV-OC43) and HKU1 (HCoV-HKU1) use 9-O-acetylated acid as their receptor on host cells [20,21]. Severe acute respiratory syndrome coronavirus (SARS-CoV) and human coronavirus NL63 (HCoV-NL63) were confirmed to infect cells by ACE2 receptor [22,23]. Amino acid sequences of seven conserved non-structural proteins domains in ORF1a/b can be used for CoV species classification [24]. Zhou et al. [24] found that these amino acid sequences of SARS-



**Fig. 1.** Several representative genome organization and structural proteins of CoVs. ORF1a and ORF1b encode two polyproteins, pp1a and pp1ab. The two polyproteins were cleaved by PL<sup>pro</sup> (Blue) and M<sup>pro</sup> (Brown) into 16 NSPs involved in viral transcription and replication, whereas the remaining ORFs are responsible for encoding accessory proteins and four structural proteins including S, M, E, and N protein. Importantly, the cleavage product of pp1ab does not contain NSP11, because NSP11 is an oligopeptide generated when the ribosome is not frameshifted. HE, hemagglutinin-esterase. Viral names: HCoV-229E, Human coronavirus 229E; MHV, mouse hepatitis virus; SARS-CoV, severe acute respiratory syndrome coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; IBV, infectious bronchitis virus; BuCoV HKU11, bulbul coronavirus HKU11.

CoV and SARS-CoV-2 were 94.4% identical, indicating that both viruses belong to SARS-related coronaviruses (SARSr-CoV). To confirm the receptor of SARS-CoV-2, Hela cells expressing ACE2, APN and DPP4 were used in the SARS-CoV-2 infection experiment respectively, and it was found that SARS-CoV-2 entered the cells through ACE2 receptor instead of other CoV receptors [24].

### 3. Mechanism of SARS-CoV-2 S protein binding with ACE2

ACE2 is a type I transmembrane glycoprotein that is widely expressed in the lungs, heart, testis and kidneys [25]. ACE2 consists of an extracellular peptidase domain (PD) and an intracellular C-terminal collectrin-like domain (CLD) [25–27]. ACE2 can not only cleave angiotensin I into Ang-(1–9), but also catalyze the conversion of angiotensin II into Ang-(1–7), thereby antagonizing the pressure boost of angiotensin II [25]. Yan et al. [28] obtained a stable ACE2-B<sup>0</sup>AT1 complex (B<sup>0</sup>AT1, a sodium-dependent neutral amino acid transporter, also known as SLC6A19) after co-expression of B<sup>0</sup>AT1 and ACE2 in HEK293F cells. After analysis by cryo-electron microscopy, it was found that PD in ACE2 homodimer has two conformations, open and closed, and each PD can bind to one SARS-CoV-2 S-RBD, that is, one ACE2 homodimer can bind to two S protein trimers [28]. Similar to SARS-CoV, SARS-CoV-2 S-RBD interacts with ACE2 mainly through polarity [28]. However, SARS-CoV-2 S-RBD had higher human ACE2 (hACE2) binding affinity than SARS-CoV S-RBD [29]. Paradoxically, the binding level of SARS-CoV-2 S protein to soluble hACE2 is equivalent to or slightly lower than that of SARS-CoV S protein [30]. The lying-down conformation of RBD in SARS-CoV-2 S protein seems to explain the decrease in S protein binding capacity [31]. Nevertheless, reducing S-RBD exposure as a conformational masking-strategy of SARS-CoV-2 will contribute to immune evasion and still have a comparable infection efficiency [32].

### 4. Potential antiviral agents acting on S protein-ACE2 interaction

As mentioned previously, the SARS-CoV-2 S protein recognizes the host cell ACE2 receptor and initiates virus-host cell membrane fusion, which is essential for viral infection. Therefore, screening inhibitors that inhibit S protein-ACE2 interaction is particularly important in the treatment of COVID-19. At present, researches on inhibiting this target mainly focus on regulating S protein, ACE2 receptor, or inhibiting S protein-ACE2 interaction. Related targets and active compounds that act on SARS-CoV and SARS-CoV-2 are reviewed in Table 1 and Fig. 2.

#### 4.1. Inhibitors acting on S protein

The diversity of CoVs is mainly reflected in the variable S proteins, which are the most important pathogenic proteins of CoVs and determine the binding of viruses to host cell receptors and the subsequent virus-cell membrane fusion process [11].

##### 4.1.1. Small molecule inhibitors of SARS-CoV-2 S-RBD/ACE2 interaction

In an attempt to discover small molecule inhibitors that target the SARS-CoV-2 S protein, we have initiated a drug screening strategy based on computer-aided drug design and biological verification [33]. *Glycyrrhiza uralensis* Fisch. (Licorice) has attracted our attention with a high frequency of use in Traditional Chinese Medicine (TCM) prescriptions for the treatment of COVID-19. Fortunately, through molecular docking technology, we found that the active ingredient of licorice, glycyrrhizic acid, could effectively bind to SARS-CoV-2 S-RBD (Fig. 3). In addition, we verified the binding activity of glycyrrhizic acid with SARS-CoV-2 S-RBD ( $K_D = 0.87 \mu\text{M}$ ) through surface plasmon resonance (SPR) technology [33]. Notably, this is the first time that NanoBit technology has been applied to determine the blocking activity of small molecules against SARS-CoV-2 S-RBD/ACE2 interaction. The results showed that glycyrrhizic acid effectively blocked SARS-CoV-2 S-RBD/ACE2

