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Potential of coronavirus 3C-like protease inhibitors for the development of new anti-SARS-CoV-2 drugs: Insights from structures of protease and inhibitors

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ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), similar to SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), which belong to the same *Betacoronavirus* genus, induces severe acute respiratory disease that is a threat to human health. Since the outbreak of infection by SARS-CoV-2 began, which causes coronavirus disease 2019 (COVID-19), the disease has rapidly spread worldwide. Thus, a search for effective drugs able to inhibit SARS-CoV-2 has become a global pursuit. The 3C-like protease (3CL^{pro}), which hydrolyses viral polyproteins to produce functional proteins, is essential for coronavirus replication and is considered an important therapeutic target for diseases caused by coronaviruses, including COVID-19. Many 3CL^{pro} inhibitors have been proposed and some new drug candidates have achieved success in preclinical studies. In this review, we briefly describe recent developments in determining the structure of 3CL^{pro} and its function in coronavirus replication and summarise new insights into 3CL^{pro} inhibitors and their mechanisms of action. The clinical application prospects and limitations of 3CL^{pro} inhibitors for COVID-19 treatment are also discussed.

1. Introduction

Since the outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) began in 2019, the infection has rapidly spread worldwide [1]. Infection with SARS-CoV-2 causes dry cough, fever, shortness of breath and acute respiratory distress syndrome (ARDS), which may lead to death [2]. As of 26 April 2020, a total of 2 774 135 SARS-CoV-2-infected cases and 190 871 related deaths have been confirmed worldwide (who.sprinklr.com/). SARS-CoV-2 has a high transmission efficiency, with the reproduction number (R_0) estimated to be 2.5 [3]. Many experts predict that SARS-CoV-2 may persist for a long time and will cause at least 500 000 deaths worldwide [4]. The World Health Organization (WHO) has announced coronavirus disease 2019 (COVID-19), the disease caused by SARS-CoV-2 infection, as a public health emergency of international concern (PHEIC) [5]. Therefore, developing safe and effective anti-SARS-CoV-2 drugs is urgently required.

SARS-CoV-2 is a member of the family Coronaviridae, which comprises the largest positive-sense, single-stranded RNA viruses [6]. These viruses are classified into four genera (α, β, γ and δ). SARS-CoV, Middle East respiratory syndrome coronavirus (MERS-CoV) and SARS-CoV-2 are betacoronaviruses [7]. Analysis of the genome sequences of these three viruses has revealed that SARS-CoV-2 has a higher identity to SARS-CoV (89.1% nucleotide similarity) than to MERS-CoV [8]. The 3C-like protease (3CL^{pro}) is a cysteine protease that hydrolyses the viral polyproteins pp1a and pp1ab to produce functional proteins during coronavirus replication. Because of its highly conserved sequence and essential functional properties, 3CL^{pro} has been validated as a potential target for the development of drugs to treat SARS, MERS and COVID-19 [9–12]. At present, a variety of natural and synthetic inhibitors targeting different sites and regions of 3CL^{pro} have been developed [13–15]. As the highly conserved catalytic sites of 3CL^{pro} are shared

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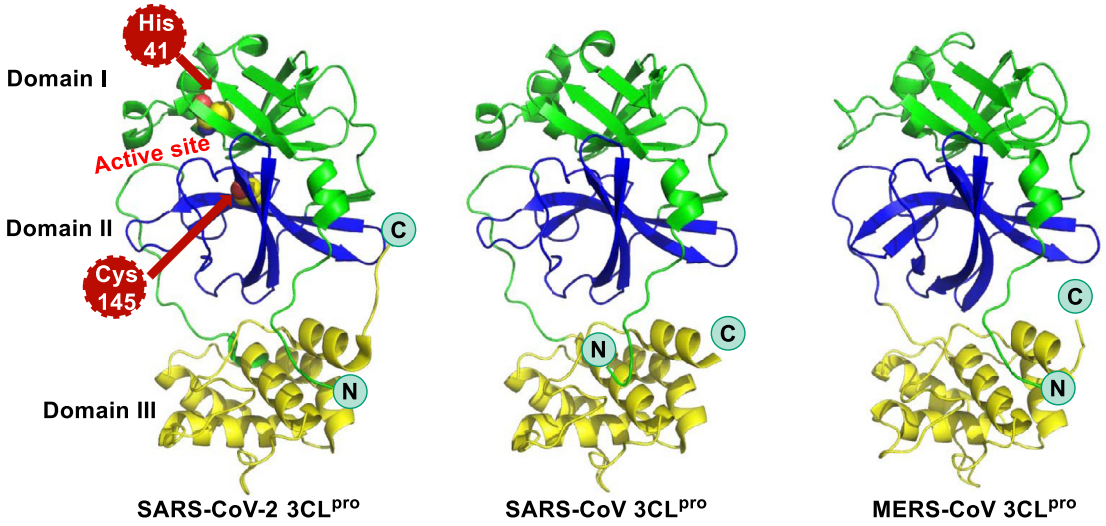

Fig. 1. Three-dimensional structures of SARS-CoV-2 3CL^{pro} (PDB ID: 6M03), SARS-CoV 3CL^{pro} (PDB ID: 2C3S) and MERS-CoV 3CL^{pro} (PDB ID: 4YLU). Domains I–III are coloured in green, blue and yellow, respectively. Two main amino acid residues (His41 and Cys145) in the catalytic site of SARS-CoV-2 3CL^{pro} are indicated as CPK and are coloured by atom types.

by the three coronaviruses [12,16], tremendous efforts have been made to study this target to speed up the search for anti-SARS-CoV-2 drugs from among previously approved drugs, clinical trial candidates and bioactive agents that were identified in preclinical studies as potential treatments for SARS and MERS [17]. These studies may provide more potential active compounds or drug candidates for the development of new drugs against COVID-19 [18].

In this review, we briefly describe recent developments in determining the crystal structure of 3CL^{pro} and highlight its structural differences among SARS-CoV-2, MERS-CoV and SARS-CoV. We further summarise new insights into 3CL^{pro} inhibitors and their mechanisms of action, with a particular focus on newly reported potential SARS-CoV-2 3CL^{pro} inhibitors discovered through virtual screening and in vitro experiments. In addition, prospective clinical applications and limitations of 3CL^{pro} inhibitors for COVID-19 treatment are discussed.

