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Review

Drug discovery and development targeting the life cycle of SARS-CoV-2

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ABSTRACT

A newly emerged coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), belongs to the β-coronavirus family and shows high similarities with SARS-CoV. On March 11, 2020, the World Health Organization (WHO) declared SARS-CoV-2 a global pandemic, and the disease was named the coronavirus disease 2019 (COVID-19). The ongoing COVID-19 pandemic has caused over 46 million infections and over one million deaths worldwide, and the numbers are still increasing. Efficacious antiviral agents are urgently needed to combat this virus. The life cycle of SARS-CoV-2 mainly includes the viral attachment, membrane fusion, genomic replication, assembly and budding of virions. Accordingly, drug development against SARS-CoV-2 currently focuses on blocking spike protein binding to ACE2, inhibiting viral membrane fusion with host cells, and preventing the viral replication by targeting 3C-like protease, papain-like protease, RNA-dependent RNA polymerase as well as some host-cell proteins. In this review, the advances of drug development in these three major areas are elaborated.

1. Introduction

The life cycle of SARS-CoV-2 begins with the binding of the receptor-binding domain (RBD) in the S1 subunit of spike protein to angiotensin converting enzyme 2 (ACE2) which is expressed on the surface of epithelial cells in the respiratory tract as well as the intestine [1,2] (Fig. 1). Upon cell contact, the virus enters into the host cells by direct fusion of the host cell and viral membranes and/or endocytosis using the S2 subunit of the spike protein [1,3]. The spike protein is synthesized as an inactive precursor and subsequent cleavages by cellular proteases cause conformational changes of the S2 subunit, making the spike protein functionally active and ready for subsequent membrane fusion [4]. In a direct fusion entry, the spike protein is cleaved by transmembrane protease serine 2 (TMPRSS2) in close to ACE2 after the formation of the spike protein-ACE2 complex, subsequently triggering the membrane fusion with the host cell and the release of the viral genome into the host cells [3]. Other proteases such as trypsin, plasmin and factor Xa may also contribute to this process [5,6]. The virus can also enter the host cell by an endocytosis-mediated process. The spike protein activation appears to take place in endosomes by the action of furin and cathepsin B/L (CatB/L) in endo-lysosomes [1,3], which eventually promotes the viral envelope fusion with the host cell membrane and the release of the viral RNA.

The genomic viral RNA in the cytoplasm of infected cells can be translated into two polyproteins, pp1a and pp1ab [7] (Fig. 1), which are processed by two viral proteases, 3C-like protease (3CLpro), also known as the main protease (Mpro), and papain-like protease (PLpro), to generate 16 mature non-structural proteins (NSPs) [2,7]. Among them, NSP12, also known as RNA-dependent RNA polymerase (RdRp), assembles with several NSPs to form a replication and transcription complex (RTC) anchored on double-membrane vesicles (DMVs), responsible for the viral genome replication and transcription [8]. The viral RNA products are localized in the DMVs and transported to the cytosol through a molecular pore complex across the double membrane [8]. Then, structural proteins including spike (S), envelope (E), membrane (M) proteins are translated in the endoplasmic reticulum (ER) and transported to the Golgi apparatus for virion assembly. The viral genomic RNA and the structural protein N are biosynthesized and assembled into the nucleocapsid in the cytoplasm and then associated with the viral structural proteins to produce new virions. In the end, virions are released from the infected cell through exocytosis [9].

Accordingly, therapeutics are being developed to block one or more events in the life cycle of SARS-CoV-2. Proteins participating in the viral life cycle can be targets for drug development. In this review, we summarize the strategies and advances of drug development targeting key steps of the viral life cycle for the treatment of COVID-19 (Fig. 1).

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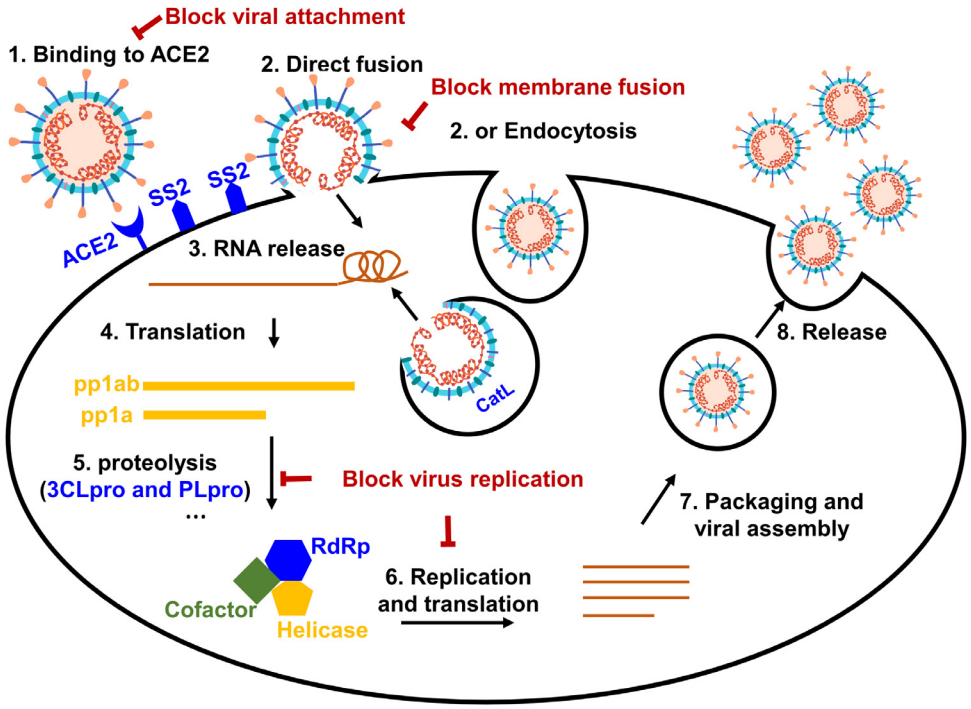

Fig. 1. Strategies to block key steps of the life cycle of SARS-CoV-2. Multiple steps in the life cycle of the virus are presented, including the binding of the spike protein to ACE2, membrane fusion between the virus and the host cell, release of the viral RNA, translation of the viral RNA, proteolysis of polyproteins (pp1ab and pp1a) by 3C-like protease together with papain-like protease, replication and translation by the RTC, packaging and assembly of viral particles, and virion release.

2. Preventing viral attachment

The entry of SARS-CoV-2 into host cells is mediated by the viral spike glycoprotein, which forms trimeric spikes on the viral surface [10]. Each monomer of the spike protein could be divided into two subunits, S1 and S2. S1 contains the N-terminal domain (NTD) and the RBD which is responsible for interacting with ACE2. Monoclonal antibodies (mAbs) blocking interactions between the RBD and ACE2 serve as prophylactics and therapeutics against COVID-19.

2.1. mAbs targeting S1 subunit

The S1 subunit mediates the binding of coronaviruses to host-cell receptors, it is thus a pivotal target for designing therapeutic agents including neutralizing antibodies. As listed in Table 1, recent studies have identified and characterized several mAbs which bind to the S1 subunit. Approaches including isolation of convalescent patients' B cells, animal immunization, *in vitro* phage display, and repurposing anti-SARS mAbs, have been applied to discover these mAbs. Among them, the isolation of convalescent patients' B cells is the most popular one due to the successful application of convalescent plasma in treating COVID-19 patients. Upon different binding sites on the S1, the developed mAbs could be classified into three categories by targeting the RBD receptor-binding subdomain (residues 438–498), the RBD core domain (residues 338–437) or the NTD domain (Table 1). For those mAbs which were able to block the interaction between the RBD and ACE2 but their structures in complex with the RBD have not been determined, we classified them into the first category. All these mAbs have been proven to be effective in combating SARS-CoV-2, even though most antibodies in the last two categories were unable to compete with the binding of ACE2.

2.1.1. mAbs targeting the RBD receptor-binding subdomain

As listed in Table 1, a majority of developed neutralizing mAbs targeted the RBD receptor-binding subdomain, which block the viral RBD-mediated binding to ACE2 directly. Among these mAbs, structures of the RBD or the entire spike protein in complex with Fabs of BD-23 [11], CB6 [12], B38 [13], PB-2F6 [14], REGN10933 [15], REGN-10987 [15], H11-D4 [16], H11-H4 [16], and 2–4 [17] were determined and are shown in Fig. 2. The binding sites of these mAbs on the RBD overlap or partly overlap with that of ACE2, therefore, these mAbs interfere with the virus–receptor interactions by both steric hindrance and direct competition with ACE2. Remarkably, some mAbs in this category exhibited satisfied efficacy in mice infected with SARS-CoV-2. BD-368-2 developed by Cao et al. showed potent therapeutic and prophylactic efficacy in SARS-CoV-2-infected hACE2 transgenic mice at a dose of 20 mg/kg [11]. 50 mg/kg of CB6 could effectively reduce viral load and alleviate infection-related lung damage in both prophylactic and treatment settings. [12]. 1.5 mg/kg of 2–15 could reduce lung viral load by 4 logs [17]. Administration of COV2-2130 at a dose of 20 mg/kg protected mice from weight loss and reduced the viral burden as well as levels of inflammation in the lungs [18].