**Table 1**  
Potential antiviral drugs or compounds and their pharmacological function.

| Target             | Drug or compound    | Pharmacological function  | Ref.   |
|--------------------|---------------------|---|--|
| S-RBD-<br>ACE2 PPI | Glycyrrhizic acid   | Anti-SARS-CoV-2, binds to S-RBD and blocks SARS-CoV-2 S-RBD/ACE2 interaction                                      | [33]   |
|                    | Kobophenol A        | Anti-SARS-CoV-2, blocks the interaction between ACE2 and SARS-CoV-2 S-RBD   | [34]   |
|                    | Cepharanthine       | Anti-SARS-CoV-2, binds to the S protein and interferes with SARS-CoV-2 S-RBD/ACE2 interaction                     | [35]   |
|                    | Demethylzeylasteral | Anti-SARS-CoV-2, binds to S-RBD or ACE2 and blocks SARS-CoV-2 S-RBD/ACE2 interaction                              | [36]   |
|                    | Heparin             | Anti-SARS-CoV-2, binds to S-RBD and causes structural change in S-RBD protein                                     | [37]   |
|                    | CR3022              | Anti-SARS-CoV-2, monoclonal antibody  | [41]   |
|                    | B38                 | Anti-SARS-CoV-2, monoclonal antibody  | [42]   |
|                    | H4                  | Anti-SARS-CoV-2, monoclonal antibody  | [42]   |
|                    | 47D11               | Anti-SARS-CoV-2 and anti-SARS-CoV, neutralizing antibody  | [43]   |
|                    | 7B11                | Anti-SARS-CoV-2, monoclonal antibody  | [44]   |
|                    | CA1                 | Anti-SARS-CoV-2, monoclonal antibody  | [45]   |
|                    | CB6                 | Anti-SARS-CoV-2, monoclonal antibody  | [45]   |
|                    | VHH-72-Fc           | Anti-SARS-CoV-2, bivalent nanobody  | [50]   |
|                    | hrsACE2             | Anti-SARS-CoV-2, inhibits virus infection of cells and reduces viral load by 1000–5000 times                      | [71]   |
|                    | SSAA09E2            | Anti-SARS-CoV, inhibits interaction between S-RBD and ACE2  | [88]   |
|                    | Membrane fusion     | EK1   | Anti-SARS-CoV, binds to HR1 of S2 and inhibits the formation of 6-HB fusion core structure |
| EK1C4              |                     | Anti-SARS-CoV-2, binds to HR1 of S2 and inhibits the formation of 6-HB fusion core structure                      | [54]   |
| IPB02              |                     | Anti-SARS-CoV-2, binds to HR1 of S2 and inhibits S protein-mediated cell–cell fusion and pseudovirus transduction | [57]   |
| ITZ                |                     | Anti-SARS-CoV-2, inhibits the formation of 6-HB and thereby inhibits virus-cell membrane fusion                   | [58]   |
| EB                 |                     |   | [58]   |

(continued on next page)

Table 1 (continued)

| Target        | Drug or compound                 | Pharmacological function  | Ref.          |
|---------------|----------------------------------|---|---------------|
|               |                                  | Anti-SARS-CoV-2, inhibits the formation of 6-HB and thereby inhibits virus-cell membrane fusion                               |               |
|               | TGG                              | Anti-SARS-CoV, binds to S2 subunit and inhibits the entry of SARS-CoV   | [59]          |
|               | Luteolin                         | Anti-SARS-CoV, binds to S2 subunit and inhibits the entry of SARS-CoV   | [59]          |
|               | SSAA09E3                         | Anti-SARS-CoV, inhibits membrane fusion   | [88]          |
| ACE2 receptor |                                  |   |               |
|               | NAAE                             | Anti-SARS-CoV, ACE2 enzyme inhibitor  | [63]          |
|               | Ephedrine                        | Anti-SARS-CoV-2, binds to ACE2 and inhibits SARS-CoV-2 pseudovirus from entering ACE2 over-expressed HEK293T cells            | [64]          |
|               | Pseudoephedrine                  | Anti-SARS-CoV-2, binds to ACE2 and inhibits SARS-CoV-2 pseudovirus from entering ACE2 over-expressed HEK293T cells            | [64]          |
|               | Methylephedrine                  | Anti-SARS-CoV-2, binds to ACE2 and inhibits SARS-CoV-2 pseudovirus from entering ACE2 over-expressed HEK293T cells            | [64]          |
|               | Berbamine                        | Anti-SARS-CoV-2, reduces the level of ACE2 on the cell surface and prevents SARS-CoV-2 from entering the host cells           | [65]          |
|               | Protoporphyrin IX                | Anti-SARS-CoV-2, binds to ACE2 and inhibits SARS-CoV-2  | [66]          |
|               | Verteporfin                      | Anti-SARS-CoV-2, binds to ACE2 and inhibits SARS-CoV-2  | [66]          |
|               | Chloroquine                      | Antiviral drugs, raises endosomal PH value, inhibits ACE2 terminal glycosylation and blocks NTD binding to attachment factors | [74–77,79,84] |
|               | Hydroxychloroquine               | Anti-SARS-CoV-2, raises endosomal PH value, inhibits ACE2 terminal glycosylation and blocks NTD binding to attachment factors | [78,79,81,84] |
| Enzyme        |                                  |   |               |
|               | SSAA09E1                         | Anti-SARS-CoV, Cathepsin L inhibitor  | [88]          |
|               | Tetrahydroquinoline oxocarbazate | Anti-SARS-CoV, Cathepsin L inhibitor  | [89]          |
|               | Camostat mesylate                | Anti-SARS-CoV-2, TMPRSS2 inhibitor  | [91]          |
|               | E-64d                            |   | [91]          |