2. Structure of 3C-like protease (3CL^{pro})

2.1. SARS-CoV 3CL^{pro} structure

In SARS-CoV, 3CL^{pro} cleaves 11 sites in the polyproteins, with the recognition sequence Leu–Gln↓(Ser, Ala, Gly), including its own N- and C-terminal autoprocessing sites, by recognising the P1′ and P1–P4 sites [19]. A recent study has indicated that 3CL^{pro} cleaves its C-terminal autoprocessing site through the subsite cooperativity of Phe P2 and Phe P3′ [15]. Three types of SARS-CoV 3CL^{pro} crystal structures have been elucidated, including the wild-type active dimer, monomeric forms with G11A, S139A or R298A mutation on the dimer interface [18], and a superactive octamer [20]. In these structures, there are three domains in each protomer, domains I (residues 8–101) and II (residues 102–184) containing N-terminal residues, and domain III (residues 201–303). N-terminal residues form a typical chymotrypsin fold, and C-terminal residues form an extra domain [21] (Fig. 1). Active residues, which are located in a gap between domain I and domain II, can be divided into subsites S1–S6. The catalytic dyad His41–Cys145 is at the S1 subsite [20]. The crucial role of the S1 subsite includes the formation of an oxyanion hole when the carboxylate anion of a conserved Gln at the cleavage site interacts with Cys145, Ser144 and Gly143, which can stabilise the transition during proteolysis [22,23]. The hydrophobic side chains are located at the S2 and S4 subsites. Subsites S5 and S6 are far from the catalytic dyad and close to the surface of the structure, thus contributing little to substrate binding [10]. In the homodimer structure, seven residues at the very N-terminus (also as known as the N-finger) are squeezed between protomers A and B and interact with the two terminal domains of each protomer. These interactions have been proven essential for dimerisation. Furthermore, the regions around residues Asn214, Glu288–Glu290 and Arg298–Gln299 at the C-terminus have been confirmed to be important for enzyme dimerisation [24].

2.2. MERS-CoV 3CL^{pro} structure

The 3CL^{pro} sequences of MERS-CoV and SARS-CoV have 51% similarity [25]. In contrast to the tightly associated dimer of SARS-CoV 3CL^{pro}, the MERS-CoV 3CL^{pro} requires a ligand to form a weakly associated dimer [26]. All of the available MERS-CoV 3CL^{pro} structures have been solved in the presence of a ligand and adopt a conformation similar to that of SARS-CoV 3CL^{pro}, with a backbone root-mean-square deviation (RMSD) of 1.06 Å over 232 Cα atoms in the protomers (Fig. 1). In the active site, a preferred small amino acid residue at the P2 position induces a narrow S2 pocket of MERS-CoV 3CL^{pro}. Consistently, none of the 11 cleavage sites contains a phenylalanine residue in MERS-CoV. Instead, leucine (Leu) is primarily favoured at the P2 position, followed by methionine (Met) [26]. These differences between the active sites in the enzyme structures may explain why previously reported inhibitors of SARS-CoV 3CL^{pro} could not potently suppress the activity of MERS-CoV 3CL^{pro} without structural modifications. On the dimer interface of SARS-CoV 3CL^{pro}, two arginine residues (Arg4 and Arg298) are required to form some indispensable interactions for dimerisation. The corresponding residues (Val4 and Met298) are not involved in dimer formation in MERS-CoV 3CL^{pro}. Substrate binding and dimer formation are affected by some non-conserved residues that are adjacent to the key residues [27].

2.3. SARS-CoV-2 3CL^{pro} structure

The similarity between the 3CL^{pro} sequences of SARS-CoV-2 and SARS-CoV has been shown to be 96%; of the 306 residues, only 12 residues are different, namely T35V, A46S, S65N, L86V, R88K, S94A, H134F, K180N, L202V, A267S, T285A and I286L [18]. As expected, the 3CL^{pro} structure of the novel coronavirus is a contact dimer,

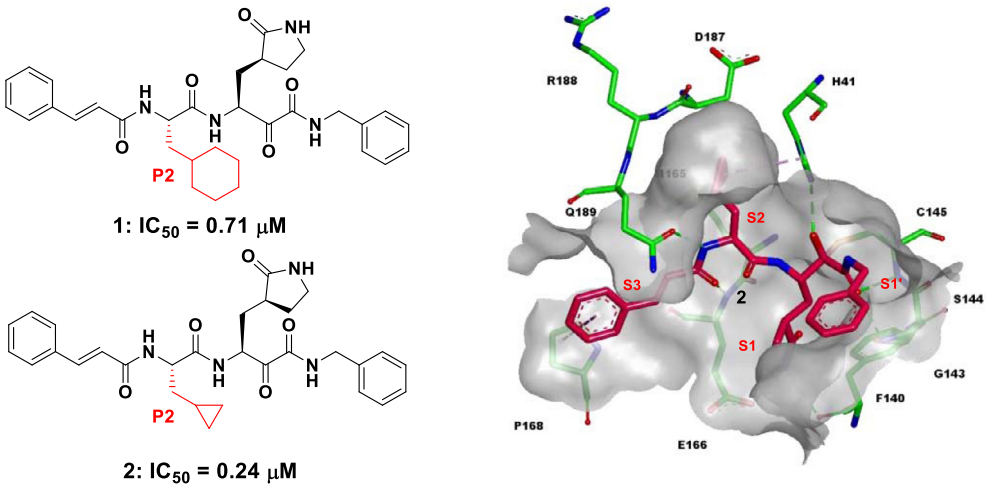

Fig. 2. Chemical structures of α-ketoamides **1** and **2** and X-ray crystallography of the complex of **2** and SARS-CoV 3CL^{pro} (PDB ID: 5N5O).

which is similar to the 3CL^{pro} structure of SARS-CoV. When overlapped, the two 3CL^{pro} structures of SARS-CoV-2 (PDB ID: 6M03) and SARS-CoV (PDB ID: 2C3S) (Fig. 1) present a RMSD of 0.53 Å over the 277 Cα atoms. Furthermore, all the residues surrounding both active sites are oriented in almost the same direction, except for Ala46 in the S2 subsite of SARS-CoV-2 3CL^{pro}. Ala46 is located close to the surface of the SARS-CoV-2 3CL^{pro} structure, but this residue is replaced by a serine in SARS-CoV 3CL^{pro}. Another striking difference is observed on the dimer interface. In the SARS-CoV 3CL^{pro} dimer, a polar interaction is formed by the hydroxyl groups of the Thr285 residue in domain III of each protomer, which is due to a hydrophobic interaction between Thr285 and Ile286, whilst in the SARS-CoV-2 3CL^{pro} dimer, Thr285 and Ile286 are substituted by alanine and leucine, respectively. These mutations lead to a slightly closer packing of the two domains III of the dimer against one another, and transitions in the catalytic centre, which gain a higher catalytic activity [28].