2.1.2. mAbs targeting the RBD core domain

Recent studies demonstrated that targeting the RBD core domain is feasible for mAbs [30–34], although the underlying mechanism is not determined. Compared to the RBD receptor-binding subdomain, the sequence identity of the RBD core domain between SARS-CoV and SARS-CoV-2 is substantially higher (86.3% versus 46.7%), providing the possibility to develop broad-spectrum antiviral antibodies.

CR3022, a developed mAb against SARS-CoV, was found to have the ability to bind to the RBD of SARS-CoV-2 [33]. Structures of CR3022 in complex with SARS-CoV-2 spike protein and the single RBD were determined. As shown in Fig. 3a, CR3022 binds to the epitope of the RBD core domain, which is accessible in the open state of the spike protein. This epitope does not overlap with the binding site for ACE2 (Fig. 3a). As a result, it could not block the binding of ACE2 to the SARS-CoV-2 RBD (Fig. 3a). However, a plaque-reduction neutralization test showed that CR3022 could neutralize pseudotyped SARS-CoV-2 with an EC_{50} value of ~114 ng/mL [33].

Another three mAbs, 47D11 [32], EY6A [34] and H014 [30], also bind to the similar epitope. All of them displayed potent efficacy against authentic SARS-CoV-2, with EC_{50} values of 570 ng/mL, 70 ng/mL and 38 nM, respectively. As shown in Fig. 3b, c, structures of SARS-CoV-2 spike protein or the RBD in complex with EY6A and H014 were determined, respectively. The epitope of EY6A is quite similar to that of CR3022. As for H014, it binds to the RBD core domain but its binding site is located at the distal edge of the RBD receptor-binding subdomain, blocking the interaction between the RBD and ACE2. H014 demonstrated good antiviral efficacy in animals. In the prophylactic and prophylactic plus therapeutic groups, treatment of H014 at a dose of 50 mg/kg resulted in a ~10-fold and

Table 1
Summary of mAbs neutralizing SARS-CoV-2 S1 subunit.

Name	Methods	Epitope	EC_{50} @ authentic SARS-CoV-2	EC_{50} @ pseudotyped SARS-CoV-2	Ref
BD-368-2	convalescent COVID-19 patients	Receptor-binding subdomain	15 ng/ml	1.2 ng/ml	[11]
CB6	convalescent COVID-19 patients	Receptor-binding subdomain	36 ng/ml	23 - 41 ng/ml	[12]
B38	convalescent COVID-19 patients	Receptor-binding subdomain	177 ng/mL	\	[13]
H4	convalescent COVID-19 patients	Receptor-binding subdomain	896 ng/ml	\	[13]
P2B-2F6	convalescent COVID-19 Patients	Receptor-binding subdomain	410 ng/ml	50 ng/ml	[14]
REGN10933	humanized mice or convalescent COVID-19 patients	Receptor-binding subdomain	0.037 nM	0.043 nM	[15]
REGN10987	humanized mice or convalescent COVID-19 patients	Receptor-binding subdomain	0.042 nM	0.041 nM	[15]
REGN10989	humanized mice or convalescent COVID-19 patients	Receptor-binding subdomain	0.007 nM	0.007 nM	[15]
REGN10934	humanized mice or convalescent COVID-19 patients	Receptor-binding subdomain	0.028 nM	0.054 nM	[15]
H11-D4	*in vitro* phage display	Receptor-binding subdomain	6 nM	\	[16]
H11-H4	*in vitro* phage display	Receptor-binding subdomain	18 nM	\	[16]
2-15	convalescent COVID-19 patients	Receptor-binding subdomain	0.7 ng/ml	5 ng/ml	[17]
2-4	convalescent COVID-19 patients	Receptor-binding subdomain	57 ng/ml	394 ng/ml	[17]
4-8	convalescent COVID-19 patients	NTD domain	9 ng/ml	32 ng/ml	[17]
2-43	convalescent COVID-19 patients	Undetermined region on spike	3 ng/ml	71 ng/ml	[17]
COV2-2196	convalescent COVID-19 patients	Receptor-binding subdomain	15 ng/ml	0.7 ng/ml	[18]
COV2-2130	convalescent COVID-19 patients	Receptor-binding subdomain	107 ng/ml	1.6 ng/ml	[18]

(*continued on next page*)

Table 1 (*continued*)

Name	Methods	Epitope	EC_{50} @ authentic SARS-CoV-2	EC_{50} @ pseudotyped SARS-CoV-2	Ref
COV2-2165	convalescent COVID-19 patients	Receptor-binding subdomain	332 ng/ml	\	[18]
ADI-55689	convalescent SARS patients	Receptor-binding subdomain	effective at 100 nM.	50 - 1400 ng/ml	[19]
311mab-31B5	convalescent COVID-19 patients	Receptor-binding subdomain	\	33.8 ng/ml	[20]
311mab-32D4	convalescent COVID-19 patients	Receptor-binding subdomain	\	69.8 ng/ml	[20]
CC12.1	convalescent COVID-19 patients	Receptor-binding subdomain	22 ng/ml;	19 ng/ml	[21]
CV30	convalescent COVID-19 patients	Receptor-binding subdomain	\	30 ng/ml	[22]
C121	convalescent COVID-19 patients	Receptor-binding subdomain	1.64 ng/ml	6.73 ng/ml	[23]
C002	convalescent COVID-19 patients	Receptor-binding subdomain	\	8.88 ng/ml	[23]
C119	convalescent COVID-19 patients	Receptor-binding subdomain	\	9.12 ng/ml	[23]
ab1	*in vitro* phage display	Receptor-binding subdomain	200 ng/ml	\	[24]
7B11	animal immunization	Receptor-binding subdomain	\	Effevtive at 10 μg/mL	[25]
rRBD-15	*in vitro* phage display	Receptor-binding subdomain	\	12.2 nM	[26]
3F11	*in vitro* phage display	Receptor-binding subdomain	372.3 ng/ml	0.9 ng/ml	[27]
COVA1-18	convalescent COVID-19 patients	Receptor-binding subdomain	7 ng/ml	8 ng/ml	[28]
COVA2-15	convalescent COVID-19 patients	Receptor-binding subdomain	9 ng/ml	8 ng/ml	[28]
S2E12	convalescent COVID-19 patients	Receptor-binding subdomain	4.2 ng/ml	2.3 ng/ml	[29]
S2M11	convalescent COVID-19 patients	RBD core domain	1.2 ng/ml	2.1 ng/ml	[29]
H014	animal immunization and *in vitro* phage display	RBD core domain and receptor-binding subdomain	38 nM	3 nM	[30]
S309	convalescent SARS patients	RBD core domain	79 ng/ml	\	[31]
47D11	animal immunization	RBD core domain	570 ng/ml	61 ng/ml	[32]
CR3022	anti-SARS antibodies	RBD core domain		~ 114 ng/ml	[33]
EY6A	convalescent COVID-19 patients	RBD core domain	70 ng/ml	\	[34]
4A8	convalescent COVID-19 patients	NTD domain	610 ng/ml	49000 ng/ml	[35]

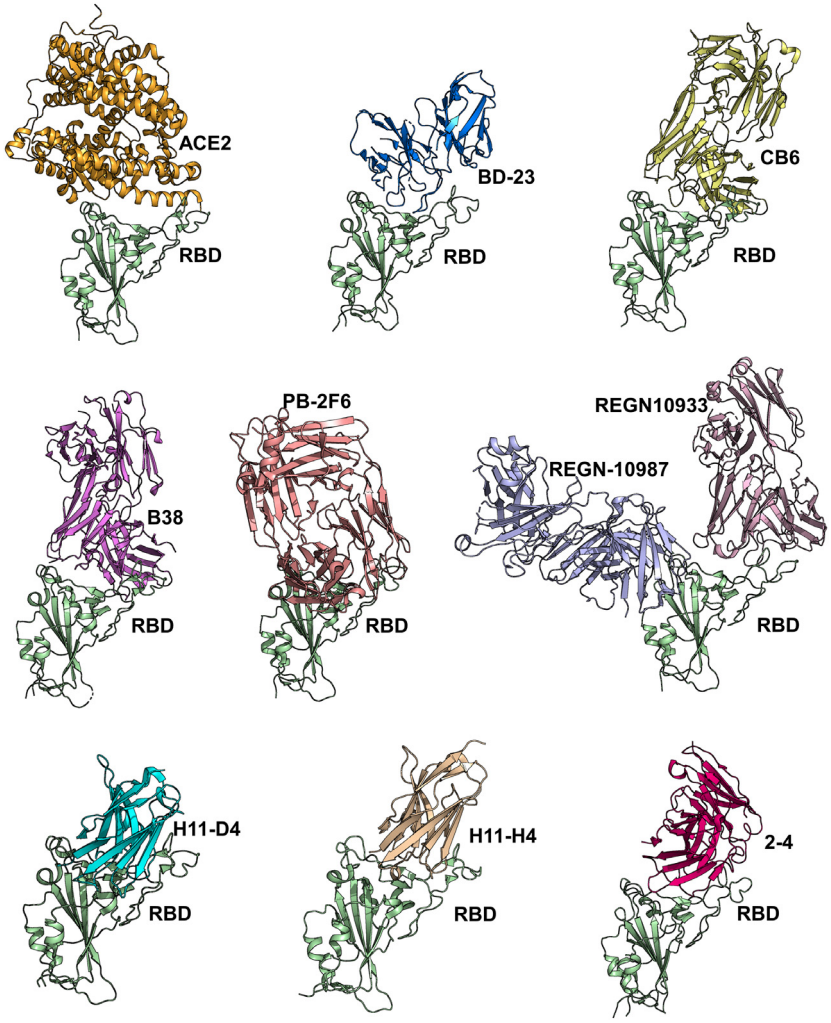

Fig. 2. Structures of the RBD bound with ACE2 and mAbs targeting the RBD receptor-binding subdomain. The binding sites of BD-23, CB6, B38, PB-2F6, REGN10933, REGN-10987, H11-D4, H11-H4, and 2-4 on the RBD overlapped or partially overlapped with that of ACE2. All structures are shown in cartoons.