Table 1 (continued)

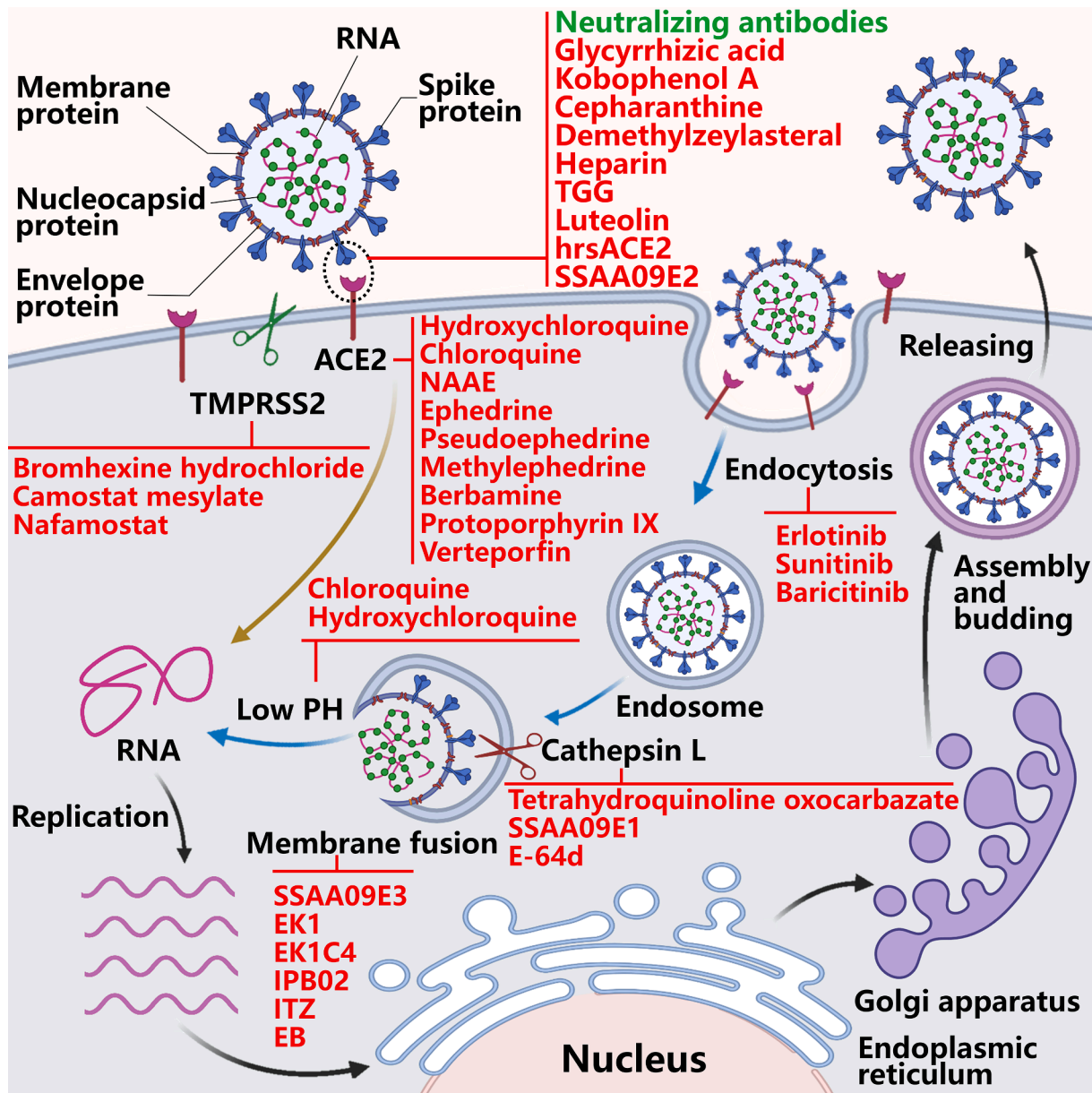
| Target | Drug or compound         | Pharmacological function                                    | Ref.    |
|--------|--------------------------|---|---------|
|        | Nafamostat               | Anti-SARS-CoV-2, Cathepsin L inhibitor                      | [77]    |
|        | Bromhexine hydrochloride | Anti-SARS-CoV-2, TMPRSS2 inhibitor                          | [92–94] |
|        | Sunitinib                | Anti-SARS-CoV-2, AAK1 inhibitor, inhibits endocytosis       | [96,97] |
|        | Erlotinib                | Anti-SARS-CoV-2, AAK1 inhibitor, inhibits endocytosis       | [96,97] |
|        | Baricitinib              | Anti-SARS-CoV-2, JAK kinase inhibitor, inhibits endocytosis | [97]    |