3. 3C-like protease (3CL^{pro}) inhibitors and their mechanisms of action

3CL^{pro} is indispensable for coronavirus replication but has not been found in host cells, making this enzyme an ideal target for antiviral agents. Based on the crystal structure of 3CL^{pro}, a variety of inhibitors have been developed in the past 5 years. Numerous 3CL^{pro} inhibitors have been reported, including peptide mimetics [29] and small molecules. However, most studies have mainly focused on small-molecule compounds, through virtual screening based on the crystal structure of 3CL^{pro}, and through verification of the effects of candidate compounds on the enzyme activity or viral load in vitro.

3.1. SARS-CoV 3CL^{pro} inhibitors

3.1.1. α-Ketoamide inhibitors

Based on the conserved active sites of the main protease of coronaviruses, a structure-based design of α-ketoamides was carried out to achieve broad-spectrum antiviral activities against alpha- and betacoronaviruses as well as enteroviruses [30]. It was found that optimisation of the functional group at the P2 site of α-ketoamides was crucial to find the best compromise for different sizes of the S2 pocket of 3CL^{pro}. The designed compound **1** (P2 = cyclohexylmethyl) showed the most potent inhibition of SARS-CoV 3CL^{pro} (IC$_{50}$ = 0.71 μM). The crystal structure (PDB ID: 5N5O) of SARS-CoV 3CL^{pro} in complex with compound **2** (P2 = cyclopropylmethyl) showed that the P2 substituent fitted snugly into the flexible S2 pocket, resulting in hydrophobic interactions with the Met49, Met165 and Asp187 residues and forming hydrogen bonds (H-bonds) between Gln189 and the P2 residue (Fig. 2). Notably, owing to the high similarity between the 3CL^{pro} enzymes of SARS-CoV and SARS-CoV-2, compound **1** is expected to be a potential antiviral candidate against SARS-CoV-2.

3.1.2. Serine derivatives

A collection of serine derivatives derived from tetrapeptide inhibitor **3** (IC$_{50}$ = 98 nM) and D-serine derivative **4**, which was identified as a non-peptidyl small-molecule inhibitor (IC$_{50}$ = 30 μM) (Fig. 3), was designed and screened against a SARS-CoV 3CL R188I mutant protease [31]. Compound **4** showed practically no cytotoxicity towards HeLa cells (CC$_{50}$ > 200 μM). A detailed docking simulation analysis (**4** with 3CL^{pro}; PDB ID: 3AW1) suggested that the P1′, P1 and P4 substituents in the D-serine skeleton fitted well into the S1′, S1 and S4 pockets, respectively. Further optimisation of **4** led to the generation of a series of new scaffolds of phenylisoserine derivatives [32]. Among them, SK80 (**5**) showed an inhibitory effect on the 3CL mutant protease (IC$_{50}$ = 43 μM) (Fig. 3). The binding mode of compound **5** with 3CL^{pro} (PDB ID: 3AW1) indicated that the functional groups at the P1′, P1, P2 and P4 sites of **5** formed hydrophobic interactions with the S1′, S1, S2 and S4 pockets of the protease, respectively. In addition, two amide groups of **5** formed H-bonds with the His164 and Gln189 residues.

3.1.3. Flavonoids

A library of flavonoids consisting of 10 different scaffolds was tested in vitro for inhibitory effects on SARS-CoV 3CL^{pro} [9]. Among the compounds, herbacetin (**6**; PubChem CID: 5280544), rhoifolin (**7**; PubChem CID: 5282150) and pectolinarin (**8**; PubChem CID: 168849) were found to exert prominent inhibitory effects, with IC$_{50}$ values of 33.2, 27.5 and 37.8 μM, respectively (Fig. 4). An induced-fit docking study of compounds **6, 7** and **8** with SARS-CoV 3CL^{pro} (PDB ID: 4WY3) showed that herbacetin (**6**) formed four H-bonds at the S2 site, and the 8-hydroxyl group was essential for the formation of H-bonds with Glu166 and Gln 189. Interestingly, the binding modes of rhoifolin (**7**) and pectolinarin (**8**) were different to that of herbacetin (**6**). The bulky carbohydrates attached to the chromen-4-one core skeleton fitted well into the S1 and S2 pockets of the protease. The binding interactions with the S1, S2 and S3′ subsites might contribute to the high affinity of rhoifolin (**7**) with SARS-CoV 3CL^{pro}.

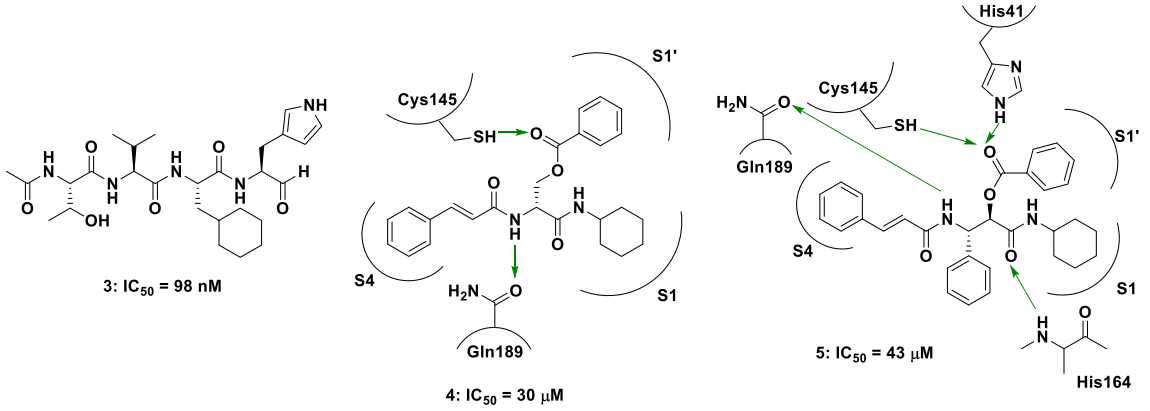

Fig. 3. Chemical structures of serine derivatives and their interactions with SARS-CoV 3CL^{pro}; the green arrows represent H-bond interactions.

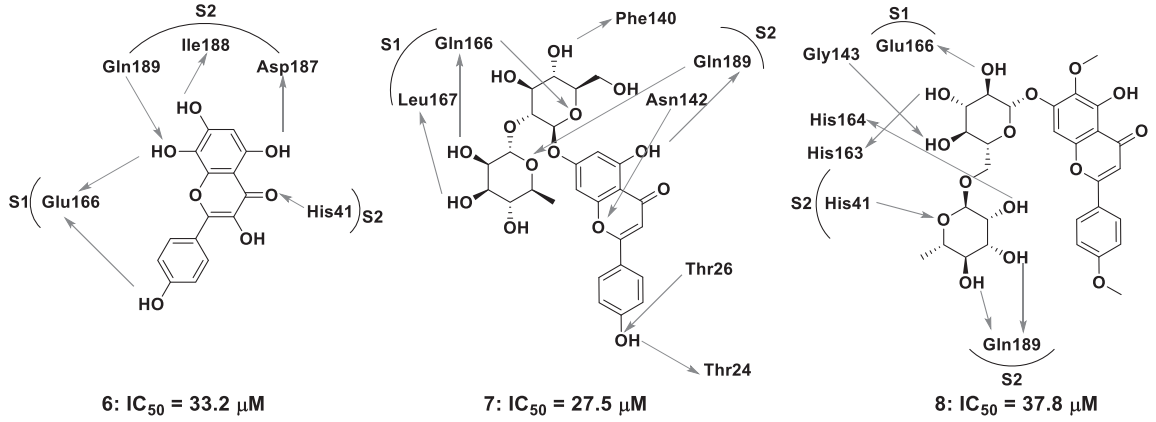

Fig. 4. Chemical structures of flavonoids and their interactions with SARS-CoV 3CL^{pro}.