100-fold reduction of viral titers in the lungs at day 5, respectively [30].

S309, a neutralizing mAb against both SARS-CoV and SARS-CoV-2 [31], recognizes a conserved glycan-containing epitope in the RBD core domain, which is on an opposite side of the epitopes for CR3022, EY6A, and H014. This epitope is accessible in both the open and closed states of the spike protein (Fig. 3d). The EC_{50} value of S309 against authentic SARS-CoV-2 is 79 ng/mL. Further mechanism studies demonstrated that the antiviral ability of S309 is associated with the inducement of antibody-dependent cell cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) [31]. In addition to S309, S2M11 developed by Tortorici [29] also targets such an epitope and achieves the antiviral efficacy by a similar mechanism (Fig. 3e).

2.1.3. mAbs targeting the NTD domain

Neutralizing antibodies binding to the NTD of the spike protein were also reported. 4A8 [35], isolated from convalescent COVID-19 patients, exhibited neutralization against both the authentic and pseudotyped SARS-CoV-2 *in vitro*, with EC_{50} values of 610 ng/mL and 49 μg/mL, respectively. However, it was unable to block the interaction between ACE2 and spike protein. Its cryo-EM structure in complex with the SARS-CoV-2 spike protein was determined, in which 4A8 binds to the NTD domain of the spike protein without directly contacting with the receptor binding site (Fig. 4). It is speculated that the binding of 4A8 may restrain conformational changes of the spike protein [35]. Another mAb, 4–8, also targets the NTD, which is revealed by a low-resolution cryo-EM structure of SARS-CoV-2 spike protein bound with 4–8 [17]. *In vitro* studies showed that 4–8 was highly effective against both the authentic and pseudotyped SARS-CoV-2, with EC_{50} values of 9 ng/mL and 32 ng/mL, respectively [17]. These together reveal that the NTD may also serve as a therapeutic target for mAbs development.

Epitopes of the mAbs targeting the RBD core domain and the NTD are distinct from the well-established receptor-blocking neutralizing antibody epitope. Combined use of several antibodies targeting the different epitopes may thereby improve the efficacy and mitigate the risk of immune escape. Indeed, a combination using of COV2-2196 targeting the RBD receptor-binding subdomain and COV2-2130 targeting a distinct epitope on RBD exhibited better efficacy than those of the individual ones [18]. Nevertheless, the detailed antiviral mechanism for mAbs binding to the RBD core domain as well as the NTD remains to be explored.

2.2. Soluble ACE2 and peptide targeting the spike RBD

Human ACE2 (hACE2) consists of three parts, an extracellular segment that contains the peptidase domain, a transmembrane region, and an intracellular portion. Since the RBD of SARS-CoV-2 spike protein binds to the extracellular domain of hACE2, soluble hACE2 lacking the transmembrane and the intracellular segments is sufficient for neutralizing the RBD and competing with the full-length ACE2 in the mem-

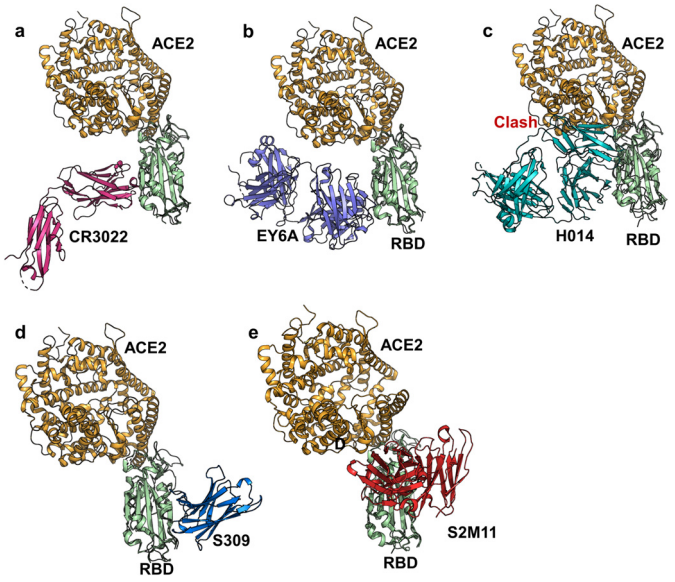

Fig. 3. A superposition of structures of the RBD core domain which contacts mAbs as well as ACE2. The RBD core domain, ACE2, CR3022, EY6A, H014, S309, and S2M11 are shown in green, orange, magenta, purple, cyan, blue, and red cartoons, respectively.

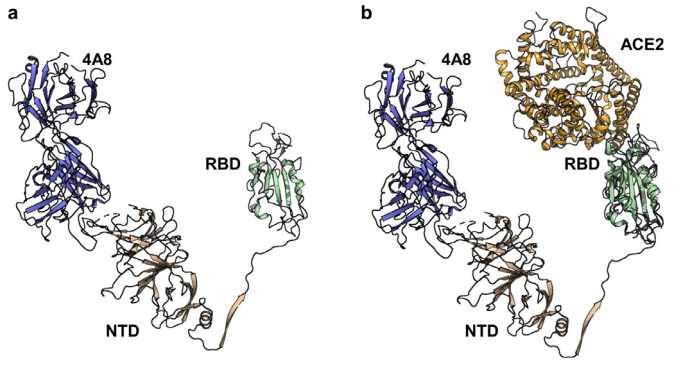

Fig. 4. 4A8 binding to the NTD of SARS-CoV-2 spike protein. (a) Structure of 4A8 in complex with SARS-CoV-2 spike protein. 4A8, the NTD and RBD of the spike protein are shown in purple, wheat and green cartoons, respectively. (b) A superposition of the structure of SARS-CoV-2 spike protein in complex with 4A8 on that of the spike-ACE2 complex. ACE2 is shown in orange cartoons. The binding site of 4A8 on the spike protein is distinct from that of ACE2.

brane surface. Monteil *et al* recently reported that recombinant soluble hACE2 (rshACE2) significantly blocked early stages of SARS-CoV-2 infections and protected the infected mice from lung injury [36]. To improve the binding affinity for the spike protein as well as the stability of rshACE2, Chan et al. carried out site-directed mutagenesis on ACE2 [37]. The binding affinity of engineered rshACE2 for the RBD of spike protein reached 600 pM, which is ~37-fold of the wild type hACE2 and comparable with that of mAbs.

The rshACE2 has some advantages over other therapeutics. First, SARS-CoV-2 has limited potential to develop resistance for escaping the rshACE2-mediated neutralization unless it also sacrifices its affinity for the native receptor ACE2. Besides, rshACE2 could potentially alleviate COVID-19 symptoms by proteolytic conversion of angiotensin peptides that regulate blood pressure and volume. In fact, the administration of rshACE2 is being tested against acute respiratory distress syndrome ARDS and acute lung injury [38,39]. Moreover, rshACE2 has proven to be safe in healthy human subjects and patients with lung diseases [40], and is being evaluated in a European phase II clinical trial for COVID-19 treatment [37].

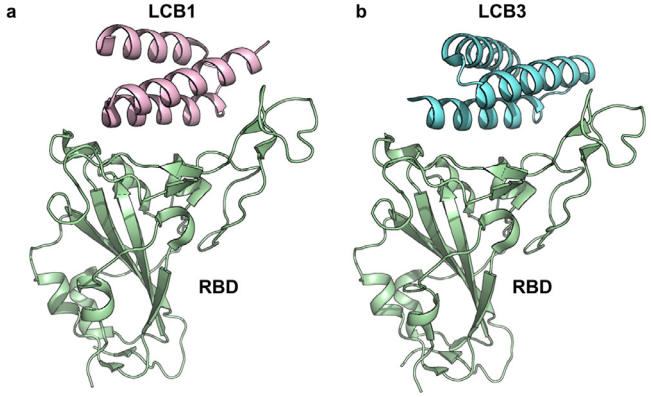

Fig. 5. Structures of the SARS-CoV-2 spike RBD in complex with miniproteins, LCB1 (a) and LCB3 (b). The RBD, LCB1 and LCB3 are shown in green, pink and cyan cartoons, respectively. Both LCB1 and LCB3 occupy the binding site of ACE2 on the RBD.

Some peptides could also interfere with the ACE2/spike interactions. Several peptides were designed and synthesized based on the complex structure of the spike protein bound with ACE2 in order to block the RBD binding to ACE2 and be used as inhaled therapeutics for topical lung delivery [41,42]. A 23-mer fragment of the ACE2 peptidase domain α1 helix was proven to engage the spike RBD with moderate binding affinity. Peptides or small molecules with improved affinity are needed to efficiently block the interaction between the spike RBD and ACE2 [41,42].