interaction and had little cytotoxicity on mouse aorta smooth muscle cells (MASMCs) and human bronchial epithelial (16HBE) cells [33]. Most recently, Gangadevi et al. found that Kobophenol A has the potential activity of blocking the interaction between ACE2 and SARS-CoV-2 S-RBD through virtual screening of natural product library, and determined the activity of the compound *in vitro* by enzyme-linked immunosorbent assay (ELISA) (Fig. 4) [34]. The results showed that Kobophenol A blocked the binding of S-RBD and ACE2 with half-maximal inhibitory concentration (IC<sub>50</sub>) value of 1.81 ± 0.04 μM [34]. More importantly, Kobophenol A inhibited SARS-CoV-2 infection of VeroE6-EGFP cells with median effective concentration (EC<sub>50</sub>) value of 71.6 μM, while Kobophenol A showed no cytotoxicity to VeroE6-EGFP cells at concentration of 100 μM, suggesting that Kobophenol A may be a lead compound against SARS-CoV-2 [34]. Cepharanthine, as a naturally occurring alkaloid screened from approved drugs, was found to inhibit SARS-CoV-2 infection of VeroE6/TMPRSS2 cells with IC<sub>50</sub> value of 0.35 μM [35]. Further *in silico* docking simulations showed that cepharanthine binds to the SARS-CoV-2 S protein and interferes with the interaction between SARS-CoV-2 S-RBD and the ACE2 receptor [35]. In addition, demethylzeylasteral exhibited the ability to bind to both S-RBD and ACE2 with K<sub>D</sub> values of 1.039 μM and 1.736 μM, respectively [36]. However, the CC<sub>50</sub> of demethylzeylasteral in 293 T-hACE2 cells was 7.67 ± 0.79 μM and it only showed slight anti-SARS-CoV-2 pseudovirus activity under the non-cytotoxic concentration [36]. Another study by Mycroft-West et al. found that Heparin, an anticoagulant drug, is able to bind to SARS-CoV-2 S-RBD, causing conformational change in S-RBD protein, and has a potential anti-viral activity [37].

#### 4.1.2. Antibodies that block the binding of ACE2 and S protein

In recent years, monoclonal antibodies (mAbs) targeting virus S protein has been shown to be therapeutic and preventive against multiple viral infections, and mAbs may be a promising class of drugs for the treatment of SARS-CoV-2 infection [38–40]. It has been reported that the specific human mAb for SARS-CoV, CR3022, could effectively bind to SARS-CoV-2 S-RBD (K<sub>D</sub> = 6.3 nM), thus blocking the binding of SARS-CoV-2 to the target cell ACE2 receptor, which can be used for the prevention and treatment of SARS-CoV-2 infection [41]. However, other mAbs acting on SARS-CoV, such as m396 and CR3014, failed to bind to SARS-CoV-2 S protein, suggesting that differences in S-RBD between SARS-CoV and SARS-CoV-2 have important effects on the cross-reactivity of mAbs [41]. Wu et al. [42] isolated four mAbs that can bind to SARS-CoV-2 S-RBD from a convalescent COVID-19 patient, and all of these antibodies showed neutralizing activity against SARS-CoV-2 *in vitro*, with IC<sub>50</sub> values ranging from 0.177 μg/mL to 1.375 μg/mL. However, only the antigen epitopes of antibody B38 and H4 are located on the S-RBD-ACE2 binding interface, and these two antibodies bind to different epitopes of S-RBD respectively, avoiding possible immune evasion in clinical applications [42]. Wang et al. [43] conducted ELISA-





**Fig. 2.** SARS-CoV-2 replication cycle and promising drugs or small molecule compounds to inhibit viral infection. The S protein of SARS-CoV-2 is cleaved on the cell surface by TMPRSS2 into S1 and S2. Subsequently, RBD of S1 binds ACE2 and leads to the fusion of viral membrane and plasma membranes, releasing viral genetic material into the cytoplasm, where RNA transcription and replication are performed (pathway 1, brown line). The new viral RNA is transported to the endoplasmic reticulum and Golgi to be assembled with structural proteins and bud into vesicles. The vesicles are then transported to the cell surface and released. SARS-CoV-2 binds to ACE2 and forms an endosome by endocytosis (pathway 2, blue line). The viral S protein is then cleaved by cathepsin L. The low PH in the endosome induces the fusion of the viral envelope with the endosome membrane, thereby releasing the viral genetic material. In this paper, the therapeutic targets for SARS-CoV-2 mainly include: 1) viral attachment (S protein, ACE2 and ACE2/S protein-protein interaction); 2) S protein cleavage (TMPRSS2, cathepsin L); 3) viral entry (endocytosis).

cross-reactivity evaluation of antibody-containing supernatants of SARS-CoV S protein hybridoma, and found that antibody 47D11 targeted the S-RBD of SARS-CoV and SARS-CoV-2, which could effectively inhibit SARS-CoV and SARS-CoV-2 infection of Vero E6 cells, showing cross-neutralizing activity. Tai et al. [44] identified 6 SARS-CoV S-RBD specific mAbs that cross-react with SARS-CoV-2 S-RBD, and two of these antibodies, 18F3 and 7B11, were able to neutralize SARS-CoV-2 pseudovirus infection at low concentrations, but only 7B11 had the ability to block the binding of SARS-CoV-2 S-RBD to ACE2. Shi et al. [45] isolated two specific human mAbs named CA1 and CB6 from a convalescent COVID-19 patient, and found that both antibodies could specifically bind to HEK293T cells transfected with SARS-CoV-2 S protein, and both

CA1 and CB6 showed neutralization activity against Vero E6 cells infected with SARS-CoV-2 pseudovirus *in vitro*. Further research found that CB6 overlaps with the ACE2 binding epitopes on SARS-CoV-2 S-RBD, which has therapeutic and preventive effects on rhesus macaques infected with SARS-CoV-2 [45].