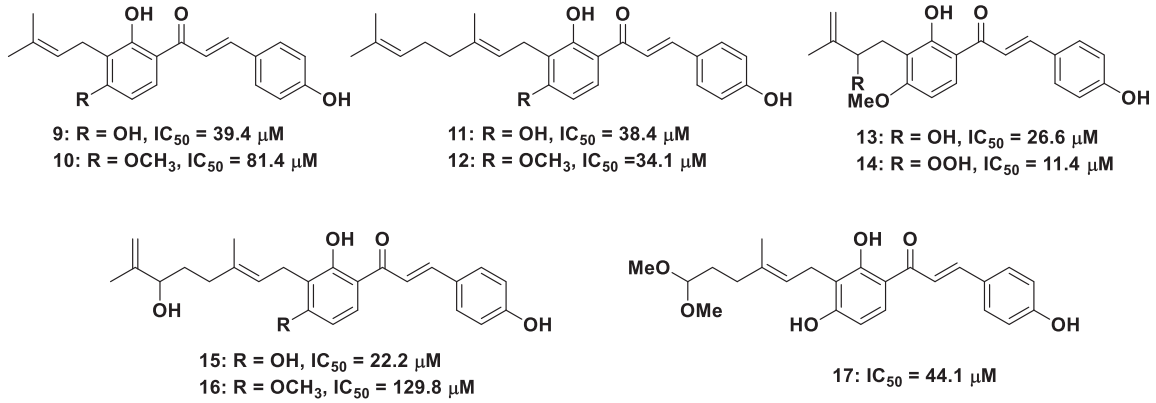

Fig. 5. Chemical structures of chalcones and their inhibitory activities against SARS-CoV 3CL^{pro}.

3.1.4. Chalcones

A series of alkylated chalcones (**9**–**17**) isolated from *Angelica keiskei* were evaluated for their inhibitory activities against SARS-CoV 3CL^{pro} [33] (Fig. 5). Among these chalcones, compound **14**, with a perhydroxyl group, showed the most potent inhibitory effect (IC_{50} = 11.4 ± 1.4 μM). These results suggested that the perhydroxyl group might be critical for binding to SARS-CoV 3CL^{pro}. Furthermore, docking studies of compound **14** with 3CL^{pro} (PDB ID: 2ZU) showed that the carbonyl and hydroxyl groups formed H-bonds with His163 and Ser144, respectively. Notably, the perhydroxyl group of **14** formed a strong H-bond with the vital residue Cys145.

3.1.5. Pyrazolones

Based on reported neuraminidase inhibitors, a library of pyrazolone derivatives was synthesised and screened against SARS-CoV 3CL^{pro} [10] (Fig. 6). Compound **18**, with a pyrazolone ring surrounded by three hydrophobic groups, showed the most potent inhibition, with an IC_{50} of 5.8 ± 1.5 μM. Structure–activity relationship analysis suggested that the phenyl pharmacophore in ring C and the carboxylate in ring A were essential for the inhibitory activity. A detailed docking simulation analysis of **18** with 3CL^{pro} (PDB ID: 2ALV) showed that the carboxylate group of ring A formed H-bond interactions with the vital residues Gly143, Ser144 and Cys145 at the S1 subsite. The furan B ring was found to inter-

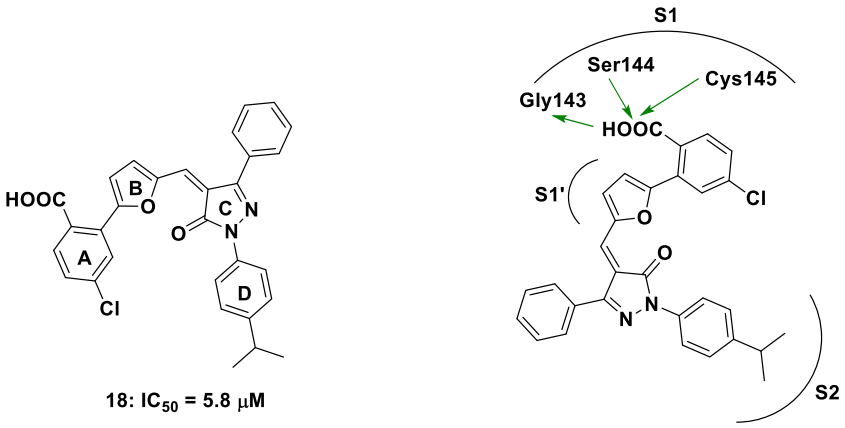

Fig. 6. Chemical structure of pyrazolone **18** and its interactions with SARS-CoV 3CL^{pro}.

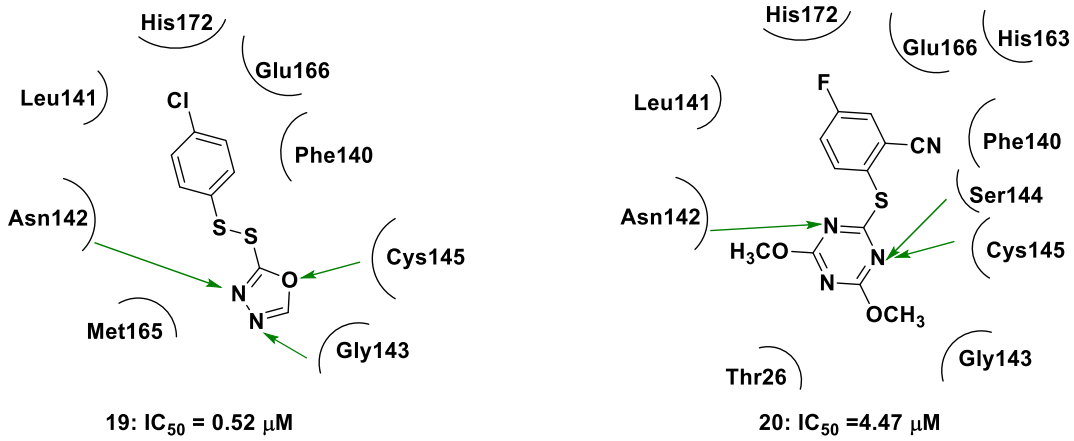

Fig. 7. Chemical structures of aromatic disulfide **19** and pyrithiobac derivative **20**.

act with the hydrophobic Leu residue in the S1′ pocket, and ring D fitted well in the hydrophobic S2 subsite.