2.3. De novo design of miniproteins targeting the RBD

In the past decades, de novo design of proteins with a variety of folds and functions from first-principles has progressed from an outrageous concept to a routine accomplishment, which is now brimming with potential for application in pharmaceuticals [43,44]. Cao et al. set out to design high-affinity miniprotein binders for the SARS-CoV-2 spike RBD in order to compete with ACE2 [45]. Large pools of designed minibinders were screened for their binding to the RBD, leading to one ACE2-helix-based design and eleven de novo designs with binding affinity to the RBD ranging from 100 nM to 2 μM. Further optimization resulted in two designs (LCB1 and LCB3), showing subnanomolar binding affinity for the RBD, which are comparable to those of mAbs. Additionally, the melting temperature of two minibinders was over 95 °C, indicating the excellent thermal stability of the miniproteins. Structures of LCB1 and LCB3 in complex with spike ectodomain trimer were determined at a resolution of 2.7 Å and 3.1 Å, respectively. As shown in Fig. 5, both LCB1 and LCB3 occupied the ACE2 binding site, blocking the RBD binding to the receptor. Compared to mAbs, LCB1 and LCB3 possess enhanced stability, smaller size and comparable binding affinity, providing a prospect for direct delivery into the respiratory system through intranasal administration, nebulization or dry powder aerosol [45].

3. Inhibiting membrane fusion

Interactions of ACE2 with the spike protein lock the RBD in an ‘up’ conformation [46], which is followed by a further activation through proteolytic cleavages of the spike protein at the S1/S2 and S2’ sites by host proteases, like TMPRSS2 and cathepsin B/L [6]. As shown in Fig. 6, the cleavage at the S2’ site results in the release of a fusion peptide that is inserted into the host cell membrane, which is followed by the formation of an antiparallel six-helix bundle (6-HB) consisting of HR1 and HR2, promoting the fusion process and allowing the uncoating as well as the release of the viral RNA into cytoplasm. Accordingly, ligands stabilizing the prefusion form of the spike protein, inhibiting the cleavage of the

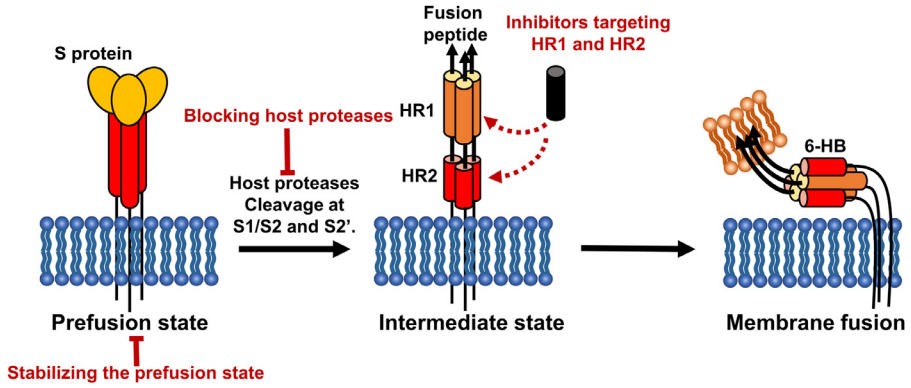

Fig. 6. Strategies used to block the proposed membrane fusion of SARS-CoV-2 with host cells. The cleavage at S2' generates a fusion peptide, which is inserted into the host cell membrane. Then, HR1 and HR2 form 6-HB to facilitate the membrane fusion.

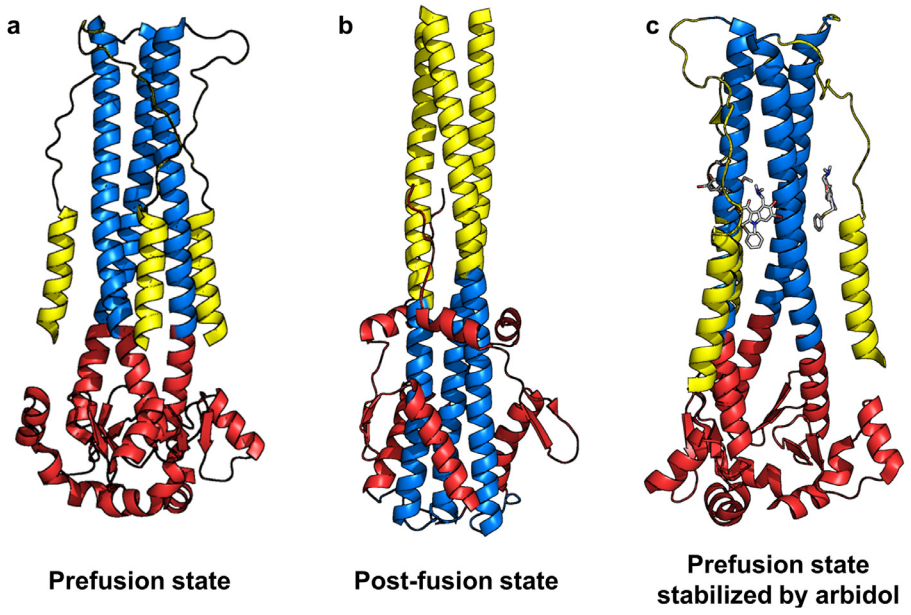

Fig. 7. Structures of influenza virus HA of H7N9 in a prefusion state (a), post-fusion state (b), and the prefusion state bound with arbidol (c).

host proteases on the spike protein, or preventing HR1 and HR2 from the rearrangement were able to block the membrane fusion of SARS-CoV-2 with the host cells, thus providing potential therapeutics against COVID-19.

3.1. Stabilizing the prefusion form of the spike protein

Arbidol (umifenovir) was used for prophylaxis and treatment of acute respiratory infections including influenza in China and Russia [47]. It is the only available antiviral drug that targets hemagglutinin (HA), the major glycoprotein involved in virus attachment to host cells. Studies have revealed that arbidol binds to HAs from various strains with K_ds ranging from 5.6 to 44.3 μM [48]. Kadam et al. determined the crystal structure of HA of H7N9 in complex with arbidol, revealing the binding mode of arbidol with HAs [49]. As shown in Fig. 7, arbidol binds into a hydrophobic cavity at the interface of the HA, which stabilizes the prefusion conformation of HA prevents its conformational rearrangement and thereby hampers the following membrane fusion. However, whether arbidol could hold the prefusion form of SARS-CoV-2 spike protein that is equivalent to HA of influenza remains to be elucidated. A clinical study in China including 69 patients with SARS-CoV-2 infection revealed that arbidol treatment showed tendency to improve the discharging rate and decrease the mortality rate [50]. In addition, an open-label randomized controlled trial in Iran showed that arbidol treatment significantly contributed to clinical and laboratory improvements such as peripheral oxygen saturation, requiring ICU admissions, duration of hospitalization, and chest CT involvements [51].

Stabilization of the prefusion form of spike protein thus serves as another promising approach for inhibiting the membrane fusion between SARS-CoV-2 and host cells.

3.2. Blocking host proteases

3.2.1. TMPRSS2

TMPRSS2, highly expressed in the respiratory epithelium cells, was reported to be required for the SARS-CoV-2 spike protein priming by Hoffmann et al. [1]. The contact of the virus with ACE2 may facilitate the S1 subunit cleavage by TMPRSS2 which is embedded in the cell membrane. Knockdown or knockout of TMPRSS2 significantly reduced the entry of SARS-CoV-2 into host cells [1,3], suggesting that TMPRSS2 is a potential target for drug development against the virus.

Three drugs including camostat mesylate, nafamostat mesylate and bromhexine hydrochloride have been found to exhibit potent inhibition against TMPRSS2 and to significantly reduce the entry of SARS-CoV-2 [1,52,53] (Fig. 8). Several clinical trials worldwide have recently been

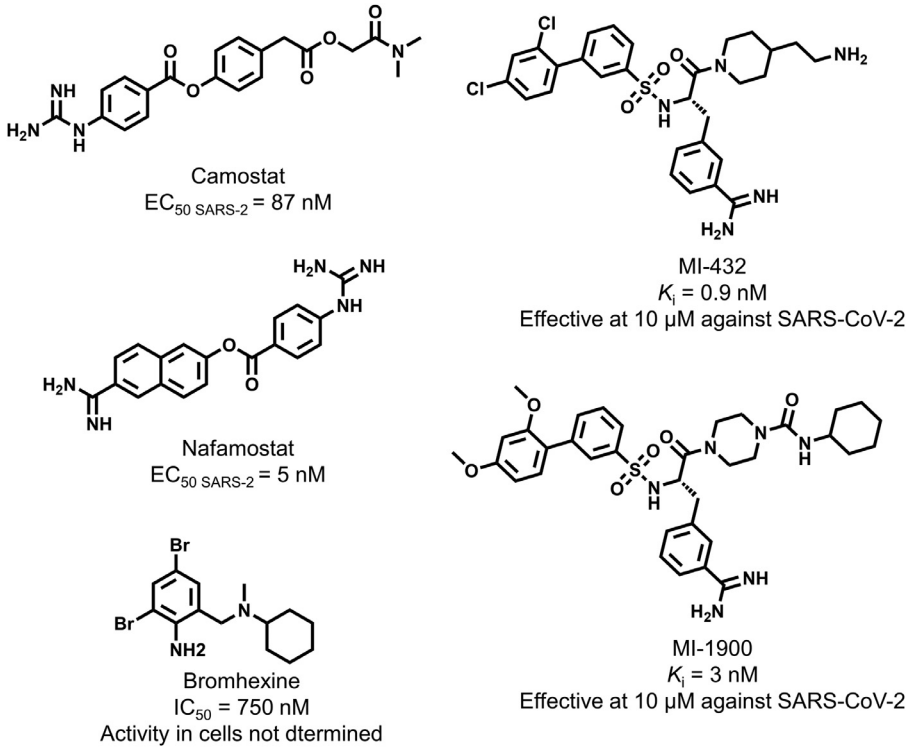

Fig. 8. Chemical structures of representative inhibitors of TMPRSS2.

initiated to assess the efficacy of camostat mesylate against COVID-19 [54].