In addition to conventional antibodies, camelids also produce a special antibody called Nanobody, which contains only a variable domain of heavy chain (VHH), but its antigen affinity and specificity are comparable to conventional antibodies and has higher thermodynamic and chemical stability [46–49]. Wrapp et al. [50] immunized a llama with the S proteins of SARS-CoV and MERS-CoV, and isolated two nanobodies, MERS VHH-55 and SARS VHH-72, which could effectively

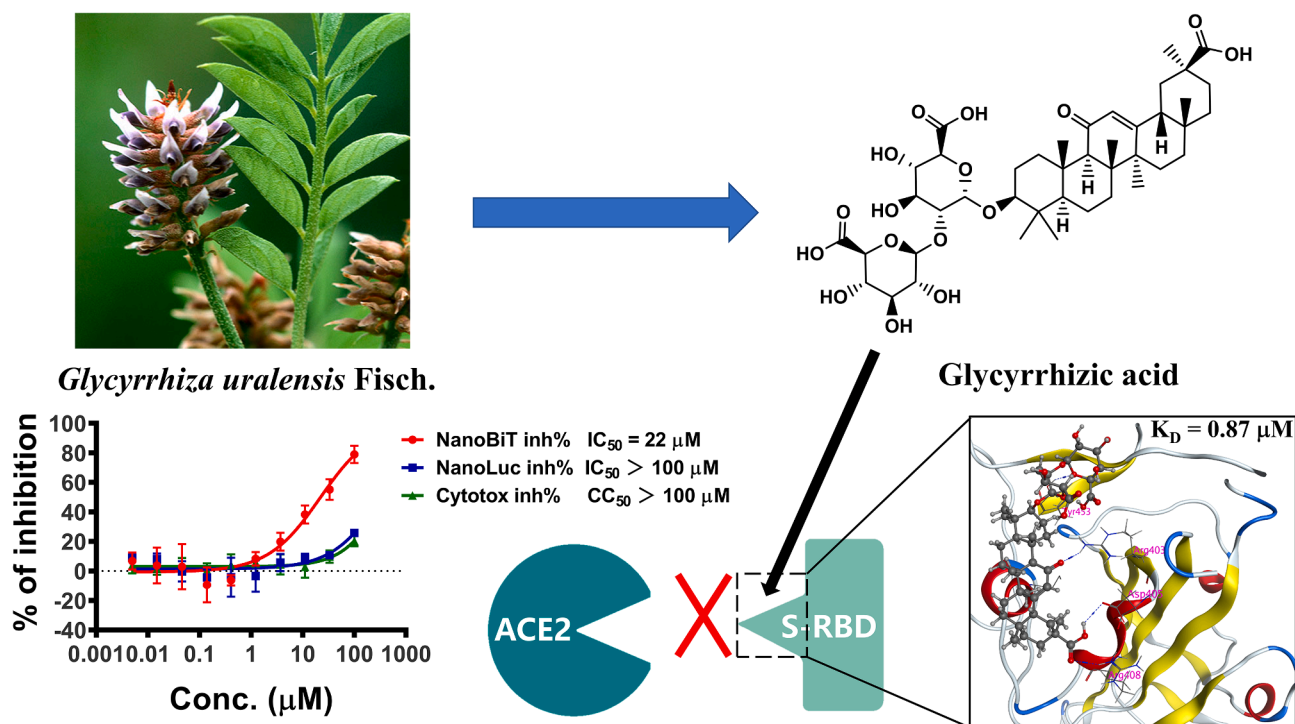


Fig. 3. The binding activity of glycyrrhizic acid with SARS-CoV-2 S-RBD and the inhibitory activity on the S-RBD/ACE2 interaction. Reproduced from ref. [33], copyright 2020, with permission from Elsevier.

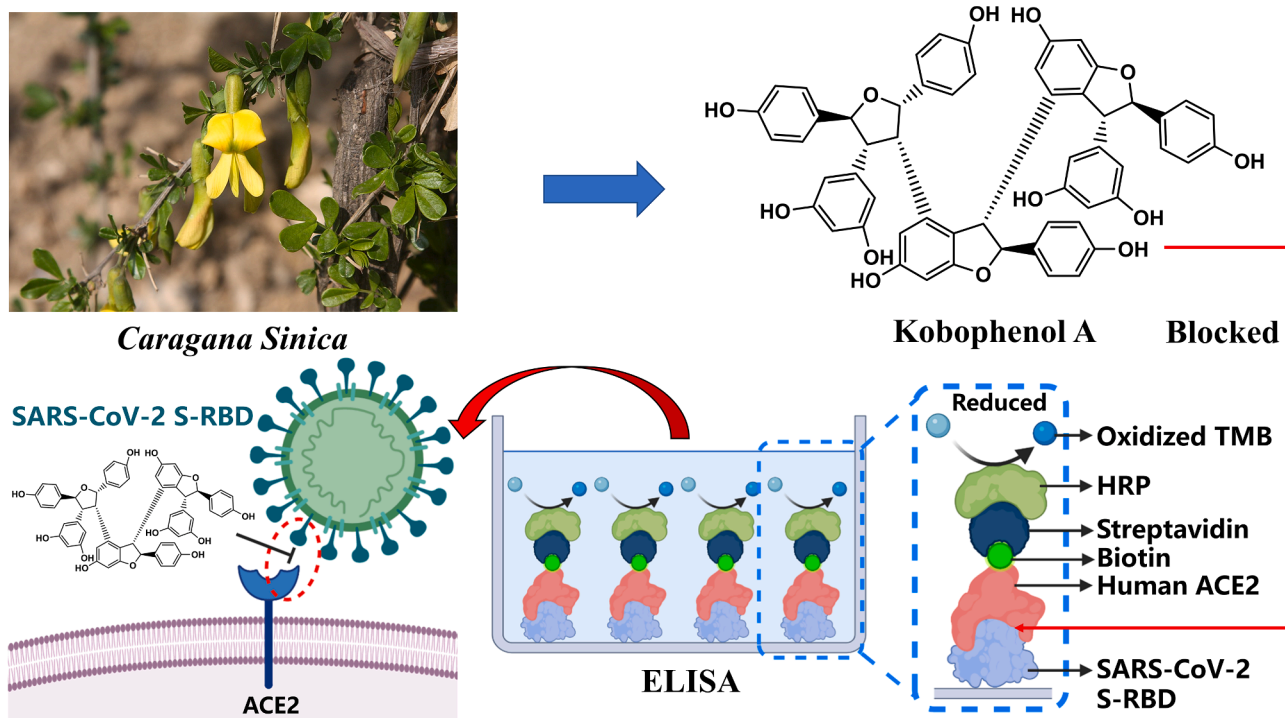


Fig. 4. The activity of Kobophenol A blocking the interaction between ACE2 and SARS-CoV-2 S-RBD was detected *in vitro* by ELISA.