3.1.6. Unsymmetrical aromatic disulfides and pyrithiobac derivatives

Wang et al. reported novel unsymmetrical aromatic disulfide inhibitors against SARS-CoV 3CL^{pro} with excellent IC_{50} values (0.52–5.9 μM) [34]. Among them, 1,3,4-oxadiazole disulfide (**19**) exhibited the most potent inhibition, with an IC_{50} value of 0.516 ± 0.06 μM (Fig. 7). Subsequent enzymatic kinetic studies showed that compound **19** acted as a reversible and non-competitive inhibitor. The binding mode of **19** was predicted using simulation models in a docking study with 3CL^{pro} (PDB ID: 2AMD). Compound **19** formed hydrophobic interactions with Phe140, Leu141, His163, Glu166 and His172, whilst the 1,3,4-oxadiazole group formed multiple H-bonds with Asn142, Gly143 and Cys145. As part of an ongoing investigation of novel inhibitors [35], a new class of 55 pyrithiobac derivatives were synthesised and evaluated for their inhibitory activities. Among these synthetic compounds, sulfide **20**, with a 1,3,5-triazin ring and a phenyl ring, exhibited promising inhibitory activity with an IC_{50} value of 4.47 μM (Fig. 7). Molecular docking studies of **20** with 3CL^{pro} (PDB ID: 2AMD) showed that multiple hydrophobic interactions were generated between **20** and the His172, Glu166, His163, Gly143, Leu141, Phe140 and Thr26 residues. Furthermore, four H-bonds were formed between the 1,3,5-triazin ring and Asn142, Ser144 and Cys145.

3.2. MERS-CoV 3CL^{pro} inhibitors

3.2.1. Dipeptidyl aldehyde bisulfite adducts

It was reported that GC376 (**21**; PubChem CID: 71481119) displayed potent inhibition toward MERS-CoV in cell-based systems [36]. Compound **21** could effectively reduce the viral load of MERS-CoV, with an EC_{50} value of 0.9 μM (Fig. 8). A cocrystal structure of MERS-CoV 3CL^{pro} (PDB ID: 5WKJ) with **21** indicated a covalent binding mode between **21** and Cys148 through the formation of a tetrahedral hemithioacetal adduct. Because of a hydrophobic-driven interaction between **21** and the lactam ring of the Gln surrogate side chain of 3CL^{pro}, a new inhibitor, GC813 (**22**), was designed and its antiviral activity was evaluated in a cell-based system (EC_{50} = 0.5 μM). Based on the above findings, attachment of a piperidine motif to the peptidyl component of the inhibitor was proposed to improve the interaction with the S1–S4 pockets. Thus, a novel class of inhibitors containing piperidine motifs as design elements was discovered. Among them, two compounds (**23** and **24**) exhibited the most potent inhibitory effects against MERS-CoV, both in terms of the 3CL^{pro} activity (IC_{50} values of 0.4 μM and 0.7 μM, respectively) and viral load (EC_{50} values of 0.5 μM and 0.8 μM, respectively) [36].

3.2.2. Peptide aldehydes

Because the Cys148 residue is highly conserved in the active sites of both proteases, enterovirus 71 3C^{pro} inhibitors were screened for their inhibitory effects against MERS-CoV 3CL^{pro}. The

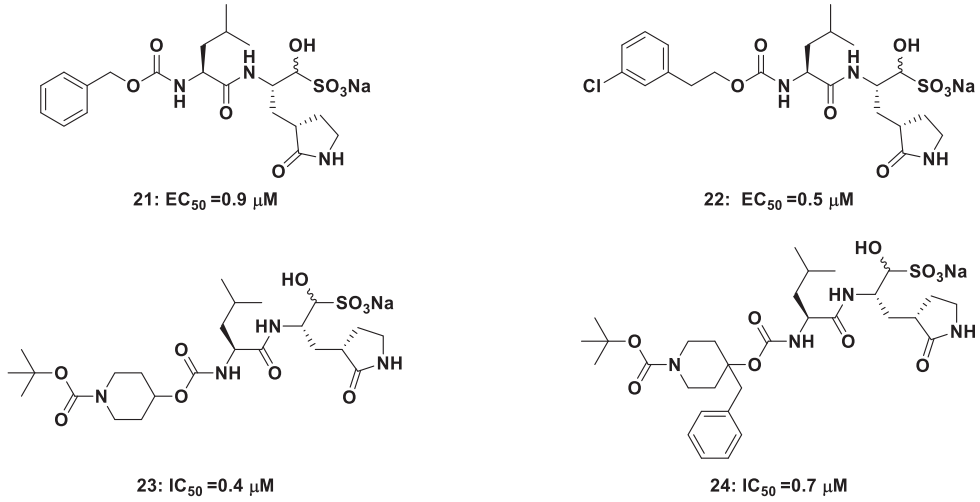

Fig. 8. Chemical structures of dipeptidyl aldehyde bisulfite adducts.

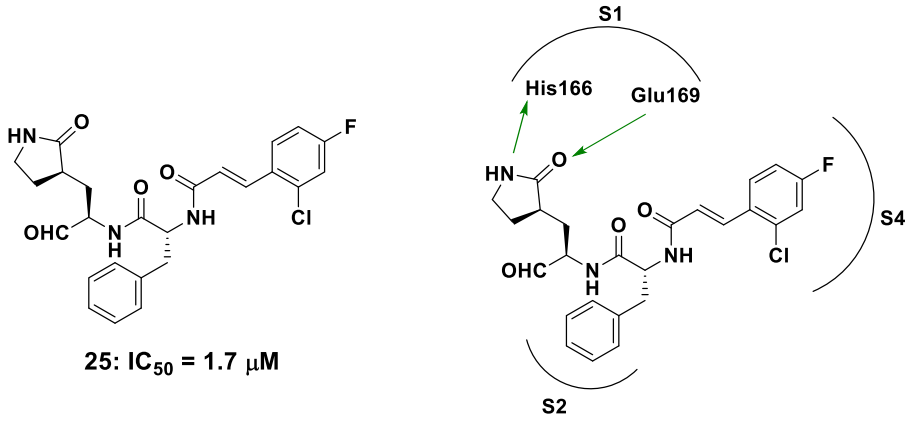

Fig. 9. Chemical structure of the peptide aldehyde **25** and its interactions with MERS-CoV 3CL^{pro}.

peptide aldehyde **25** exhibited prominent inhibitory activity, with an IC_{50} value of 1.7 ± 0.3 μM, and also suppressed viral replication, with an EC_{50} value of 0.6 ± 0.0 μM (Fig. 9). A docking model revealed that a covalent bond was formed between the γ-sulfur of Cys148 and the aldehyde carbon of **25**, and the generated oxyanion was stabilised by His41. Moreover, multiple H-bonds were formed to enhance the direct binding, e.g. the P1 lactam moiety of **25** interacted with His166 and Glu169 at the S1 subsite, and the amide group between the cinnamoyl and phenylalanine groups interacted with Gln192 and Glu169 [16].