MI-432 and MI-1900, two peptidomimetics developed in 2013, showed extremely potent inhibition against TMPRSS2, with K_i values of 0.9 nM and 3 nM, respectively [55] (Fig. 8). Bestle et al. evaluated the antiviral efficacy of these two inhibitors against SARS-CoV-2 in the Calu-3 cell line [3]. At a concentration of 10 μM, MI-432 and MI-1900 suppressed the multicycle replication of SARS-CoV-2.

3.2.2. Cathepsin B and L

Cathepsin B and L (CatB/L) belong to the papain family. Once SARS-CoV-2 reaches intracellular endosomes, CatB/L execute the proteolysis of the S1 subunit in the acidic endosome and lysosome compartments. E-64d, a non-selective and covalent cathepsin inhibitor using an epoxide ring as a warhead, efficiently blocked the spike-driven entry of SARS-CoV-2 into the TMPRSS2 knockout HEK293T cell line. Besides, the combined use of E-64d and camostat has been found to enhance the antiviral activity against SARS-CoV-2 in cell lines and to block the infection completely [1], providing evidence to consider CatB/L as host targets against COVID-19.

As SARS-CoV and MERS-CoV prefer the proteolysis performed by CatL when entering host cells, more attention has been paid to develop CatL inhibitors for anti-CoVs. In addition to E-64d, three CatL inhibitors including MDL-28170 [56], oxocarbazate [57], and K11777 [58] (Fig. 9) displayed antiviral activities against SARS-CoV. However, none of them have been proved effective against SARS-CoV-2. In addition, because CatL in the endosome and lysosome has been considered to be pH-sensitive, agents which are able to regulate the pH of the endosome or lysosome environment provide another option to block the membrane fusion. Chloroquine and hydroxychloroquine used for malaria treatment were capable of neutralizing endolysosomal pH, thus preventing the protease activities of CatL and inhibiting the membrane fusion. Recently, several clinical trials successively showed that hydroxychloroquine was significantly associated with viral load reduction or disappearance in patients with COVID-19 [59–62]. In contrast, results are negative in a multicenter, randomized, open-label, three-group, controlled clinical trial for the treatment of COVID-19 with chloroquine or hydroxychloroquine [63].

Additionally, the SARS-CoV-2 spike protein was also cleaved by other proteases like furin [3,5]. These proteases could be potential targets for combating SARS-CoV-2. However, attention should be paid to reducing toxicities of these inhibitors because the host proteases usually play important roles in many physiological processes.

3.3. Inhibitors targeting HR1 and HR2

As shown in Fig. 6, HR1 and HR2 of the S2 subunit play a key role in viral fusion by forming the 6-HB. This step brings the membrane of the host cell closer to that of a virus. Accordingly, peptides derived from HR1 and HR2 could theoretically block this event and thus prevent the membrane fusion. Several peptides derived from the HR2 region that could bind the HR1 have been previously reported for inhibition of enveloped viruses. For example, enfuvirtide, the first fusion inhibitor with 36 amino acids derived from the HR2 domain, has been approved for the treatment of HIV. In contrast, peptides derived from the HR1 domain tended to aggregate in the absence of HR2, and thereby did not display antiviral activity.

Previous studies reported that EK1, an optimized peptide derived from the HR2 region of HCoV-OC43, worked as a pan-coronavirus fusion inhibitor and was effective in inhibiting infections of five HCoVs, including SARS-CoV, MERS-CoV and 3 SARS-related CoVs. *In vivo* studies demonstrated that mice treated with EK1 were protected from HCoV-OC43 or MERS-CoV infections [64]. In a recent study, a crystal structure of the 6-HB fusion core of SARS-CoV-2 formed by HR1 and HR2 domains was determined at a resolution of 2.9 Å [65]. As shown in Fig. 10, the overall structure of SARS-CoV-2 6-HB is quite similar to that of other HCoVs, such as SARS-CoV and MERS-CoV, suggesting the potential of EK1 in disturbing the 6-HB formation of SARS-CoV-2. EK1 effectively inhibited the pseudotyped SARS-CoV-2 spike protein mediated membrane fusion, with an EC_{50} value of 2468 nM. To improve the inhibitory activity of EK1, a series of cholesteryl EK1 with multiple linkers were designed and synthesized, eventually leading to the

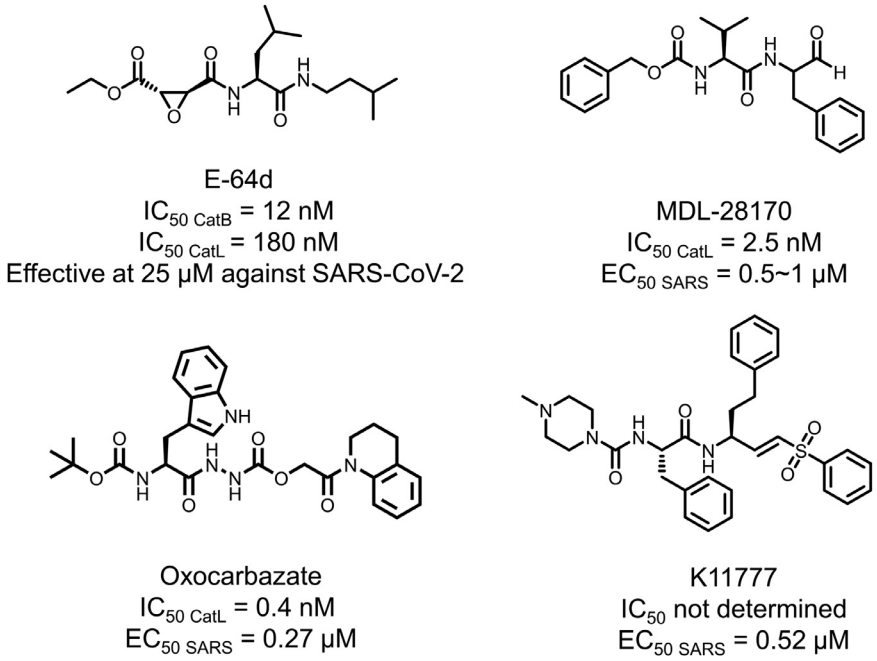

Fig. 9. Chemical structures of representative inhibitors of CatL.

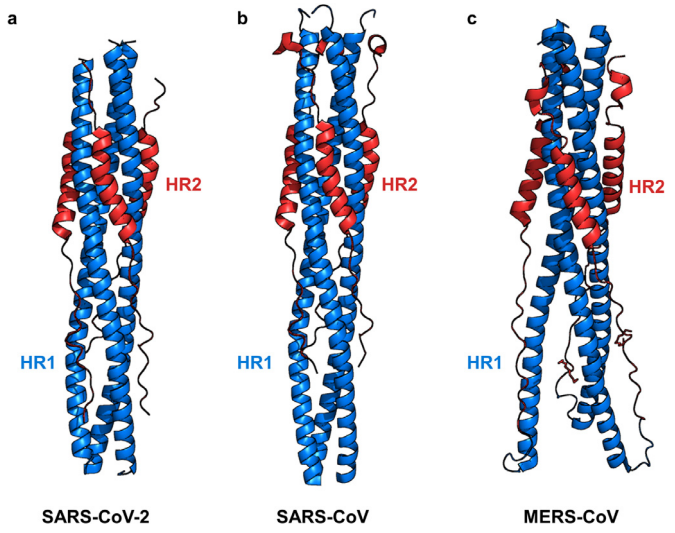

Fig. 10. Structures of SARS-CoV-2 (a), SARS-CoV (b), and MERS-CoV (c) 6-HB. HR1 and HR2 are shown in blue and red cartoons, respectively. The structures of 6-HB of SARS-CoV-2, SARS-CoV and MERS-CoV are quite similar.

discovery of lipopeptide EK1C4, which exhibited the most potent activity against the membrane fusion mediated entry of various pseudotyped coronaviruses including SARS-CoV-2. In cells, EK1C4 blocked the pseudotyped SARS-CoV-2 infection in a dose-dependent manner with an EC_{50} value of 36.5 nM, 67-fold more potent than that of EK1. Studies used to assess the *in vivo* efficacy of EK1C4 against SARS-CoV-2 are required [65].

A lipopeptide fusion inhibitor, termed IPB02, was designed based on the sequence of SRARS-CoV-2 HR2. Compared to IPB01 which is in the absence of cholesterol modification, IPB02 exhibited greatly increased α-helical stability and target-binding affinity. The EC_{50} values of IPB01 and IBP02 against the pseudotyped SARS-CoV-2 infection were reported to be 33700 nM and 80 nM, respectively [66].