neutralize the pseudoviruses of MERS-CoV and SARS-CoV *in vitro*. Characterization analysis of these two nanobodies revealed that they bound to S-RBD with high affinity, but their mechanism of action was different: MERS VHH-55 directly hindered the binding of MERS-CoV S-RBD to DPP4 by occupying the binding site of DPP4 on MERS-CoV S-RBD; SARS VHH-72 did not occupy the site on SARS-CoV S-RBD that binds to ACE2, but the collision with ACE2 indirectly affected the

binding of SARS-CoV S-RBD to ACE2 [50]. On this basis, the researchers further found that by engineering the SARS VHH-72 into a bivalent Fc-fusion, VHH-72-Fc, the SARS-CoV-2 pseudovirus could be effectively neutralized (IC<sub>50</sub> = 0.2 μg/mL), indicating that the unique biophysiological properties and effective neutralization capacity of the nanobody could make it a candidate drug for the treatment of SARS-CoV-2 infection [50].

#### 4.2. Inhibitors of virus-cell membrane fusion mediated by spike S2 subdomain

As described, the S2 subunit plays an important role in the process of virus-cell membrane fusion. In addition, unlike S1 subunit, the low variability of S2 subunit and the highly conserved HR domain make HR1 and HR2 ideal targets for anti-SARS-CoV-2 [51,52]. Xia et al. [53] designed a peptide fusion inhibitor EK1 targeting the HR1 domain of CoV S protein, which could effectively inhibit five pseudotyped CoVs infections including SARS-CoV and MERS-CoV. On this basis, the researchers used X-ray diffraction technology to solve the 6-HB core structure in SARS-CoV-2 S2 subunit, and found that there were 8 residues mutations in the fusion core region of HR1, which could enhance the interaction between HR1 and HR2, resulting in stronger membrane fusion ability and more stable 6-HB conformation of SARS-CoV-2 than SARS-CoV [54]. Therefore, the development of CoV membrane fusion inhibitors can be one of the effective means to inhibit SARS-CoV-2 infection. Given that lipidation strategy has been reported to effectively improve the antiviral activity of membrane fusion inhibitory peptides [55,56], the researchers covalently attached cholesterol molecules to the C-terminal of EK1 sequence and constructed a series of corresponding lipopeptides, and found that EK1C4, one of the lipopeptides, was the most effective fusion inhibitor for SARS-CoV-2 S protein-mediated membrane fusion and pseudovirus infection [54]. Moreover, the antiviral activity of EK1C4 was stronger than that of EK1, indicating that lipidation of EK1 was a promising modification strategy and could improve the fusion inhibition activity of EK1 against SARS-CoV-2 infection [54]. Zhu et al. [57] designed a HR2 sequence-based lipopeptide fusion inhibitor IPB02, which can effectively inhibit SARS-CoV-2 S protein-mediated cell-cell fusion ( $IC_{50} = 0.025 \mu M$ ) and SARS-CoV-2 pseudovirus transduction ( $IC_{50} = 0.08 \mu M$ ). Recent studies have shown that the two clinically approved drugs, itraconazole (ITZ) and estradiol benzoate (EB), inhibit the SARS-CoV-2 S protein-mediated intercellular fusion by affecting the formation of 6-HB [58]. Furthermore, ITZ and EB exhibited inhibitory activity against authentic SARS-CoV-2 infection of Vero E6 cells with  $EC_{50}$  values of 3.25 and 6.72  $\mu M$  [58], respectively. Frontal affinity chromatography-mass spectrometry (FAC/MS) was used to perform high-throughput screening of small molecule libraries to discover inhibitors that target the S2 subunit [59]. The results showed that tetra-O-galloyl- $\beta$ -D-glucose (TGG) and luteolin had high affinity with SARS-CoV S2 subunit thus could inhibit the entry of SARS-CoV pseudotyped virus and wild-type SARS-CoV into Vero E6 cells [59].

#### 4.3. Inhibitors acting on ACE2 receptor

In the lungs of normal people, ACE2 is intensively expressed in a small number of type II alveolar cells, which are capable of producing surfactants that reduce surface tension to prevent alveolar collapse and essential for lung gas exchange functions [60–62]. Therefore, type II alveolar cell damage is an important cause of lung tissue damage after SARS-CoV-2 invasion. Theoretically, blocking ACE2 can prevent SARS-CoV-2 infection. Therefore, searching for drug targets and designing drugs through the structure of ACE2 binding to viral S protein has become a current research hotspot [28–30]. Based on the crystal structure of ACE2, Huentelman et al. [63] screened out N-(2-aminoethyl)-1 aziridine-ethanamine (NAAE) with strong binding ability to ACE2 by using molecular docking technology, and analyzed its ability to inhibit ACE2 enzyme activity. The results showed that NAAE could inhibit ACE2 enzyme activity in a dose-dependent manner ( $IC_{50} = 57 \pm 7 \mu M$ ). Furthermore, the researchers applied NAAE to inhibit SARS-CoV membrane fusion experiment, and found that NAAE could shift the residues bound to SARS-CoV S protein on ACE2, thus inhibiting the binding of ACE2 to SARS-CoV S protein [63]. Recently, Lv et al. [64] used the ACE2/CMC-HPLC-IT-TOF-MS system to screen the active ingredients of the TCM Ephedra and identified three compounds, ephedrine,