3.2.3. Pyrazolones

The neuraminidase inhibitor 3k (**26**) is also known as the best inhibitor of MERS-CoV 3CL^{pro} (IC_{50} = 5.8 ± 1.6 μM) (Fig. 10). The carboxylate group of **26** was shown to interact with Ser147 and His166 to destabilise the oxyanion hole at the S1 subsite. Moreover, the lactam group in the pyrazolone core interacted with Glu169 through H-bonds, and the phenyl group in ring C formed a π–π stacking interaction with His41. The S2 pocket of MERS-CoV 3CL^{pro} is smaller than that of SARS-CoV 3CL^{pro}, making the phenyl group in ring C of **26** deeply incorporated into the S2 pocket of MERS-CoV 3CL^{pro} [10].

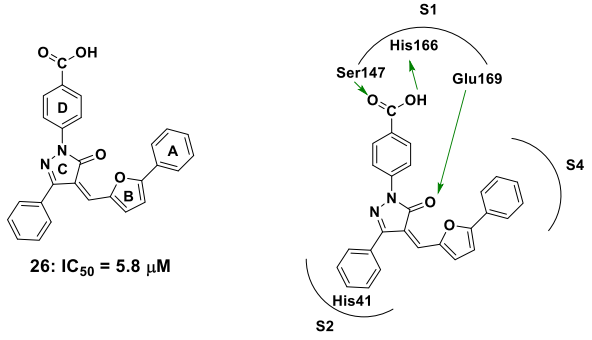

Fig. 10. Chemical structure of the neuraminidase inhibitor 3k (**26**) and its interactions with MERS-CoV 3CL^{pro}.

3.3. SARS-CoV-2 3CL^{pro} inhibitors

3.3.1. Drug repurposing

The emergence of SARS-CoV-2 has put drug repurposing on the fast track. The anti-human immunodeficiency virus (HIV) drug combination lopinavir/ritonavir (PubChem CID: 11979606) has been approved for phase III clinical trials for COVID-19 [37–40]. In virtual docking experiments, lopinavir (**27**; PubChem CID: 92727) and ritonavir (**28**; PubChem CID: 392622) were predicted to bind to the key residues Thr24, Thr26 and Asn119 in the 3CL^{pro} pocket to form two H-bonds each [41]. In addition, ASC09F, as a fixed-dose combination of ASC09 (**29**) and ritonavir for HIV infection, is undergoing phase III clinical trials in combination with oseltamivir (**30**; PubChem CID: 65028) (ClinicalTrials.gov ID: NCT04261270) (Fig. 11). Given the similarity of the genomic sequences encoding the 3CL^{pro} catalytic sites of SARS-CoV-2, MERS-CoV and SARS-

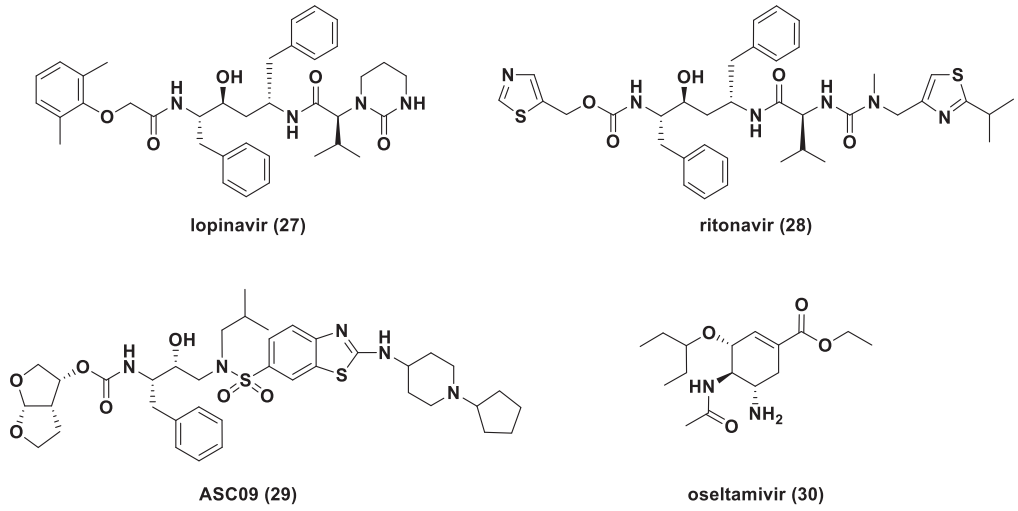

Fig. 11. Chemical structures of lopinavir, ritonavir, ASC09 and oseltamivir.

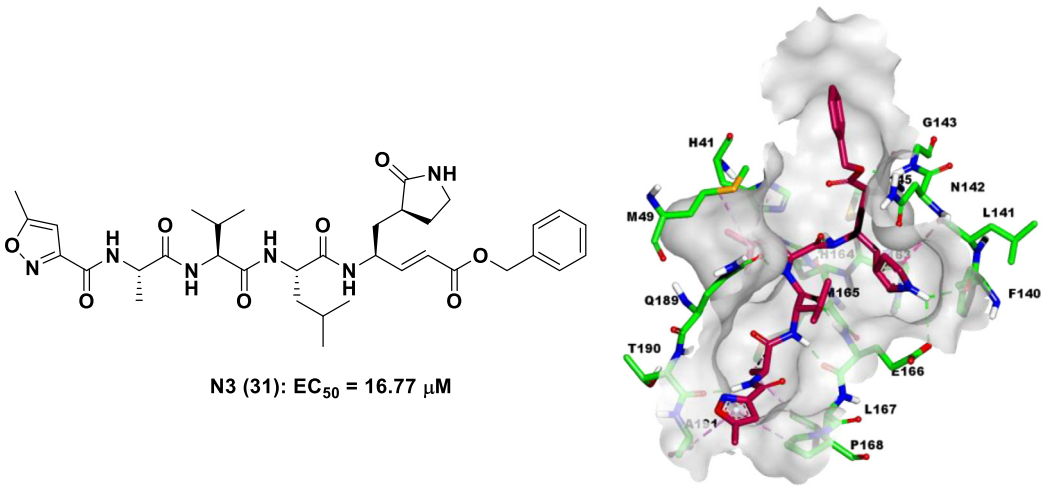

Fig. 12. Chemical structure of the Michael acceptor inhibitor N3 (**31**) and X-ray crystallography of the complex of N3 and SARS-CoV-2 3CL^{pro} (PDB ID: 6LU7).