4. Inhibiting the viral genome replication

Upon entry into host cells, the viral genome is uncoated and released into the host cells. As described in Introduction, 3CLpro, PLpro, RdRP and NSPs involved in the formation of DMVs are essential for the replication of SARS-CoV-2. Some host enzymes like DHODH that catalyzes the fourth step in the de novo pyrimidine biosynthesis pathway also play important roles in the replication of SARS-CoV-2 [67,68]. Therefore, inhibitors of these target proteins could efficiently reduce the replication of SARS-CoV-2, and thus resist COVID-19.

4.1. 3CLpro

Following an autolytic cleavage of itself from polyproteins pp1a and pp1ab, 3CLpro digests the polyproteins at 11 conserved sites to generate 13 mature NSPs (NSP4–16). The important function of 3CLpro in the viral life cycle together with the absence of closely related homologues in humans makes 3CLpro an attractive target for drug development against SARS-CoV-2 as well as other coronaviruses. Crystal structures of the SARS-CoV-2 3CLpro in the absence and presence of inhibitors have been determined. The SARS-CoV-2 3CLpro is composed of three domains (I-III), and the substrate-binding site is located in a cleft between domain I and domain II (Fig. 11). It shares features with previously reported 3CLpro of other coronaviruses. The high structure similarity suggests that previously reported 3CLpro inhibitors may inhibit the SARS-CoV-2 3CLpro too [69]. Since the outbreak of COVID-19, tremendous efforts have been made in identifying inhibitors of the SARS-CoV-2 3CLpro, which are mainly classified into peptidic and small molecule inhibitors.

4.1.1. Peptidic inhibitors/peptidomimetics

Substrates of 3CLpro are peptides, and therefore peptidic inhibitors derived from the substrates have been developed against 3CLpro [70]. To enhance the binding affinity of peptidic inhibitors to 3CLpro, amino acids of the substrate were substituted to fit the binding pocket perfectly. In addition, an irreversible or reversible warhead was attached to the peptidic inhibitors so as to form a covalent bond with the catalytic cysteine of 3CLpro, resulting in improved potency of the inhibitors.

Several peptidic inhibitors of 3CLpro from other coronaviruses, including N3 [69], 13b [71], GC373 [72–74], GC376 [72–74], bocepre-

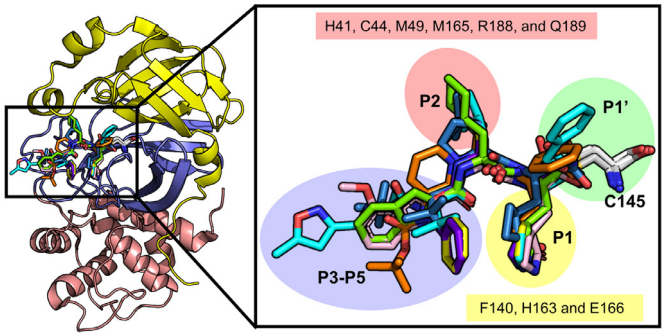

Fig. 11. An overlay of complex structures of the SARS-CoV-2 3CLpro with reported peptidic inhibitors. The SARS-CoV-2 3CLpro is shown in cartoons. Domains I - III are shown in yellow, purpleblue and pink, respectively. Peptide inhibitors are shown in sticks.

vir[73], and PF-00835231[75], have been proved effective against the SARS-CoV-2 3CLpro. The first five compounds were also reported to exhibit potent inhibition against SARS-CoV-2 replication, with EC_{50} values of 16.77, 4, 1.50, 0.90, and 15.57 μM, respectively. In addition, a series of newly designed peptidomimetics including 11a and 11b exhibited potent inhibitory activities against the SARS-CoV-2 3CLpro, with IC_{50} values of 0.053 and 0.040 μM, respectively [76]. The two compounds also had good anti–SARS-CoV-2 infection activity in cell culture, with EC_{50} values of 0.53 and 0.72 μM, respectively (Fig. 12) [76].

Crystal structures of the SARS-CoV-2 3CLpro in complex with the peptidomimetics including N3, 13b, GC373, GC376, boceprevir, PF-00835231, 11a, and 11b were also determined (Fig. 11). An overlay of these complex structures revealed that binding modes of the peptidomimetics were quite similar at the P1', P1 and P2 sites. At the P1' site, inhibitors were covalently linked to the catalytic C145 of the protease using the warheads such as α,β-unsaturated ketone (N3), α-ketoamide (13b, and boceprevir), aldehyde (GC373, GC376, 11a, and 11b) and hydroxymethylketone (PF-00835231). The bisulfite group of GC376 was first removed to generate an aldehyde group which further creates a covalent bond to C145. In addition, most of these inhibitors occupied the P1 site with an (*S*)-γ-lactam ring and formed hydrogen-bonds with the main chain of F140 as well as the side chain of H163/E166. Some hydrophobic groups were introduced to occupy the P2 site and establish hydrophobic interactions with H41, C44, M49, M165, R188, and Q189. However, at the P3, P4 and P5 sites, these inhibitors adopted different binding modes.

4.1.2. *Small molecule inhibitors*

A high-throughput screening of a library including ~10,000 compounds was performed, which identified two approved drugs, disulfiram and carmofur, and four drug candidates including ebselen, shikonin, PX-12, and tideglusib [69] (Fig. 13). The determined crystal structure of the SARS-CoV-2 3CLpro in complex with carmofur revealed that it covalently linked to C145 while its fatty acid tail occupied the hydrophobic P2 site [77]. The carbonyl oxygen of carmofur formed hydrogen bonds with the backbone amides of G143 and C145. The fatty acid tail formed hydrophobic interactions with H41, M49 and M165 (Fig. 14a).

A recent study by Su et al. identified baicalin and baicalein, two ingredients of a traditional Chinese medicine (Shuanghuanglian), as the noncovalent, nonpeptidomimetic inhibitors of the SARS-CoV-2 3CLpro (Fig. 14b). The IC_{50} values of baicalin and baicalein against the SARS-CoV-2 3CLpro were 6.41 and 0.94 μM, respectively. Baicalin and baicalein also exhibited antiviral activities in a cell-based system, with EC_{50} values of 27.87 and 2.94 μM, respectively. Remarkably, the binding mode of baicalein with the SARS-CoV-2 3CLpro determined by X-ray protein crystallography was distinctly different from those of known 3CLpro inhibitors. Baicalein was productively ensconced in the core of the substrate-binding pocket by interacting with two catalytic residues, the crucial P1/P2 sites and the oxyanion loop, acting as a "shield" in front of the catalytic dyad to effectively prevent substrate access to the catalytic dyad at the active site (Fig. 14b) [78]. Three phenolic hydroxyl groups of baicalein made multiple hydrogen bonds with the main chains of L141/G143 as well as the side chains of S144/H163 with or without the aid of a buried water molecule. The only carbonyl group established a hydrogen bond with the main chain of E166, while the free phenyl ring was inserted into the P2 subsite, making hydrophobic interactions with multiple residues including Q189, R188, M49, C44, and H41(Fig. 14b).

4.2. *PLpro*

The papain-like protease (PLpro) of SARS-CoV-2 recognizes an LXGG motif and cleaves the peptide bond on the second glycine, leading to the release of NSP1−3, which are essential for viral replication. In addition to the cleavages of the viral polyproteins, PLpro could help the virus suppress host immune response. Once the host cells are infected, the innate immune system generates an antiviral state through post-translational modification of host-cell proteins by ubiquitin (Ub) and interferon-stimulated gene product 15 (ISG15), which prompts the NFκB inflammation and IFN-I responses [80]. PLpro could efficiently remove the ISG15 and ubiquitin modifications from the proteins by its catalytic cysteine cleavage domain, dampening inflammation and antiviral signaling [80]. The essential roles of PLpro in SARS-CoV-2 replication and immune suppression made it a promising target for the development of antiviral drugs.

The amino sequence of the SARS-CoV-2 PLpro displays 83% sequence identity to that of the SARS-CoV PLpro [80]. Accordingly, the solved crystal structure of the SARS-CoV-2 PLpro is similar to that of the SARS-CoV PLpro. It consists of a ubiquitin-like domain, a thumb domain, a Zn-binding domain, and a palm domain (Fig. 15). The active site including the catalytic triad, C111–H272–D286, is located at the interface between the thumb and palm domains. As the substrate binding pocket of PLpro is conserved, inhibitors of the SARS-CoV PLpro usually inhibit the catalytic activity of the SARS-CoV-2 PLpro too.

In 2008, Ratia et al. reported a class of naphthalene derivatives identified as inhibitors of the SARS-CoV PLpro through a high-throughput screening of 50080 compounds [81]. Structure–activity relationship analysis and chemical optimization on these hits lead to the discovery of potent, noncovalent, competitive naphthalene inhibitors of the SARS-CoV PLpro, the IC_{50} values of which reached submicromolar. GRL-0617, one of the naphthalene inhibitors, was found to inhibit SARS-CoV replication in Vero cells with single digit micromolar potency [79]. Due to the good affinity of naphthalene inhibitors to the SARS-CoV PLpro and satisfactory antiviral activity in cells, investigators embarked on exploring their inhibitory effect on SARS-CoV-2. It was reported that GRL-0617 could also inhibit the activity of the SARS-CoV-2 PLpro with an IC_{50} value of 2.4 μM and the replication of SARS-CoV-2 in cells with an EC_{50} value of 27.6 μM [80]. The crystal structure of the SARS-CoV-2 PLpro in complex with GRL-0617 revealed that GRL-0617 bound to an allosteric site adjacent to the active site through an induced-fit mechanism, where the flexible blocking loop 2, which contains Y268, adopts a closed conformation in order to interact well with the inhibitor [80,82,83]. The amide bond of GRL-0617 formed hydrogen bonds with the side chain of D164 and the main chain of Q269.