pseudoephedrine, and methylephedrine that bind to human ACE2 and have anti-SARS-CoV-2 pseudovirus activity. Berbamine, a bis-benzylisoquinoline alkaloid, was found to reduce the expression of ACE2 on the surface of Huh7 cells by inhibiting transient receptor potential mucolipin channels (TRPMLs)-mediated  $Ca^{2+}$  release from lysosomes, disrupting the endolysosomal trafficking of ACE2, and increasing the secretion of ACE2 via exosomes, thereby blocking SARS-CoV-2 from entering the host cells [65]. In addition, both protoporphyrin IX and verteporfin were found to disrupt the S-RBD/ACE2 interaction by binding to ACE2 [66]. Notably, protoporphyrin IX and verteporfin not only inhibited the cytopathic effect of SARS-CoV-2 in Vero E6 cells with  $IC_{50}$  values of 1.25  $\mu M$  and 0.31  $\mu M$ , respectively, but also effectively prevented SARS-CoV-2 infection in the mouse model expressing human ACE2 [66].

In fact, ACE2 is considered as a protective factor for lungs and kidneys, and inhibition of ACE2 enzyme activity will lead to poor prognosis of lung injury and kidney injury [67,68]. In a recent retrospective cohort study, angiotensin-converting enzyme inhibitor (ACE-I) or angiotensin II receptor blocker (ARB) therapy was associated with the occurrence of severe complications and increased hospital mortality in patients with severe COVID-19 [69]. Therefore, these potential ACE2 inhibitors may not be suitable for the treatment of SARS-CoV-2 infection. Based on this, it is believed that providing excessive soluble ACE2 can be used as a potential treatment for COVID-19 [70]. Because it not only protects the lungs from damage, but also prevents SARS-CoV-2 from entering the target cells [71]. At present, human recombinant soluble ACE2 (hrsACE2) has entered phase I and phase II clinical trials [72,73].

Chloroquine was originally used as an antimalarial agent, but it was later found to have a direct antiviral effect by increasing the endosomal pH and eliminating virus-endosome fusions to inhibit the entry and replication of many viruses [74,75]. Vincent et al. [76] used Vero E6 cells to study the anti-SARS-CoV infection effect of chloroquine, and found that chloroquine could impair the terminal glycosylation of ACE2, reduce the interaction efficiency of ACE2-SARS-CoV, and inhibit the entry of virus into cells. In addition, chloroquine showed significant antiviral effects before and after Vero E6 infection with SARS-CoV, suggesting that chloroquine can be used for the prevention and treatment of SARS-CoV infection [76]. In a recent *in vitro* study, chloroquine was found to inhibit SARS-CoV-2 infection in Vero E6 cells, indicating that chloroquine may be a potential drug for the treatment of SARS-CoV-2 infection [77]. Hydroxychloroquine is a derivative of chloroquine, which can also increase the pH value of the endosome and impair the terminal glycosylation of ACE2 [78,79], but hydroxychloroquine is less toxic than chloroquine in animals [80]. Yao et al. [81] used Vero cells infected with SARS-CoV-2 to compare the antiviral activity of chloroquine and hydroxychloroquine, and found that both of them inhibited the viral replication in a concentration-dependent manner, but hydroxychloroquine ( $EC_{50} = 0.72 \mu M$ ) was more effective than chloroquine ( $EC_{50} = 5.47 \mu M$ ). In addition, sialic acids linked to glycoproteins and gangliosides have been reported as receptors or attachment factors for CoV entry into cells [82,83]. Fantini et al. [84] clarified the new mechanism of action of chloroquine and hydroxychloroquine through structural and molecular modeling methods, and found that they could bind to sialic acids and gangliosides on the host cell membrane, thereby blocking the binding of SARS-CoV-2 NTD to the host cell surface attachment factors.

#### 4.4. Potential enzyme targets and promising antiviral compounds

Although the binding of CoV S protein to host cell receptor is the first step in establishing infection, the proteolytic activation step plays an important role in subsequent viral fusion [85]. Studies have shown that SARS-CoV enters cells through two different pathways, one is mediated by TMPRSS2 on the cell surface, and the other is mediated by cathepsin L in the endosome [14,85–87]. Therefore, searching for drugs that inhibit TMPRSS2 or cathepsin L activity is also a potential treatment for