CoV, previously reported 3CL^{pro} inhibitors for SARS and MERS have been widely investigated for their action in COVID-19 [42]. Examples include pyrithiobac derivatives, unsymmetrical aromatic disulfides and SK80 (**5**) for SARS and GC813 (**22**) for MERS, as wells as GC376 (**21**), a peptidomimetic inhibitor (**1**) [15,16] and an neuraminidase inhibitor analogue (**26**) [10] for SARS and MERS, which are being investigated in preclinical studies as potential candidates for COVID-19 treatment [43].

3.3.2. Peptidomimetic inhibitors

A competitive inhibitor that binds to the active site of a protease may block the enzyme activity by competing with a specific substrate. Peptidic inhibitors usually mimic natural substrates and can be further optimised by attaching chemical agents such as epoxy ketones, aldehydes, Michael acceptors and halomethyl ketones [44]. The Michael acceptor inhibitor N3 (**31**), based on specific computer-aided drug design for inhibiting 3CL^{pro}, is a potent irreversible inhibitor that fits inside the substrate-binding pocket of the enzyme [45]. Compound **31** showed multiple H-bonds with the main chain of the residues in the substrate-binding pocket and significantly prevented (at a concentration of 10 μM) the SARS-CoV-2-induced cytopathic effects in infected cells [46] (Fig. 12). Two preferred substrates of SARS-CoV-2 3CL^{pro} (Ac–Abu–Tle–Leu–Gln–ACC and Ac–Thz–Tle–Leu–Gln–ACC) were identified through HyCoSuL screening and the best recognised natural amino acid substrate was Ac–Val–Lys–Leu–Gln–ACC, with a kinetic parameter of 228.4 ± 9.9 μM [47]. The poor oral bioavailability and metabolic stability of peptides or peptidomimetics are major obstacles for their drug development. Therefore, further studies of small molecules with favourable pharmacokinetic properties may offer a promising alternative.

3.3.3. Other natural and synthetic agents

The natural product 5,7,3′,4′-tetrahydroxy-2′-(3,3-dimethylallyl)isoflavone (**32**) was extracted from *Psorothamnus arborescens* (Fig. 13). Homology modelling and molecular dynamic simulations showed that compound **32** formed H-bonds with the Cys145 and His41 catalytic dyad of SARS-CoV-2 3CL^{pro} and interacted with Gln189, Glu166, His164, Gly143, Asn142, Met49, Ser46, Thr45, Cys44, Thr26, Thr25 and Thr24 receptor-binding sites, with a docking score of −16.35 and a binding affinity of −29.57 kcal/mol [25]. Docking procedures based on a crystal structure of SARS-CoV-2 3CL^{pro} (PDB ID: 6LU7) demonstrated that two hydropyrimidinedione compounds, CP-1 (**33**) and CP-2 (**34**), had binding energies of −70.6 ± 3.9 kcal/mol and −69.9 ± 4.0 kcal/mol, respectively. These compounds formed H-bonds with

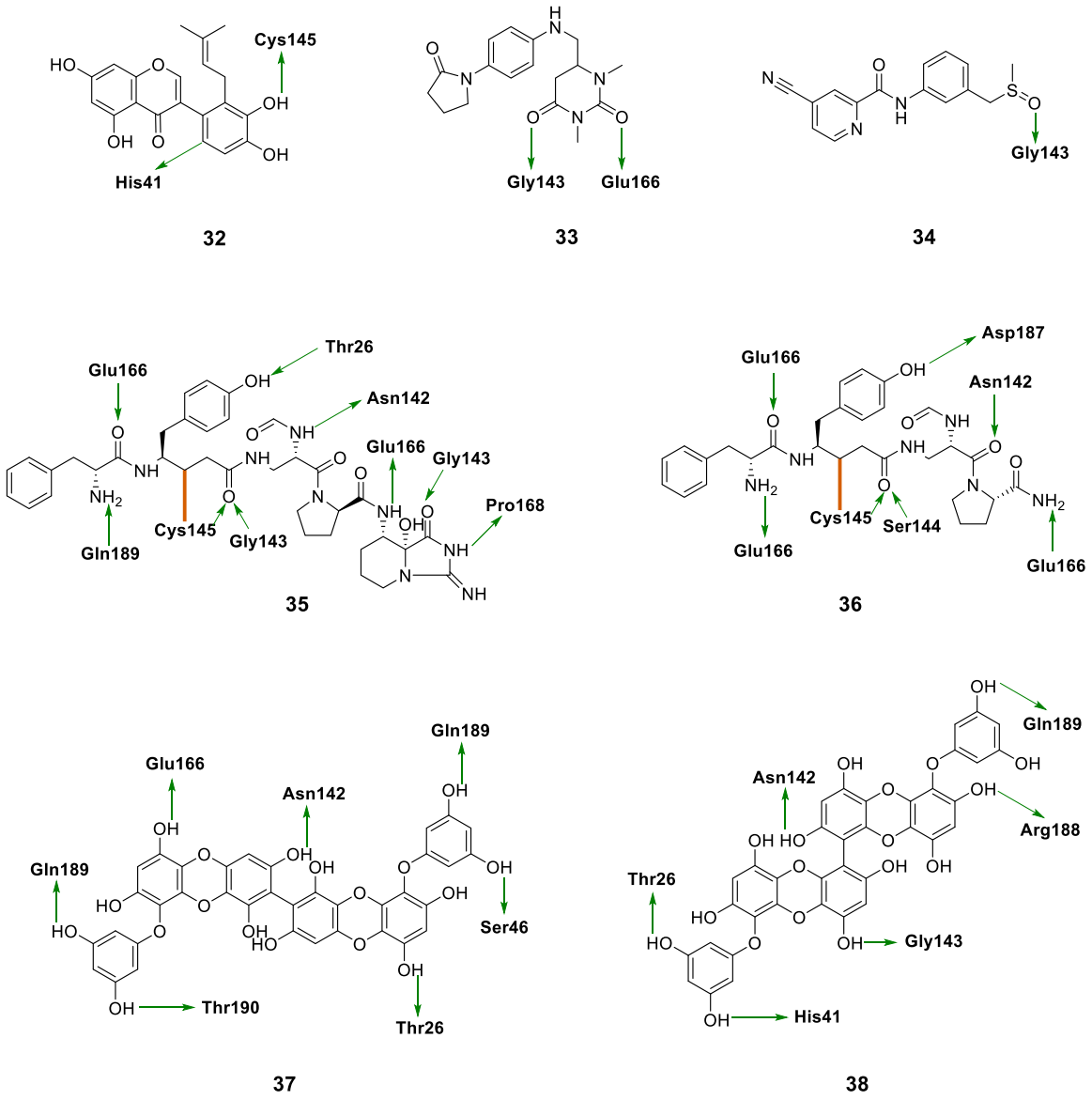

Fig. 13. Chemical structures of potent SARS CoV-2 3CL^{pro} inhibitors and their interactions with SARS-CoV-2 3CL^{pro}.