In addition, two peptide-like compounds, VIR250 and VIR251, were developed as covalent inhibitors of the SARS-CoV-2 PLpro on the basis of an in-depth study on the substrate preference of the SARS-CoV-2 PLpro. The crystal structure of SARS-CoV-2 PLpro in complex with VIR250 and VIR251 showed that the catalytic C111 of the SARS-CoV-2 PLpro engaged in Michael addition to the carbon of the vinyl group of the vinylmethyl ester, resulting in formation of a covalent thioether linkage. In addition to the allosteric site, these peptide-like inhibitors also occupy the catalytic site formed by C111, H272 and D286. However, VIR250 and VIR251 showed a moderate inhibitory effect on the SARS-CoV-2 PLpro, with IC_{50} values of two-digit micromolar [84].

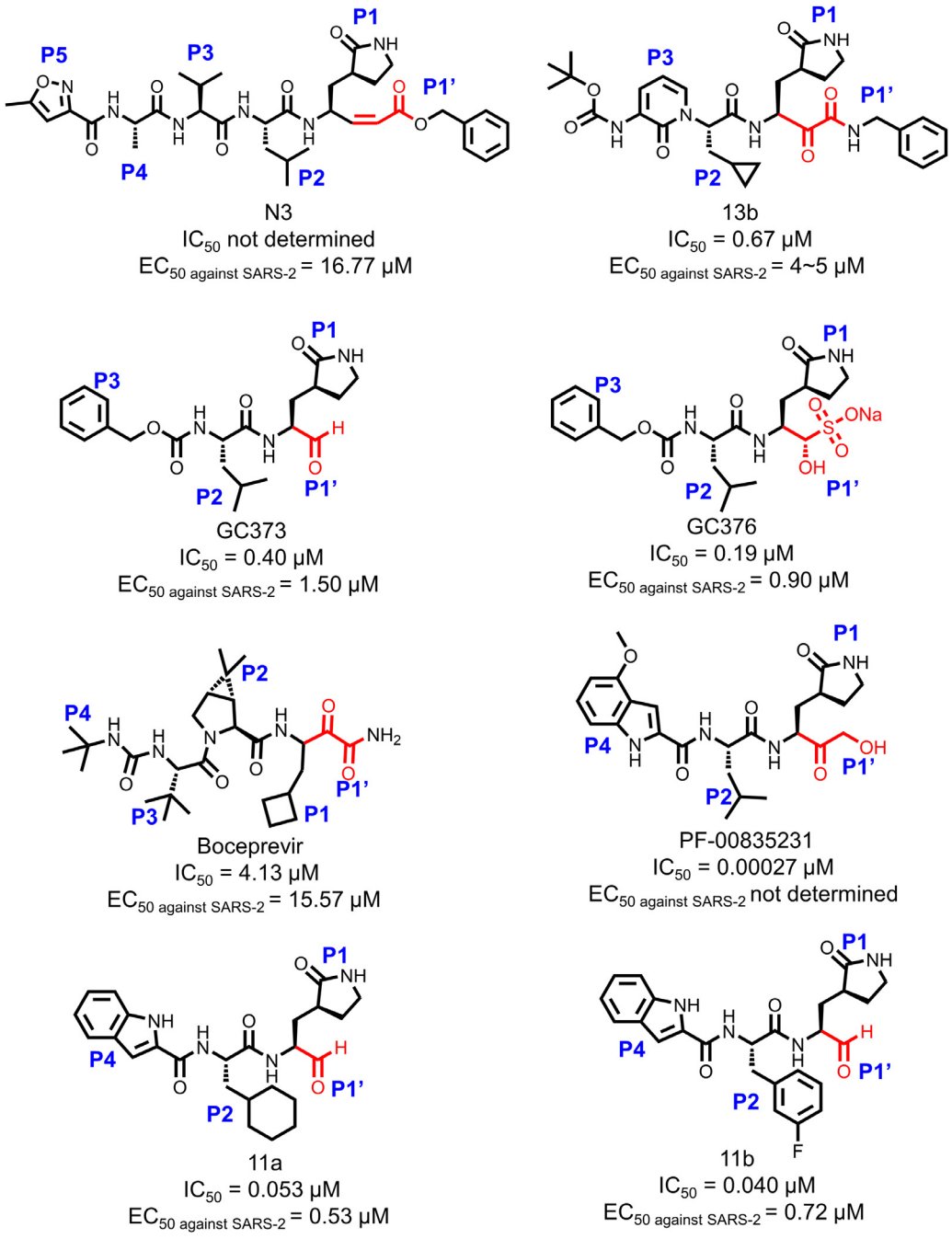

Fig. 12. Chemical structures of representative peptidic inhibitors of the SARS-CoV-2 3CLpro.

4.3. RdRp

RdRp is another crucial viral enzyme that assists in translation and replication of SARS-CoV-2, and it has no host-cell homolog. RdRp has a common function across several viral species, including influenza, hepatitis C virus, Zika, and CoVs, owing to its conserved overall structure as well as the active site. Thus, antiviral agents acting on RdRp of other RNA viruses may block the viral replication of SARS-CoV-2. Several research groups have determined the cryo-EM structures of SARS-CoV-2 RdRp, which confirmed that the SARS-CoV-2 RdRp has a conserved structure as other viral RdRps have [8,85,86]. The structure similarity makes drug repurposing an effective strategy to shorten the time of drug development. Over the past decade, several pharmaceutical companies focused on developing inhibitors against the RdRp of RNA viruses. Nucleotide and its analogs are an important class of developed RdRp inhibitors. Among them, favipiravir, ribavirin, sofosbuvir, baloxavir, and dasabuvir have been approved for the treatment of viral infection while another four nucleoside analogs including remdesivir, galidesivir, pimodivir, and beclabuvir moved to clinical trials [87]. Remdesivir which was originally developed for treating Ebola virus (EBOV) [88] was found to have a positive, therapeutic effect in patients with COVID-19. On October 22, 2020, remdesivir was approved by the FDA for the treatment of COVID-19.

Remdesivir is a prodrug that will be active when converted to a triphosphate form (GS-441524, the active form of remdesivir) in cells. GS-441524 shows broad-spectrum antiviral activity against vari-

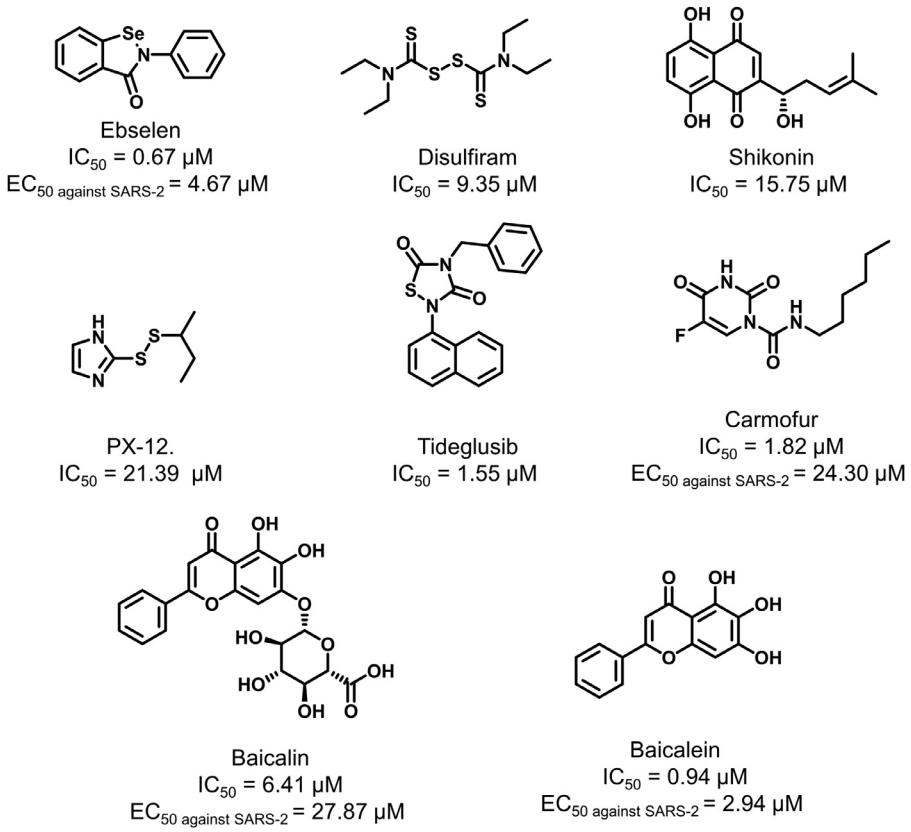

Fig. 13. Chemical structures of representative small molecule inhibitors of the SARS-CoV-2 3CLpro.