SARS-CoV-2 infection. Adedeji et al. [88] screened the Maybridge Hit-Finder small-molecule chemical library using the SARS/HIV-luc pseudotyped virus infection assay, and after excluding the compounds that significantly reduced the luciferase activity due to cytotoxicity or luciferase inhibition, finally screened three compounds that could specifically inhibit the entry of SARS-CoV into cells, SSAA09E1, SSAA09E2 and SSAA09E3. Further studies showed that only SSAA09E1 inhibited the activity of cathepsin L ( $IC_{50} = 5.33 \pm 0.61 \mu\text{M}$ ), SSAA09E2 could interfere with the interaction between SARS-CoV S-RBD and ACE2, and SSAA09E3 inhibited the fusion of viral membrane and host cellular membrane. In addition, the cathepsin L inhibitor tetrahydroquinoline oxocarbazate also has the activity of inhibiting SARS-CoV pseudotype virus infection ( $IC_{50} = 273 \pm 49 \text{ nM}$ ) [89]. Studies have shown that the expression of TMPRSS2 in host cells can significantly increase the number of CoVs entering target cells [90]. Hoffmann et al. [91] found that SARS-CoV-2 requires the priming of TMPRSS2 during its binding to the ACE2 receptor, and experiments confirmed that the TMPRSS2 inhibitor, camostat mesylate, could partially inhibit SARS-CoV-2 invasion of Caco-2 cells and Vero cells. However, when camostat mesylate was used together with cathepsin L inhibitor E-64d, it was able to completely inhibit SARS-CoV-2 invasion, indicating that SARS-CoV-2 entry into host cells can be simultaneously mediated by cathepsin L and TMPRSS2. Wang et al. [77] found that TMPRSS2 inhibitor Nafamostat could also block membrane fusion and thus inhibit SARS-CoV-2 infection ( $EC_{50} = 22.5 \mu\text{M}$ ). In addition, the FDA approved bromhexine hydrochloride is a selective inhibitor of TMPRSS2 with an  $IC_{50}$  of  $0.75 \mu\text{M}$  [92], and was once proposed as a candidate drug for the treatment of SARS-CoV and MERS-CoV infections [90]. Due to its extensive clinical application and high safety, it has been believed that bromhexine hydrochloride can be used as a drug to treat COVID-19 or prevent SARS-CoV-2 infection [93,94].

AP2-associated protein kinase 1 (AAK1) is one of the important regulators involved in endocytosis, so the disruption of AAK1 may interrupt the passage of the virus into the cells [95]. The screening of BenevolentAI's knowledge graph showed that the anti-tumor drugs sunitinib and erlotinib can inhibit viral infection of cells by inhibiting AAK1 activity, but these drugs have strong side effects [96,97]. In contrast, the safer janus kinase inhibitor baricitinib may be a potential drug for the treatment of COVID-19 because it inhibits not only AAK1 activity, but also the activity of cyclin G-associated kinase, another regulator of endocytosis [97].

## 5. Discussion

The high infectivity and lethality of SARS-CoV-2 is an urgent requirement for the development of new antiviral interventions. To prevent and treat COVID-19, the most potent strategy of inhibiting the viral entry is targeting host or virus-related components [98]. Therefore, a better understanding of COVID-19 pathogenesis and the structure–function relationships of drug targets will improve the success rate of new antiviral drug development. Currently, one of the most effective methods is to directly block or indirectly interfere with the interaction between SARS-CoV-2 S protein and human ACE2. In addition, inhibition of important proteases associated with viral infection and spread, such as  $M^{\text{pro}}$ ,  $PL^{\text{pro}}$ , RdRp, Helicase, TMPRSS2 and Cathepsin L, also provides the effective approach for novel antiviral drug discovery. Although SARS-CoV-2 has high sequence homology with SARS-CoV, the lack of advances in anti-SARS-CoV drug research and the high mutagenicity of SARS-CoV-2 have limited the number of candidate drugs available for screening anti-SARS-CoV-2 [99,100]. Therefore, it is urgently needed to rapidly discover and develop effective and low-toxic agents to avoid the risk of a pandemic, and this experience will play an important role in the face of large-scale infectious diseases in the future.

Recently, several SARS-CoV-2 vaccines have achieved good results in clinical trials, such as BBIBP-CorV, mRNA-1273 and CoronaVac [101–103]. However, SARS-CoV-2 has now been replaced by some new

mutated strains including the mutation D614G (Asp614 to Gly) in the viral S protein, which makes this variant more capable of replication and faster spreading [104]. Notably, there is growing evidence that human convalescent and postvaccination serum has decreased neutralizing activity against SARS-CoV-2 variants, such as E484K and N439K [105,106]. These SARS-CoV-2 variants can evade antibody-mediated immunity and adversely affect current responses to reinfection, vaccines, and antibody therapeutics [106]. Nevertheless, immune evasion usually comes at the cost of the biological fitness of the virus, and sera with high neutralization titers are still able to play a protective role against the spread of SARS-CoV-2 infection [105,107]. Therefore, vaccinating as many people as possible with a vaccine that can produce high neutralizing antibody titers seems to be one of the effective ways to deal with the variants circulating globally [105].

Developing a new targeted drug would theoretically show better anti-CoV activity [108], but the development of new drugs requires not only a huge amount of money, but also decades of research [109]. In addition, the discovery of new drug targets for the treatment of SARS-CoV-2 will also contribute to the generation of new therapies. Recently, a research group discovered that aryl hydrocarbon receptor (AHR) is a candidate therapeutic target for the treatment of respiratory failure, the primary cause of death in severe COVID-19 patients, caused by hypoxia in patients with SARS-CoV-2 infection [110]. Inhibition of AHR can not only enhance the patient's antiviral immunity, but also directly improve lung pathology, indicating that AHR-targeted therapies have outstanding effects and potential therapeutic value in virus-induced diseases [110]. Moreover, the establishment of a new animal model for COVID-19 will also help us have a deeper understanding of the pathogenesis of COVID-19 and the host response to SARS-CoV-2 infection [111]. We believe that with the deepening understanding of the mechanism of SARS-CoV-2 infection, more targeted preventive measures and therapeutic drugs will be explored and clarified in follow-up studies.

## CRedit authorship contribution statement

**Yusen Xiang:** Investigation, Writing – original draft. **Mengge Wang:** Writing - review & editing. **Hongzhuan Chen:** Conceptualization, Supervision. **Lili Chen:** Conceptualization, Supervision.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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