the Gly143 and Glu166 residues and a π–π stacking interaction with His41 [48]. Pseudotheonamides C (**35**) and D (**36**), isolated from the marine sponge *Theonella swinhoei*, formed a covalent bond with the Cys145 residue of SARS-CoV-2 3CL^{pro}. These two compounds occupied a similar position within the catalytic site and formed H-bonds with the Asn142, Ser144 and Glu166 residues, whilst their benzyl groups fitted into the hydrophobic pockets in the enzyme structure. These compounds acted similarly to Michael acceptor covalent inhibitors, with binding energies of −14.4 kcal/mol and −14.9 kcal/mol, respectively [49]. The phlorotannins 8,8′-bieckol (**37**) and 6,6′-bieckol (**38**) from *Ecklonia cava* formed an extensive network of H-bonds with the His41 and Cys145 residues of SARS-CoV-2 3CL^{pro}, with binding energies of −12.9 kcal/mol and −12.1 kcal/mol, respectively [49]. In the case of an improved α-ketoamide inhibitor (**39**), its amide oxygen interacted with the main-chain amides of Cys145, Ser144 and Gly143 to form an oxyanion hole and to bind to the P3–P2 amide bond integrated into a pyridone ring. Compound **39** showed inhibition of purified recombinant 3CL^{pro} enzymes from SARS-CoV-2, MERS-CoV and SARS-CoV, with IC_{50} values of 0.58–0.90 μM [28] (Fig. 14).

4. Conclusion and perspectives

SARS-CoV-2, which is causing the current pandemic of COVID-19, a severe respiratory illness, has spread globally to more than 200 countries since its emergence in late 2019 in China. Thus far, no drug or vaccine has been shown to be effective for COVID-19 treatment. Slow detection of SARS-CoV-2 and limited therapeutic options for COVID-19 are the major challenges. Some countries are urgently carrying out drug development research for treatment of this disease. A variety of antiviral drug targets have drawn widespread interest among scientists, including (i) RNA-dependent RNA polymerase, (ii) papain-like protease, (iii) 3CL^{pro}, (iv) spike glycoproteins and their receptors [angiotensin-converting enzyme 2 (ACE2) for SARS-CoV and dipeptidyl peptidase 4 for MERS-CoV] and (v) helicase [50]. High-resolution crystal structures of 3CL^{pro} exhibited highly conserved cleavage sites, and numerous 3CL^{pro} inhibitors have been reported, which may hasten the process of anti-COVID-19 drug discovery. Molecular docking methods and binding mode analyses are feasible and practical options for virtual screening of inhibitors targeting the key sites of SARS-CoV-2 3CL^{pro}. Many

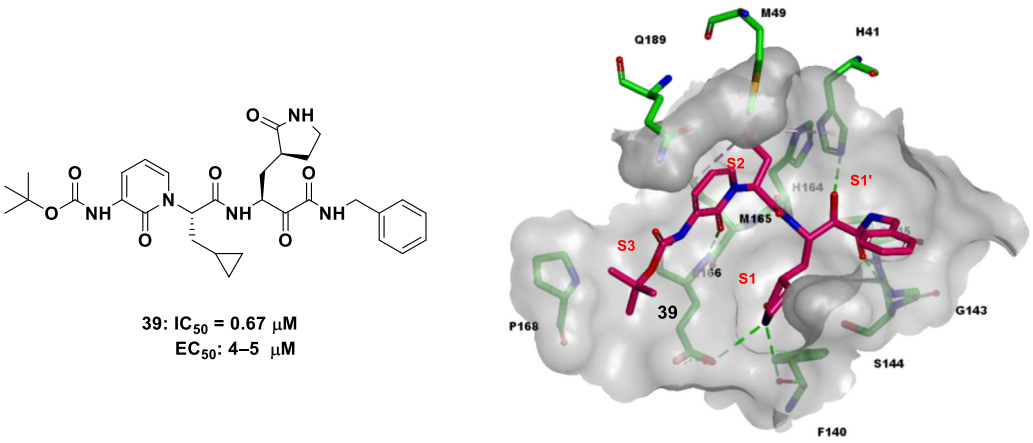

Fig. 14. X-ray crystallography of the complex of the improved α-ketoamide inhibitor **39** and SARS-CoV-2 3CL^{pro} (PDB ID: 6Y2F).

3CL^{pro} inhibitors that were previously reported for SARS and MERS may be candidate inhibitors for SARS-CoV-2 3CL^{pro}.

Repurposing of antiviral drugs, as well as screening and modification of existing 3CL^{pro} inhibitors, may provide a fast-track approach for COVID-19 treatment. Kaletra® (lopinavir/ritonavir), an anti-HIV drug, has received much attention amid the current COVID-19 outbreak [42,51]. In addition, ASC09F, lopinavir and ritonavir, which are currently in clinical trials, and GC376, GC813 and SK80, which are in preclinical studies, may deserve even more attention. Studies on 3CL^{pro} inhibitors are usually focused on substrate-binding sites (S1′–S1–S2–S3–S4), cleavage sites (P1–P4 and P1′–P4′), the catalytic dyad (His41 and Cys145) and other key residues, such as Thr190, Gln189, Glu166, Met165, Ser144, Gly143, Asn142, Leu141 and Phe140. Because the catalytic sites are highly conserved in the SARS-CoV, SARS-CoV-2 and MERS-CoV 3CL^{pro} structures, existing inhibitors of the 3CL^{pro} enzymes of the two other coronaviruses may also be effective against SARS-CoV-2 3CL^{pro}. Although virtual screening makes it possible to discover inhibitor molecules within a relatively short time, the antiviral activities of these agents still need to be experimentally tested in relevant cell and animal models. In addition, cocrystallisation experiments would provide insights into the mechanisms of inhibitor binding to 3CL^{pro} of SARS-CoV-2.

Taken together, 3CL^{pro} inhibitors have a great potential for the development of new drugs against SARS-CoV-2. Although some known 3CL^{pro} inhibitors will accelerate the discovery and development of anti-SARS-CoV-2 drug candidates, there is a long way to go.

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