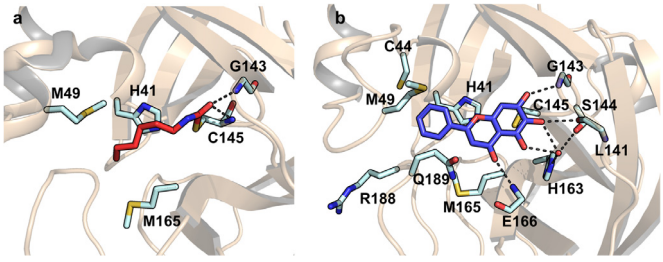

Fig. 14. Crystal structures of the SARS-CoV-2 3CLpro in complex with carmofur (a) and baicalein (b). (a) Carmofur covalently bound to C145 of the SARS-CoV-2 3CLpro. (b) Baicalein adopted a unique binding mode to non-covalently interact with the SARS-CoV-2 3CLpro. Proteins are shown in cartoons, and inhibitors as well as key residues are shown in sticks. Hydrogen bonds are shown as dash lines.

ous pathogenic RNA viruses, including multiple variants of EBOV, other filoviruses and human CoVs, in both cultured cells and animal models [89–92]. Wang et al. first explored the efficacy of remdesivir on SARS-CoV-2 infection and revealed that remdesivir had excellent inhibitory effect on replication of SARS-CoV-2, with an EC_{50} value of 0.77 μM [93], prompting the studies of remdesivir in COVID-19 clinical trials. The first reported case in the United States demonstrated that the treatment of remdesivir apparently improved symptoms of the COVID-19 patient on the eighth day [94]. Since then, more clinical trials were carried out to determine the safety and efficacy of remdesivir in treating COVID-19. Recently, a double-blind, randomized, placebo-controlled trial involved in 1062 COVID-19 patients revealed that remdesivir was superior to placebo in shortening the time to recovery and had evidence of lower respiratory tract infection [95].

4.4. NSPs involved in formation of DMVs

For coronaviruses such as MERS-CoV and SARS-CoV, the RTCs are anchored on DMVs derived from ER [96]. NSPs such as NSP3, NSP4 and NSP6 are thought to have the ability to induce DMV formation [96]. Inhibitors of these three NSPs may be capable of preventing DMV formation and thus impairing viral RNA synthesis. A pre-clinical compound named K22 was reported to impair DMV formation and viral RNA synthesis of a broad range of coronaviruses including SARS-CoV and MERS-CoV by interacting with NSP6 [97,98]. Studies are required to explore the inhibitory effect of K22 on RNA synthesis of SARS-CoV-2.

4.5. Host targets

It is widely known that viruses are unable to complete their replication without the help of the host. They will hijack various host proteins or pathways throughout their life cycles to facilitate the replication. The inhibition or knockdown of such host proteins will block the viral replication, providing potential antiviral targets for drug development. Recently, novel and potent inhibitors of human dihydroorotate dehydrogenase (DHODH) were reported as broad-spectrum antiviral agents against RNA viruses including SARS-CoV-2 [99]. DHODH is a rate-limiting enzyme in the de novo pyrimidine nucleotide biosynthetic pathway that catalyzes the stereospecific oxidation of (S)-dihydroorotate to orotic acid and finally generates uridine and cytosine, providing nucleotide resources for host cells as well as the viruses. Therefore, inhibition of DHODH affords a good strategy to block the replication of the viruses. Xiong *et al.* identified two novel and potent DHODH inhibitors, S312 and S416. These two inhibitors exhibited broad-spectrum antiviral activity against influenza A (H1N1, H3N2 and H9N2), Zika, Ebola, and SARS-CoV-2 in cells. Notably, the EC_{50} value of S416 against the SARS-CoV-2 replication in the cells was 0.017 μM and the resulting se-

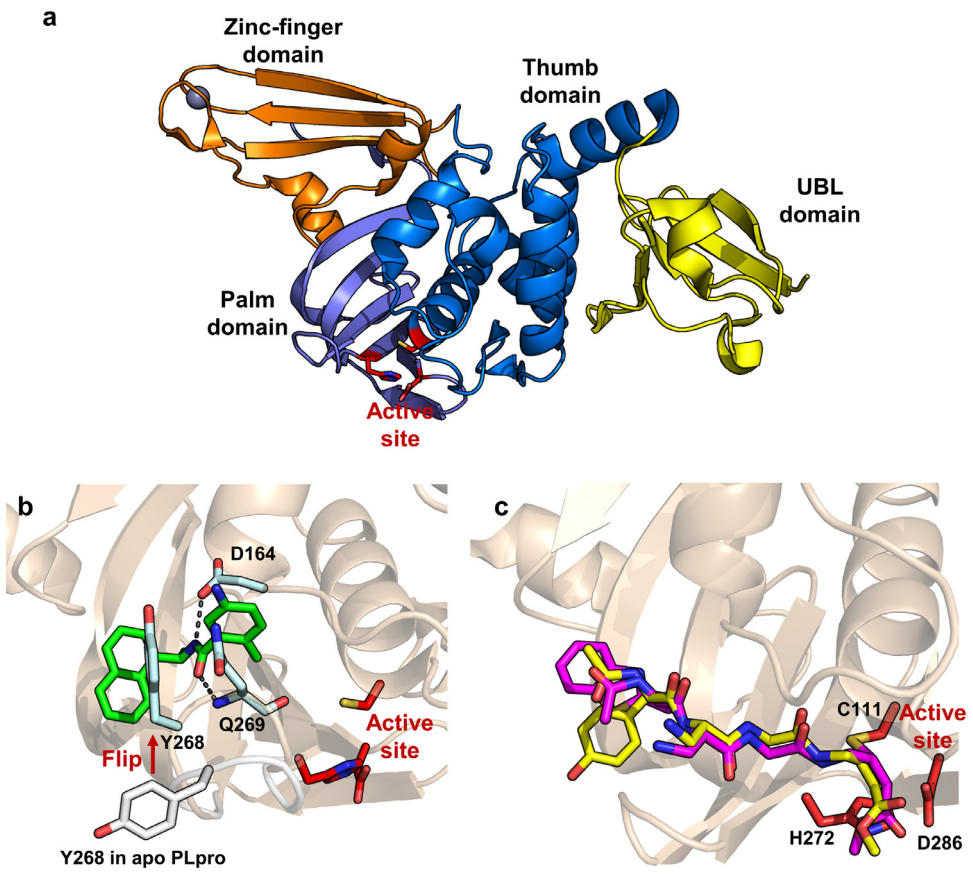

Fig. 15. Structures of the SARS-CoV-2 PLpro and the binding mode of its inhibitors in the substrate binding pocket. (a) The crystal structure of the SARS-CoV-2 PLpro. The ubiquitin-like (UBL), thumb, zinc-finger, and palm domains are shown in yellow, blue, orange, and purple, respectively. The active site is highlighted in red. (b) The binding mode of GRL-0617 in the SARS-CoV-2 PLpro. GRL-0617 is shown in green sticks. (c) The binding mode of VIR250 (yellow) and VIR251 (magentas) in the SARS-CoV-2 PLpro.

lectivity index (SI = CC_{50}/EC_{50}) reaches 10506. The *in vivo* study revealed that S312 had excellent antiviral efficacy in influenza A virus-infected mice. S312/S416 and the approved drugs targeting DHODH (leflunomide/teriflunomide) thus have therapeutic potentials in combating SARS-CoV-2 [99,100].

5. Conclusion and perspective

As a positive-sense, single-stranded RNA virus, SARS-CoV-2 utilizes host cells to accomplish various physiological processes, including the viral attachment, membrane fusion, genomic replication, assembly and budding of virions, ultimately leading to pathological damage of the host. Therefore, each stage of the viral life cycle could be a potential target for developing antiviral agents. The first step in the cycle is the viral attachment. A large number of agents, including antibodies, soluble ACE2, de novo designed miniproteins, and peptides, have been identified to hamper the spike RBD-mediated binding to ACE2. Following this receptor binding process, SARS-CoV-2 enters the host cell by membrane fusion with the help of some host proteases. Inhibitors of host proteases such as TMPRSS2 and CatL have been proved effective against SARS-CoV-2. In addition to the inhibitors of host proteases, small molecules stabilizing the prefusion state of the spike protein such as arbidol, and peptides derived from the HR2 region of the spike protein are able to prevent the membrane fusion between SARS-CoV-2 and the host cells, thus providing promising therapeutics for COVID-19. Once SARS-CoV-2 enters the host cells, the viral genome is released and replicated. Viral proteins, including 3CLpro, PLpro and RdRP, and some host targets such as DHODH, are essential for the replication of SARS-CoV-2. A number of inhibitors of these virial and host proteins have been developed to efficiently reduce the replication of SARS-CoV-2 genome. Therefore, many agents targeting various stages of the life cycle of SARS-CoV-2 have been developed. In the future, more preclinical and clinical studies are required to explore efficacy as well as safety of these agents in the treatment of COVID-19.

SARS-CoV-2 is a single-stranded RNA virus and has inherently higher rates of mutation than DNA viruses [101]. If the mutation changes the virus phenotype and host biological consequences, it could have profound implications for combating the epidemic [102]. Recently, mutations in the spike protein such as D614G and N501Y were found, which might induce its conformational changes and enhance its binding affinity to ACE2 [103–105]. To overcome this problem, a combination use of mAbs targeting different epitopes or agents simultaneously blocking several key events in the viral life cycle may prevent the evolution of viral escape mutants and achieve better efficacy [106].

Author contributions

Haixia Su drafted the paper. Yechun Xu and Hualiang Jiang revised the draft.

Declaration of Competing Interest

The authors declare no conflict of interest.

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